A materials science framework for connecting protein stability with surface adsorption and coating applications

Ma, Gamaliel Junren

2022

Ma, G. J. (2022). A materials science framework for connecting protein stability with surface adsorption and coating applications. Doctoral thesis, Nanyang Technological University, Singapore. <https://hdl.handle.net/10356/154985>

<https://hdl.handle.net/10356/154985>

<https://doi.org/10.32657/10356/154985>

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

Downloaded on 26 Apr 2023 22:04:43 SGT

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**A MATERIALS SCIENCE FRAMEWORK FOR CONNECTING
PROTEIN STABILITY WITH SURFACE ADSORPTION AND
COATING APPLICATIONS**

MA JUNREN, GAMALIEL

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2022

**A MATERIALS SCIENCE FRAMEWORK FOR CONNECTING
PROTEIN STABILITY WITH SURFACE ADSORPTION AND
COATING APPLICATIONS**

MA JUNREN, GAMALIEL

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2022

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

22 January 2022

.....

Date

NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU

Ma Junren, Gamaliel

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

22 January 2022

.....
Date

NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
Prof Cho Nam-Joon

Authorship Attribution Statement

This thesis contains material from 3 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 4 is published as G.J. Ma, A.R. Ferhan, T.N. Sut, J.A. Jackman, and N.-J. Cho. Understanding How Natural Sequence Variation in Serum Albumin Proteins Affects Conformational Stability and Protein Adsorption. *Colloids and Surfaces B: Biointerfaces* **194**, 111194 (2020). DOI: 10.1016/j.colsurfb.2020.111194.

The contributions of the co-authors are as follows:

- Prof. Cho provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Asst. Prof. Jackman and Dr. Ferhan.
- I co-designed the study with Dr. Ferhan and Asst. Prof. Jackman and performed all the laboratory work at the Centre for Biomimetic Sensor Science at the School of Materials Science and Engineering. I also analyzed the data.
- Mr. Tun Naw Sut assisted in the collection of the quartz crystal microbalance-dissipation data.
- Dr. Ferhan assisted in the interpretation of the quartz crystal microbalance-dissipation and localized surface plasmon resonance data.

Chapter 5 and 7 is published as G.J. Ma, A.R. Ferhan, J.A. Jackman, and N.-J. Cho. Conformational Flexibility of Fatty Acid-Free Bovine Serum Albumin Proteins Enables Superior Antifouling Coatings. *Communications Materials* **1**, 45 (2020). DOI: 10.1038/s43246-020-0047-9.

The contributions of the co-authors are as follows:

- Prof. Cho provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Asst. Prof. Jackman and Dr. Ferhan.

- I co-designed the study with Dr. Ferhan and Asst. Prof. Jackman.
- I prepared all samples, collected localized surface plasmon resonance data, obtained Fourier transform infrared spectroscopy data, carried out enzyme-linked immunosorbent assays and Western blot experiments and conducted data evaluation.
- Mr. Tun Naw Sut assisted in the collection of the quartz crystal microbalance-dissipation data.

Chapter 6 is published as G.J. Ma, A.R. Ferhan, J.A. Jackman, and N.-J. Cho. Elucidating How Different Amphipathic Stabilizers Affect BSA Protein Conformational Properties and Adsorption Behavior. *Langmuir* **36**, 35, 10606–10614 (2020). DOI: 10.1021/acs.langmuir.0c02048.

The contributions of the co-authors are as follows:

- Prof. Cho provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Asst. Prof. Jackman and Dr. Ferhan.
- I co-designed the study with Dr. Ferhan and Asst. Prof. Jackman.
- I prepared all samples, collected localized surface plasmon resonance data, obtained fluorescence spectroscopy and Fourier transform infrared spectroscopy data, and conducted data evaluation.
- Mr. Tun Naw Sut assisted in the collection of the quartz crystal microbalance-dissipation data.

22 January 2022

.....
Date

ITU NTU NTU NTU NTU NTU NTU NTU
NTU NTU NTU NTU NTU NTU NTU NTU
ITU NTU NTU NTU NTU NTU NTU NTU
ITU NTU NTU NTU NTU NTU NTU NTU
.....
Ma Junren, Gamaliel

Abstract

The formation of protein coatings at the solid-liquid interface via protein adsorption is broadly relevant for various materials science and biomedical applications such as implantable biomedical devices, biosensors and nanomedicine. The noncovalent adsorption behavior of protein molecules is governed by various interfacial forces between the protein molecule in solution and the solid surface, as well as by intramolecular forces which determine a protein's folded structure and solution-phase conformational stability. Although a protein's conformational stability has been closely linked to its extent of unfolding in the adsorbed state, it remains to be understood how this relationship may be exploited in order to control a protein's adsorption behavior and adlayer properties to suit specific application needs. The objective of this thesis is to systematically interrogate the relationship between protein conformational stability and protein adsorption behavior such as adsorption uptake, adsorption rate, and the degree of adsorption-related denaturation and spreading. The overall hypothesis is that rationally tuning the conformational stability of protein molecules based on materials science concepts can influence protein adsorption and that the insights gained from this framework can be used to engineer improved protein coating applications. To test this hypothesis, serum albumins as model proteins with modulated conformational stabilities based on their structure and processing, in line with the materials science paradigm, were comparatively evaluated using an experimental approach that characterized the proteins' solution-phase conformational stabilities by measuring their temperature-dependent aggregation profiles and degree of secondary structural unfolding, and also that which characterized the proteins' adsorption behavior by measuring their adsorption uptake, adsorption rate and extent of surface-induced denaturation. It was revealed that primary structure variations between serum albumin proteins from different species affected protein conformational stability and adsorption behavior, and that bovine serum albumin (BSA) possessed the lowest conformational stability and greatest adsorption-related denaturation compared to that of human and rat origin. This provided mechanistic reasons for why BSA is so widely used in surface passivation ("blocking") protocols for many biotechnological applications. A further investigation on several commercially available and differently processed BSA proteins

revealed that they possessed significantly different conformational stabilities and adsorption behavior, depending on whether or not a purification step which removed fatty acids was performed. It was further determined that the doping of BSA proteins with fatty acids and other related amphipathic molecules generally increased protein conformational stability and could either increase or decrease adsorption uptake in a concentration-dependent manner, depending on the chemical structure of the amphipathic molecules and their binding strength with BSA protein. Finally, the experimentally observed differences in conformational properties and adsorption behavior of certain types of BSA proteins were correlated with their surface passivation performance. In summary, the findings in this thesis presented how materials science concepts can be used to rationalize the relationship between protein conformational stability and adsorption behavior and offer a broadly applicable approach to engineer protein coatings for various useful applications.

Lay Summary

Proteins in solution spontaneously adsorb onto solid surfaces in a very common process called protein adsorption. There is growing interest in understanding how to control this process in order to fabricate protein-coated materials that can be used in various biomedical technologies to improve human health. While it is known that proteins adsorb differently depending on the stability of the protein's folded structure, which is called conformational stability, how differences in conformational stability affects specific adsorption behaviors, and how that affects the resulting protein coating are all outstanding questions. Therefore, the objective of this thesis is to study the fabrication of coatings from proteins with different conformational stabilities. To do this, the conformational stability of a model protein: serum albumin, was deliberately changed based on materials science concepts (*i.e.*, structure and processing). A set of experiments was developed to study this change in conformational stability and to also study how that, in turn, affects adsorption behavior. Using this framework, it was discovered that bovine serum albumin (BSA) had different conformational properties compared to human and rat serum albumin and was therefore likely to be more suitable for coating applications requiring high surface coverage. It was further discovered that amphipathic molecules such as fatty acids had a profound impact on BSA conformational stability and adsorption behavior. Importantly, it was discovered that BSA proteins with different conformational stabilities and adsorption behaviors exhibited different coating performances, which highlighted the usefulness of this experimental approach in studying and predicting how protein conformational stability can affect protein coatings. In summary, the findings in this thesis demonstrate how a materials science framework can be utilized to study protein coating fabrication and how a similar approach would also be applicable to study and fabricate various types of useful protein coatings.

Acknowledgements

I thank God for making all things possible and setting me on this path towards pursuing a Ph.D.

I am very grateful to Nanyang Technological University for providing an excellent and conducive academic environment and the Graduate Research Officer Scheme for financially supporting my studies.

I would also like to express my sincere gratitude to my supervisor Prof. Nam-Joon Cho for encouraging me to pursue my doctoral studies and granting me the amazing opportunity to be part of his research group. His drive, positivity and dedication to research are traits which I aspire to emulate in my future career. I would also like to thank members of my Thesis Advisory Committee, Prof. Bo Liedberg, Prof. Juha Song and Prof. Dalton Tay for their guidance.

I am exceptionally grateful to my mentors Prof. Joshua Jackman and Dr. Ferhan Abdul Rahim for their patience, guidance, and mentorship, for the many meaningful discussions and advice on both work and life, and for showing me the joy and fun in science. Their encouragements have been invaluable throughout the course of my doctoral studies, and I am extremely fortunate to have them as my mentors.

It has been a great pleasure to work with my fellow group members in the Engineering in Translational Science group. I would like to thank my seniors Dr. Min Chul Kim, Dr. Bo Kyeong Yoon and Dr. Jae Hyeon Park for their teaching, guidance, and advice on various essential laboratory skills, as well as Ms. Haram Jung, Ms. Shu An, Ms. Young Kyoung “Kayla” Kim and Ms. Yun Yang for their kind administrative support. I would like to especially thank Mr. Tun Naw Sut for being my closest companion in this group, for his contribution to experiments and discussions in this project, for encouraging me not to worry too much, and for the laughter he brings. I would also like to express my sincere thanks and appreciation to my dear colleagues Dr. Gaia Ferracci, Dr. Soo Hyun Park, Dr.

Elba Valle, Dr. Teng Fei Fan, Dr. Youngkyu Hwang, Dr. Zhao Ze, Mr. Mohammed Shahrudin Bin Ibrahim, Ms. Jingyu Deng and Mr. Hyunhyuk Tae for their joy, warmth, kindness, and support.

To my family, thank you for your loving support.

To my dearest wife, thank you for being there for me. I love you very much.

Table of Contents

Abstract	i
Lay Summary	iii
Acknowledgements	v
Table of Contents	vii
Table Captions	xv
Figure Captions	xvii
Abbreviations	xxxvii
 Chapter 1 Introduction.....	 1
1.1 Hypothesis/Problem Statement	2
1.2 Objectives and Scope	5
1.3 Dissertation Overview	7
1.4 Findings and Outcomes/Originality	9
References.....	11
 Chapter 2 Literature Review	 17
2.1 Protein Adsorption	18
2.1.1 Physicochemical Principles of Protein Adsorption.....	18
2.1.2 Environmental and Material Surface Parameters Affecting Protein Adsorption.....	19

2.1.3	Conformational Stability and its Influence on Protein Adsorption	22
2.1.4	Experimental Approaches to Characterize Protein Conformational Stability and Protein Adsorption	24
2.1.4.1	Characterization of Solution-Phase Conformational Stability	24
2.1.4.2	Characterization of Protein Adsorption.....	26
2.1.5	Examples of Functional Protein Coatings.....	28
2.2	Serum Albumin	31
2.2.1	Structure and Biological Function of Serum Albumin	31
2.2.2	Solution and Surface Properties of Serum Albumin.....	36
2.2.2.1	Solution Properties	36
2.2.2.2	Adsorption Properties.....	37
2.2.3	Purification and Processing Conditions of Serum Albumin	39
2.2.4	Applications of Serum Albumin Coatings.....	43
2.3	Outstanding Questions	44
	References.....	46
Chapter 3 Experimental Methodology		69
3.1	Rationale for the Selection of Materials and Experimental Methods	70
3.2	Materials.....	73
3.2.1	Serum Albumin Samples	73
3.2.2	Other Reagents.....	74
3.3	Sample Preparation	75
3.4	Characterization Techniques	76
3.4.1	Dynamic Light Scattering.....	76
3.4.2	Circular Dichroism Spectroscopy	79

3.4.3	Fluorescence Spectroscopy	82
3.4.4	Quartz Crystal Microbalance-Dissipation.....	84
3.4.5	Localized Surface Plasmon Resonance Sensing	87
3.4.6	Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy	91
3.4.7	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot.....	95
3.4.8	Silica Nanoparticle Preparation and Enzyme-Linked Immunosorbent Assay for Complement Activation	99
	References	100

Chapter 4 Understanding How Natural Sequence Variation in Serum Albumin Proteins Affects Conformational Stability and Protein Adsorption109

4.1	Introduction	110
4.2	Materials and Methods	111
4.2.1	Reagents	111
4.2.2	Sample Preparation	112
4.2.3	Structural Modeling	112
4.2.4	Multiple Sequence Alignment	112
4.2.5	Dynamic Light Scattering	113
4.2.6	Circular Dichroism Spectroscopy	113
4.2.7	Quartz Crystal Microbalance-Dissipation.....	114
4.2.8	Localized Surface Plasmon Resonance Sensing	115
4.2.9	Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy ...	115
4.2.10	Statistical Analysis.....	116
4.3	Results and Discussion.....	116

4.3.1 Comparing the Amino Acid Sequence Between Serum Albumin Species.....	116
4.3.2 Solution-Phase Conformational Stability of Serum Albumin Proteins	118
4.3.3 Comparing the Adsorption Behavior of Serum Albumin Proteins	123
4.3.4 Adsorption-Related Protein Conformational Changes	127
4.3.5 Mechanistic Picture of BSA, HSA and RSA Adsorption Behavior	131
4.4 Conclusion.....	132
References.....	133
 Chapter 5 Quantitative Assessment of Bovine Serum Albumin Proteins from Different Purification and Processing Strategies	 141
5.1 Introduction	142
5.2 Materials and Methods	143
5.2.1 Bovine Serum Albumin Proteins	143
5.2.2 Reagents	143
5.2.3 Sample Preparation	144
5.2.4 Dynamic Light Scattering	144
5.2.5 Circular Dichroism Spectroscopy	145
5.2.6 Quartz Crystal Microbalance-Dissipation.....	146
5.2.7 Localized Surface Plasmon Resonance Sensing	146
5.2.8 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy ...	147
5.2.9 Statistical Analysis.....	148
5.3 Results	149
5.3.1 Evaluation Strategy	149
5.3.2 Thermal Stability of Fatted and Defatted BSA Proteins.....	154
5.3.3 Adsorption Behavior of Fatted and Defatted BSA Proteins	161

5.3.4 Adsorption-Related Conformational Changes	164
5.3.5 Addition of Caprylic Acid to Defatted and Fatted BSA Proteins	168
5.4 Discussion	173
5.5 Conclusion.....	176
References.....	176

Chapter 6 Elucidating How Different Amphipathic Stabilizers Affect BSA Protein Conformational Properties and Adsorption Behavior185

6.1 Introduction	186
6.2 Materials and Methods	188
6.2.1 Reagents.....	189
6.2.2 Sample Preparation	189
6.2.3 Fluorescence Spectroscopy	190
6.2.4 Dynamic Light Scattering.....	190
6.2.5 Circular Dichroism Spectroscopy	190
6.2.6 Quartz Crystal Microbalance-Dissipation.....	191
6.2.7 Localized Surface Plasmon Resonance Sensing.....	192
6.2.8 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy ...	192
6.2.9 Statistical Analysis.....	193
6.3 Results and Discussion.....	194
6.3.1 Binding Interaction Analysis	194
6.3.2 Solution-Phase Conformational Stability.....	197
6.3.3 Nature of Amphiphile Headgroup-BSA Interactions.....	204
6.3.4 Real-Time Adsorption Behavior	206
6.3.5 Adsorption-Related Protein Conformational Changes	210

6.3.6 Schematic Overview of Amphiphile Effects	213
6.4 Conclusion.....	215
References	216
 Chapter 7 Evaluating the Surface Passivation Performance of BSA Proteins for Antifouling Applications	223
7.1 Introduction	224
7.2 Materials and Methods	225
7.2.1 Reagents	225
7.2.2 Sample Preparation	226
7.2.3 Quartz Crystal Microbalance-Dissipation.....	227
7.2.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot.....	228
7.2.5 Silica Nanoparticle Preparation and Enzyme-Linked Immunosorbent Assay for Complement Activation	229
7.2.6 Statistical Analysis.....	230
7.3 Results and Discussion.....	230
7.3.1 Inhibiting Serum Biofouling	230
7.3.2 Inhibiting Nonspecific Adsorption in Western Blots.....	233
7.3.3 Inhibiting Nanoparticle-Induced Complement Activation	237
7.4 Conclusion.....	241
References	241
 Chapter 8 Conclusions and Recommendations.....	245
8.1 Discussion and Conclusion	246
8.2 Future Directions.....	249

8.2.1 Alternative Methods for Modulating Protein Conformational Properties .	249
8.2.2 Application of Experimental Framework to Alternative Proteins	253
References	257
List of Publications	263

Table Captions

- Table 2.1** Summary of common techniques used to characterize protein conformational stability in solution.
- Table 2.2** Summary of common techniques used to characterize protein adsorption uptake, adsorption kinetics and adsorption-related conformational changes.
- Table 2.3** A summary of the process conditions for cold ethanol fractionation, heat-shock fractionation, and charcoal defatting of serum albumin.
- Table 5.1** Summary of BSA protein reagents. The albumin content percentage by agarose electrophoresis and the percentage of free fatty acids were obtained from the manufacturer's certificates of analysis. The amount of free fatty acid in BSA 1-3 were not reported because they were "fatted" reagents while the free fatty acid percentage for BSAs 4-6 verifies that they were "defatted" reagents.
- Table 5.2** Secondary structure percentage of BSA proteins 1-6 in solution and in the adsorbed state from ATR-FTIR spectroscopy measurements. Data are reported as mean $\pm$ s.d. ($n=3$).
- Table 5.3** Secondary structure percentage of BSA 5 and CA-BSA 5 in solution and in the adsorbed state from ATR-FTIR spectroscopy measurements. Data are reported as mean $\pm$ s.d. ($n=3$).
- Table 6.1** Summary of binding interaction data from fluorescence spectroscopy experiments. Stern-Volmer constant (K_{SV}), bimolecular quenching rate constant (k_q), effective binding constant (K_b), and number of different binding site types (n) for the amphiphile-doped BSA samples are shown. Calculations of K_b and n were omitted for ME samples due to insignificant

quenching effects related to negligible binding. K_{SV} values are presented as fitted slope value $\pm$ standard error of the slope, while the values and uncertainty for k_q were derived from K_{SV} based on Eq 6.3.

Figure Captions

Figure 1.1 The materials science tetrahedron. A material's structure, processing, properties, and performance are interrelated. These interrelations are elucidated through characterization.

Figure 2.1 Molecular structure of BSA. **a**, Protein ribbon diagram with “ghost” surface showing domain I in red, domain II in blue and domain III in green with their individual A and B subdomains in related colors. **b**, Solid surface of the protein showing charge distribution from amino acid side groups. Blue represents positive charge, red represents negative charge and white represents neutral charge. The yellow line indicates the protein dipole moment. Adapted with permission from ref. 205. Copyright 2017, American Chemical Society

Figure 2.2 Aligned molecular structures of HSA (burgundy), BSA (cyan), serum albumin from horse (blue and violet) and from rabbit (green). Disulphide bridges are depicted as spheres. Adapted with permission from ref. 190. Copyright 2012, International Union of Crystallography.

Figure 2.3 Electrostatic surface potential ($K_B T/e_c$) of serum albumins from **a,b**, sheep, **c,d**, goat, and **e,f**, BSA. Adapted with permission from ref. 192. Copyright 2012, International Union of Crystallography.

Figure 2.4 The isomerism of BSA from N form at neutral pH, to F form at pH 4 and E form at below pH 3. Adapted with permission from ref. 207. Copyright 2012, Elsevier.

Figure 3.1 The process flow for the manufacture of the six BSA types from bovine plasma tested in this study. BSAs that have undergone cold ethanol fractionation, heat shock fractionation, and cold ethanol followed by heat

shock fractionation have been designated BSA 1, BSA 2 and BSA 3, respectively. BSAs 4, 5 and 6 have been designated as the defatted versions of BSAs 1, 2 and 3.

Figure 3.2 DLS measurement principle. Light from a laser source is scattered by protein particles in solution. The scattered light is analyzed as a function of time and converted into a correlation curve. Differences in intensity fluctuations due to differences in diffusivity are interpreted as differences in particle size. Adapted with permission from ref. 30. Copyright 2021, Nanoscience Instruments

Figure 3.3 CD spectra of poly-L-lysine at pH 11.1 exhibiting (1) α -helical and (2) antiparallel β -sheet secondary structures, and at pH 5.7 exhibiting (3) extended conformation. Collagen exhibiting (4) triple helix and (5) denatured conformations. Adapted with permission from ref. 34. Copyright 2007, Nature Publishing Group.

Figure 3.4 QCM-D measurement principle. **a**, An AC voltage is applied across the quartz crystal sensor, producing a resonance frequency that decreases upon any addition of mass on the sensor surface. **b**, When the AC power is cut off, the sensor's oscillation decays, and rigidly attached adsorbates on the sensor surface result in longer decay patterns, while **c**, viscoelastic adsorbates result in shorter decay curves. Adapted with permission from ref. 50. Copyright 2021, Nanoscience Instruments.

Figure 3.5 Schematic of the LSPR measurement setup. Optical extinction is recorded in transmission mode and the shifts in wavelength of the maximum optical extinction due to the presence of biomolecules are monitored. Adapted with permission from ref. 52. Copyright 2017, Royal Society of Chemistry.

Figure 3.6 Schematic of ATR-FTIR optical setup. An infrared beam is totally internally

reflected at the interface between the ATR crystal and the sample, which covers the top of the crystal. An evanescent wave is created and is absorbed by the sample at areas of total internal reflectance. Information on the absorbance by the sample is collected by the detector. Adapted with permission from ref. 61. Copyright 2015, IOS Press.

Figure 3.7 Schematic of Western blot process flow. Proteins from a sample are separated by SDS-PAGE, and then transferred to a nitrocellulose membrane. After blocking with BSA blocking solution, membranes are incubated with primary followed by HRP-conjugated secondary antibodies. Upon addition of a chemiluminescent substrate, which reacts with the HRP, Western blot bands can be imaged. Adapted with permission from ref. 73. Copyright 2019, Elsevier.

Figure 3.8 Comparison of native and heat-denatured BSA proteins as blocking agents. **a**, Schematic illustration of Western blot blocking protocol using a blocking solution made from either native or heat-denatured BSA. Gels were loaded with SiNP-treated serum (lanes 1-4), untreated serum (lane 5) and buffer solution (lane 6) and after transfer to nitrocellulose membrane, were blocked with **b**, native BSA monomers or **c**, heat-denatured BSA oligomers/aggregates prior to antibody incubation. The differences in band intensity indicate the superior blocking performance of denatured BSA aggregates Adapted with permission from ref. 27. Copyright 2018, American Chemical Society.

Figure 4.1 **a**, Experimental strategy to characterize how structural variations between serum albumin proteins affect conformational stability and adsorption behavior. **b**, Modeled 3D structures of BSA, HSA, and RSA. **c**, Multiple sequence alignment of BSA, HSA, and RSA. Amino acids are classified based on their physicochemical properties according to the Zappo color scheme. **d**, List of different physicochemical property pairs and respective

number of amino acid substitutions between BSA, HSA, and RSA. Substitutions involving changes in hydrophobic interactions and electrostatic interactions were designated as Type 1 and Type 2, respectively.

Figure 4.2 DLS characterization of BSA, HSA, and RSA protein size distributions. DLS measurements of intensity-weighted size distributions for **a**, BSA, **b**, HSA, and **c**, RSA proteins at 25 °C (n=5). The mean hydrodynamic diameter (labeled as D) and polydispersity index (labeled as PDI) are indicated.

Figure 4.3 Hydrodynamic diameter of BSA, HSA, and RSA measured by dynamic light scattering (DLS) as a function of temperature. Data are presented as mean $\pm$ standard deviation (s.d.) [n=5, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (versus data at 25 °C)].

Figure 4.4 Time-dependent changes in the mean hydrodynamic diameters of BSA, HSA, and RSA proteins at 60 °C. Values are presented as mean $\pm$ s.d., where s.d. is defined as full-width-at-half-maximum (FWHM)/2.355 (n=5).

Figure 4.5 Temperature-dependent circular dichroism (CD) spectra of BSA, HSA, and RSA proteins. CD spectra of **a**, BSA, **b**, HSA, and **c**, RSA proteins were measured at 25, 50, 55, 60, 65, 70, and 75 °C and are reported in molar residue ellipticity units $[\theta]$.

Figure 4.6 α -helical percentage of BSA, HSA, and RSA as a function of temperature from circular dichroism (CD) spectroscopy experiments. Data are reported as mean $\pm$ s.d. (n=3). Mean values are presented on top of each column.

Figure 4.7 **a**, Time-resolved QCM-D ΔF shifts and **b**, corresponding $|\Delta F_{\max}|$ values at adsorption saturation. **c**, Time-resolved QCM-D ΔD shifts and **d**, corresponding $\Delta D_{\max}$ values at adsorption saturation. Data in **b** and **d** are presented as mean $\pm$ s.d. (n=3, one-way ANOVA with Tukey's multiple

comparisons test). Test results are reported for HSA and RSA (versus BSA). Dots represent individual data points.

Figure 4.8 QCM-D frequency-energy dissipation curves for comparing the adsorption profiles of each protein onto silica surface. Time-independent frequency-energy dissipation (F-D) curves are derived from QCM-D frequency and energy dissipation shifts for BSA, HSA, and RSA protein adsorption onto silica surfaces at 25 °C.

Figure 4.9 **a**, Time-resolved LSPR $\Delta\lambda$ shifts and **b**, corresponding $\Delta\lambda_{\text{max}}$ shifts at adsorption saturation. Data in **b**, are presented as mean $\pm$ s.d. (n=3, one-way ANOVA with Tukey's multiple comparisons test). Test results are reported for HSA and RSA (versus BSA). Dots represent individual data points.

Figure 4.10 **a**, Rate of change of LSPR peak shift during the initial stage of adsorption ($d\Delta\lambda/dt$) as a function of time and **b**, corresponding maximum rate of change of LSPR peak shift during the initial stage of adsorption $(d\Delta\lambda/dt)_{\text{max}}$. **c**, Comparison between the rate of size-related diffusion-limited adsorption and the maximum rate of change in LSPR peak shift data from **b**. Rates are normalized to the BSA case. **d**, α -helical percentage of BSA, HSA, and RSA proteins in solution and upon adsorption from ATR-FTIR spectroscopy experiments. Data in **b**, **c**, and **d** are presented as mean $\pm$ s.d. [n=3, one-way ANOVA in **b** and two-way ANOVA in **d** with Tukey's multiple comparisons test]. Test results in **b** are reported for HSA and RSA (versus BSA). Test results in **d** are reported for HSA and RSA (versus BSA) in solution and are separately reported for HSA and RSA (versus BSA) in the adsorbed state. Dots in **b** and **d** represent individual data points.

Figure 4.11 Quantification of adsorption-related changes in secondary structure for BSA, HSA, and RSA proteins by ATR-FTIR measurements. Amide I regions from ATR-FTIR spectrograms of **a**, BSA, **b**, HSA, and **c**, RSA proteins in solution

(left panels) and in the adsorbed state (right panels). Experimentally obtained absorbance spectra were resolved into individual component curves, which were each assigned to a specific secondary structure element.

Figure 4.12 Schematic illustration of BSA, HSA, and RSA proteins in solution and conformational stability-related adsorption behavior trends. **a**, BSA and HSA proteins exist as monomers while RSA proteins are assembled as multimers in solution. **b**, BSA, HSA, and RSA display different adsorption behaviors. BSA's lower conformational stability than HSA leads to greater surface-induced denaturation and larger footprint size. Adsorption of larger RSA multimers results in denaturation of only the molecules in contact with the surface (darker colored) while other molecules remain in their native state.

Figure 5.1 Evaluation of BSA protein reagents for blocking applications. **a**, Overview of basic steps in BSA blocking protocols. (i) A target surface is identified to coat with BSA for surface passivation ("blocking"). (ii) BSA proteins in aqueous solution are incubated with the target surface. (iii) BSA proteins adsorb and denature on the target surface. (iv) A washing step is performed to remove weakly bound BSA protein molecules. (v) A firmly attached monolayer of adsorbed BSA protein molecules is formed on the surface. **b**, Overall experimental strategy to analyze BSA reagent properties. A series of BSA protein reagents, which were purified by different methods, were selected and their conformational, adsorption, and blocking properties were evaluated. A series of solution-phase and surface-sensitive biophysical measurement approaches were employed along with surface- and nanoparticle-based bioassays. **c**, Outline of purification routes to isolate BSA protein molecules from bovine plasma. Fatty acid-containing BSA proteins (referred to as "fatted") were prepared by (1) cold ethanol fractionation, (2) heat-shock fractionation, and (3) cold ethanol fractionation followed by heat-shock fractionation and these reagents are designated as BSA 1, 2, 3, respectively. Fatty acid-free versions (referred to as "defatted") of BSA 1, 2, 3 were also

prepared and are designated as BSA 4, 5, 6, respectively.

Figure 5.2 Quantification of ATR-FTIR spectral features sensitive to the presence of bound fatty acids in BSA proteins. ATR-FTIR spectrograms of (a-f) BSA proteins 1-6 in solution. The peak absorbance intensity ratio of the asymmetric stretch of methylene groups ($\nu_{\text{as}}(\text{CH}_2)$, 2936 cm^{-1}) to the asymmetric stretch of methyl groups ($\nu_{\text{as}}(\text{CH}_3)$, 2961 cm^{-1}) was quantified as an indication of the amount of bound fatty acids for each BSA protein. Insets show an expanded view of the region enclosed within the dashed box where the peaks are located.

Figure 5.3 DLS characterization of BSA protein size distribution. a-f, DLS measurements of BSA proteins 1-6 at $25\text{ }^{\circ}\text{C}$ ($n=5$). The mean hydrodynamic diameter (D) and polydispersity index (PDI) are indicated in each panel.

Figure 5.4 Hydrodynamic diameter of BSA protein molecules as a function of temperature, as measured in DLS experiments. Data are reported as mean $\pm$ standard deviation (s.d.), where s.d. is defined as the full-width-at-half-maximum (FWHM)/2.355 [$n=5$, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (versus data at $25\text{ }^{\circ}\text{C}$)]. Dots represent individual data points.

Figure 5.5 Time-dependent changes in protein size at $60\text{ }^{\circ}\text{C}$ incubation temperature. Time-dependent changes in the mean hydrodynamic diameters of BSA proteins 1-6 at $60\text{ }^{\circ}\text{C}$. Values are presented as mean $\pm$ s.d. ($n=5$) where s.d. is defined as full-width-at-half-maximum (FWHM)/2.355.

Figure 5.6 CD spectroscopy characterization of BSA proteins. a, Circular dichroism (CD) spectra of BSA proteins 1-6 at $25\text{ }^{\circ}\text{C}$ are reported in molar residue ellipticity units $[\theta]$. b-g, CD spectra of BSA proteins 1-6 at $25, 50, 55, 60, 65, 70$ and $75\text{ }^{\circ}\text{C}$ are reported in molar residue ellipticity units $[\theta]$. All elevated

temperature measurements were recorded after a 5-min temperature equilibration period. All measurements were conducted in triplicate.

Figure 5.7 Fractional percentage of α -helicity in BSA protein molecules as a function of temperature, as measured in CD spectroscopy experiments. Mean values are presented on top of each column. Values were computed from molar residue ellipticity data and data are reported as mean $\pm$ s.d. ($n=3$). Dots represent individual data points. The grey horizontal line in each graph corresponds to 50% helicity.

Figure 5.8 QCM-D experiments were conducted to measure frequency (ΔF) and energy dissipation (ΔD) signals related to the protein adsorption process. **a**, Time-resolved QCM-D ΔF shifts and **b**, corresponding $|\Delta F_{\max}|$ shifts at saturation. **c**, Time-resolved QCM-D ΔD shifts. **d**, $|\Delta F_{\max}/\Delta D_{\max}|$ ratios obtained from saturation data in panels **a** and **c**. A buffer washing step is performed at around $t=35$ min and is indicated by an arrow in panels **a** and **c**. Data in **b** and **d** are reported as mean $\pm$ s.d. ($n=3$, one-way ANOVA with Tukey's multiple comparisons test). P values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method). Dots represent individual data points.

Figure 5.9 Comparison of QCM-D frequency-energy dissipation curves for BSA adsorption onto silica surfaces. Time-independent frequency-energy dissipation (F-D) curves derived from QCM-D frequency and energy dissipation shifts related to the adsorption of BSA proteins 1-6 onto silica surfaces at 25°C.

Figure 5.10 LSPR experiments were conducted to measure $\Delta\lambda_{\max}$ signals related to the protein adsorption process. **a**, Time-resolved LSPR wavelength shifts ($\Delta\lambda$) and **b**, corresponding $\Delta\lambda_{\max}$ shifts at saturation. Data in **b** are reported as mean $\pm$ s.d. ($n=3$, one-way ANOVA with Tukey's multiple comparisons test). P

values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method). Dots represent individual data points.

Figure 5.11 Time-derivative plot of LSPR-tracked BSA adsorption kinetics. Time-derivative of wavelength shifts ($d\Delta\lambda/dt$) corresponding to the LSPR-tracked adsorption of BSA proteins 1-6 onto silica-coated sensor surfaces.

Figure 5.12 Evaluation of adsorption-related conformational changes in BSA protein structure. **a**, Maximum rate of change in the LSPR wavelength shift $(d\Delta\lambda/dt)_{\max}$ during the initial adsorption stage. Values are computed from data in Figure 5.10. Data are reported as mean $\pm$ s.d. ($n=3$, one-way ANOVA with Tukey's multiple comparisons test). **b**, Fractional percentage of α -helicity in BSA protein molecules in solution and in the adsorbed state, as determined in ATR-FTIR spectroscopy experiments. Data are reported as mean $\pm$ s.d. ($n=3$, two-way ANOVA with Tukey's multiple comparisons test). *P* values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method) in **a**, and are separately reported for defatted BSA proteins in solution (versus fatted BSA proteins in solution from the same fractionation method) and for defatted BSA proteins in the adsorbed state (versus fatted BSA proteins in the adsorbed state from the same fractionation method) in **b**. Dots represent individual data points.

Figure 5.13 Quantification of adsorption-related protein conformational changes in the secondary structure of BSA proteins based on ATR-FTIR measurements. Amide I regions from attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectrograms of **a-f**, BSA proteins 1-6 in solution (left panels) and in the adsorbed state (right panels). Experimentally obtained absorbance spectra were resolved into individual component curves, which were each assigned to a secondary structure element.

Figure 5.14 Verification of the role of fatty acid in causing differences in the conformational and adsorption properties between fatted and defatted BSAs via caprylic acid doping of defatted BSA 5. **a**, DLS measurements of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5) at 25 °C ($n=5$). The mean hydrodynamic diameter (D) and polydispersity index (PDI) are indicated in each panel. **b**, Hydrodynamic diameter of BSA 5 and CA-BSA 5 as a function of temperature, as measured in DLS experiments. Data are reported as mean $\pm$ s.d. [$n=5$, one-way ANOVA with Dunnett's multiple comparisons test (versus data at 25 °C) for CA-BSA 5 data]. Dots represent individual data points. **c**, Circular dichroism (CD) spectra of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5) at 25 °C are reported in molar residue ellipticity units $[\theta]$ ($n=3$). **d**, CD spectra of BSA 5 and CA-BSA 5 at 25, 50, 55, 60, 65, 70 and 75 °C are reported in molar residue ellipticity units $[\theta]$ ($n=3$). All elevated temperature measurements were recorded after a 5-min temperature equilibration period. **e**, Fractional percentage of α -helicity in BSA 5 and CA-BSA 5 protein molecules as a function of temperature, as measured in CD spectroscopy experiments. Mean values are presented on top of each column. Values were computed from molar residue ellipticity data and data are reported as mean $\pm$ s.d. ($n=3$). Dots represent individual data points. **f**, Time-resolved QCM-D ΔF shifts and **g**, corresponding $|\Delta F_{\max}|$ shifts at saturation. **h**, Time-resolved QCM-D ΔD shifts. **i**, $|\Delta F_{\max}/\Delta D_{\max}|$ ratios obtained from saturation data in panels **f** and **h**. **j-k**. LSPR experiments were conducted to measure $\Delta\lambda_{\max}$ signals related to the protein adsorption process. **j**, Time-resolved LSPR wavelength shifts ($\Delta\lambda$) and **k**, corresponding $\Delta\lambda_{\max}$ shifts at saturation. Data in **g**, **i**, **k** are reported as mean $\pm$ s.d. ($n=3$, unpaired t-test). Dots represent individual data points. **l**, Time-independent frequency-energy dissipation (F-D) curves derived from QCM-D frequency and energy dissipation shifts related to the adsorption of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5) onto silica surfaces at 25°C. **m**, Time-derivative of wavelength shifts ($d\Delta\lambda/dt$) corresponding to the LSPR-tracked adsorption of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5) onto silica-coated sensor

surfaces. **n-o.** Amide I regions from attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectrograms of **n.** BSA 5 and **o.** caprylic acid-doped BSA 5 (CA-BSA 5) in solution (left panels) and in the adsorbed state (right panels). Experimentally obtained absorbance spectra were resolved into individual component curves, which were each assigned to a secondary structure element. **p,** Maximum rate of change in the LSPR wavelength shift $(d\Delta\lambda/dt)_{\max}$ during the initial adsorption stage of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5). Data are reported as mean $\pm$ s.d. ($n=3$, unpaired t-test). **q,** Fractional percentage of α -helicity in BSA 5 and CA-BSA 5 protein molecules in solution and in the adsorbed state, as determined in ATR-FTIR spectroscopy experiments. Data are reported as mean $\pm$ s.d. ($n=3$, two-way ANOVA with Sidak's multiple comparisons test). Dots represent individual data points.

Figure 5.15 Influence of caprylic acid doping on the adsorption of fatted BSA proteins 1-3, as measured by QCM-D experiments. **a,** Time-resolved QCM-D ΔF and **b,** ΔD shifts along with **c,** corresponding $|\Delta F_{\max}|$ shifts at saturation (from panel **a**) and **d,** $|\Delta F_{\max}/\Delta D_{\max}|$ ratios obtained from saturation data in panels **a** and **b** for BSA 1 without and with caprylic acid doping (labeled as BSA 1 and CA-BSA 1, respectively). **(e-h)** Equivalent data for BSA 2. **(i-l)** Equivalent data for BSA 3. Data in **c, d, g, h, k, l** are reported as mean $\pm$ s.d. ($n=3$, unpaired t-test). Dots represent individual data points.

Figure 5.16 Effect of fatty acids on BSA conformational and adsorption properties and implications for blocking applications. **a,** Schematic illustration of fatted and defatted BSA protein molecules in solution. The presence of fatty acids leads to higher conformational stability in solution. **b,** Schematic illustration of fatted and defatted BSA protein molecules in the adsorbed state. Higher conformational stability of fatted BSA leads to less adsorption-induced denaturation, greater protein-protein repulsion, and thus less well-packed protein coatings. On the other hand, the lower conformational stability of

defatted BSA leads to greater adsorption-induced denaturation, less protein-protein repulsion, and thus more well-packed protein coatings. As a result, defatted BSA proteins form more effective surface passivation coatings that could be more useful for blocking applications.

Figure 6.1 Overview of experimental strategy. The binding interactions of BSA with different amphipathic molecules, namely CA, MC, and ME, were quantitatively characterized by fluorescence spectroscopy along with dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy measurements to evaluate solution-phase conformational stability. Quartz crystal microbalance-dissipation (QCM-D), localized surface plasmon resonance (LSPR), and attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy experiments were conducted to determine how binding interactions with the tested amphipathic molecules affected BSA protein adsorption behavior in terms of adsorption uptake and kinetics along with related conformational changes.

Figure 6.2 Evaluation of CA, MC, and ME binding interactions with BSA proteins. Intrinsic fluorescence emission spectra of 10 μ M BSA in the absence and presence of **a**, CA, **b**, MC, and **c**, ME amphiphiles. **d**, Stern-Volmer plots corresponding to the data in panels A-C. Data are presented as mean $\pm$ standard deviation (s.d.). Linear fits and corresponding K_{SV} values of CA, MC, and ME binding to BSA are presented for each data set. **e**, Plot of $\log((F_0 - F)/F)$ versus $\log[S]$. Linear fits and corresponding K_b values of CA and MC binding to BSA are presented for each data set.

Figure 6.3 DLS-measured size distributions of BSA proteins in the absence and presence of the three tested amphiphiles. Mean hydrodynamic diameter (labeled as D) and polydispersity index (labeled as PDI) of **a**, defatted BSA, **b**, CA 10, **c**, CA 100, **d**, MC 10, **e**, MC 100, **f**, ME 10, and **g**, ME 100 samples were measured at 25 $^{\circ}$ C and are indicated in each panel.

Figure 6.4 Temperature-dependent DLS and CD spectroscopy measurements to evaluate solution-phase conformational stability of amphiphile-doped BSA proteins. **a**, Hydrodynamic diameter of BSA proteins alone and in the presence of CA, MC, and ME at amphiphile:BSA molar ratios of 10:1 and 100:1 as a function of temperature. Data are presented as mean $\pm$ standard deviation (s.d.), where s.d. is defined as the full-width-at-half-maximum (FWHM)/2.355 [$n=5$, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (versus data at 25 °C)]. Dots represent individual data points.

Figure 6.5 Time-dependent changes in BSA protein size in the absence and presence of the three tested amphiphiles at 60 °C incubation temperature. Data were obtained by DLS measurements and are presented as mean $\pm$ s.d. ($n=5$) where s.d. is defined as full-width-at-half-maximum (FWHM)/2.355.

Figure 6.6 Temperature-dependent CD spectra of BSA proteins in the absence and presence of the three tested amphiphiles. CD spectra of **a**, defatted BSA, **b**, CA 10, **c**, CA 100, **d**, MC 10, **e**, MC 100, **f**, ME 10, and **g**, ME 100 samples were measured at 25, 50, 55, 60, 65, 70, and 75 °C and are reported in molar residue ellipticity units [θ].

Figure 6.7 Degree of α -helical secondary structure, expressed in percentage values, for BSA alone and in the presence of CA, MC, and ME at amphiphile:BSA molar ratios of 10:1 and 100:1 as a function of temperature obtained from CD spectroscopy experiments. Data were computed from molar residue ellipticity values and are reported as mean $\pm$ s.d. ($n=3$). Dots represent individual data points. Mean values are presented on top of each column.

Figure 6.8 Temperature-dependent DLS measurements of amphiphile-doped BSA samples in different ionic strength conditions. Hydrodynamic diameter of **a**, CA 10, **b**, MC 10, and **c**, ME 10 samples in 1.5, 150, and 500 mM NaCl Tris

buffer solutions as a function of temperature. Data are presented as mean $\pm$ s.d. ($n=5$) where s.d. is defined as full-width-at-half-maximum (FWHM)/2.355.

Figure 6.9 QCM-D and LSPR measurements for tracking real-time adsorption of amphiphile-doped BSA proteins onto silica surfaces. **a**, QCM-D frequency (ΔF) shifts as a function of time and **b**, corresponding $|\Delta F_{\max}|$ shifts at adsorption saturation. **c**, QCM-D energy dissipation (ΔD) shifts as a function of time. **d**, $|\Delta F_{\max}/\Delta D_{\max}|$ values obtained from the data in panels **a** and **c** at adsorption saturation. Data in panels **b** and **d** are presented as mean $\pm$ s.d. [$n=3$ biological replicates, one-way ANOVA with Dunnett's multiple comparisons test (versus defatted BSA)]. Dots represent individual data points.

Figure 6.10 **a**, LSPR wavelength ($\Delta\lambda$) shifts as a function of time and **b**, corresponding $\Delta\lambda_{\max}$ shifts at adsorption saturation. Data in panel **b** are presented as mean $\pm$ s.d. [$n=3$ biological replicates, one-way ANOVA with Dunnett's multiple comparisons test (versus defatted BSA)]. Dots represent individual data points.

Figure 6.11 Rates of change of LSPR peak shift ($d\Delta\lambda/dt$) corresponding to the adsorption of amphiphile-doped BSA proteins onto silica surfaces as a function of time. The rates of change were recorded during the initial stage of adsorption.

Figure 6.12 Evaluation of adsorption-related protein conformational changes for amphiphile-doped BSA proteins. **a**, Maximum rate of change in the $\Delta\lambda$ shift during the initial stage of adsorption ($(d\Delta\lambda/dt)_{\max}$). **b**, Fractional percentage of α -helical secondary structure for BSA proteins in solution and in the adsorbed state, as measured by ATR-FTIR spectroscopy experiments. Data in panels **a** and **b** are presented as mean $\pm$ s.d. [$n=3$ biological replicates, one-way ANOVA with Dunnett's multiple comparisons test (versus defatted BSA) in

panel **a** and two-way ANOVA with Dunnett's multiple comparisons test for all samples in solution (versus defatted BSA in solution) and separately for all samples in the adsorbed state (versus defatted BSA in the adsorbed state) in panel **b**]. Dots represent individual data points.

Figure 6.13 Quantification of adsorption-related protein conformational changes of amphiphile-doped BSA proteins by ATR-FTIR spectroscopy measurements. Amide I regions from ATR-FTIR spectrograms of **a**, defatted BSA, **b**, CA 10, **c**, CA 100, **d**, MC 10, **e**, MC 100, **f**, ME 10, and **g**, ME 100 samples in solution (left panels) and in the adsorbed state (right panels). Experimentally obtained absorbance spectra were resolved into individual component curves, which were each assigned to a secondary structure element.

Figure 6.14 Schematic illustration summarizing how CA, MC, and ME amphiphiles affect the solution-phase conformational stability and adsorption properties of BSA proteins. ME had low binding affinity to BSA proteins and hence largely negligible effects. MC moderately enhanced conformational stability, which reduced adsorption-related denaturation while the nonionic character of MC allowed MC-doped BSA protein adlayers to have relatively higher packing densities. Likewise, CA strongly enhanced conformational stability, which also reduced adsorption-related denaturation. However, the anionic character of CA created more repulsive interactions between CA-doped BSA proteins, which led to CA-doped BSA protein adlayers with relatively lower packing densities.

Figure 7.1 Schematic illustration of serum biofouling assay. Pristine and BSA-coated silica surfaces were incubated in 100% fetal bovine serum (FBS) followed by a buffer washing step, and the amount of serum biofouling was measured. The passivation efficiency of the BSA coatings was calculated relative to the uncoated surface.

Figure 7.2 Quantification of BSA surface passivation performance against serum biofouling based on QCM-D measurements. Time-resolved QCM-D frequency shifts (ΔF) evaluating the passivation efficiency of BSA protein coatings against fetal bovine serum (FBS) fouling of silica surfaces for **a**, BSA protein 1 and **b**, BSA protein 5. The protocol steps involved (1) 100 μ M BSA addition, (2) buffer washing step, (3) addition of undiluted FBS, and (4) a buffer washing step. Bare surface control refers to a control experiment whereby FBS was added to an uncoated silica surface (no BSA coating). The difference in ΔF values due to BSA adsorption alone (post-washing) and after FBS incubation (post-washing) was computed ($\Delta F_{\text{FBS-BSA}}$, indicated by dashed lines) and the passivation efficiency percentage was calculated from this value compared to the equivalent value computed in the control experiment without BSA coating

Figure 7.3 Passivation efficiencies of BSA coatings to inhibit serum biofouling ($n=3$ biological replicates). Data are reported as mean $\pm$ standard error of the mean (s.e.m.) (one-way ANOVA with Tukey's multiple comparisons test). P values are reported for defatted BSA proteins (versus fatty BSA proteins from the same fractionation method). Dots represent individual data points.

Figure 7.4 Blocking performance of BSA protein reagents in Western blot experiments. **a**, Schematic illustration of the BSA blocking step in a Western blot experiment. **b**, Three Western blot lanes consisted of a colored molecular weight marker (lane 1) and normal human serum (NHS) samples (lanes 2-3). Each membrane was individually blocked with BSA 1 (fatty) or BSA 5 (defatted) prior to C3b antibody incubation. Red arrows indicate the position of nonspecific bands located near the 250, 75, and 50 kDa molecular weight markers. Black arrows indicate the position of specific bands corresponding to C3b (104 kDa) and iC3b (40 kDa). The objective of the blocking step is to

minimize signal noise coming from nonspecific bands in order to focus analysis on specific bands.

Figure 7.5 Quantification of Western blot band intensities. **a**, Greyscale digital images of Western blots with the three lanes selected for the extraction of lane intensity profiles. Lane 1 is from molecular weight markers while lanes 2 and 3 are from NHS samples. Left and right panels show membranes blocked by BSA 1 and BSA 5 proteins, respectively. **b**, Intensity profiles from lanes 1, 2, and 3 are shown in the top, middle, and bottom rows, respectively. Bands in the digital image appear as peaks in the intensity profile. Baselines were established by connecting the two minima on both sides of a selected peak. The intensity values were obtained based on the area under the curves bounded by the established baseline. Left and right panels show membranes blocked by BSA proteins 1 and 5, respectively. Arrows indicate the position of nonspecific bands located near the 250, 75, and 50 kDa molecular weight markers.

Figure 7.6 Comparison of nonspecific band intensities resulting from the use of fatted and defatted BSA proteins in Western blot experiments. Intensity values of the nonspecific bands that are located near the 250, 75, and 50 kDa molecular weight markers from membranes that were blocked with a blocking solution that included BSA 1 (fatted) or BSA 5 (defatted) ($n=8$ biological replicates, unpaired t-test). Data were obtained from Fiji/ImageJ software program and are reported as mean $\pm$ standard error of the mean (s.e.m.). Dots represent individual data points and intensities are in arbitrary units.

Figure 7.7 Schematic illustration of nanoparticle-triggered complement activation assay. Pristine and BSA-coated silica nanoparticles were incubated in 100% normal human serum (NHS), followed by ELISA measurements to determine the extent of nanoparticle-triggered complement activation. The degree of protection for BSA-coated nanoparticles was calculated relative to uncoated

nanoparticles.

Figure 7.8 Reduction in silica nanoparticle-induced complement activation due to BSA coatings, as quantified by normalized SC5b-9 levels. The level of complement activation for each sample was determined by measuring SC5b-9 concentrations by enzyme-linked immunosorbent assay (ELISA) and normalized against SC5b-9 levels in NHS without nanoparticles [$n=5$ biological replicates, one-way ANOVA with Dunnett's multiple comparisons test (versus uncoated nanoparticles)]. Data are reported as mean $\pm$ s.e.m. and dots represent individual data points.

Figure 7.9 Protection efficiencies of BSA coatings to inhibit nanoparticle-induced complement activation ($n=5$ biological replicates). Data are reported as mean $\pm$ standard error of the mean (s.e.m.) (one-way ANOVA with Tukey's multiple comparisons test). P values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method). Dots represent individual data points.

Figure 8.1 QCM-D measurement of 100 μ M BSA protein adsorption onto silica surfaces in water-ethanol mixtures. **a**, Frequency (ΔF) and **b**, energy dissipation (ΔD) shifts are reported as a function of time. The baseline was recorded in the appropriate water-ethanol mixture and then BSA in the equivalent solvent mixture was added at $t = 5$ min followed by a washing step in the same solvent mixture (without protein) at $t = 35$ min. The insets in panel **a**, represent normalized data for the washing step and the baselines correspond to the adsorbed protein layers before washing. Reproduced with permission from ref. 8. Copyright 2020, American Chemical Society.

Figure 8.2 LSPR measurement of 100 μ M BSA protein adsorption onto silica-coated gold nanodisk arrays in water-ethanol mixtures. **a**, LSPR peak shifts ($\Delta\lambda$) signal are reported as a function of time. The baseline was recorded in the

appropriate water-ethanol mixture and then BSA in the equivalent solvent mixture was added at $t = 5$ min followed by a washing step in the same solvent mixture (without protein) at $t = 35$ min. **b**, Magnified view of normalized $\Delta\lambda$ data from panel a for the washing step and the baselines correspond to the adsorbed protein layers before washing. Reproduced with permission from ref. 8. Copyright 2020, American Chemical Society.

Figure 8.3 QCM-D tracking of lysozyme adsorption in water-ethanol mixtures. **a**, Time-resolved ΔF shifts for lysozyme adsorption onto a silica surface in different water-ethanol mixtures as a function of ethanol fraction. The baseline was recorded in the appropriate water-ethanol mixture, followed by 50 μM lysozyme addition from $t = 5$ min onwards and then a washing step in the equivalent water-ethanol mixture (without protein) from $t = 25$ min. The inset shows a magnified view of the normalized Δf shifts due to solvent washing. **b**, Corresponding data for the time-resolved ΔD shifts that were measured simultaneously in the QCM-D measurements. Reproduced from ref. 22.

Figure 8.4 LSPR tracking of lysozyme adsorption in water-ethanol mixtures. **a**, Time-resolved $\Delta\lambda$ shifts for lysozyme adsorption onto a silica-coated nanodisk surface in different water-ethanol mixtures as a function of ethanol fraction. The baseline was recorded in the appropriate water-ethanol mixture, followed by 50 μM lysozyme addition from $t = 5$ min onwards and then a washing step in the equivalent water-ethanol mixture (without protein) from $t = 25$ min. **b**, Magnified view of the normalized $\Delta\lambda$ shifts due to solvent washing. Reproduced from ref. 22.

Abbreviations

ANOVA	Analysis of Variance
ATR	Attenuated Total Reflection
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CA	Caprylic Acid
CARPA	Complement Activation-Related Pseudoallergy
CD	Circular Dichroism
DLS	Dynamic Light Scattering
DSC	Differential Scanning Calorimetry
ECL	Enhanced chemiluminescence
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared
FWHM	Full Width at Half Maximum
HRP	Horseradish Peroxidase
HSA	Human Serum Albumin
IEP	Isoelectric Point
IgG	Immunoglobulin G
ITC	Isothermal Titration Calorimetry
LSPR	Localized Surface Plasmon Resonance
MC	Monocaprylin
MCT	Mercury Cadmium Telluride
ME	Methyl Caprylate
MRE	Molar Residue Ellipticity
NHS	Normal Human Serum
PDI	Polydispersity Index
PEG	Polyethylene glycol
PES	Polyethersulfone
PTFE	Polytetrafluoroethylene

RI	Refractive Index
RSA	Rat Serum Albumin
s.d.	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SiNP	Silica Nanoparticles
SPR	Surface Plasmon Resonance
SV	Stern-Volmer
TBS	Tris Buffered Saline
TBST	TBS solution with 0.1% (vol/vol) Tween 20
TEMED	tetramethylethylenediamine
TIRF	Total Internal Reflection Fluorescence
UV	Ultraviolet
QCM-D	Quartz Crystal Microbalance-Dissipation

Chapter 1

Introduction

Protein adsorption is a common phenomenon that can potentially be engineered to form functional biointerfaces for nanobiotechnological devices that can improve human health, although it can also hinder the performance of such devices if uncontrolled. In order to control the protein adsorption process to fabricate functional protein coatings, a detailed understanding of how various factors affect protein adsorption is needed. While several material and environmental parameters that affect protein adsorption have been identified, much less is understood on how a protein's solution-phase conformational stability can be modulated to affect adsorption behavior. Along this line, materials science concepts together with robust characterization of a protein's solution-phase and adsorption properties offer a new approach for studying protein adsorption. This chapter introduces the background and significance of protein adsorption which motivates the hypothesis of this thesis and outlines the scope as well as the specific hypotheses and aims to be addressed.

1.1 Hypothesis/Problem Statement

Protein adsorption is a process in which proteins spontaneously adsorb onto interfaces on account of their amphiphilic nature.^{1,2} This phenomenon is pertinent to several disciplines including materials science, biochemistry, biomedical engineering and nanotechnology. For example, protein adsorption onto implantable biomedical devices represents a crucial first step in dictating their biocompatibility. That is, the interactions between the host body and the adsorbed proteins on the biomaterial may either result in complete integration of the implant or may elicit immune responses that result in medical complications such as thrombosis.³⁻⁶ In another example, biosensors and immunoassays typically rely on biorecognition elements including enzymes and antigens appropriately immobilized, for example through adsorption, onto the sensor surface and their specific interactions with target markers from biologically complex media such as blood or serum.⁷ Although, the nonspecific adsorption of other proteins onto the sensing surface from the biologically complex medium results in fouling and can diminish biosensing performance, various surface passivation strategies – which include covalent and noncovalent attachment of synthetic polymers and bioinert proteins onto surfaces – have been developed to prevent or reduce fouling.⁸⁻¹⁰ With the development of nanotechnology in recent years, protein-nanomaterial interactions have gained tremendous interest due to the combination of the unique properties of materials and interfaces at the nanoscale, together with the huge potential for diverse practical applications through nanomaterial biofunctionalization via protein adsorption.¹¹⁻¹³ To date, improved anticancer, antiviral, vaccine and diagnostic capabilities are some of the clinically-relevant applications that have been reported arising from functional protein coatings onto nanomaterials.¹⁴⁻¹⁷ However, nanomaterials can also elicit immune responses arising from the adsorption of proteins from the immune system *in vivo*, such as complement activation¹⁸ as well as complement activation-related pseudoallergy (CARPA).^{19,20}

These reasons highlight the importance of protein adsorption in various fields and why it has been long been studied with the goal of achieving a fundamental understanding of the protein adsorption process in order that it might be controlled to meet specific application

needs.^{6, 21} To date, numerous studies have been conducted in order to investigate how surface properties, such as surface charge, atomic composition, hydrophobicity and roughness, as well as environmental conditions, including solution pH, ionic strength, type of buffer and temperature, influence protein adsorption behavior in terms of adsorption uptake, adsorption kinetics and the degree of adsorption-related conformational change.²²⁻²⁸ Within this scope, one other study approach is to investigate how a protein's solution-phase conformational stability may influence adsorption behavior.

One of the earliest to discuss how protein conformational stability affects adsorption behavior was Professor Willem Norde in the late 1980s when he demonstrated that proteins with lower conformational stabilities – due to weaker intramolecular interactions – undergo more extensive conformational changes, or unfolding, upon adsorption. This leads to larger increases in conformational entropy and thus, adsorption is favored even in scenarios when the protein and surface have similar net charges, *i.e.*, where there are electrostatic forces of repulsion.^{1, 29} On the other hand, proteins with relatively higher conformational stabilities tend to undergo less extensive adsorption-related unfolding and their adsorption is more strongly governed by electrostatic interactions. Despite these discussions, systematic investigations on how modulating protein conformational stability might affect protein adsorption have scarcely been conducted. One study demonstrated the feasibility of this concept and reported that the conformational stability of a human enzyme could be modulated through point-mutations and that some of these engineered variants with lower conformational stability displayed more irreversible adsorption.³⁰ This warranted new experimental study designs that modulate protein conformational stability changes, followed by characterizing protein adsorption behavior and coating performance. Indeed more recent studies demonstrated how the conformational stability of bovine serum albumin (BSA), a protein widely used in various areas of biotechnology for surface passivation applications, could be affected by ionic strength or temperature in order to form adlayers with greater surface passivation efficiency.^{28, 31, 32} Moving forward, there is an outstanding need to go beyond particular experimental approaches and to develop a comprehensive analytical framework to systematically modulate protein conformational

stability and to establish a relationship between conformational stability, adsorption behavior and coating performance.

Figure 1.1 The materials science tetrahedron. A material's structure, processing, properties, and performance are interrelated. These interrelations are elucidated through characterization.

To this end, the materials science paradigm, often illustrated as the materials science tetrahedron presented in **Figure 1.1**, is highly useful in guiding the development of this framework. Based on this paradigm, this thesis seeks to systematically investigate how protein conformational stability may be modulated based on the Structure and Processing components of the materials science tetrahedron, how that affects protein adsorption behavior *i.e.*, the Properties component, and how understanding the relationship between these three components can help develop strategies to improve coating applications, which is the Performance component. These relationships are established by using robust characterization techniques. Thus, the overall hypothesis of this thesis is that rationally tuning the conformational stability of protein molecules based on materials science concepts can influence protein adsorption and that the insights gained from this framework can be used to engineer improved protein coating applications.

To test this hypothesis, a model protein with useful coating applications when adsorbed, such as lysozyme which has antibacterial properties,³³⁻³⁸ as well as gelatin, casein and whey proteins for food packaging applications may be considered.³⁹ Ultimately, serum albumin protein was selected for use in this thesis and was determined to be a suitable model protein for several reasons. First, BSA protein coatings formed via surface adsorption has long

been used for surface passivation or antifouling applications in several biological assays including enzyme-linked immunosorbent assays (ELISA), blots, immunohistochemistry and polymerase chain reaction,^{10, 40-44} although efforts are scant to improve its surface passivation performance on both flat and nanomaterial surfaces. Second, serum albumin has several homologs originating from several other mammalian species, which collectively serves as a useful study approach for understanding how natural sequence variations (*i.e.*, protein primary structure) may influence conformational stability and adsorption behavior. Third, various alternative purification strategies which involve disparate environmental parameters such as temperature and the use of additives such as fatty acids, are currently utilized to manufacture serum albumin proteins, which serves as a useful approach to study how different processing pathways may influence conformational stability and adsorption behavior. In light of this, the following specific hypotheses will be addressed in this thesis:

- Natural sequence variation in the primary structure of serum albumin proteins cause differences in their solution-phase conformational stability that result in different adsorption behavior.
- BSA purified through different processing strategies possess different solution-phase conformational stabilities and adsorption behavior that relate to the differences in the temperatures used and/or the inclusion of fatty acid stabilizers during their isolation and purification processes.
- The physicochemical properties of fatty acids and related amphipathic molecules critically determine their modulation of BSA protein conformational stability and adsorption behavior.
- The extent of the changes in protein conformational stability and adsorption behavior can be related to differences in surface passivation performance, which can be evaluated using a combination of *in vitro* and biological assays.

1.2 Objectives and Scope

While protein coatings can be fabricated to perform various functions, attention is drawn in this thesis to protein coatings for antifouling or surface passivation functionality. Indeed, the nonspecific adsorption of proteins onto solid surfaces often hinders the performance of devices in contact with biological media such as eliciting immune responses from biomedical implants and nanomaterials or fouling on biosensors and immunoassays that lead to non-meaningful results. The poor performance of these biomedical devices by protein adsorption is an impediment on the research progress for the improvement of human health. Motivated by the need for bioinert surfaces, alternative strategies such as the synthesis of polymer coatings like polyethylene glycol (PEG) and various polymer brushes have been developed to prevent protein adsorption.⁴⁵ However, PEG can undergo enzymatic and oxidative degradation *in vivo*^{46, 47} and polymer brushes often involve multiple-step synthesis and require specific surface chemistry for chemical immobilization on the surface.⁴⁸

Interestingly, while the ultimate goal is to prevent non-specific adsorption of proteins to surfaces, this phenomenon can be turned into an advantage by fabricating coatings formed by protein adsorption for the prevention of additional protein adsorption. In this regard, BSA protein adsorption has long been used to form bioinert coatings to prevent further nonspecific adsorption in various immunoassays,⁴⁹⁻⁵² and BSA coatings have also been shown to improve nanoparticle stability and biocompatibility.^{53, 54} Indeed, one of the advantages of BSA coatings over synthetic polymer coatings is its versatility and simplicity; no prior surface modification is needed due to the nonspecific nature of BSA adsorption, and only contact between the BSA solution and the solid surface is required for adsorption to take place and the coating to form. These advantages make it an attractive and promising alternative to synthetic polymer coatings for tackling the protein adsorption problem. However, few attempts have been made towards improving its surface passivation performance.

To date, it is known that structural differences in terms of natural sequence variations from serum albumin homologs,^{55, 56} and processing differences in terms of the addition of fatty acid stabilizers,^{57, 58} can affect the solution-phase conformational stability of serum

albumin proteins although their effects on adsorption behavior and surface passivation performance is less understood. To address these outstanding questions, this thesis seeks to systematically investigate and rationalize how protein sequence variation and environmental processing conditions can modulate serum albumin protein conformational stability, how such modulations affect adsorption behavior and how these insights may be applied to fabricate improved surface passivation protein coatings. To achieve this objective, appropriate serum albumin samples were chosen, and a robust suite of experimental techniques were selected that characterized their solution-phase conformational stability and adsorption behavior, and how these correlates to their surface passivation performance. In particular, studies were carried out in this thesis with the following specific aims:

- To determine how natural sequence variation in the different species of serum albumin proteins can affect protein conformational stability and adsorption behavior.
- To investigate how different processing strategies for BSA purification, such as different fractionation methods and whether or not a fatty acid removal step was performed, can affect BSA conformational stability and adsorption behavior.
- To elucidate how fatty acids and other fatty acid-derived amphiphiles stabilize BSA conformational stability and adsorption behavior.
- To evaluate the surface passivation performance of BSA proteins with different conformational flexibilities using an *in vitro* experimental model consisting of biophysical and biological assays.

1.3 Dissertation Overview

The thesis addresses the largely unexplored concept of tuning protein conformational stability based on materials science concepts to influence protein adsorption behavior with the goal of improving protein coating applications. The thesis is organized into the following chapters:

Chapter 1 “Introduction” presents the background information and motivation for the research topic. The hypothesis, scope and specific aims of the project are provided.

Chapter 2 “Literature Review” introduces the background on the physical principles of protein folding, conformational stability and protein adsorption, experimental approaches to characterize conformational stability and adsorption as well as surface passivation methods to prevent biofouling, including a critical discussion of how existing methods using synthetic polymers compare with protein coatings for various biotechnological applications. A general background for serum albumin proteins is then introduced, including protein structure, biological function, purification, and processing strategies, as well as the various examples of serum albumin protein coatings and their applications. In closing, outstanding questions are highlighted that motivate the research goals of this thesis.

Chapter 3 “Experimental Methodology” provides a description of the reagents used, the characterization techniques employed, the physical principles behind the techniques as well as justification for their use.

Chapter 4 “Understanding How Natural Sequence Variation in Serum Albumin Proteins Affects Conformational Stability and Protein Adsorption” presents the first empirical observations on how serum albumins with natural sequence variations, namely BSA, human serum albumin (HSA) and rat serum albumin (RSA) exhibit distinct protein adsorption profiles. Through bioinformatics analysis and various experimental techniques, this chapter establishes how differences in protein conformational stability arising from different amino acid sequences relates to protein adsorption behavior and provides grounds for why BSA is the most commonly used for surface passivation applications as well as why it is the most suitable protein for further study.

Chapter 5 “Quantitative Assessment of Bovine Serum Albumin Proteins from Different Purification and Processing Strategies” presents the fundamental characterization of BSA proteins that have gone through different purification routes to determine how processing strategies can influence BSA conformational stability and adsorption behavior. This

chapter identifies a specific trend between fatty acid-containing and fatty acid-free BSA proteins which is verified by further control experiments. Mechanistic details for how fatty acids affect BSA adsorption and surface passivation performance are discussed.

Chapter 6 “Elucidating How Different Amphipathic Stabilizers Affect BSA Protein Conformational Properties and Adsorption Behavior” discusses how different fatty acid-derived amphiphiles, namely monoglyceride and fatty acid methyl ester, influences BSA conformational stability and adsorption behavior in comparison to fatty acid. This chapter also introduces a method to characterize the binding affinity of fatty acid and its derivatives to BSA. The correlation between surfactant chemistry, surfactant-protein interaction, binding affinity and stabilizing effectiveness is discussed.

Chapter 7 “Evaluating the Surface Passivation Performance of BSA Proteins for Antifouling Applications” presents the characterization of serum albumin proteins for various antifouling applications on both flat and nanomaterial surfaces. This chapter correlates the fundamental characterization of a protein’s adsorption behavior to its surface passivation performance.

Chapter 8 “Discussion and Future Work” summarizes the key findings from the various studies in this project and draws out significant insights that advance the field. Future research opportunities are discussed, particularly on understanding how environmental conditions such as the addition of other surfactant variants or the use of aqueous-organic solvent mixtures can influence protein conformational stability and adsorption behavior, as well as applying this materials science framework to other protein systems.

1.4 Findings and Outcomes/Originality

The findings in this thesis led to several novel outcomes that advance the understanding of how protein conformational stability can be tuned to influence specific protein adsorption behavior and how such insights provide clues to design improved protein coatings. Some of the key findings in this thesis are summarized below:

1. It was established, on the basis of protein structure, conformational stability and colloidal properties, why BSA could be more suitable for surface passivation applications than their human and rat homologues. A robust characterization protocol as well as theoretical and experimental analytical approaches were presented which enabled the solution-phase properties and adsorption behavior of BSA, HSA and RSA proteins to be distinguished. These findings enabled a detailed mechanistic picture of the final adsorbed layer to be constructed which revealed how differences in the conformational and colloidal properties of the proteins – induced by sequence variation – can influence the nature of the resultant adlayer.

2. Different industrial processing strategies – which can involve the use of vastly different temperatures, depending on the processing method – that result in commercially available BSA proteins with varying amounts of bound fatty acids were identified. Using the established characterization protocol, it was discovered that the degree of conformational stability and adsorption behavior heavily depended on the amount of bound fatty acids. This finding represents a significant advance because: BSA is one of the most widely used surface passivation agents in the world, and while there are many commercially available versions of BSA proteins that differ depending on how they were purified, there has been minimal discussion on how such differences may influence surface passivation performance, which is intimately linked to adsorption behavior. Notably, the difference between how fatty acid-modulated conformational stability versus sequence variation-modulated conformational stability influenced adsorption behavior was rationalized by taking into account the additional electrostatic charge conferred by the fatty acids.

3. Building upon the previous finding, it was elucidated how different fatty acid-derived amphipathic stabilizers affect BSA conformational stability and adsorption behavior. It was identified that the degree of the stabilizing effect is intimately linked to the binding affinity of the amphiphiles to BSA protein and is strongly dependent on the polarity of the amphiphile headgroup. BSA proteins doped with different amphipathic

stabilizers exhibited different adsorption behavior which was rationalized by taking into account their binding affinity, and their ability to confer additional electrostatic charge to the BSA protein molecule.

4. Appropriate *in vitro* experimental approaches were developed to evaluate the antifouling performance of BSA proteins with different levels of bound fatty acids and conformational flexibility obtained through different industrial processing strategies. General trends between the extent of adsorption uptake from the previous results was correlated with the surface passivation performance. Importantly, these evaluations confirm that different commercially available BSA proteins have different surface passivation capabilities, which can be rationalized based on how differences in conformational stability and adsorption behavior give rise to different adlayer properties.

References

1. Norde, W., Adsorption of proteins from solution at the solid-liquid interface. *Adv. Colloid Interface Sci.* **1986**, 25, 267-340.
2. Nakanishi, K.; Sakiyama, T.; Imamura, K., On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. *J. Biosci. Bioeng.* **2001**, 91 (3), 233-244.
3. Meyers, S. R.; Grinstaff, M. W., Biocompatible and bioactive surface modifications for prolonged in vivo efficacy. *Chem. Rev.* **2011**, 112 (3), 1615-1632.
4. Anderson, J. M., Biological responses to materials. *Annu. Rev. Mater. Res.* **2001**, 31 (1), 81-110.
5. Gorbet, M. B.; Sefton, M. V., Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. *Biomaterials* **2004**, 25 (26), 5681-5703.
6. Gray, J. J., The interaction of proteins with solid surfaces. *Curr. Opin. Struct. Biol.* **2004**, 14 (1), 110-115.

7. Bhakta, S. A.; Evans, E.; Benavidez, T. E.; Garcia, C. D., Protein adsorption onto nanomaterials for the development of biosensors and analytical devices: A review. *Anal. Chim. Acta* **2015**, *872*, 7-25.
8. Wisniewski, N.; Reichert, M., Methods for reducing biosensor membrane biofouling. *Colloids Surf., B* **2000**, *18* (3-4), 197-219.
9. Chen, S.; Li, L.; Zhao, C.; Zheng, J., Surface hydration: Principles and applications toward low-fouling/nonfouling biomaterials. *Polymer* **2010**, *51* (23), 5283-5293.
10. Sweryda-Krawiec, B.; Devaraj, H.; Jacob, G.; Hickman, J. J., A new interpretation of serum albumin surface passivation. *Langmuir* **2004**, *20* (6), 2054-2056.
11. Lynch, I.; Dawson, K. A., Protein-nanoparticle interactions. *Nano Today* **2008**, *3* (1-2), 40-47.
12. Nel, A. E.; Mädler, L.; Velegol, D.; Xia, T.; Hoek, E. M.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M., Understanding biophysicochemical interactions at the nano–bio interface. *Nat. Mater.* **2009**, *8* (7), 543-557.
13. Kopac, T., Protein corona, understanding the nanoparticle–protein interactions and future perspectives: A critical review. *Int. J. Biol. Macromol.* **2021**, *169*, 290-301.
14. Shi, J.; Kantoff, P. W.; Wooster, R.; Farokhzad, O. C., Cancer nanomedicine: progress, challenges and opportunities. *Nat. Rev. Cancer* **2017**, *17* (1), 20.
15. Jackman, J. A.; Yoon, B. K.; Ouyang, L.; Wang, N.; Ferhan, A. R.; Kim, J.; Majima, T.; Cho, N. J., Biomimetic nanomaterial strategies for virus targeting: Antiviral therapies and vaccines. *Adv. Funct. Mater.* **2020**, 2008352.
16. Zhou, X.; Jiang, X.; Qu, M.; Aninwene, G. E.; Jucaud, V.; Moon, J. J.; Gu, Z.; Sun, W.; Khademhosseini, A., Engineering Antiviral Vaccines. *ACS Nano* **2020**.
17. Kairdolf, B. A.; Qian, X.; Nie, S., Bioconjugated nanoparticles for biosensing, in vivo imaging, and medical diagnostics. *Anal. Chem.* **2017**, *89* (2), 1015-1031.
18. Moghimi, S.; Simberg, D., Complement activation turnover on surfaces of nanoparticles. *Nano Today* **2017**, *15*, 8-10.
19. Szebeni, J., Complement activation-related pseudoallergy: a stress reaction in blood triggered by nanomedicines and biologicals. *Mol. Immunol.* **2014**, *61* (2), 163-173.

20. Kleine-Tebbe, J.; Klimek, L.; Hamelmann, E.; Pfaar, O.; Taube, C.; Wagenmann, M.; Werfel, T.; Worm, M., Severe allergic reactions to the COVID-19 vaccine—statement and practical consequences. *Allergol. Sel.* **2021**, *5*, 26.
21. Rabe, M.; Verdes, D.; Seeger, S., Understanding protein adsorption phenomena at solid surfaces. *Adv. Colloid Interface Sci.* **2011**, *162* (1-2), 87-106.
22. Calatayud, M. P.; Sanz, B.; Raffa, V.; Riggio, C.; Ibarra, M. R.; Goya, G. F., The effect of surface charge of functionalized Fe₃O₄ nanoparticles on protein adsorption and cell uptake. *Biomaterials* **2014**, *35* (24), 6389-6399.
23. Givens, B. E.; Xu, Z.; Fiegel, J.; Grassian, V. H., Bovine serum albumin adsorption on SiO₂ and TiO₂ nanoparticle surfaces at circumneutral and acidic pH: a tale of two nano-bio surface interactions. *J. Colloid Interface Sci.* **2017**, *493*, 334-341.
24. Roach, P.; Farrar, D.; Perry, C. C., Interpretation of protein adsorption: surface-induced conformational changes. *J. Am. Chem. Soc.* **2005**, *127* (22), 8168-8173.
25. Dolatshahi-Pirouz, A.; Rechendorff, K.; Hovgaard, M. B.; Foss, M.; Chevallier, J.; Besenbacher, F., Bovine serum albumin adsorption on nano-rough platinum surfaces studied by QCM-D. *Colloids Surf., B* **2008**, *66* (1), 53-59.
26. Park, J. H.; Sut, T. N.; Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Cho, N.-J., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19* (13), 8854-8865.
27. Wei, T.; Kaewtathip, S.; Shing, K., Buffer effect on protein adsorption at liquid/solid interface. *J. Phys. Chem. C* **2009**, *113* (6), 2053-2062.
28. Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Park, J. H.; Zhdanov, V. P.; Cho, N.-J., Indirect nanoplasmonic sensing platform for monitoring temperature-dependent protein adsorption. *Anal. Chem.* **2017**, *89* (23), 12976-12983.
29. Arai, T.; Norde, W., The behavior of some model proteins at solid-liquid interfaces 1. Adsorption from single protein solutions. *Colloids Surf.* **1990**, *51*, 1-15.
30. Karlsson, M.; Ekeröth, J.; Elwing, H.; Carlsson, U., Reduction of irreversible protein adsorption on solid surfaces by protein engineering for increased stability. *J. Biol. Chem.* **2005**, *280* (27), 25558-25564.

31. Park, J.; Sut, T.; Jackman, J.; Ferhan, A.; Yoon, B.; Cho, N., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19*.
32. Park, J. H.; Jackman, J. A.; Ferhan, A. R.; Ma, G. J.; Yoon, B. K.; Cho, N.-J., Temperature-induced denaturation of BSA protein molecules for improved surface passivation coatings. *ACS Appl. Mater. Interfaces* **2018**, *10* (38), 32047-32057.
33. Derde, M.; Vié, V.; Walrant, A.; Sagan, S.; Lechevalier, V.; Guérin-Dubiard, C.; Pezennec, S.; Cochet, M. F.; Paboeuf, G.; Pasco, M., Antimicrobial activity of lysozyme isoforms: key molecular features. *Biopolymers* **2017**, *107* (12), e23040.
34. Beaussart, A.; Retourney, C.; Quilès, F.; Morais, R. D. S.; Gaiani, C.; Fiérobe, H.-P.; El-Kirat-Chatel, S., Supported lysozyme for improved antimicrobial surface protection. *J. Colloid Interface Sci.* **2021**, *582*, 764-772.
35. Bayazidi, P.; Almasi, H.; Asl, A. K., Immobilization of lysozyme on bacterial cellulose nanofibers: characteristics, antimicrobial activity and morphological properties. *Int. J. Biol. Macromol.* **2018**, *107*, 2544-2551.
36. Buruaga-Ramiro, C.; Valenzuela, S. V.; Valls, C.; Roncero, M. B.; Pastor, F. J.; Díaz, P.; Martinez, J., Development of an antimicrobial bioactive paper made from bacterial cellulose. *Int. J. Biol. Macromol.* **2020**, *158*, 587-594.
37. Sarkar, S.; Gulati, K.; Mishra, A.; Poluri, K. M., Protein nanocomposites: special inferences to lysozyme based nanomaterials. *Int. J. Biol. Macromol.* **2020**, *151*, 467-482.
38. Das, S.; Pabba, M.; Dhushyandhun, M. E.; Patra, C. R., Theranostic Applications of Lysozyme-Based Nanoparticles. In *Nanoparticles in Medicine*, Shukla, A. K., Ed. Springer Singapore: Singapore, 2020; pp 1-23.
39. Mihalca, V.; Kerezsi, A. D.; Weber, A.; Gruber-Traub, C.; Schmucker, J.; Vodnar, D. C.; Dulf, F. V.; Socaci, S. A.; Fărcaș, A.; Mureșan, C. I., Protein-based films and coatings for food industry applications. *Polymers* **2021**, *13* (5), 769.
40. Jeyachandran, Y.; Mielczarski, J.; Mielczarski, E.; Rai, B., Efficiency of blocking of non-specific interaction of different proteins by BSA adsorbed on hydrophobic and hydrophilic surfaces. *J. Colloid Interface Sci.* **2010**, *341* (1), 136-142.

41. Steinitz, M., Quantitation of the blocking effect of tween 20 and bovine serum albumin in ELISA microwells. *Anal. Biochem.* **2000**, 282 (2), 232-238.
42. Mahmood, T.; Yang, P.-C., Western blot: technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* **2012**, 4 (9), 429.
43. Buchwalow, I. B.; Böcker, W., Background Staining, Autofluorescence and Blocking Steps. In *Immunohistochemistry: Basics and Methods*, Springer Berlin Heidelberg: Berlin, Heidelberg, 2010; pp 41-46.
44. Besecker, J.; Cornell, K. A.; Hampikian, G., Dynamic passivation with BSA overcomes LTCC mediated inhibition of PCR. *Sens. Actuators, B* **2013**, 176, 118-123.
45. Wei, Q.; Becherer, T.; Angioletti-Uberti, S.; Dzubiella, J.; Wischke, C.; Neffe, A. T.; Lendlein, A.; Ballauff, M.; Haag, R., Protein interactions with polymer coatings and biomaterials. *Angew. Chem. Int. Edit.* **2014**, 53 (31), 8004-8031.
46. Sharma, S.; Johnson, R. W.; Desai, T. A., Evaluation of the stability of nonfouling ultrathin poly (ethylene glycol) films for silicon-based microdevices. *Langmuir* **2004**, 20 (2), 348-356.
47. Kryuk, T.; Mikhal'chuk, V.; Petrenko, L.; Nelepova, O.; Nikolaevskii, A., Promising inhibitors of poly (ethylene glycol) oxidation in aqueous solutions. *Pharm. Chem. J.* **2002**, 36 (1), 32-35.
48. Wei, Q.; Haag, R., Universal polymer coatings and their representative biomedical applications. *Mater. Horiz.* **2015**, 2 (6), 567-577.
49. Engvall, E.; Perlmann, P., Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry* **1971**, 8 (9), 871-874.
50. Avrameas, S.; Guilbert, B., Enzyme-immunoassay for the measurement of antigens using peroxidase conjugates. *Biochimie* **1972**, 54 (7), 837-842.
51. Towbin, H.; Staehelin, T.; Gordon, J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* **1979**, 76 (9), 4350-4354.
52. Burnette, W. N., "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and

- radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **1981**, *112* (2), 195-203.
53. Brewer, S. H.; Glomm, W. R.; Johnson, M. C.; Knag, M. K.; Franzen, S., Probing BSA binding to citrate-coated gold nanoparticles and surfaces. *Langmuir* **2005**, *21* (20), 9303-9307.
54. Mariam, J.; Sivakami, S.; Dongre, P. M., Albumin corona on nanoparticles—a strategic approach in drug delivery. *Drug Deliv.* **2016**, *23* (8), 2668-2676.
55. Carter, D. C.; Ho, J. X., Structure of serum albumin. In *Advances in Protein Chemistry*, Elsevier: 1994; Vol. 45, pp 153-203.
56. Kosa, T.; Maruyama, T.; Otagiri, M., Species differences of serum albumins: II. Chemical and thermal stability. *Pharm. Res.* **1998**, *15* (3), 449-454.
57. Arakawa, T.; Kita, Y., Stabilizing effects of caprylate and acetyltryptophanate on heat-induced aggregation of bovine serum albumin. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2000**, *1479* (1-2), 32-36.
58. Anraku, M.; Tsurusaki, Y.; Watanabe, H.; Maruyama, T.; Kragh-Hansen, U.; Otagiri, M., Stabilizing mechanisms in commercial albumin preparations: octanoate and N-acetyl-L-tryptophanate protect human serum albumin against heat and oxidative stress. *Biochim. Biophys. Acta, Proteins Proteomics* **2004**, *1702* (1), 9-17.

Chapter 2

Literature Review

The formation of protein coatings through protein adsorption is a highly complex and widely studied subject for its current and potential impact on nanobiomedical applications. Among the many useful applications of protein coatings, which include diagnostics and therapeutics, surface passivation is one of the most widely used, in which biomedical devices and surfaces are coated with a protective layer to prevent further non-specific adsorption of proteins. However, few studies focus on improving protein coatings for surface passivation applications despite this approach being a widely adopted protocol in the blocking step of immunoassays such as ELISA or Western blot. Serum albumin coatings show promise as a candidate for improving surface passivation due to its wide use, availability, and its effectiveness in immunoassays and nanobiomedical devices. A review of the literature finds gaps in understanding the structure-properties-processing relationship for serum albumin and few attempts to improve its surface passivation performance with an engineering approach.

2.1 Protein Adsorption

2.1.1 Physicochemical Principles of Protein Adsorption

Proteins are highly complex, amphiphilic, biological macromolecules, consisting of a long chain of amino acids joined together in a sequence by peptide linkages. There are 20 standard amino acids, all of which consists of amino and carboxyl functional groups – which are responsible for the peptide linkages – as well as a unique side group which distinguishes one amino acid from another in terms of physicochemical properties such as hydrophobicity, polarity, electrostatic charge, and the nature of its chemical structure *e.g.*, aliphatic or aromatic. Each protein consists of a unique sequence of amino acids known as the primary structure of the protein. A protein's complexity comes from the various types of intramolecular interactions (covalent bonding, hydrogen bonding, electrostatic, hydrophobic, and van der Waals forces) between individual amino acids along its primary structure chain on account of their different physicochemical properties such as polarity, electric charge and hydrophobicity. Hydrogen bonding exists between peptide linkages and also between relevant amino acid side groups. Hydrogen bonds are also responsible for causing the polypeptide chain to form secondary structures such as α -helices and β -sheets. Side groups with unlike charges attract while those with like charges repel. Side groups containing a sulfhydryl molecule can also pair up to form disulfide linkages. Hydrophobic side groups associate through hydrophobic interactions and tend to be surrounded by hydrophilic polar side groups in order to minimize their exposure to the aqueous environment. The combination of these attractive and repulsive interactions between amino acids under physiological conditions thermodynamically drives the flexible amino acid chain to fold and assume various secondary, tertiary and quaternary folded structures, to eventually form a stable three-dimensional native conformation.¹ The sequence of the amino acid chain determines the arrangement of these interactions and therefore dictates the final shape of the folded three-dimensional native structure or conformation.²

Non-covalent interactions are abundant in biomacromolecules and are essential for biological functions.³ In the case of proteins, non-covalent interactions enable protein

conformational changes, a hallmark of dynamic biological processes such as ligand binding. The native structure of a protein is critical to its biological function as unfolded or improperly folded proteins may be biologically inactive or even functionally abnormal, both of which can lead to adverse health effects.⁴ Indeed, the weaker non-covalent interactions which are critical to protein function can at the same time be easily overcome by external stresses, which causes the protein to unfold and lose its native structure in a process known as denaturation.^{5, 6}

The presence of a liquid-solid interface is one such external stress, that is, proteins undergo surface-induced denaturation upon adsorption to a solid surface.⁷ Moreover, proteins are prone to adsorption onto interfaces due to their amphipathic character, which makes them highly surface active.⁸ In the adsorption process, protein molecules are first transported to the surface from the bulk solution either by diffusion or under the influence of a force field. The proteins then attach onto the surface, mediated by protein-surface interactions which include various interaction forces depending on the nature of the protein and the surface, such as electrostatic interaction, hydrophobic interaction, hydrogen bonding and van der Waals forces.^{9, 10} This is followed by a surface-induced denaturation step where protein molecules unfold and spread to occupy a larger surface area on the solid surface.^{6, 9, 11, 12} The extent of unfolding depends on the protein-surface interaction, lateral protein-protein interactions between adsorbed protein molecules, as well as the stability of the protein molecule's folded conformation, which is termed as conformational stability. The numerous studies on protein adsorption conducted to date have investigated how the adsorption phenomenon and the nature of the resulting protein film can be affected by environmental parameters including solution pH, temperature, and ionic strength; by material surface parameters including atomic composition, surface charge and topography; as well as by protein properties, in particular, protein conformational stability.

2.1.2 Environmental and Material Surface Parameters Affecting Protein Adsorption

Solution pH can affect adsorption by influencing the electrostatic interactions of protein-surface and protein-protein interactions. Certain amino acids contain acidic side groups

(*i.e.*, Asp and Glu) while others have basic side groups (*i.e.*, Lys, Arg, His), which have isoelectric points (IEP) higher or lower than physiological pH, respectively. There often exists a combination of acidic and basic side groups at various locations within the same protein, giving rise to different charges in localized areas and producing an overall net charge. Proteins possess a net negative or positive charge when the solution pH is above or below the IEP of most of the side groups, respectively. When the solution pH is much higher or lower than the IEP, there is significant electrostatic repulsion between adsorbed protein molecules which results in a lower adsorption amount as compared to conditions when pH is near the IEP of the protein.¹³⁻¹⁶ At the same time, pH conditions in which the net charge of a protein is opposite to that of the surface result in greater protein-surface interactions and cause more extensive adsorption-related conformational changes on the surface in proteins with lower conformational stability.¹⁷⁻¹⁹

The electrostatics in the system can also be affected by the concentration of ions in the aqueous solution, expressed as ionic strength. A higher ionic strength decreases the Debye length, a measure of the net electrostatic effect of particles in a solution.²⁰ This means that increasing ionic strength dampens both attractive and repulsive forces between opposite and like-charges respectively, which can lead to an increase in adsorption uptake of charged proteins to like-charged surfaces and a decrease in adsorption to oppositely-charged surfaces.^{15, 21} At the same time, some studies have reported an opposite effect in which the adsorption uptake of a negatively charged protein onto a negatively charged surface increased with increasing ionic strength.^{22, 23} This can be explained by taking into account that under certain experimental conditions, increasing ionic strength increases protein-surface interactions which leads to more extensive adsorption-related conformational change and spreading, resulting in adsorbed protein molecules occupying a greater surface area and leaving less available space for subsequent adsorbing proteins.

Temperature can also affect the adsorption behavior of proteins. At higher temperature, proteins in solution undergo temperature-induced denaturation due to the destabilization of intramolecular hydrogen bonds.^{24, 25} Proteins with such increased conformational flexibility would be more prone to surface-induced denaturation which results in increased

adsorption uptake.²⁶⁻²⁸ Indeed, surface-induced denaturation, or the unfolding of protein molecules on the surface, is one of the major driving forces for irreversible protein adsorption. Thermodynamically, adsorption is favored because a protein molecule loses its folded conformational structure upon adsorption and increases in conformational entropy,²⁹ while from an interfacial science perspective, both protein adsorption and structural unfolding occurs due to protein-surface interactions being more favorable than intramolecular protein interactions.^{9, 10}

Surface chemistry can also affect various types of protein-surface interactions. In particular, the effects of surface charge on protein adsorption has been extensively demonstrated on different surface types or through different surface functionalization strategies. Intuitively, proteins generally adsorb in greater amounts to surfaces of opposite charges, and vice versa.³⁰⁻³⁴ It is important to note here that proteins have anisotropic charge distributions amounting to a net charge, and therefore, proteins with a net negative charge can still adsorb onto negatively charged surfaces by orienting their positively charged regions towards the surface,^{33, 35, 36} and irreversible adsorption can then be achieved through surface-induced denaturation. Additionally, the contribution of van der Waals forces from different surface types have also been theoretically and experimentally verified to play a non-trivial role in promoting the adsorption process.³⁷⁻⁴⁰ The hydrophobicity of the surface is another significant parameter, in which protein adsorption is often more irreversible on a hydrophobic rather than a hydrophilic surface due to the interactions between the hydrophobic interior of proteins and the surface which causes extensive protein conformational changes, coupled with the large entropy gain from the release of highly ordered water molecules near the surface.^{29, 41} Although other types of interactions such as electrostatics and van der Waals forces can also influence adsorption behavior, hydrophobic interactions is the major driving force for protein adsorption to hydrophobic surfaces.^{12, 40, 42-44} On the other hand, hydrogen bonding plays a more dominant role in the adsorption of proteins onto hydrophilic surfaces.⁴⁵⁻⁴⁹

In addition to surface chemistry, surface topography such as roughness and particle curvature can also impact protein adsorption uptake and the extent of adsorption-related

conformational change. Several studies investigating the effects of particle curvature on protein adsorption generally agree that proteins undergo less extensive conformational changes when adsorbed onto particles with higher curvature *i.e.*, smaller-sized particles.⁵⁰⁻⁵³ On the other hand, surface roughness has a more profound impact on protein adsorption uptake as well as adsorption-related conformational changes with a number of studies reporting either unaltered or altered adsorption uptake and conformational changes which appears to be related to the size of the protein in relation to the length scale of the roughness features.⁵⁴⁻⁶²

Taken together, these studies have revealed crucial information on how environmental and surface parameters affect the adsorption behavior of protein molecules and has greatly contributed to advancing the understanding of the protein adsorption process. However, it is also apparent that these parameters alone do not fully account for a protein's adsorption behavior. Differences in the protein itself can give rise to different adsorption behavior. One example would be how larger-sized proteins generally have slower adsorption kinetics and exhibit greater adsorption uptake than smaller-sized proteins.^{63, 64} In particular, the extent of adsorption-related conformational changes to the proteins has also been shown to be critical in dictating the outcome of the adsorption process and is intimately linked to the conformational stability of a protein. Indeed, the environmental parameters *i.e.*, ionic strength, pH and temperature, not only influence protein adsorption by modulating protein-surface intermolecular interactions, but also by modulating intramolecular interactions which impacts protein conformational stability.^{28, 65-67}

2.1.3 Conformational Stability and its Influence on Protein Adsorption

Proteins exist as a polypeptide chain with folded conformations which are essential for specific interactions to occur in order for proteins to carry out their biological functions. The stability of the protein's folded conformation, *i.e.*, the conformational stability of proteins, which is often described based on the free energy change between the folded and the unfolded state or the native and the denatured state,⁶⁷ is a result of competing attractive and repulsive intramolecular forces. Attractive forces stabilize the folded conformation of

the protein and include hydrophobic interactions, hydrogen bonding, electrostatic attraction, van der Waals forces as well as disulfide bonds while repulsive forces destabilize the folded conformation and is mainly driven by conformational entropy and electrostatic repulsion.^{68,69} The difference between the attractive and repulsive forces result in a relatively small free energy difference between the folded and unfolded state which is approximately between 5 and 15 kcal/mol for most proteins,⁶⁷ which makes it easy for proteins to be unfolded by chemical denaturants such as urea, guanidine hydrochloride or organic solvents,⁷⁰⁻⁷⁴ by high temperature,^{75, 76} or by adsorption.^{7, 77}

The conformational stability of proteins has long been recognized to influence protein adsorption behavior. Indeed, the unfolding or denaturation of a protein upon adsorption causes the protein's polypeptide backbone and side groups to have greater degrees of rotational and translational freedom than in its native state. This contributes to large increases in conformational entropy which thermodynamically drives the adsorption process by largely decreasing the associated Gibbs free energy. A protein with greater conformational stability would undergo less extensive denaturation upon adsorption, which results in a smaller increase in conformational entropy and therefore adsorption would be less favored compared to a protein with lower conformational stability, all other parameters being equal. Moreover, the more extensive adsorption-related unfolding of less conformationally stable proteins results in a larger protein-surface contact area which further favors adsorption. Early demonstrations of this concept was conducted by comparing the adsorption of relatively less conformationally stable or 'soft' proteins such as BSA, α -lactalbumin, myoglobin and ovalbumin against relatively more conformationally stable or 'hard' proteins such as lysozyme, cytochrome c and RNase A, which showed that the 'soft' proteins underwent more extensive surface-induced denaturation.^{29, 42, 78-80}

A more engineering approach was also taken to demonstrate this concept, in which samples that differ only in a few parameters were compared. Billsten *et al.* compared wild type and mutant variants of bacteriophage T4 lysozyme –differing by a single amino acid through point mutation – which had different conformational stabilities and demonstrated that less

stable mutants underwent more extensive surface-induced denaturation upon adsorption,⁸¹ while McGuire *et al.* demonstrated that these less stable mutants were more irreversibly adsorbed onto both hydrophilic and hydrophobic surfaces.⁸² Adopting a similar approach using mutant variants of human carbonic anhydrase II with varying conformational stabilities, Karlsson *et al.* demonstrated that less stable variants exhibited a faster rate of adsorption-related conformational change⁸³ and displayed more irreversible adsorption onto a variety of surfaces with different hydrophobicity and charge.⁸⁴ In line with this concept but adopting a different experimental approach, Park *et al.* demonstrated how the conformational properties of BSA proteins could be modulated through environmental parameters such as ionic strength as well as temperature-induced denaturation and aggregation, how these affect the conformation of the adsorbed proteins in terms of adsorption-related conformational changes and adsorption irreversibility, and how such findings provide insight on strategies that improve surface passivation or antifouling performance.^{66, 85} Taken together, these studies highlight the significance of protein conformational stability and its effects on protein adsorption behavior. In particular, the different strategies to modulate protein conformational stability used in the literature such as directed amino acid point mutations and various environmental parameters warrants further exploration of how other experimental approaches, guided by an overarching and rational framework, may be adopted as well.

2.1.4 Experimental Approaches to Characterize Protein Conformational Stability and Protein Adsorption

The characterization of protein conformational stability in solution as well as protein adsorption behavior is a critical step towards unraveling their relationship. While a vast number of methods and techniques to characterize both properties have been reported in the literature, this section briefly reviews some of the more commonly used ones.

2.1.4.1 Characterization of Solution-Phase Conformational Stability

The conformational stability of proteins can be characterized by measuring the Gibbs free energy change between the native (*i.e.*, folded) and the denatured (*i.e.*, unfolded) state of a protein in solution. A simple two-state model (*i.e.*, completely folded and completely unfolded) is commonly used to approximate the protein unfolding profile. One important assumption for this model is that the two states are in thermodynamic equilibrium, that is, unfolding is fully reversible. One way of inducing protein unfolding is by using chemical denaturants such as urea or guanidine hydrochloride.^{71, 86, 87} Urea denatures proteins by preferentially solvating the polypeptide chain of the protein,^{70, 88} and although the exact mechanism of how guanidine chloride denatures protein is still uncertain,⁸⁹⁻⁹¹ its ionic nature plays an important role in distinguishing its action of denaturation from that of urea.^{72, 92} One of several spectroscopic techniques such as ultraviolet (UV) absorbance spectroscopy, circular dichroism (CD) spectroscopy, fluorescence spectroscopy or Fourier transform infrared (FTIR) spectroscopy are then used to track the extent of unfolding as a function of the concentration of the chemical denaturant.

Protein conformational stability can also be determined through thermal unfolding and although the occurrence of irreversible temperature-induced protein aggregation invalidates the direct application of the two-state model,⁹³ protein conformational stability may still be assessed semi-quantitatively based on the extent of temperature-induced secondary structure loss as a function of temperature by CD and FTIR techniques.^{94, 95} Calorimetric methods such as differential scanning calorimetry (DSC) is another commonly used method which can directly assess the thermodynamic parameters of protein conformational stability and therefore does not require the use of model assumptions.⁹⁶ The extent of temperature-induced protein aggregation can also be used as an indication of protein conformational stability, in which less stable proteins are more prone to aggregation.^{97, 98} Indeed, temperature-induced protein aggregation occurs when the hydrophobic interior of protein molecules in the solution phase are sufficiently exposed due to thermal unfolding which eventually triggers intermolecular hydrophobic interactions.^{66, 99} Techniques like dynamic light scattering (DLS) can be used to qualitatively assess protein conformational stability by monitoring changes in the hydrodynamic diameter of protein molecules in solution as a function of temperature which

occurs due to temperature-induced aggregation.¹⁰⁰⁻¹⁰² The measurement principles, and the type of information that can be obtained through these various techniques are summarized in **Table 2.1**.

Table 2.1 Summary of common techniques used to characterize protein conformational stability in solution.

Technique	Measurement Principle	Information Obtained	References
UV absorbance spectroscopy	Exposure of aromatic side chains of amino acids to different environments due to protein denaturation causes changes in UV absorbance spectra	<ul style="list-style-type: none"> Gibbs free energy, enthalpy and entropy of denaturation Relative extent of denaturation 	71, 86, 103-107
Fluorescence spectroscopy	Exposure of aromatic side chains of amino acids to different environments due to protein denaturation causes changes in fluorescence spectra	<ul style="list-style-type: none"> Gibbs free energy, enthalpy and entropy of denaturation 	71, 86, 103, 108-111
CD spectroscopy	Changes in protein secondary structure due to protein denaturation causes changes in CD spectra	<ul style="list-style-type: none"> Gibbs free energy, enthalpy and entropy of denaturation Secondary structural content Midpoint transition temperature Relative extent of denaturation 	71, 86, 94, 103, 110-113
FTIR spectroscopy	Changes in protein secondary structure due to protein denaturation causes changes in FTIR spectra	<ul style="list-style-type: none"> Secondary structural content Midpoint transition temperature Relative extent of denaturation 	95, 114-118
DSC	Changes in the heat capacity of a protein due to protein unfolding is recorded as a function of temperature	<ul style="list-style-type: none"> Enthalpy of denaturation Midpoint transition temperature 	96, 114, 119-121
DLS	Changes in the hydrodynamic size of a protein due to aggregation is recorded as a function of temperature	<ul style="list-style-type: none"> Hydrodynamic size of native and aggregated state Onset temperature of aggregation 	100-102

2.1.4.2 Characterization of Protein Adsorption

The adsorption of proteins onto a solid-liquid interface can be characterized broadly in terms of three aspects: 1) the amount of proteins adsorbed/adsorption uptake, 2) the rate of protein adsorption/adsorption kinetics, and 3) the extent of surface-induced denaturation/adsorption-related conformational changes. Historically, the adsorption uptake was typically measured by the so-called depletion method in which the adsorption

uptake was determined based on the loss in the concentration of the protein solution after adsorption to a solid surface. Micro or nanoparticles were added into the solution which provided a large surface area onto which proteins may adsorb in order to improve the accuracy of the measurement.¹²²⁻¹²⁴ Adsorption isotherms were determined by measuring the amount of adsorption uptake as a function of the protein solution concentration. The concentration of the protein solution may be measured by any established assay including the Lowry assay, bicinchoninic acid (BCA) assay, Bradford assay or UV absorbance assay.¹²⁵

Developments in label-free surface-sensitive techniques, which produce a change in signal readout upon an adsorption event at an interface, led to their use for studying protein adsorption. These techniques are especially useful due to their ability to not only measure adsorption uptake but to also track adsorption events in real time, thus allowing for the measurement of protein adsorption kinetics. Some of these techniques such as ellipsometry,¹²⁶⁻¹²⁸ surface plasmon resonance (SPR)¹²⁹⁻¹³¹ and localized surface plasmon resonance (LSPR)^{28, 132, 133} make use of optical principles such as refractive index changes to measure the adsorption uptake of proteins to a variety of surfaces in real time. On the other hand, the quartz crystal microbalance-dissipation (QCM-D)¹³⁴⁻¹³⁶ technique is acoustic-based and makes use of an oscillating quartz crystal sensor to provide information on the mass and the viscoelasticity of the adsorbed film as well as on the amount of hydrodynamically-coupled solvent.^{137, 138}

Adsorption-related conformational changes can be characterized by CD in a similar fashion to the depletion method, in which the measure of the change in secondary structure upon the adsorption of protein molecules onto particles in solution indicate the extent of surface-induced denaturation.^{81, 83, 139} Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy is another technique that can probe the extent of surface-induced denaturation by characterizing the secondary structure change of protein molecules that are adsorbed onto a bare or coated ATR crystal.^{49, 140-142} At the same time, it has also been demonstrated that the LSPR technique is capable of determining the extent of protein conformational changes upon adsorption due to its very short penetration depth of ~20 nm

which is comparable to the size of protein molecules and its sensitivity to the spatial proximity of adsorbates to the sensor surface.^{28, 132, 143, 144} The measurement principles, and the type of information that can be obtained through these various techniques are summarized in **Table 2.2**.

Table 2.2 Summary of common techniques used to characterize protein adsorption uptake, adsorption kinetics and adsorption-related conformational changes.

Technique	Measurement Principle	Information Obtained	References
Depletion method	Changes in protein solution concentration correlates to the amount of protein adsorbed	• Adsorption uptake	122-125
Ellipsometry	Changes in the polarization of light upon reflection from a sample surface correlates to changes in film thickness and refractive index due to protein adsorption	• Adsorption uptake • Adsorption kinetics • Film thickness	126-128
SPR	Changes in the refractive index near the surface due to protein adsorption causes changes in the resonance angle	• Adsorption uptake • Adsorption kinetics • Film thickness	129-131
LSPR	Changes in the refractive index near the nanoparticle transducer due to protein adsorption causes changes in the extinction wavelength peak	• Adsorption uptake • Adsorption kinetics • Film thickness • Relative extent of surface-induced denaturation	28, 132, 133
QCM-D	Changes in the resonance frequency and energy dissipation of the quartz sensor correlates to mass and viscoelasticity of adsorbed proteins	• Adsorption uptake • Adsorption kinetics • Film thickness and mechanical properties • Protein-surface interaction	134-138
CD spectroscopy	Changes in protein secondary structure due to protein adsorption causes changes in CD spectra	• Relative extent of surface-induced denaturation	81, 83, 139
ATR-FTIR spectroscopy	Changes in protein secondary structure due to protein adsorption causes changes in FTIR spectra	• Relative extent of surface-induced denaturation	49, 140-142

2.1.5 Examples of Functional Protein Coatings

The study of how and why proteins adsorb to solid surfaces is of great interest due to the vast potential of fabricating protein coatings with distinct functions for use in a variety of applications including food science and biomedical technologies.¹⁴⁵⁻¹⁴⁸ Focusing on the latter example, more specific biomedical applications of functional protein coatings

including surface passivation or stealth functionality, coatings for sensors and diagnostics, and coatings for medicine such as antibacterial/antiviral, vaccine and cancer therapy will be briefly reviewed.

A major thrust in the development of advanced biomedical devices and one that is central to this thesis is the prevention of unwanted protein adsorption onto solid surfaces. This process, also known as fouling, has been implicated in a variety of problems in the biomedical and biotechnology fields, and although several strategies have been developed to address this problem,^{149, 150} the use of protein films shows excellent promise in terms of biocompatibility and its vast potential for engineered functionality. One of the most well-known examples on the use of antifouling protein films is the ‘blocking’ step in typical protocols for immune assays such as enzyme-linked immunosorbent assay (ELISA) and Western blots where purified protein such as BSA and casein or protein mixtures such as non-fat milk and serum are coated onto a target surface to prevent nonspecific adsorption that can interfere with the assays’ signal readouts, which rely on specific binding interactions between an antigen and an antibody.^{151, 152} These problems are similarly encountered in biosensing platforms for *in vitro* diagnostic applications,^{153, 154} and several studies have demonstrated how the adsorption of BSA as well as various other proteins such as chicken serum albumin, hemoglobin and lysozyme and non-fat milk can improve the performance of biosensors.¹⁵⁵⁻¹⁵⁸ A number of fundamental studies have also demonstrated how other types of coatings formed by soy protein isolates,¹⁵⁹ or fish proteins¹⁶⁰ exhibit antifouling or surface passivating properties in general.

In another example of functional protein coatings via protein adsorption for biosensing applications, Rao and co-workers have demonstrated how rational protein engineering cause anisotropic orientation of nonspecifically adsorbed mutant protein receptors with improved capture rate over wild type protein,¹⁶¹ and how protein engineering approaches to also improve the specificity of a protein receptor¹⁶² can improve the performance of protein-based biosensors.

Surface passivating protein coatings have also been fabricated onto the surface of

biomedical implants following the general strategy that preventing the adsorption of proteins that are responsible for clotting or generating immune responses onto the implant surface would help with biocompatibility.^{163, 164} A parallel strategy involves the formation of bioactive protein coatings such as elastin-like proteins, silk proteins or other proteins derived from the extracellular matrix such as collagen and gelatin, onto implants that actively aid with integration, reduce inflammation and, when combined with antimicrobial peptides such as magainin, fight against bacterial infections.¹⁶⁵⁻¹⁶⁹ Regarding the latter point, the antimicrobial properties and applications of lysozyme protein-based coatings have also been demonstrated in numerous studies.¹⁷⁰⁻¹⁷⁶

Protein adsorption can also be used to functionalize nanoparticles¹⁷⁷ whereby a layer of proteins is formed on the nanoparticle surface called the protein corona, which alters the biological identity of the nanoparticles.¹⁷⁷⁻¹⁷⁹ While this can lead to undesirable immune response such as complement activation,¹⁸⁰ rational pre-coating of protein molecules including BSA, Factor H, clusterin and CD47 onto nanoparticles have been demonstrated to effectively act as stealth coatings for nanoparticles to prevent immune response and cellular uptake.¹⁸¹⁻¹⁸⁵ Numerous other bioactive applications have also been reported for various protein-nanoparticle systems including drug delivery, diagnostics, therapy for antibacterial, antiviral and anticancer applications.^{175, 186-188}

Taken together, these features demonstrate the central role of protein adsorption in various biomedical applications and highlight the importance of a fundamental understanding in order to engineer functional protein coatings. In particular, BSA protein is often used in many surface passivation applications on both flat and nanomaterial surfaces, although few studies have been conducted to improve its passivation performance. There is, therefore, great motivation to develop superior serum albumin coatings through engineering approaches for surface passivation applications. From a materials science and engineering perspective, a fundamental understanding of its structure, properties and processing are critical to improving its passivation performance.

2.2 Serum Albumin

2.2.1 Structure and Biological Function of Serum Albumin

Serum albumin is the most abundant (~55%) of the blood proteins in mammals, consisting of a single polypeptide chain of about 66 kDa folded into three mostly α -helical domains named as domains I, II and III which are stabilized by 17 disulfide bonds. Each domain is further divided into A and B subdomains (**Figure 2.1**). The three-dimensional structure of human serum albumin (HSA) was revealed through X-ray crystallography to be cardioid in shape.¹⁸⁹ Serum albumin from other mammals such as cow, sheep, goat, rabbit, horse, pig and rat share similar primary structures with the former five having their crystal structures confirmed to be similarly cardioid in shape (**Figures 2.2 & 2.3**), and the latter two likely also having similar shape properties although formal crystallographic studies for these proteins have not yet been conducted.¹⁹⁰⁻¹⁹³

Albumin is synthesized in the liver and has two main biological functions. The first is to maintain colloidal osmotic pressure, also known as oncotic pressure. The second is to bind and transport a variety of small molecules, which is of particular interest in the drug delivery field and has sparked numerous binding studies. These include long-chain fatty acid anions which are insoluble but with important roles in lipid metabolism, toxic waste molecules like bilirubin, hormones, bile acids as well as amino acids.¹⁹⁴⁻¹⁹⁶ Albumin's binding function extends to exogenous compounds such as drug molecules which can affect the efficacy of intravenously administered drugs.¹⁹⁷ Studies on the binding capacity of albumin revealed that these binding events are reversible and tend to be driven by hydrophobic association. The association constants of drug binding depends on the species of albumin due to their differences in primary structure.¹⁹⁸ Of note, the binding of certain molecules may affect albumin conformation in terms of secondary structural or shape changes, as well as the stability of albumin in terms of resistance to thermal denaturation, aggregation or oxidative stress.¹⁹⁹⁻²⁰¹

While serum albumins from different species perform the same general functions and share some similarities in secondary structure and overall shape, certain fundamental differences still exist stemming from their variation in primary structure. For example, BSA and HSA are two widely studied serum albumins but a high-resolution crystal structure study revealed only a 75.8% correlation between them.¹⁹⁰ Other differences in their solution and adsorption properties have also been reported.^{47, 119, 202-204} In one example, BSA as well as serum albumin from sheep and goat were shown to have different surface charge distributions (**Figure 2.3**).¹⁹² Indeed, while serum albumin as a class of proteins has been studied for decades and used as a model protein for numerous studies, no systematic studies have been undertaken towards how parameters like primary structure and molecular binding can affect albumin adsorption and much remains to be understood in this area. Of particular interest is the existence of other homologous serum albumins essentially acting as a natural library of different primary structures which can be studied to establish a correlation between structure (primary, secondary etc.) and properties (solution and adsorption) and ultimately to surface passivation performance.

Figure 2.1 Molecular structure of BSA. **a**, Protein ribbon diagram with “ghost” surface showing domain I in red, domain II in blue and domain III in green with their individual A and B subdomains in related colors. **b**, Solid surface of the protein showing charge distribution from amino acid side groups. Blue represents positive charge, red represents negative charge and white represents neutral charge. The yellow line indicates the protein dipole moment. Adapted with permission from ref. 205. Copyright 2017, American Chemical Society.

Figure 2.2 Aligned molecular structures of HSA (burgundy), BSA (cyan), serum albumin from horse (blue and violet) and from rabbit (green). Disulphide bridges are depicted as spheres. Adapted with permission from ref. 190. Copyright 2012, International Union of Crystallography.

Figure 2.3 Electrostatic surface potential ($K_B T / e_c$) of serum albumins from **a,b**, sheep, **c,d**, goat, and **e,f**, BSA. Adapted with permission from ref. 192. Copyright 2012, International Union of Crystallography.

2.2.2 Solution and Surface Properties of Serum Albumin

2.2.2.1 Solution Properties

Although the primary structures vary between the different species of serum albumin, mammalian serum albumin possess similar charge distributions with domains I and II having negative charge and domain III having a small positive charge, resulting in a high net negative charge (<-10) at physiological pH, granting albumin high solubility in water (**Figure 2.1**).¹⁹⁴ The isoelectric point of serum albumin is around 4.8 to 5.3.²⁰⁶ Serum albumin molecules are also able to undergo extensive reversible conformational changes with changes in solution pH, adopting different isomeric forms. These forms are classified as N for normal form at neutral pH, F for the partially unfolded fast migrating form at pH 4, E for extended form below pH 3, B for basic form at pH greater than 8 and A for aged form at pH near 10 with time (**Figure 2.4**). The unfolding of serum albumin with pH changes is attributed to electrostatic repulsion between like-charged amino acid side groups. Unfolding of the serum albumin molecule can be characterized by measuring its hydrodynamic size using dynamic light scattering (DLS). The hydrodynamic size of serum albumin dictates its diffusion-limited transport through solution which can affect protein adsorption kinetics. Characterization of protein charge can be conducted by measurement of zeta potential and electrophoretic mobility.

Figure 2.4 The isomerism of BSA from N form at neutral pH, to F form at pH 4 and E form at below pH 3. Adapted with permission from ref. 207. Copyright 2012, Elsevier.

Information on a protein's conformational stability can be obtained by determining its resistance to denaturation. The denaturation of serum albumin in solution has been extensively studied and can be induced either through heating or through chemical denaturants such as urea or guanidine hydrochloride. However, protein stability is more often assessed in correlation to thermal stability, hence only temperature-induced denaturation will be covered herein. Increasing temperature causes the serum albumin to unfold due to the disruption of hydrogen bonds. This unfolding process is reversible up to around 50 °C.²⁰⁸ At higher temperatures, conformational changes become extensive such that their interior hydrophobic regions are sufficiently exposed, causing serum albumin molecules to aggregate by hydrophobic interaction.^{75, 95} The thermodynamics of the denaturation process in solution are typically quantified by differential scanning calorimetry (DSC).²⁰⁹ The aggregation event can be tracked by temperature-dependent DLS measurements. The change in secondary structure can be measured by circular dichroism (CD) spectroscopy or Fourier transform infrared (FTIR) spectroscopy.^{75, 95} The change in tertiary structure can be measured by UV-vis spectroscopy.²¹⁰

2.2.2.2 Adsorption Properties

As previously mentioned, serum albumin is considered a 'soft' protein as irreversible adsorption occurs even on hydrophilic and negatively charged surfaces like silica or titania,

and serum albumin readily undergoes surface-induced denaturation in which the serum albumin molecule is thermodynamically driven to deform and spread across the surface, forming additional protein-surface contacts and resulting in irreversible adsorption.²¹¹ As with most other proteins, serum albumin is more strongly and more quickly bound to hydrophobic than hydrophilic surfaces and also undergo more extensive conformational change compared to hydrophilic surfaces. This is because adsorption to hydrophobic surfaces require the serum albumin molecule's hydrophobic interior to be in contact with it. On the other hand, serum albumin adsorption to hydrophilic surfaces like silica is weaker and has been observed to be a series of adsorption-desorption cycles where conformational changes take place with each cycle with a net adsorption rate.¹³⁹ The rate of spreading on the surface depends on the surface chemistry and also on the conformational stability of the serum albumin. The extent of the spreading is affected by the rate of spreading relative to the rate of adsorption. The amount adsorbed is consequently affected by the rate of spreading since an adsorbed albumin molecule that is more spread out would occupy a larger surface area, thereby decreasing the remaining available space for other molecules.

Adsorption amount and kinetics are typically monitored by surface sensitive techniques such as the acoustic-based quartz crystal microbalance-dissipation (QCM-D) technique, and the optically based surface plasmon resonance (SPR) and ellipsometry.¹¹ Total internal reflection fluorescence (TIRF) can also be used to measure adsorption kinetics and amount adsorbed.²¹¹ Conformational changes by surface-induced denaturation can be studied by CD spectroscopy of proteins adsorbed to particles¹³⁹ and by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy.²¹² The thermodynamics of the adsorption event can be studied by isothermal titration calorimetry (ITC).²¹³

Just as solving any engineering problem first requires a fundamental understanding of the system, so too does engineering serum albumin adsorption for improved surface passivation applications first require a fundamental understanding of serum albumin's properties both in solution and upon adsorption to solid surfaces. This approach has been applied in the study of human carbonic anhydrase II⁸⁴ and bacteriophage T4 lysozyme⁸¹ in

which protein variants determined to have poorer conformational stability in solution are shown to undergo more extensive surface-induced denaturation upon adsorption. However, despite decades of research and its abundant use, serum albumin's solution and surface properties have largely been studied separately. Additionally, no attempt has been made thus far in studying how differences in serum albumin primary structure or even their processing techniques may affect their conformational stability and thereby their adsorption behavior. Additionally, certain processing techniques can affect the stability of serum albumin proteins and will be reviewed in the following section.

2.2.3 Purification and Processing Conditions of Serum Albumin

The need for the mass production of pure serum albumin came during World War II when a stable substitute for blood plasma was needed to treat injured soldiers against hypovolemic shock.²¹⁴ The cold ethanol fractionation method was developed by Cohn and co-workers that purified serum albumin from bovine and human plasma.²¹⁵ This method involved adjusting ethanol concentration, temperature, pH and ionic strength of the plasma together with centrifugation and filtration in order to precipitate and separate various constituent proteins into five fractions. Fractions I to V are fibrinogen, γ -globulin, β -globulin, α -globulin, and serum albumin, respectively. Serum albumin is sometimes referred to as Fraction V for this reason. Cohn reported six methods to obtain the five fractions that differed mainly in the workable range of solution pH and ethanol concentrations. Method 6 was developed with the goal of fractionating as much of the plasma proteins as possible and the processing conditions are briefly described herein.²¹⁶ A mixture of ethanol and sodium acetate-acetic acid buffer is added to plasma to adjust ethanol concentration, pH and ionic strength conditions. The plasma is first cooled to $-3\text{ }^{\circ}\text{C}$, adjusted to pH 7.2, 8% ethanol and 0.14 M ionic strength. Here, fibrinogen is precipitated and removed by centrifugation as Fraction I. Next, the temperature is brought to $-5\text{ }^{\circ}\text{C}$ while solution conditions are adjusted to pH 6.9, 25% ethanol and 0.09 M ionic strength to precipitate β - and γ - globulins as Fractions II and III which are removed by centrifugation. Next, maintaining the temperature and ionic strength, pH is adjusted to 5.2 and ethanol concentration to 18% to precipitate α -globulins and lipids which are removed by

centrifugation as Fraction IV-1. Keeping the same temperature and ionic strength, pH is adjusted to 5.8 and ethanol concentration to 40% where α - and β -globulins and some serum albumin are precipitated and removed by centrifugation as Fraction IV-4. The supernatant is filtered followed by adjusting the pH to 4.8 and ionic strength to 0.11 M while maintaining temperature and ethanol concentration to precipitate serum albumin which is removed by centrifugation. Some companies today like Sigma-Aldrich,²¹⁷ Gemini Bio-Products,²¹⁸ and Equitech-Bio²¹⁹ still sell serum albumin fractionated based on Cohn's cold ethanol fractionation method although certain steps are likely to have been modified.²²⁰⁻²²²

Another fractionation method reported in companies like Sigma-Aldrich,²¹⁷ Rocky Mountain Biologicals,²²³ Bovogen,²²⁴ Gemini Bio-Products,²¹⁸ and Equitech-Bio²²⁵ is heat-shock fractionation. The earliest found record describing a method to purify serum albumin from plasma by heating is in a patent by Reid dated 1955.²²⁶ This method of fractionation relies on heat to denature, precipitate and remove all other proteins in the serum except for albumin, which is conferred heat resistance through the addition of the sodium salts of fatty acids (hereafter referred to as fatty acids) such as oleic, capric, or caprylic acid. The fatty acids are then removed by ion exchange resins and/or by dialysis. This phenomenon of fatty acids conferring thermal stability to serum albumin was first reported by Ballou *et al.*²²⁷ in 1944 and was quickly followed up with a series of studies by Boyer *et al.*²²⁸⁻²³⁰ They showed that the fatty acids prevented heat-induced aggregation in a concentration-dependent manner and that the stabilizing effect increases with increasing fatty acid chain length. The eight-carbon chain caprylic acid was one of the most effective stabilizers as larger concentrations of it could be added due to its relatively higher solubility compared to the longer-chain fatty acids. The first journal publication detailing the use of ethanol, caprylic acid, and heat for serum albumin fractionation was by Schneider *et al.* and was published in 1975.²³¹ Schneider's processing conditions are briefly described herein. All pH changes were made with either hydrochloric acid (HCl) or sodium hydroxide (NaOH). Plasma as the source material is made to pH 6.5, with an ethanol concentration of 9% and sodium caprylate is added to 0.004 M. The plasma solution is then stirred and heated to 68 °C for over 3 h, then cooled to 10 °C for over 4 h while still stirring. Solution pH is then adjusted to 4.4 and left overnight for precipitates to form. The

precipitates are then separated by centrifugation, the supernatant is made to pH 4.8 and filtered. Purified serum albumin is then precipitated from the solution using 22% PEG 4000 at 16 °C for 0.5 h and removed by centrifugation. Serum albumin purified by heat-shock fractionation reportedly tends to be of higher purity than cold ethanol fractionated ones. The stabilizing effect of caprylic acid has also been used for the heat inactivation of viruses for therapeutic HSA, post-purification. This process typically consists of adding appropriate amounts of caprylic acid as stabilizers followed by heating to 60 °C for 10 h.²²⁰

Certain commercially available serum albumins are described to be fatty acid free which implies that processing steps were taken to ensure the removal of any bound fatty acids to the serum albumin. As mentioned before, serum albumins are capable of binding fatty acids and other small molecules. The binding of molecules to serum albumin was heavily studied and the presence of pre-bound fatty acids was found to interfere with the binding of other molecules. For this reason Chen reported a simple process to remove the bound fatty acids from serum albumin through charcoal treatment, in which fatty acids are preferentially bound to the charcoal surface, and has since been widely used.²³² The processing conditions for this charcoal treatment are briefly described herein. 0.5 mg of activated charcoal powder is added per mg of serum albumin in aqueous solution. The temperature is then lowered to 0 °C and solution pH is adjusted to 3 while stirring for 1 h. The charcoal is then removed by centrifugation and the pH is adjusted back to neutral to yield defatted serum albumin with no reported changes to its secondary structure. A summary of cold ethanol fractionation, heat-shock fractionation and the fatty acid removal process is summarized in **Table 2.3**.

Table 2.3 A summary of the process conditions for cold ethanol fractionation, heat-shock fractionation, and charcoal defatting of serum albumin.

Cold Ethanol Fractionation	<ol style="list-style-type: none"> 1. Plasma is cooled. Ethanol and sodium acetate-acetic acid buffer is added to adjust ethanol concentration, pH and ionic strength. 2. pH 7.2, 8% ethanol, -3°C, 0.14 M ionic strength. Fibrinogen is precipitated and removed by centrifugation (Fraction I). 3. pH 6.9, 25% ethanol, -5 °C, 0.09 M ionic strength. β- and γ- globulins are precipitated and removed by centrifugation (Fraction II + III). 4. pH 5.2, 18% ethanol, -5 °C, 0.09 M ionic strength. α-globulins and lipids are removed by centrifugation (Fraction IV-1). 5. pH 5.8, 40% ethanol, -5 °C, 0.09 M ionic strength. α- and β-globulins and some albumin are precipitated and removed by centrifugation (Fraction IV-4). The supernatant is filtered. 6. pH 4.8, 40% ethanol, -5 °C, 0.11 M ionic strength. Albumin is precipitated and removed by centrifugation (Fraction V).
Heat-Shock Fractionation	<ol style="list-style-type: none"> 1. Plasma is made to pH 6.5, 9% ethanol, 0.004 M sodium caprylate 2. Stirred and heated to 68 °C within 3 h. 3. Temperature is gradually lowered to 10 °C over 4 h while stirring. 4. pH is adjusted to 4.4 and plasma solution is kept overnight. 5. Precipitates are separated by centrifugation. Supernatant is made to pH 4.8 and filtered. 6. Albumin is precipitated using 22% PEG 4000 at 16 °C for 0.5 h and removed by centrifugation.
Charcoal Defatting	<ol style="list-style-type: none"> 1. 0.5 mg activated charcoal powder is added per mg albumin in aqueous solution. 2. Conditions made to pH 3 and 0 °C. Solution is stirred for 1 h. 3. Charcoal is removed by centrifugation. pH adjusted back to 7.

It can therefore be seen that the processing conditions of serum albumin can significantly affect serum albumin properties,²³³ particularly how the presence of fatty acids would affect the solution properties of albumin. It is therefore intuitive that the presence of fatty acids would affect the adsorption behavior of serum albumin as indeed, fatty acid-bound serum albumins have greater conformational stability than their fatty acid-free counterparts²³⁴ and, as established previously, the conformational stability of proteins correlates to their adsorption behavior and stability against surface-induced denaturation. To date, only two studies by Pitt *et al.* have reported this effect by demonstrating how HSA with bound stearic acid exhibited reduced adsorption onto a polyurethane surface compared

to defatted HSA although this was in the context of studying the adsorption behavior of HSA on a stearic acid-functionalized surface.^{235, 236} However, a lack in a direct application of this knowledge towards understanding how processing conditions, such as the presence of bound fatty acids, can affect BSA protein adsorption and surface passivation performance is striking considering the decades of serum albumin adsorption studies that followed and its widespread use today in biological assays such as for blocking non-specific adsorption. Moreover, Xiao *et al.* reported that blocking solutions made from different BSAs obtained from Sigma-Aldrich and used for ELISA yielded different results,²³⁷ although, the BSAs chosen in their study were not based on their different fractionation methods or presence of fatty acids and no comprehensive study on their adsorption behavior were conducted.

In summary, understanding the interplay between structure, properties and processing is key to engineering improved surface passivation applications. The next section will review the existing studies in the literature on albumin coatings for surface passivation and any strategies to improve its performance.

2.2.4 Applications of Serum Albumin Coatings

Serum albumin has been used as a coating in various biomedical applications. Indeed, BSA is highly advantageous for surface passivation applications due to its low cost and wide availability from its well-established purification methods, as well as its bioinertness and general high affinity for adsorbing onto various surfaces. Aside from the abovementioned uses in ELISA or Western blot assays, serum albumin has also been used to coat various medical devices such as catheters, arterial grafts, tympanostomy tubes and bone grafts to improve their biocompatibility and effectiveness.²³⁸⁻²⁴² Pre-coating nanoparticles with albumin has also been reported to reduce nanotoxicity and immune response on account of their non-toxicity and non-immunogenicity.²⁴³ While there are numerous studies correlating serum albumin adsorption behavior on various surfaces to surface chemistry, few studies have taken an engineering approach which focuses instead on tuning the properties of the serum albumin molecule itself for improved passivation performance. Of

these few studies, Park *et al.* investigated the ionic strength dependence on the amount of BSA adsorbed to silica surface at physiological pH using QCM-D measurements and showed that the greatest amount adsorbed and least protein spreading on the surface was achieved at zero-salt conditions whereas higher ionic strength conditions (150 mM NaCl) yielded films of BSA molecules that are more spread out on the surface with a lower amount adsorbed.²³ Surface passivation performance was evaluated by using the QCM-D to monitor changes in mass upon incubation of the film with fetal bovine serum (FBS) as a fouling agent. Despite the greater amount adsorbed at zero-salt conditions, the amount of desorbed proteins was correspondingly large upon buffer rinsing, resulting in a slightly poorer blocking efficiency of the zero-salt BSA film compared to that at higher ionic strength conditions. They then exploited the increased adsorption amount at zero-salt conditions by an additional glutaraldehyde crosslinking step to stabilize the adsorbed amount resulting in an improved blocking efficiency. In another study, Park *et al.* made use of a simple and versatile alternative strategy to improve passivation performance by denaturing the BSA through a heat treatment before surface adsorption.⁶⁶ This heat treatment altered its conformational properties and increased the size of the adsorbing species, resulting in highly irreversible adsorption onto silica surface on account of its increased contact surface area per adsorbing species. In addition to a better blocking efficiency against FBS, the denatured BSA also performed better as a blocking agent in a Western blot application by significantly reducing the intensity of non-specific bands. Of note, these studies include the holistic investigation of both solution and adsorption properties in explaining its passivation performance.

2.3 Outstanding Questions

The use of serum albumin as an antifouling protein coating for biomedical applications appears to be a promising alternative to polymer coatings. An in-depth understanding on its solution and adsorption properties in relation to its structure and processing must be acquired in order to develop them as effective surface passivating agents. Towards this goal, numerous outstanding questions need to be addressed.

Serum albumin from different species possess different amino acid sequences and solution properties such as ligand binding, solution-phase conformational stability and thermal denaturation profiles despite having similar three-dimensional crystal structures. This provides motivation for asking how sequence variation between natural homologs of serum albumin proteins may give rise to different adsorption behaviors and how such properties are linked with conformational stability. Furthermore, BSA and HSA are the two most studied serum albumins and their adsorption behaviors have been the only ones tested to date. The aforementioned gaps in the literature provide motivation to systematically investigate serum albumin proteins from other species as well.

The second major question is how processing conditions could potentially affect serum albumin's adsorption behavior. To date, no systematic study has been conducted comparing any physicochemical differences between the multitude of differently processed serum albumins, particularly on whether the different fractionation methods or the presence of fatty acids could affect conformational stability and adsorption profile. This is particularly important as BSA remains widely used today for its adsorption onto surfaces for the prevention of non-specific binding in biological immunoassays and other biosensing applications, yet no empirically-backed guidelines on which BSA type is better for blocking applications exist. A deeper study on the mechanistic aspects of how such differences in either processing conditions such as temperature or the nature of the bound amphiphiles such as fatty acids affect conformational stability and adsorption behavior is also warranted.

Finally, flowing from the previous questions, it is reasonable to then ask how the experimentally observed trends in conformational stability and adsorption behavior correlate to surface passivation performance. Related questions include how surface passivation performance is to be assessed and whether similar trends may still be observed on different material surfaces with different properties such as hydrophilic vs hydrophobic surfaces or bulk vs nanomaterial surfaces.

Taken together, these provide the motivation for this project in identifying how structural variation and different processing conditions could affect serum albumin adsorption with the goal of developing a strategy to maximize albumin surface passivation performance. At the same time, an opportunity is presented through this project to establish and develop a comprehensive analytical framework for the study of protein adsorption onto solid surfaces.

References

1. Dobson, C. M.; Šali, A.; Karplus, M., Protein folding: a perspective from theory and experiment. *Angew. Chem. Int. Edit.* **1998**, *37* (7), 868-893.
2. Anfinsen, C. B., Principles that govern the folding of protein chains. *Science* **1973**, *181* (4096), 223-230.
3. Lodish, H.; Berk, A.; Zipursky, S.; Matsudaira, P.; Baltimore, D.; Darnell, J., Noncovalent bonds. *Mol. Cell Biol.* **2000**.
4. Dobson, C. M., Protein folding and misfolding. *Nature* **2003**, *426* (6968), 884.
5. Tanford, C., Protein denaturation. In *Advances in Protein Chemistry*, Elsevier: 1968; Vol. 23, pp 121-282.
6. Norde, W., Adsorption of proteins from solution at the solid-liquid interface. *Adv. Colloid Interface Sci.* **1986**, *25*, 267-340.
7. Fernández, A.; Ramsden, J. J., On adsorption-induced denaturation of folded proteins. *J. Biol. Phys. Chem.* **2001**, *1* (2), 81-84.
8. Hlady, V.; Buijs, J., Protein adsorption on solid surfaces. *Curr. Opin. Biotechnol.* **1996**, *7* (1), 72-77.
9. Tsapikouni, T. S.; Missirlis, Y. F., Protein–material interactions: from micro-to-nano scale. *Mater. Sci. Eng., B* **2008**, *152* (1-3), 2-7.
10. Yano, Y. F., Kinetics of protein unfolding at interfaces. *J. Phys.: Condens. Matter* **2012**, *24* (50), 503101.
11. Rabe, M.; Verdes, D.; Seeger, S., Understanding protein adsorption phenomena at solid surfaces. *Adv. Colloid Interface Sci.* **2011**, *162* (1-2), 87-106.

12. van der Veen, M.; Stuart, M. C.; Norde, W., Spreading of proteins and its effect on adsorption and desorption kinetics. *Colloids Surf., B* **2007**, *54* (2), 136-142.
13. Höök, F.; Rodahl, M.; Kasemo, B.; Brzezinski, P., Structural changes in hemoglobin during adsorption to solid surfaces: effects of pH, ionic strength, and ligand binding. *Proc. Natl. Acad. Sci.* **1998**, *95* (21), 12271-12276.
14. Jachimska, B.; Tokarczyk, K.; Łapczyńska, M.; Puciul-Malinowska, A.; Zapotoczny, S., Structure of bovine serum albumin adsorbed on silica investigated by quartz crystal microbalance. *Colloids Surf., A* **2016**, *489*, 163-172.
15. Meissner, J.; Prause, A.; Bharti, B.; Findenegg, G. H., Characterization of protein adsorption onto silica nanoparticles: influence of pH and ionic strength. *Colloid Polym. Sci.* **2015**, *293* (11), 3381-3391.
16. Moerz, S. T.; Huber, P., pH-dependent selective protein adsorption into mesoporous silica. *J. Phys. Chem. C* **2015**, *119* (48), 27072-27079.
17. Quiquampoix, H.; Staunton, S.; Baron, M.-H.; Ratcliffe, R., Interpretation of the pH dependence of protein adsorption on clay mineral surfaces and its relevance to the understanding of extracellular enzyme activity in soil. *Colloids Surf., A* **1993**, *75*, 85-93.
18. Demanèche, S.; Chapel, J.-P.; Monrozier, L. J.; Quiquampoix, H., Dissimilar pH-dependent adsorption features of bovine serum albumin and α -chymotrypsin on mica probed by AFM. *Colloids Surf., B* **2009**, *70* (2), 226-231.
19. Lee, W.-K.; Ko, J.-S.; Kim, H.-M., Effect of electrostatic interaction on the adsorption of globular proteins on octacalcium phosphate crystal film. *J. Colloid Interface Sci.* **2002**, *246* (1), 70-77.
20. Israelachvili, J. N., *Intermolecular and Surface Forces*. Academic press: 2011.
21. Jones, K. L.; O'Melia, C. R., Protein and humic acid adsorption onto hydrophilic membrane surfaces: effects of pH and ionic strength. *J. Membr. Sci.* **2000**, *165* (1), 31-46.
22. Ramsden, J.; Prenosil, J., Effect of ionic strength on protein adsorption kinetics. *J. Phys. Chem.* **1994**, *98* (20), 5376-5381.
23. Park, J. H.; Sut, T. N.; Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Cho, N.-J., Controlling adsorption and passivation properties of bovine serum albumin on silica

- surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19* (13), 8854-8865.
24. Vogt, G.; Woell, S.; Argos, P., Protein thermal stability, hydrogen bonds, and ion pairs. *J. Mol. Biol.* **1997**, *269* (4), 631-643.
 25. Pace, C. N.; Fu, H.; Lee Fryar, K.; Landua, J.; Trevino, S. R.; Schell, D.; Thurlkill, R. L.; Imura, S.; Scholtz, J. M.; Gajiwala, K., Contribution of hydrogen bonds to protein stability. *Protein Sci.* **2014**, *23* (5), 652-661.
 26. Jackler, G.; Steitz, R.; Czeslik, C., Effect of temperature on the adsorption of lysozyme at the silica/water interface studied by optical and neutron reflectometry. *Langmuir* **2002**, *18* (17), 6565-6570.
 27. Kiesel, I.; Paulus, M.; Nase, J.; Tiemeyer, S.; Sternemann, C.; Rüster, K.; Wirkert, F. J.; Mende, K.; Büning, T.; Tolan, M., Temperature-driven adsorption and desorption of proteins at solid-liquid interfaces. *Langmuir* **2014**, *30* (8), 2077-2083.
 28. Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Park, J. H.; Zhdanov, V. P.; Cho, N.-J., Indirect nanoplasmonic sensing platform for monitoring temperature-dependent protein adsorption. *Anal. Chem.* **2017**, *89* (23), 12976-12983.
 29. Norde, W. In *Driving forces for protein adsorption at solid surfaces*, Macromolecular Symposia, Wiley Online Library: 1996; pp 5-18.
 30. Pasche, S.; Vörös, J.; Griesser, H. J.; Spencer, N. D.; Textor, M., Effects of ionic strength and surface charge on protein adsorption at PEGylated surfaces. *J. Phys. Chem. B* **2005**, *109* (37), 17545-17552.
 31. Gessner, A.; Lieske, A.; Paulke, B. R.; Müller, R. H., Influence of surface charge density on protein adsorption on polymeric nanoparticles: analysis by two-dimensional electrophoresis. *Eur. J. Pharm. Biopharm.* **2002**, *54* (2), 165-170.
 32. Guo, S.; Zhu, X.; Li, M.; Shi, L.; Ong, J. L. T.; Jańczewski, D.; Neoh, K. G., Parallel control over surface charge and wettability using polyelectrolyte architecture: effect on protein adsorption and cell adhesion. *ACS Appl. Mater. Interfaces* **2016**, *8* (44), 30552-30563.
 33. Hartvig, R. A.; van de Weert, M.; Østergaard, J.; Jorgensen, L.; Jensen, H., Protein adsorption at charged surfaces: the role of electrostatic interactions and interfacial charge regulation. *Langmuir* **2011**, *27* (6), 2634-2643.

34. Rezwan, K.; Meier, L. P.; Gauckler, L. J., Lysozyme and bovine serum albumin adsorption on uncoated silica and AlOOH-coated silica particles: the influence of positively and negatively charged oxide surface coatings. *Biomaterials* **2005**, *26* (21), 4351-4357.
35. Ladam, G.; Schaaf, P.; Decher, G.; Voegel, J.-C.; Cuisinier, F. J., Protein adsorption onto auto-assembled polyelectrolyte films. *Biomol. Eng.* **2002**, *19* (2-6), 273-280.
36. Kubiak-Ossowska, K.; Jachimska, B.; Al Qaraghuli, M.; Mulheran, P. A., Protein interactions with negatively charged inorganic surfaces. *Curr. Opin. Colloid Interface Sci.* **2019**, *41*, 104-117.
37. Roth, C. M.; Neal, B. L.; Lenhoff, A. M., Van der Waals interactions involving proteins. *Biophys. J.* **1996**, *70* (2), 977-987.
38. Zhdanov, V.; Kasemo, B., Van der Waals interaction during protein adsorption on a solid covered by a thin film. *Langmuir* **2001**, *17* (18), 5407-5409.
39. Moerz, S. T.; Huber, P., Protein adsorption into mesopores: a combination of electrostatic interaction, counterion release, and van der Waals forces. *Langmuir* **2014**, *30* (10), 2729-2737.
40. Hähl, H.; Evers, F.; Grandthyll, S.; Paulus, M.; Sternemann, C.; Loskill, P.; Lessel, M.; Hüsecken, A. K.; Brenner, T.; Tolan, M., Subsurface influence on the structure of protein adsorbates as revealed by in situ X-ray reflectivity. *Langmuir* **2012**, *28* (20), 7747-7756.
41. Poncin-Epaillard, F.; Vrlnic, T.; Debarnot, D.; Mozetic, M.; Coudreuse, A.; Legeay, G.; El Moualij, B.; Zorzi, W., Surface treatment of polymeric materials controlling the adhesion of biomolecules. *J. Funct. Biomater.* **2012**, *3* (3), 528-543.
42. Haynes, C. A.; Norde, W., Globular proteins at solid/liquid interfaces. *Colloids Surf., B* **1994**, *2* (6), 517-566.
43. Guo, S.; Pranantyo, D.; Kang, E.-T.; Loh, X. J.; Zhu, X.; Jańczewski, D.; Neoh, K. G., Dominant albumin–surface interactions under independent control of surface charge and wettability. *Langmuir* **2018**, *34* (5), 1953-1966.
44. Tilton, R. D.; Robertson, C. R.; Gast, A. P., Manipulation of hydrophobic interactions in protein adsorption. *Langmuir* **1991**, *7* (11), 2710-2718.

45. Svensson, O.; Kurut, A.; Skepö, M., Adsorption of β -casein to hydrophilic silica surfaces. Effect of pH and electrolyte. *Food Hydrocolloids* **2014**, *36*, 332-338.
46. Yoon, J.-Y.; Kim, J.-H.; Kim, W.-S., The relationship of interaction forces in the protein adsorption onto polymeric microspheres. *Colloids Surf., A* **1999**, *153* (1-3), 413-419.
47. Kurrat, R.; Prenosil, J.; Ramsden, J., Kinetics of human and bovine serum albumin adsorption at silica–titania surfaces. *J. Colloid Interface Sci.* **1997**, *185* (1), 1-8.
48. Kubiak-Ossowska, K.; Jachimska, B.; Mulheran, P. A., How negatively charged proteins adsorb to negatively charged surfaces: a molecular dynamics study of BSA adsorption on silica. *J. Phys. Chem. B* **2016**, *120* (40), 10463-10468.
49. Givens, B. E.; Xu, Z.; Fiegel, J.; Grassian, V. H., Bovine serum albumin adsorption on SiO₂ and TiO₂ nanoparticle surfaces at circumneutral and acidic pH: a tale of two nano-bio surface interactions. *J. Colloid Interface Sci.* **2017**, *493*, 334-341.
50. Roach, P.; Farrar, D.; Perry, C. C., Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. *J. Am. Chem. Soc.* **2006**, *128* (12), 3939-3945.
51. Goy-López, S.; Juárez, J.; Alatorre-Meda, M.; Casals, E.; Puentes, V. F.; Taboada, P.; Mosquera, V., Physicochemical characteristics of protein–NP bioconjugates: the role of particle curvature and solution conditions on human serum albumin conformation and fibrillogenesis inhibition. *Langmuir* **2012**, *28* (24), 9113-9126.
52. Shrivastava, S.; McCallum, S. A.; Nuffer, J. H.; Qian, X.; Siegel, R. W.; Dordick, J. S., Identifying specific protein residues that guide surface interactions and orientation on silica nanoparticles. *Langmuir* **2013**, *29* (34), 10841-10849.
53. Lundqvist, M.; Sethson, I.; Jonsson, B.-H., Protein adsorption onto silica nanoparticles: conformational changes depend on the particles' curvature and the protein stability. *Langmuir* **2004**, *20* (24), 10639-10647.
54. Cai, K.; Bossert, J.; Jandt, K. D., Does the nanometre scale topography of titanium influence protein adsorption and cell proliferation? *Colloids Surf., B* **2006**, *49* (2), 136-144.

55. Minelli, C.; Kikuta, A.; Tsud, N.; Ball, M. D.; Yamamoto, A., A micro-fluidic study of whole blood behaviour on PMMA topographical nanostructures. *J. Nanobiotechnol.* **2008**, *6* (1), 1-11.
56. Lord, M.; Cousins, B.; Doherty, P.; Whitelock, J.; Simmons, A.; Williams, R.; Milthorpe, B., The effect of silica nanoparticulate coatings on serum protein adsorption and cellular response. *Biomaterials* **2006**, *27* (28), 4856-4862.
57. Rechendorff, K.; Hovgaard, M. B.; Foss, M.; Zhdanov, V.; Besenbacher, F., Enhancement of protein adsorption induced by surface roughness. *Langmuir* **2006**, *22* (26), 10885-10888.
58. Hovgaard, M. B.; Rechendorff, K.; Chevallier, J.; Foss, M.; Besenbacher, F., Fibronectin adsorption on tantalum: the influence of nanoroughness. *J. Phys. Chem. B* **2008**, *112* (28), 8241-8249.
59. Janco, E. W.; Hulander, M.; Andersson, M., Curvature-dependent effects of nanotopography on classical immune complement activation. *Acta Biomater.* **2018**, *74*, 112-120.
60. Yang, Y.; Knust, S.; Schwiderek, S.; Qin, Q.; Yun, Q.; Grundmeier, G.; Keller, A., Protein adsorption at nanorough titanium oxide surfaces: the importance of surface statistical parameters beyond surface roughness. *Nanomaterials* **2021**, *11* (2), 357.
61. Cacciafesta, P.; Hallam, K. R.; Watkinson, A. C.; Allen, G. C.; Miles, M. J.; Jandt, K. D., Visualisation of human plasma fibrinogen adsorbed on titanium implant surfaces with different roughness. *Surf. Sci.* **2001**, *491* (3), 405-420.
62. Lord, M. S.; Foss, M.; Besenbacher, F., Influence of nanoscale surface topography on protein adsorption and cellular response. *Nano Today* **2010**, *5* (1), 66-78.
63. Yang, Q.; Kaul, C.; Ulbricht, M., Anti-nonspecific protein adsorption properties of biomimetic glycocalyx-like glycopolymer layers: effects of glycopolymer chain density and protein size. *Langmuir* **2010**, *26* (8), 5746-5752.
64. Slack, S. M.; Horbett, T. A., The Vroman effect: a critical review. **1995**.
65. Ugwu, S. O.; Apte, S. P., The effect of buffers on protein conformational stability. *Pharm. Technol.* **2004**, *28* (3), 86-109.

66. Park, J. H.; Jackman, J. A.; Ferhan, A. R.; Ma, G. J.; Yoon, B. K.; Cho, N.-J., Temperature-induced denaturation of BSA protein molecules for improved surface passivation coatings. *ACS Appl. Mater. Interfaces* **2018**, *10* (38), 32047-32057.
67. Pace, C. N., Conformational stability of globular proteins. *Trends Biochem. Sci.* **1990**, *15* (1), 14-17.
68. Pace, C. N.; Shirley, B. A.; McNutt, M.; Gajiwala, K., Forces contributing to the conformational stability of proteins. *FASEB J.* **1996**, *10* (1), 75-83.
69. Pace, C. N., Measuring and increasing protein stability. *Trends Biotechnol.* **1990**, *8*, 93-98.
70. Bennion, B. J.; Daggett, V., The molecular basis for the chemical denaturation of proteins by urea. *Proc. Natl. Acad. Sci.* **2003**, *100* (9), 5142-5147.
71. Shirley, B. A., Urea and guanidine hydrochloride denaturation curves. In *Protein Stability and Folding: Theory and Practice*, Shirley, B. A., Ed. Humana Press: Totowa, NJ, 1995; pp 177-190.
72. Monera, O. D.; Kay, C. M.; Hodges, R. S., Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. *Protein Sci.* **1994**, *3* (11), 1984-1991.
73. Griebenow, K.; Klibanov, A. M., On protein denaturation in aqueous-organic mixtures but not in pure organic solvents. *J. Am. Chem. Soc.* **1996**, *118* (47), 11695-11700.
74. Magsumov, T.; Ziyang, L.; Sedov, I., Comparative study of the protein denaturing ability of different organic cosolvents. *Int. J. Biol. Macromol.* **2020**, *160*, 880-888.
75. Militello, V.; Vetri, V.; Leone, M., Conformational changes involved in thermal aggregation processes of bovine serum albumin. *Biophys. Chem.* **2003**, *105* (1), 133-141.
76. Leuenberger, P.; Ganscha, S.; Kahraman, A.; Cappelletti, V.; Boersema, P. J.; von Mering, C.; Claassen, M.; Picotti, P., Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. *Science* **2017**, *355* (6327).
77. Roach, P.; Farrar, D.; Perry, C. C., Interpretation of protein adsorption: surface-induced conformational changes. *J. Am. Chem. Soc.* **2005**, *127* (22), 8168-8173.

78. Norde, W.; Favier, J. P., Structure of adsorbed and desorbed proteins. *Colloids Surf.* **1992**, *64* (1), 87-93.
79. Haynes, C. A.; Norde, W., Structures and stabilities of adsorbed proteins. *J. Colloid Interface Sci.* **1995**, *169* (2), 313-328.
80. Norde, W., My voyage of discovery to proteins in flatland... and beyond. *Colloids Surf., B* **2007**, *61* (1), 1-9.
81. Billsten, P.; Wahlgren, M.; Arnebrant, T.; McGuire, J.; Elwing, H., Structural changes of T4 lysozyme upon adsorption to silica nanoparticles measured by circular dichroism. *J. Colloid Interface Sci.* **1995**, *175* (1), 77-82.
82. McGuire, J.; Krisdhasima, V.; Wahlgren, M. C.; Arnebrant, T., Comparative adsorption studies with synthetic, structural stability and charge mutants of bacteriophage T4 lysozyme. In *Proteins at Interfaces II*, American Chemical Society: 1995; Vol. 602, pp 52-65.
83. Karlsson, M.; Mårtensson, L.-G.; Jonsson, B.-H.; Carlsson, U., Adsorption of human carbonic anhydrase II variants to silica nanoparticles occur stepwise: binding is followed by successive conformational changes to a molten-globule-like state. *Langmuir* **2000**, *16* (22), 8470-8479.
84. Karlsson, M.; Ekeröth, J.; Elwing, H.; Carlsson, U., Reduction of irreversible protein adsorption on solid surfaces by protein engineering for increased stability. *J. Biol. Chem.* **2005**, *280* (27), 25558-25564.
85. Park, J.; Sut, T.; Jackman, J.; Ferhan, A.; Yoon, B.; Cho, N., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19*.
86. Shaw, K. L.; Scholtz, J. M.; Pace, C. N.; Grimsley, R. G., Determining the conformational stability of a protein using urea denaturation curves. In *Protein Structure, Stability, and Interactions*, Shriver, J. W., Ed. Humana Press: Totowa, NJ, 2009; pp 41-55.
87. Privalov, P. L., Stability of proteins small globular proteins. *Adv. Protein Chem.* **1979**, *33*, 167-241.

88. Hua, L.; Zhou, R.; Thirumalai, D.; Berne, B., Urea denaturation by stronger dispersion interactions with proteins than water implies a 2-stage unfolding. *Proc. Natl. Acad. Sci.* **2008**, *105* (44), 16928-16933.
89. A Qasim, M.; Taha, M., Investigation of the mechanism of protein denaturation by guanidine hydrochloride-induced dissociation of inhibitor-protease complexes. *Protein Pept. Lett.* **2013**, *20* (2), 187-191.
90. Huerta-Viga, A.; Woutersen, S., Protein denaturation with guanidinium: a 2D-IR study. *J. Phys. Chem. Lett.* **2013**, *4* (20), 3397-3401.
91. Heyda, J.; Okur, H. I.; Hladílková, J.; Rembert, K. B.; Hunn, W.; Yang, T.; Dzubiella, J.; Jungwirth, P.; Cremer, P. S., Guanidinium can both cause and prevent the hydrophobic collapse of biomacromolecules. *J. Am. Chem. Soc.* **2017**, *139* (2), 863-870.
92. Gupta, R.; Yadav, S.; Ahmad, F., Protein stability: urea-induced versus guanidine-induced unfolding of metmyoglobin. *Biochemistry* **1996**, *35* (36), 11925-11930.
93. Benjwal, S.; Verma, S.; Röhm, K. H.; Gursky, O., Monitoring protein aggregation during thermal unfolding in circular dichroism experiments. *Protein Sci.* **2006**, *15* (3), 635-639.
94. Greenfield, N. J., Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nat. Protoc.* **2006**, *1* (6), 2527.
95. Murayama, K.; Tomida, M., Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy. *Biochemistry* **2004**, *43* (36), 11526-11532.
96. Freire, E., Thermal denaturation methods in the study of protein folding. In *Methods in Enzymology*, Academic Press: 1995; Vol. 259, pp 144-168.
97. Costanzo, J. A.; O'Brien, C. J.; Tiller, K.; Tamargo, E.; Robinson, A. S.; Roberts, C. J.; Fernandez, E. J., Conformational stability as a design target to control protein aggregation. *Protein Eng., Des. Sel.* **2014**, *27* (5), 157-167.
98. Anand, S.; Ganaie, A. A.; Sharma, C., Differential thermal stability, conformational stability and unfolding behavior of Eis proteins from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. *PLoS One* **2019**, *14* (3), e0213933.

99. Philo, J. S.; Arakawa, T., Mechanisms of protein aggregation. *Curr. Pharm. Biotechnol.* **2009**, *10* (4), 348-351.
100. Ahrer, K.; Buchacher, A.; Iberer, G.; Jungbauer, A., Thermodynamic stability and formation of aggregates of human immunoglobulin G characterised by differential scanning calorimetry and dynamic light scattering. *J. Biochem. Biophys. Methods* **2006**, *66* (1), 73-86.
101. Nobbmann, U.; Connah, M.; Fish, B.; Varley, P.; Gee, C.; Mulot, S.; Chen, J.; Zhou, L.; Lu, Y.; Sheng, F.; Yi, J.; Harding, S. E., Dynamic light scattering as a relative tool for assessing the molecular integrity and stability of monoclonal antibodies. *Biotechnol. Genet. Eng. Rev.* **2007**, *24* (1), 117-128.
102. Samra, H. S.; He, F., Advancements in high throughput biophysical technologies: applications for characterization and screening during early formulation development of monoclonal antibodies. *Mol. Pharmaceutics* **2012**, *9* (4), 696-707.
103. Bechtel, W. J.; Schellman, J. A., Protein stability curves. *Biopolymers* **1987**, *26* (11), 1859-1877.
104. Donovan, J. W., Changes in ultraviolet absorption produced by alteration of protein conformation. *J. Biol. Chem.* **1969**, *244* (8), 1961-1967.
105. Poklar, N.; Vesnaver, G., Thermal denaturation of proteins studied by UV spectroscopy. *J. Chem. Educ.* **2000**, *77* (3), 380.
106. Schmid, F. X., Biological macromolecules: UV-visible spectrophotometry. *eLS* **2001**.
107. Nikolaidis, A.; Moschakis, T., Studying the denaturation of bovine serum albumin by a novel approach of difference-UV analysis. *Food Chem.* **2017**, *215*, 235-244.
108. Garidel, P.; Hegyi, M.; Bassarab, S.; Weichel, M., A rapid, sensitive and economical assessment of monoclonal antibody conformational stability by intrinsic tryptophan fluorescence spectroscopy. *Biotechnol. J.* **2008**, *3* (9-10), 1201-1211.
109. Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*. Springer Science & Business Media: 2013.

110. Patra, M.; Mukhopadhyay, C.; Chakrabarti, A., Probing conformational stability and dynamics of erythroid and nonerythroid spectrin: effects of urea and guanidine hydrochloride. *PLoS One* **2015**, *10* (1), e0116991.
111. Patel, S.; Chaffotte, A. F.; Goubard, F.; Pauthe, E., Urea-induced sequential unfolding of fibronectin: a fluorescence spectroscopy and circular dichroism study. *Biochemistry* **2004**, *43* (6), 1724-1735.
112. Yao, H.; Wynendaele, E.; De Spiegeleer, B., Thermal sensitivity as a quality control attribute for biotherapeutics: The L-asparaginase case. *Drug Test. Anal.* **2020**, *12* (1), 67-77.
113. Grimsley, G. R.; Trevino, S. R.; Thurlkill, R. L.; Scholtz, J. M., Determining the conformational stability of a protein from urea and thermal unfolding curves. *Curr. Protoc. Protein Sci.* **2013**, *71* (1), 28.4. 1-28.4. 14.
114. Matheus, S.; Friess, W.; Mahler, H.-C., FTIR and nDSC as analytical tools for high-concentration protein formulations. *Pharm. Res.* **2006**, *23* (6), 1350-1363.
115. Haris, P. I.; Severcan, F., FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *J. Mol. Catal. B: Enzym.* **1999**, *7* (1-4), 207-221.
116. From, N. B.; Bowler, B. E., Urea denaturation of staphylococcal nuclease monitored by Fourier transform infrared spectroscopy. *Biochemistry* **1998**, *37* (6), 1623-1631.
117. González, M.; Bagatolli, L. A.; Echabe, I.; Arrondo, J. L.; Argaraña, C. E.; Cantor, C. R.; Fidelio, G. D., Interaction of biotin with streptavidin: thermostability and conformational changes upon binding. *J. Biol. Chem.* **1997**, *272* (17), 11288-11294.
118. Natalello, A.; Ami, D.; Brocca, S.; Lotti, M.; Doglia, S. M., Secondary structure, conformational stability and glycosylation of a recombinant *Candida rugosa* lipase studied by Fourier-transform infrared spectroscopy. *Biochem. J.* **2005**, *385* (2), 511-517.
119. Michnik, A.; Michalik, K.; Kluczevska, A.; Drzazga, Z., Comparative DSC study of human and bovine serum albumin. *J. Therm. Anal. Calorim.* **2005**, *84* (1), 113-117.

120. Wen, J.; Arthur, K.; Chemmalil, L.; Muzammil, S.; Gabrielson, J.; Jiang, Y., Applications of differential scanning calorimetry for thermal stability analysis of proteins: qualification of DSC. *J. Pharm. Sci.* **2012**, *101* (3), 955-964.
121. Prabhu, N. V.; Sharp, K. A., Heat capacity in proteins. *Annu. Rev. Phys. Chem.* **2005**, *56*, 521-548.
122. Norde, W.; Lyklema, J., The adsorption of human plasma albumin and bovine pancreas ribonuclease at negatively charged polystyrene surfaces: I. Adsorption isotherms. Effects of charge, ionic strength, and temperature. *J. Colloid Interface Sci.* **1978**, *66* (2), 257-265.
123. Norde, W.; MacRitchie, F.; Nowicka, G.; Lyklema, J., Protein adsorption at solid-liquid interfaces: reversibility and conformation aspects. *J. Colloid Interface Sci.* **1986**, *112* (2), 447-456.
124. Fair, B. D.; Jamieson, A. M., Studies of protein adsorption on polystyrene latex surfaces. *J. Colloid Interface Sci.* **1980**, *77* (2), 525-534.
125. Olson, B. J. S. C.; Markwell, J., Assays for determination of protein concentration. *Curr. Protoc. Protein Sci.* **2007**, *48* (1), 3.4.1-3.4.29.
126. Elwing, H., Protein absorption and ellipsometry in biomaterial research. *Biomaterials* **1998**, *19* (4), 397-406.
127. Seitz, R.; Brings, R.; Geiger, R., Protein adsorption on solid-liquid interfaces monitored by laser-ellipsometry. *Appl. Surf. Sci.* **2005**, *252* (1), 154-157.
128. Malmsten, M., Ellipsometry studies of the effects of surface hydrophobicity on protein adsorption. *Colloids Surf., B* **1995**, *3* (5), 297-308.
129. Liedberg, B.; Nylander, C.; Lunström, I., Surface plasmon resonance for gas detection and biosensing. *Sens. Actuators* **1983**, *4*, 299-304.
130. Liedberg, B.; Nylander, C.; Lundström, I., Biosensing with surface plasmon resonance—how it all started. *Biosens. Bioelectron.* **1995**, *10* (8), i-ix.
131. Green, R.; Davies, J.; Davies, M.; Roberts, C.; Tendler, S., Surface plasmon resonance for real time in situ analysis of protein adsorption to polymer surfaces. *Biomaterials* **1997**, *18* (5), 405-413.
132. Jackman, J. A.; Ferhan, A. R.; Cho, N.-J., Nanoplasmonic sensors for biointerfacial science. *Chem. Soc. Rev.* **2017**, *46* (12), 3615-3660.

133. Frost, R.; Wadell, C.; Hellman, A.; Molander, S.; Svedhem, S.; Persson, M.; Langhammer, C., Core-shell nanoplasmonic sensing for characterization of biocorona formation and nanoparticle surface interactions. *ACS Sensors* **2016**, *1* (6), 798-806.
134. Laatikainen, M.; Lindström, M., Determination of adsorption isotherms with quartz crystal microbalance in liquid phase. *J. Colloid Interface Sci.* **1988**, *125* (2), 610-614.
135. Rodahl, M.; Höök, F.; Fredriksson, C.; Keller, C. A.; Krozer, A.; Brzezinski, P.; Voinova, M.; Kasemo, B., Simultaneous frequency and dissipation factor QCM measurements of biomolecular adsorption and cell adhesion. *Faraday Discuss.* **1997**, *107*, 229-246.
136. Höök, F.; Rodahl, M.; Brzezinski, P.; Kasemo, B., Energy dissipation kinetics for protein and antibody- antigen adsorption under shear oscillation on a quartz crystal microbalance. *Langmuir* **1998**, *14* (4), 729-734.
137. Höök, F.; Kasemo, B.; Nylander, T.; Fant, C.; Sott, K.; Elwing, H., Variations in coupled water, viscoelastic properties, and film thickness of a Mefp-1 protein film during adsorption and cross-linking: a quartz crystal microbalance with dissipation monitoring, ellipsometry, and surface plasmon resonance study. *Anal. Chem.* **2001**, *73* (24), 5796-5804.
138. Vörös, J., The density and refractive index of adsorbing protein layers. *Biophys. J.* **2004**, *87* (1), 553-561.
139. Giacomelli, C. E.; Norde, W., The adsorption-desorption cycle. Reversibility of the BSA-silica system. *J. Colloid Interface Sci.* **2001**, *233* (2), 234-240.
140. Chittur, K. K., FTIR/ATR for protein adsorption to biomaterial surfaces. *Biomaterials* **1998**, *19* (4-5), 357-369.
141. Zeng, H.; Chittur, K. K.; Lacefield, W. R., Analysis of bovine serum albumin adsorption on calcium phosphate and titanium surfaces. *Biomaterials* **1999**, *20* (4), 377-384.
142. Giacomelli, C. E.; Bremer, M. G.; Norde, W., ATR-FTIR study of IgG adsorbed on different silica surfaces. *J. Colloid Interface Sci.* **1999**, *220* (1), 13-23.

143. Ferhan, A. R.; Jackman, J. A.; Sut, T. N.; Cho, N.-J., Quantitative comparison of protein adsorption and conformational changes on dielectric-coated nanoplasmonic sensing arrays. *Sensors* **2018**, *18* (4).
144. Ferhan, A. R.; Spackova, B.; Jackman, J. A.; Ma, G. J.; Sut, T. N.; Homola, J.; Cho, N.-J., Nanoplasmonic ruler for measuring separation distance between supported lipid bilayers and oxide surfaces. *Anal. Chem.* **2018**, *90* (21), 12503-12511.
145. Ramos, O. L.; Pereira, R. N.; Rodrigues, R.; Teixeira, J. A.; Vicente, A. A.; Xavier Malcata, F., Physical effects upon whey protein aggregation for nano-coating production. *Food Res. Int.* **2014**, *66*, 344-355.
146. Vahedikia, N.; Garavand, F.; Tajeddin, B.; Cacciotti, I.; Jafari, S. M.; Omid, T.; Zahedi, Z., Biodegradable zein film composites reinforced with chitosan nanoparticles and cinnamon essential oil: physical, mechanical, structural and antimicrobial attributes. *Colloids Surf., B* **2019**, *177*, 25-32.
147. Silva-Bermudez, P.; Rodil, S., An overview of protein adsorption on metal oxide coatings for biomedical implants. *Surf. Coat. Technol.* **2013**, *233*, 147-158.
148. Spicer, C. D.; Jumeaux, C.; Gupta, B.; Stevens, M. M., Peptide and protein nanoparticle conjugates: versatile platforms for biomedical applications. *Chem. Soc. Rev.* **2018**, *47* (10), 3574-3620.
149. Hucknall, A.; Rangarajan, S.; Chilkoti, A., In pursuit of zero: polymer brushes that resist the adsorption of proteins. *Adv. Mater.* **2009**, *21* (23), 2441-2446.
150. Wei, Q.; Becherer, T.; Angioletti-Uberti, S.; Dzubiella, J.; Wischke, C.; Neffe, A. T.; Lendlein, A.; Ballauff, M.; Haag, R., Protein interactions with polymer coatings and biomaterials. *Angew. Chem. Int. Edit.* **2014**, *53* (31), 8004-8031.
151. Gibbs, J., Effective blocking procedures. *ELISA Technical Bull* **2001**, *3*, 1-6.
152. Mahmood, T.; Yang, P.-C., Western blot: technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* **2012**, *4* (9), 429.
153. Wisniewski, N.; Reichert, M., Methods for reducing biosensor membrane biofouling. *Colloids Surf., B* **2000**, *18* (3-4), 197-219.
154. Liu, N.; Xu, Z.; Morrin, A.; Luo, X., Low fouling strategies for electrochemical biosensors targeting disease biomarkers. *Anal. Methods* **2019**, *11* (6), 702-711.

155. Riquelme, M. V.; Zhao, H.; Srinivasaraghavan, V.; Pruden, A.; Vikesland, P.; Agah, M., Optimizing blocking of nonspecific bacterial attachment to impedimetric biosensors. *Sens. Biosens. Res.* **2016**, *8*, 47-54.
156. Park, S.; Kim, M.; Kim, D.; Kang, S. H.; Lee, K. H.; Jeong, Y., Interfacial charge regulation of protein blocking layers in transistor biosensor for direct measurement in serum. *Biosens. Bioelectron.* **2020**, *147*, 111737.
157. Ravalli, A.; da Rocha, C. G.; Yamanaka, H.; Marrazza, G., A label-free electrochemical affisensor for cancer marker detection: the case of HER2. *Bioelectrochemistry* **2015**, *106*, 268-275.
158. del Río, J. S.; Henry, O. Y.; Jolly, P.; Ingber, D. E., An antifouling coating that enables affinity-based electrochemical biosensing in complex biological fluids. *Nat. Nanotechnol.* **2019**, *14* (12), 1143-1149.
159. Ma, C.; Wu, B.; Zhang, G., Protein–protein resistance investigated by quartz crystal microbalance. *Colloids Surf., B* **2013**, *104*, 5-10.
160. Pillai, S.; Arpanaei, A.; Meyer, R. L.; Birkedal, V.; Gram, L.; Besenbacher, F.; Kingshott, P., Preventing protein adsorption from a range of surfaces using an aqueous fish protein extract. *Biomacromolecules* **2009**, *10* (10), 2759-2766.
161. Cruz-Teran, C. A.; Carlin, K. B.; Efimenko, K.; Genzer, J.; Rao, B. M., Targeted mutagenesis and combinatorial library screening enables control of protein orientation on surfaces and increased activity of adsorbed proteins. *Langmuir* **2016**, *32* (34), 8660-8667.
162. Tiruthani, K.; Mischler, A.; Ahmed, S.; Mahinthakumar, J.; Haugh, J. M.; Rao, B. M., Design and evaluation of engineered protein biosensors for live-cell imaging of EGFR phosphorylation. *Sci. Signaling* **2019**, *12* (584).
163. Wei, Q.; Li, B.; Yi, N.; Su, B.; Yin, Z.; Zhang, F.; Li, J.; Zhao, C., Improving the blood compatibility of material surfaces via biomolecule-immobilized mussel-inspired coatings. *J. Biomed. Mater. Res., Part A* **2011**, *96* (1), 38-45.
164. Hu, X.; Tian, J.; Li, C.; Su, H.; Qin, R.; Wang, Y.; Cao, X.; Yang, P., Amyloid-like protein aggregates: a new class of bioinspired materials merging an interfacial anchor with antifouling. *Adv. Mater.* **2020**, *32* (23), 2000128.

165. Raphel, J.; Karlsson, J.; Galli, S.; Wennerberg, A.; Lindsay, C.; Haugh, M. G.; Pajarinen, J.; Goodman, S. B.; Jimbo, R.; Andersson, M., Engineered protein coatings to improve the osseointegration of dental and orthopaedic implants. *Biomaterials* **2016**, *83*, 269-282.
166. Zhang, D.; Chen, Q.; Shi, C.; Chen, M.; Ma, K.; Wan, J.; Liu, R., Dealing with the foreign-body response to implanted biomaterials: strategies and applications of new materials. *Adv. Funct. Mater.* **2021**, *31* (6), 2007226.
167. Sakala, G. P.; Reches, M., Peptide-based approaches to fight biofouling. *Adv. Mater. Interfaces* **2018**, *5* (18), 1800073.
168. Jo, Y. K.; Seo, J. H.; Choi, B.-H.; Kim, B. J.; Shin, H. H.; Hwang, B. H.; Cha, H. J., Surface-independent antibacterial coating using silver nanoparticle-generating engineered mussel glue. *ACS Appl. Mater. Interfaces* **2014**, *6* (22), 20242-20253.
169. Acosta, S.; Ibañez-Fonseca, A.; Aparicio, C.; Rodríguez-Cabello, J. C., Antibiofilm coatings based on protein-engineered polymers and antimicrobial peptides for preventing implant-associated infections. *Biomater. Sci.* **2020**, *8* (10), 2866-2877.
170. Gu, J.; Su, Y.; Liu, P.; Li, P.; Yang, P., An environmentally benign antimicrobial coating based on a protein supramolecular assembly. *ACS Appl. Mater. Interfaces* **2017**, *9* (1), 198-210.
171. Derde, M.; Vié, V.; Walrant, A.; Sagan, S.; Lechevalier, V.; Guérin-Dubiard, C.; Pezennec, S.; Cochet, M. F.; Paboeuf, G.; Pasco, M., Antimicrobial activity of lysozyme isoforms: key molecular features. *Biopolymers* **2017**, *107* (12), e23040.
172. Beaussart, A.; Retourney, C.; Quilès, F.; Morais, R. D. S.; Gaiani, C.; Fiérobe, H.-P.; El-Kirat-Chatel, S., Supported lysozyme for improved antimicrobial surface protection. *J. Colloid Interface Sci.* **2021**, *582*, 764-772.
173. Bayazidi, P.; Almasi, H.; Asl, A. K., Immobilization of lysozyme on bacterial cellulose nanofibers: characteristics, antimicrobial activity and morphological properties. *Int. J. Biol. Macromol.* **2018**, *107*, 2544-2551.

174. Buruaga-Ramiro, C.; Valenzuela, S. V.; Valls, C.; Roncero, M. B.; Pastor, F. J.; Díaz, P.; Martinez, J., Development of an antimicrobial bioactive paper made from bacterial cellulose. *Int. J. Biol. Macromol.* **2020**, *158*, 587-594.
175. Sarkar, S.; Gulati, K.; Mishra, A.; Poluri, K. M., Protein nanocomposites: special inferences to lysozyme based nanomaterials. *Int. J. Biol. Macromol.* **2020**, *151*, 467-482.
176. Das, S.; Pabba, M.; Dhushyandhun, M. E.; Patra, C. R., Theranostic Applications of Lysozyme-Based Nanoparticles. In *Nanoparticles in Medicine*, Shukla, A. K., Ed. Springer Singapore: Singapore, 2020; pp 1-23.
177. Cagliani, R.; Gatto, F.; Bardi, G., Protein adsorption: a feasible method for nanoparticle functionalization? *Materials* **2019**, *12* (12), 1991.
178. Lundqvist, M.; Stigler, J.; Cedervall, T.; Berggård, T.; Flanagan, M. B.; Lynch, I.; Elia, G.; Dawson, K., The evolution of the protein corona around nanoparticles: a test study. *ACS Nano* **2011**, *5* (9), 7503-7509.
179. Lynch, I.; Dawson, K. A., Protein-nanoparticle interactions. *Nano Today* **2008**, *3* (1-2), 40-47.
180. Szebeni, J., Complement activation-related pseudoallergy: a stress reaction in blood triggered by nanomedicines and biologicals. *Mol. Immunol.* **2014**, *61* (2), 163-173.
181. Peng, Q.; Zhang, S.; Yang, Q.; Zhang, T.; Wei, X.-Q.; Jiang, L.; Zhang, C.-L.; Chen, Q.-M.; Zhang, Z.-R.; Lin, Y.-F., Preformed albumin corona, a protective coating for nanoparticles based drug delivery system. *Biomaterials* **2013**, *34* (33), 8521-8530.
182. Belling, J. N.; Jackman, J. A.; Yorulmaz Avsar, S.; Park, J. H.; Wang, Y.; Potroz, M. G.; Ferhan, A. R.; Weiss, P. S.; Cho, N.-J., Stealth immune properties of graphene oxide enabled by surface-bound complement Factor H. *ACS Nano* **2016**, *10* (11), 10161-10172.
183. Park, J. H.; Jackman, J. A.; Ferhan, A. R.; Belling, J. N.; Mokrzecka, N.; Weiss, P. S.; Cho, N.-J., Cloaking silica nanoparticles with functional protein coatings for reduced complement activation and cellular uptake. *ACS Nano* **2020**, *14* (9), 11950-11961.

184. Schöttler, S.; Becker, G.; Winzen, S.; Steinbach, T.; Mohr, K.; Landfester, K.; Mailänder, V.; Wurm, F. R., Protein adsorption is required for stealth effect of poly (ethylene glycol)- and poly(phosphoester)-coated nanocarriers. *Nat. Nanotechnol.* **2016**, *11* (4), 372.
185. Fam, S. Y.; Chee, C. F.; Yong, C. Y.; Ho, K. L.; Mariatulqabtiah, A. R.; Tan, W. S., Stealth coating of nanoparticles in drug-delivery systems. *Nanomaterials* **2020**, *10* (4), 787.
186. Chakraborty, D.; Ethiraj, K.; Mukherjee, A., Understanding the relevance of protein corona in nanoparticle-based therapeutics and diagnostics. *RSC Adv.* **2020**, *10* (45), 27161-27172.
187. Jackman, J. A.; Yoon, B. K.; Ouyang, L.; Wang, N.; Ferhan, A. R.; Kim, J.; Majima, T.; Cho, N. J., Biomimetic nanomaterial strategies for virus targeting: Antiviral therapies and vaccines. *Adv. Funct. Mater.* **2020**, 2008352.
188. Sun, W.; Lu, Y.; Gu, Z., Advances in anticancer protein delivery using micro-/nanoparticles. *Part. Part. Syst. Charact.* **2014**, *31* (12), 1204-1222.
189. Sugio, S.; Kashima, A.; Mochizuki, S.; Noda, M.; Kobayashi, K., Crystal structure of human serum albumin at 2.5 Å resolution. *Protein Eng.* **1999**, *12* (6), 439-446.
190. Bujacz, A., Structures of bovine, equine and leporine serum albumin. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2012**, *68* (10), 1278-1289.
191. Majorek, K. A.; Porebski, P. J.; Dayal, A.; Zimmerman, M. D.; Jablonska, K.; Stewart, A. J.; Chruszcz, M.; Minor, W., Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. *Mol. Immunol.* **2012**, *52* (3-4), 174-182.
192. Bujacz, A.; Talaj, J. A.; Zielinski, K.; Pietrzyk-Brzezinska, A. J.; Neumann, P., Crystal structures of serum albumins from domesticated ruminants and their complexes with 3, 5-diiodosalicylic acid. *Acta Crystallogr., Sect. D: Struct. Biol.* **2017**, *73* (11), 896-909.
193. Carter, D. C.; Ho, J. X., Structure of serum albumin. In *Advances in Protein Chemistry*, Elsevier: 1994; Vol. 45, pp 153-203.
194. Peters Jr, T., *All About Albumin: Biochemistry, Genetics, and Medical Applications*. Academic press: 1995.

195. Bal, W.; Sokołowska, M.; Kurowska, E.; Faller, P., Binding of transition metal ions to albumin: sites, affinities and rates. *Biochim. Biophys. Acta, Gen. Subj.* **2013**, *1830* (12), 5444-5455.
196. Fanali, G.; Di Masi, A.; Trezza, V.; Marino, M.; Fasano, M.; Ascenzi, P., Human serum albumin: from bench to bedside. *Mol. Aspects Med.* **2012**, *33* (3), 209-290.
197. Kragh-Hansen, U., Molecular aspects of ligand binding to serum albumin. *Pharmacol. Rev.* **1981**, *33* (1), 17-53.
198. Kosa, T.; Maruyama, T.; Otagiri, M., Species differences of serum albumins: I. Drug binding sites. *Pharm. Res.* **1997**, *14* (11), 1607-1612.
199. Radibratovic, M.; Minic, S.; Stanic-Vucinic, D.; Nikolic, M.; Milcic, M.; Velickovic, T. C., Stabilization of human serum albumin by the binding of phycocyanobilin, a bioactive chromophore of blue-green alga spirulina: molecular dynamics and experimental study. *PloS One* **2016**, *11* (12), e0167973.
200. Barreca, D.; Laganà, G.; Toscano, G.; Calandra, P.; Kiselev, M. A.; Lombardo, D.; Bellocco, E., The interaction and binding of flavonoids to human serum albumin modify its conformation, stability and resistance against aggregation and oxidative injuries. *Biochim. Biophys. Acta, Gen. Subj.* **2017**, *1861* (1), 3531-3539.
201. Ahmad, B.; Parveen, S.; Khan, R. H., Effect of albumin conformation on the binding of ciprofloxacin to human serum albumin: a novel approach directly assigning binding site. *Biomacromolecules* **2006**, *7* (4), 1350-1356.
202. Kosa, T.; Maruyama, T.; Otagiri, M., Species differences of serum albumins: II. Chemical and thermal stability. *Pharm. Res.* **1998**, *15* (3), 449-454.
203. Iafisco, M.; Sabatino, P.; Lesci, I. G.; Prat, M.; Rimondini, L.; Roveri, N., Conformational modifications of serum albumins adsorbed on different kinds of biomimetic hydroxyapatite nanocrystals. *Colloids Surf., B* **2010**, *81* (1), 274-284.
204. Voicescu, M.; Ionescu, S.; Angelescu, D. G., Spectroscopic and coarse-grained simulation studies of the BSA and HSA protein adsorption on silver nanoparticles. *J. Nanopart. Res.* **2012**, *14* (10), 1174.
205. Kubiak-Ossowska, K.; Tokarczyk, K.; Jachimska, B.; Mulheran, P. A., Bovine serum albumin adsorption at a silica surface explored by simulation and experiment. *J. Phys. Chem. B* **2017**, *121* (16), 3975-3986.

206. Foster, J. F., Some aspects of the structure and conformational properties of serum albumin. In *Albumin: Structure, Function and Uses*, Elsevier: 1977; pp 53-84.
207. Jachimska, B.; Pajor, A., Physico-chemical characterization of bovine serum albumin in solution and as deposited on surfaces. *Bioelectrochemistry* **2012**, *87*, 138-146.
208. Wallevik, K., Reversible denaturation of human serum albumin by pH, temperature, and guanidine hydrochloride followed by optical rotation. *J. Biol. Chem.* **1973**, *248* (8), 2650-2655.
209. Shrake, A.; Finlayson, J.; Ross, P., Thermal stability of human albumin measured by differential scanning calorimetry: I. effects of caprylate and N-acetyltryptophanate. *Vox Sang.* **1984**, *47* (1), 7-18.
210. Su, R.; Qi, W.; He, Z.; Zhang, Y.; Jin, F., Multilevel structural nature and interactions of bovine serum albumin during heat-induced aggregation process. *Food Hydrocolloids* **2008**, *22* (6), 995-1005.
211. Wertz, C. F.; Santore, M. M., Effect of surface hydrophobicity on adsorption and relaxation kinetics of albumin and fibrinogen: single-species and competitive behavior. *Langmuir* **2001**, *17* (10), 3006-3016.
212. Jeyachandran, Y.; Mielczarski, E.; Rai, B.; Mielczarski, J., Quantitative and qualitative evaluation of adsorption/desorption of bovine serum albumin on hydrophilic and hydrophobic surfaces. *Langmuir* **2009**, *25* (19), 11614-11620.
213. Rodler, A.; Beyer, B.; Ueberbacher, R.; Hahn, R.; Jungbauer, A., Hydrophobic interaction chromatography of proteins: studies of unfolding upon adsorption by isothermal titration calorimetry. *J. Sep. Sci.* **2018**.
214. Kendrick, D. B., *Blood Program in World War II*. Office of the Surgeon General, Department of the Army: 1964.
215. Cohn, E. J.; Strong, L. E.; Hughes, W.; Mulford, D.; Ashworth, J.; Melin, M.; Taylor, H., Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.* **1946**, *68* (3), 459-475.
216. Denizli, A., Plasma fractionation: conventional and chromatographic methods for albumin purification. *Hacettepe J. Biol. Chem.* **2011**, *39* (4), 315-341.

217. Sigma-Aldrich Bovine Serum Albumins. <https://www.sigmaaldrich.com/catalog/substance/bovineserumalbumin12345904846811?lang=en®ion=SG> (accessed Apr 2021).
218. Gemini Bio BSA: Cohn Cold EtOH vs. Heat-Shock. <https://www.gembio.com/post/bsa-cohn-cold-etoh-vs-heat-shock> (accessed Apr 2021).
219. Equitech-Bio Bovine Serum Albumin. <https://www.equitech-bio.com/product-tag/bovine-serum-albumin/> (accessed Apr 2021).
220. Burnouf, T., Modern plasma fractionation. *Transfus. Med. Rev.* **2007**, *21* (2), 101-117.
221. Hao, Y. I., A simple method for the preparation of human serum albumin 1. *Vox Sang.* **1979**, *36* (5), 313-320.
222. Tanaka, K.; Shigueoka, E.; Sawatani, E.; Dias, G.; Arashiro, F.; Campos, T.; Nakao, H., Purification of human albumin by the combination of the method of Cohn with liquid chromatography. *Braz. J. Med. Biol. Res.* **1998**, *31* (11), 1383-1388.
223. Rocky Mountain Biologicals Bovine Serum Albumin Standard Heat Shock - BSA. <https://www.rmbio.com/bovine-serum-albumin-heat-shock> (accessed Apr 2021).
224. Bovogen Biologicals Purified Proteins Bovine Serum Albumin Bovostar. <https://bovogen.com/our-products/purified-proteins/bovine-serum-albumin/> (accessed Apr 2021).
225. Equitech-Bio Heat Shock BSA. <https://www.equitech-bio.com/albumins/bsa-bovine-serum-albumin/heat-shock-bsa.html> (accessed 1 Feb 2019).
226. Reid, A. F. Method of purifying albumin. 2,705,230, 1955.
227. Ballou, G. A.; Boyer, P. D.; Luck, J. M.; Lum, F. G., The heat coagulation of human serum albumin. *J. Biol. Chem.* **1944**, *153* (2), 589-605.
228. Boyer, P. D.; Lum, F. G.; Ballou, G. A.; Luck, J. M.; Rice, R. G., The combination of fatty acids and related compounds with serum albumin I. stabilization against heat denaturation. *J. Biol. Chem.* **1946**, *162* (2), 181-198.

229. Boyer, P. D.; Ballou, G. A.; Luck, J. M., The combination of fatty acids and related compounds with serum albumin II. Stabilization against urea and guanidine denaturation. *J. Biol. Chem.* **1946**, *162* (2), 199-208.
230. Boyer, P. D.; Ballou, G. A.; Luck, J. M., The combination of fatty acids and related compounds with serum albumin III. The nature and extent of the combination. *J. Biol. Chem.* **1947**, *167* (2), 407-424.
231. Schneider, W.; Lefevre, H.; Fiedler, H.; McCarty, L. J., An alternative method of large scale plasma fractionation for the isolation of serum albumin. *Blut* **1975**, *30* (2), 121-134.
232. Chen, R. F., Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **1967**, *242* (2), 173-181.
233. Mannuzza, F. J.; Montalto, J. G., Is bovine albumin too complex to be just a commodity? *BioProcess Int.* **2010**, *8* (4), 42-49.
234. Michnik, A., Thermal stability of bovine serum albumin DSC study. *J. Therm. Anal. Calorim.* **2003**, *71* (2), 509-519.
235. Pitt, W.; Cooper, S., Albumin adsorption on alkyl chain derivatized polyurethanes: I. The effect of C-18 alkylation. *J. Biomed. Mater. Res.* **1988**, *22* (5), 359-382.
236. Pitt, W.; Grasel, T.; Cooper, S., Albumin adsorption on alkyl chain derivatized polyurethanes: II. The effect of alkyl chain length. *Biomaterials* **1988**, *9* (1), 36-46.
237. Xiao, Y.; Isaacs, S. N., Enzyme-linked immunosorbent assay (ELISA) and blocking with bovine serum albumin (BSA)—not all BSAs are alike. *J. Immunol. Methods* **2012**, *384* (1-2), 148-151.
238. Brokke, P.; Dankert, J.; Carballo, J.; Feijen, J., Adherence of coagulase-negative staphylococci onto polyethylene catheters in vitro and in vivo: a study on the influence of various plasma proteins. *J. Biomater. Appl.* **1991**, *5* (3), 204-226.
239. Marois, Y.; Chakfé, N.; Guidoin, R.; Duhamel, R. C.; Roy, R.; Marois, M.; King, M. W.; Douville, Y., An albumin-coated polyester arterial graft: in vivo assessment of biocompatibility and healing characteristics. *Biomaterials* **1996**, *17* (1), 3-14.
240. Kinnari, T. J.; Rihkanen, H.; Laine, T.; Salonen, E. M.; Jero, J., Role of albumin coating of tympanostomy tubes: long-term clinical evaluation. *Laryngoscope* **2007**, *117* (12), 2213-2217.

241. Skaliczki, G.; Schandl, K.; Weszl, M.; Major, T.; Kovács, M.; Skaliczki, J.; Szendrői, M.; Dobó-Nagy, C.; Lacza, Z., Serum albumin enhances bone healing in a nonunion femoral defect model in rats: a computer tomography micromorphometry study. *Int. Orthop.* **2013**, *37* (4), 741-745.
242. Horvathy, D. B.; Vacz, G.; Szabó, T.; Szigyarto, I. C.; Toro, I.; Vamos, B.; Hornyák, I.; Renner, K.; Klára, T.; Szabo, B. T., Serum albumin coating of demineralized bone matrix results in stronger new bone formation. *J. Biomed. Mater. Res., Part B* **2016**, *104* (1), 126-132.
243. Mariam, J.; Sivakami, S.; Dongre, P. M., Albumin corona on nanoparticles—a strategic approach in drug delivery. *Drug Deliv.* **2016**, *23* (8), 2668-2676.

Chapter 3

Experimental Methodology

The serum albumin samples used in this study were chosen as they possess different primary structure sequences or were differently processed based on their fractionation methods as well as whether they were subjected to an additional fatty acid removal step. Other reagents used as well as protein sample preparation methods are also described. The measurement principles, signal readout and the rationale for the use of the characterization techniques in this thesis for obtaining information on serum albumin solution-phase conformational stability and adsorption behavior, namely dynamic light scattering, circular dichroism spectroscopy, fluorescence spectroscopy, quartz crystal microbalance-dissipation monitoring, localized surface plasmon resonance sensing and ,attenuated total reflection-Fourier transform infrared spectroscopy was described. Biophysical and biological assays to assess BSA blocking efficiency as a measure of their surface passivation performance using quartz crystal microbalance-dissipation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis together with Western blotting, and enzyme-linked immunosorbent assay was also described.

3.1 Rationale for the Selection of Materials and Experimental Methods

The characterization methods employed, and the samples used in this project was selected based on the motivation of developing a comprehensive analytical framework with which to study the solution-phase conformational stability and adsorption behavior of serum albumin proteins. A materials science and engineering approach was employed with the goal of establishing the structure-processing-properties relationship for serum albumin proteins and assessing their performance.

In line with the experimental objectives, bovine serum albumin (BSA), human serum albumin (HSA) and rat serum albumin (RSA) were selected to understand how sequence variation can influence solution-phase conformational stability and adsorption behavior. These serum albumin proteins were all defatted and purchased from the same supplier to minimize any variation in protein properties caused by different processing techniques so that any experimentally observed differences can be largely attributed to be originating from their sequence variation.

To study the effects of how different processing conditions could influence solution and adsorption properties, six commercially available BSA types were chosen. BSA was chosen over the other serum albumins for this study due to their current wide use, low cost, as well as the availability of various BSA types that have been subject to different processing (>40 types of BSA are commercially available on the Sigma-Aldrich website¹). Specifically, BSA proteins that were purified by (1) cold ethanol fractionation², (2) heat-shock fractionation,^{3, 4} and (3) cold ethanol fractionation followed by heat-shock fractionation. The two fractionation methods were chosen as they are the two most commonly used fractionation methods for serum albumin purification.^{1, 5-13} These first three types of BSA proteins (BSA 1-3) could be further subjected to a fatty acid removal processing step¹⁴ to yield defatted BSA proteins (BSA 4-6) and these defatted BSA proteins were chosen to determine if the thermal stability that fatty acids confer to serum albumin extends to confer stability against surface-induced denaturation.¹⁵ The effects of fatty acids

on BSA properties was confirmed by doping defatted BSA with caprylic acid, a commonly used fatty acid to confer thermal stability to BSA in heat-shock fractionation.^{3, 4, 12}

To further elucidate the mechanistic aspects of the stabilizing effect that fatty acids have on BSA, different fatty acid-derived amphiphiles with different physicochemical properties, namely, a monoglyceride and a methyl ester, were selected, doped with defatted BSA, and their solution-phase and adsorption properties were compared with fatty acid-doped BSA. These two other classes of amphiphiles were selected as they can bind to serum albumin proteins as well,¹⁶⁻²⁰ but a systematic evaluation of their binding strengths to BSA as well as their influence on solution-phase conformational stability and adsorption behavior remain poorly understood.

In order to achieve these experimental objectives, accurate and consistent preparation of contaminant-free serum albumin solution samples is required before any characterization can be conducted. Herein, protein solutions were filtered using a 200 nm pore size filter in order to remove large aggregates and contaminants, and BSA protein concentration was determined by measuring the ultraviolet (UV) absorbance of the solution at 280 nm using a spectrophotometer and correlating it with its molar extinction coefficient according to the Beer-Lambert law,²¹ followed by dilution to the desired concentration.

Next, the solution-phase conformational stabilities of the serum albumin proteins were characterized so that they can be analyzed together with their adsorption behavior. Specifically, the hydrodynamic size and secondary structures of the serum albumin proteins were measured as a function of temperature by dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy measurements, respectively. The onset temperature of aggregation, which was determined as the lowest temperature at which a noticeable increase in hydrodynamic size is observed (usually accompanied by an increase in the DLS count rate) as well as the extent of α -helicity secondary structure loss are taken as key indicators of a protein's solution-phase conformational stability. This is because high temperature can cause the disruption of a protein's intramolecular bonds, leading to thermal unfolding. Sufficiently extensive thermal unfolding exposes a protein's

hydrophobic interior, resulting in intermolecular hydrophobic association and leading to temperature-induced aggregation. A more conformationally stable protein would therefore undergo less extensive unfolding and exhibit a lower onset temperature of aggregation.

Next, BSA adsorption studies were conducted by monitoring BSA adsorption onto silica surface using various complementary surface-sensitive techniques. Silica surface was chosen due to its wide use in many biomedical applications,²² as well as its use in several other serum albumin adsorption studies, which serves as good grounds for comparison with existing works on the effect of processing conditions.²³⁻²⁷ BSA adsorption kinetics and adsorption uptake were measured by two complementary surface-sensitive techniques: quartz crystal microbalance-dissipation (QCM-D) and localized surface plasmon resonance (LSPR). Given its different sensing principle, LSPR measurements strengthen the overall observations on protein adsorption when analyzed together with QCM-D measurements. Additionally, the higher surface sensitivity of the LSPR sensing platform provides information on the extent of protein conformational changes during the initial stages of adsorption.²⁸ This was further supported with information on BSA secondary structural changes from solution to adsorption state obtained from attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy experiments.

Lastly, a combination of surface sensitive tools and biological assays were employed to evaluate the surface passivation performance of different BSA proteins. QCM-D experiments were conducted to determine the blocking efficiency of irreversibly adsorbed BSA films on silica surface against biofouling by fetal bovine serum (FBS), relative to biofouling on an uncoated silica surface as a control experiment. Western blot experiments were conducted with the blocking step performed using different blocking solutions made with the different BSA proteins to evaluate and compare their surface passivation performance on a hydrophobic nitrocellulose membrane surface. In order to further compare their performance on nanomaterial surfaces, BSA proteins were coated onto silica nanoparticles (SiNP) and their surface passivation performance was evaluated based on the extent of complement activation elicited by the different BSA-coated SiNP relative to that elicited by uncoated SiNP as a control experiment.

3.2 Materials

3.2.1 Serum Albumin Samples

Defatted BSA (catalog no. A7030), defatted HSA (catalog no. A3782) and defatted RSA (catalog no. A6414) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used for the study in Chapter 4. Six types of commercially available BSA proteins used for experiments in Chapter 5 and 7 were categorized into their processing conditions of fractionation methods and fatty acid removal. Lyophilized powders of BSA purified by cold ethanol fractionation, heat-shock fractionation, and cold ethanol followed by heat-shock fractionation (catalog no. A2153, A3059 and A7638 respectively), and their essentially fatty acid-free equivalents (catalog no. A6003, A7030 and A0281 respectively) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The former three are designated as BSA 1, BSA 2 and BSA 3 and termed as fatted BSAs while the latter three are designated BSA 4, BSA 5 and BSA 6 and termed as defatted BSAs. **Figure 3.1** illustrates the process flow of the six BSAs. Defatted BSA (catalog no. A7030) was used in Chapter 6.

Figure 3.1 The process flow for the manufacture of the six BSA types from bovine plasma tested in this study. BSAs that have undergone cold ethanol fractionation, heat shock fractionation, and cold ethanol followed by heat shock fractionation have been designated BSA 1, BSA 2 and BSA 3, respectively. BSAs 4, 5 and 6 have been designated as the defatted versions of BSAs 1, 2 and 3.

3.2.2 Other Reagents

Sodium dodecyl sulfate (SDS, catalog no. L4390), sodium chloride (NaCl, catalog no. 746398), sodium hydroxide (NaOH, catalog no. S5881), octanoic acid (caprylic acid, CA, catalog no. C2875), 1-monocapryloyl-rac-glycerol (monocaprylin, MC, catalog no. M2265), methyl octanoate (methyl caprylate, ME, catalog no. 260673) were purchased from Sigma-Aldrich. Tris(hydroxymethyl)aminomethane (Tris, catalog no. 0497) was purchased from Amresco (Solon, OH, USA). Ethanol (95%) was purchased from Aik Moh (Singapore), hydrochloric acid (HCl, catalog no. 100317) was purchased from Merck (Burlington, MA, USA) and fetal bovine serum (FBS, catalog no. SV30160.03, lot no. RC35960) was purchased from HyClone Laboratories (Logan, UT, USA) and stored at -

20 °C until experiment. Normal human serum (catalog no. NHS, lot no. 38) was obtained from Complement Technology (Tyler, TX, USA) and stored at -80 °C until experiment. 30% Acrylamide/Bis Solution 29:1 (catalog no. 1610156), ammonium persulfate (catalog no. 1610700), tetramethylethylenediamine (TEMED, catalog no. 1610800), 4× Laemmli sample buffer (catalog no. 1610747), 2-mercaptoethanol (catalog no. 1610710), 10× Tris buffered saline (TBS, catalog no. 1706435), 10× Tris/glycine buffer (catalog no. 1610734), 10× Tris/glycine/SDS buffer (catalog no. 1610732), Tween 20 (catalog no. 1706531), nitrocellulose membranes (catalog no. 1620112), Precision Plus Protein Standards (catalog no. 1610375), Clarity Max Western enhanced chemiluminescent (ECL) substrate (catalog no. 1705062), and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (catalog no. 1721011, batch no. 64109318) were all purchased from Bio-Rad Laboratories (Hercules, CA, USA). Complement C3b monoclonal antibody (catalog no. MA1-40155, lot no. SH2428445) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Methanol (catalog no. M/4000/17) was obtained from Fisher Scientific (Loughborough, UK) and 100-nm diameter silica nanoparticles (catalog no. SISN100) were obtained from nanoComposix (San Diego, CA, USA).

3.3 Sample Preparation

An aqueous buffer solution of 10 mM Tris and 150 mM NaCl was prepared by dissolving the appropriate amount of Tris and NaCl powder in Milli-Q-treated water (18.2 MΩ.cm). The pH of the solution was made to 7.5 by adding 1 M HCl and the resulting buffer solution was filtered through a 0.2 µm polyethersulfone (PES) membrane filter (Thermo Fisher Scientific, catalog no. 595-4520). All serum albumin solutions were prepared by dissolving the lyophilized protein powder in Tris buffer and then filtered through a 0.2 µm syringe filter (Pall, catalog no. PN-4612,). In Chapter 4, BSA, HSA and RSA's molar extinction coefficient value was 42,925, 34,445, and 38,915 M⁻¹ cm⁻¹, respectively, which was calculated according to the number of tryptophan, tyrosine and cystine residues.²⁹ In Chapters 5 to 7, molar concentrations of BSA in solution were determined by measuring the absorbance at 280 nm and using a molar extinction coefficient value of 43,824 M⁻¹·cm⁻¹.

¹. The prepared BSA solutions were then diluted in Tris buffer to the desired concentrations. To prepare CA-doped BSA, CA was dissolved in protein-free Tris buffer, followed by NaOH titration, to make a 50 mM CA solution at pH 7.5. The CA solution was then added to appropriate amounts of BSA solution in order to yield CA-doped BSA samples with a CA:BSA molar ratio of 10:1 (CA 10) or 100:1 (CA 100). MC and ME solutions were made by dissolving appropriate amounts in equivalent buffer solution to make 20 mM MC or ME solutions, followed by heating to 70 °C for 30 min to aid dissolution. The samples were then cooled down to room temperature before adding the appropriate amounts of BSA solution in order to yield MC or ME-doped BSA samples with MC:BSA and ME:BSA molar ratios of 10:1 (MC 10 and ME 10) or 100:1 (MC 100 and ME 100). In addition, the Western blot running buffer was prepared by diluting 10× Tris/glycine/SDS buffer with water to 1× concentration, and the transfer buffer was prepared by diluting the 10× Tris/glycine buffer with water and methanol to 1× concentration and 20% (vol/vol) of methanol. The BSA-containing surface passivation (“blocking”) solutions for Western blot experiments were prepared by first diluting 10× TBS with water, followed by the addition of Tween 20 to make a 1× TBS solution with 0.1% (vol/vol) Tween 20 (TBST). BSA was then dissolved in TBST to make a 3% (wt/vol) BSA blocking solution.

3.4 Characterization Techniques

3.4.1 Dynamic Light Scattering

DLS determines the size distribution and the corresponding mean hydrodynamic diameter of the BSA samples. A NanoBrook 90Plus particle size analyser (Brookhaven Instruments, NY, USA) directs a 658.0 nm monochromatic laser at the BSA solution sample and measures at a 90° angle the fluctuations of light scattered by individual BSA molecules which are under constant Brownian motion. The BIC Particle Sizing software analyses the intensity autocorrelation function to obtain information on the diffusivity of the molecules. This is because particles with slower diffusivity have higher correlation compared to faster diffusing particles. This is schematically described in **Figure 3.2**. Particle size can then be

calculated from diffusivity for a given temperature and solution viscosity using the Stokes-Einstein equation given by:

$$D = \frac{k_B T}{6\pi\eta r} \quad (\text{Eq 3.1})$$

where D is the diffusion coefficient, k_B is the Boltzmann constant, T is absolute temperature, η is the solvent viscosity and r is the radius of the particle. The intensity-weighted Gaussian size distributions were obtained from each measurement run. The temperature of the measurement chamber can be controlled with a feedback loop between the heating element and a temperature sensor in the measurement chamber. Temperature-dependent size measurements were performed by conducting size measurements at 25 °C, and then from 50 up to 75 °C at every 5 °C increment with an equilibration time of 5 min at every temperature point. Temperature equilibration was important for such measurements as diffusivity is strongly dependent on temperature; an overestimation of sample temperature can lead to an overestimation of particle size, and vice versa. All reported values on the protein size distribution in solution were obtained from five measurements.

Figure 3.2 DLS measurement principle. Light from a laser source is scattered by protein particles in solution. The scattered light is analyzed as a function of time and converted into a correlation curve. Differences in intensity fluctuations due to differences in diffusivity are interpreted as differences in particle size. Adapted with permission from ref. 30. Copyright 2021, Nanoscience Instruments

The solvent viscosity of the aqueous buffer solution can be safely assumed to be identical to water. Size measurement reported by DLS are more accurate for particles that are monodisperse and spherical in shape. This is because intensity-weighted distributions are more sensitive to larger sized particles and the Stokes-Einstein equation holds for spherical particles. For this reason, DLS is particularly useful in detecting the aggregation of protein samples and is well-suited to study the differences in BSA's conformational properties as less conformationally stable BSAs in solution are more prone to conformational changes and would therefore be more susceptible to aggregation, which can be easily detected by

the DLS. The sensitivity of the DLS instrument to larger sized particles is also a limitation just as it is advantageous; impurities in the sample solution or on the sample cuvette can interfere with the actual readings. Therefore, all BSA solutions are always filtered, and cuvettes are wiped and visually inspected for dust before measurements. A volume of 200-250 μL of sample solution was sufficient for experiments. A smaller concentration of 150 μM (~ 10 mg/mL) was used for serum albumin samples in Chapters 4 and 6 due to the limited RSA sample available and the low solubility of MC, respectively. A larger concentration of 451 μM (~ 30 mg/mL) BSA solution was used in Chapter 5 so that larger changes in hydrodynamic size due to aggregation could be observed. It is important to note here that despite the difference in concentrations used between the different Chapters, the onset temperature of aggregation for defatted BSA A7030 remained the same because changes in protein concentration at this magnitude do not affect a protein's solution-phase conformational stability but its colloidal stability, the latter of which is measured by the extent of aggregation rather than the temperature at which aggregation begins.

In addition to temperature-dependent experiments, DLS measurements were conducted at a constant temperature of 60 $^{\circ}\text{C}$ as a function of time. These measurements were conducted to confirm that the onset temperature of aggregation occurred at 60 $^{\circ}\text{C}$ for the defatted BSA samples (especially for the lower concentration samples) as well as to support the difference in conformational stabilities between the different tested serum albumin samples since less conformationally stable proteins would be thermally unfolded to a greater extent and would therefore be more prone to temperature-induced aggregation.

3.4.2 Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was used to monitor the secondary structures of the tested serum albumin proteins in solution at room temperature and changes in secondary structure as a function of temperature. Measurements were recorded by an AVIV Model 420 CD spectrometer (AVIV Biomedical, NJ, USA). The instrument directs left and right circularly polarized light onto an optically active sample that absorbs left and right circularly polarized light to different extents. Such a sample is said to exhibit CD and the

instrument records the difference in absorbance between left and right circularly polarized light (ΔA) across a spectrum of wavelengths to produce a CD spectrum. For historical reasons, CD data is often converted from ΔA to ellipticity in millidegrees based on the ellipse formed by differences in magnitudes of the electric field vectors of the left and right circularly polarized light by the following equation:³¹

$$\text{millidegrees} = 32,982 \Delta A \quad (\text{Eq 3.2})$$

Secondary structures of proteins exhibit distinct CD spectral signatures due to electrons in the amides undergoing varying extents of $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions depending on their conformation.³² For example, α -helix structures have negative peaks at 222 and 208 nm and a positive peak at 193 nm³³ while β -sheet structures have a negative peak at 215 nm and a positive peak at 198 nm.³² Examples of the CD spectra of different secondary structures are presented in **Figure 3.3**. Quantification of the secondary structures of proteins can be based on a linear combination of the different structural elements from reference proteins using computer programs or by comparing to a reference polypeptide with a defined secondary structure.^{34, 35} The latter method is more well established for helical structures and is more easily performed. Since BSA and other serum albumins are mostly helical, this is the method of choice in this project.

Figure 3.3 CD spectra of poly-L-lysine at pH 11.1 exhibiting (1) α -helical and (2) antiparallel β -sheet secondary structures, and at pH 5.7 exhibiting (3) extended conformation. Collagen exhibiting (4) triple helix and (5) denatured conformations. Adapted with permission from ref. 34. Copyright 2007, Nature Publishing Group.

2.5 μM serum albumin solutions in a 1 mm path length cuvette with a polytetrafluoroethylene (PTFE) stopper (Hellma 110-QS) was used. Spectral measurements from 190 to 260 nm in 0.5 nm intervals with an averaging time of 0.1 s were recorded in triplicate. Similar to the DLS measurement protocol, measurements were conducted at 25 $^{\circ}\text{C}$, and then from 50 up to 75 $^{\circ}\text{C}$ at every 5 $^{\circ}\text{C}$ increment with an equilibration time of 5 min at every temperature point. All resultant spectra were subtracted by a background spectrum of buffer solution and presented in terms of molar residue ellipticity (MRE) by the following equation:

$$[\theta](\text{degree cm}^2\text{dmol}^{-1}) = \frac{\text{millidegrees}}{n \times c \times l \times 10} \quad (\text{Eq 3.3})$$

where n is the number of amino acid residues, c is the protein concentration, and l is the cuvette path length. The percentage helicity of each BSA protein was calculated from the MRE at 222 nm, $[\theta]_{222}$, based on the following equation:³⁶

$$\% \text{ Helicity} = \frac{([\theta]_{222} - 3000)}{(-36000 - 3000)} \times 100 \quad (\text{Eq 3.4})$$

As such, CD spectroscopy is highly useful in determining the presence of secondary structure in proteins and protein conformational changes to environmental conditions *i.e.*, temperature.

While the method of helicity quantification chosen herein is merely an estimation rather than an objective determination, the data obtained is nevertheless meaningful as the focus is on the comparison between different serum albumins and the extent of their conformational change relative to each other to assess differences in conformational stability. Another challenge of CD spectroscopy is its sensitivity to protein sample concentrations, in which a larger concentration will give rise to larger ΔA and millidegree values, which can then cause errors with the quantification of percentage helicity if differences in protein concentration are not correctly accounted for. Protein concentration and secondary structural measurements should therefore be accurately determined. This was validated in this project by conducting CD measurements on three separate BSA solutions of the same BSA type with different concentrations, which gave similar percentage α -helicity values when the concentrations of the three samples were used for MRE calculations.

3.4.3 Fluorescence Spectroscopy

Fluorescence spectroscopy experiments were conducted to assess the binding affinity of CA, MC, and ME onto defatted BSA proteins. BSA possesses intrinsic fluorescence with major contributions from two tryptophan residues (Trp-134 and Trp-213) and steady-state fluorescence spectroscopy can be used to monitor changes in the localized environment

around these tryptophan residues, particularly, upon the binding of ligands such as fatty acids and other surfactants.^{37, 38} The intrinsic fluorescence of BSA protein samples was characterized by fluorescence spectroscopy measurements using a Tecan Spark plate reader (Tecan, Switzerland) with the temperature set to 25 °C, an excitation wavelength of 280 nm, and the emission spectra recorded from 325 to 450 nm. For each sample, the BSA concentration was fixed at 10 µM while CA, MC, and ME concentrations were varied. All spectra of BSA samples were recorded in triplicate and subtracted by the background spectra of equivalent test compound concentrations without BSA protein to remove any possible contribution from the CA, MC, or ME background signal alone. The emission maxima for all BSA samples were located at 340 nm and fluorescence intensity values at this wavelength were used for binding analyses.

Since all fluorescence datasets were subtracted by background spectra, the observed quenching is due to the interaction between the surfactant and BSA protein molecules, either as a result of static or dynamic quenching. Static quenching is due to the formation of a non-fluorescent complex between the quencher (*i.e.*, surfactant) and the fluorophore (*i.e.*, BSA) in ground state whereas dynamic quenching is due to the interaction between the quencher and the excited state of the fluorophore. To analyze the quenching data, the Stern-Volmer (SV) equation was used:³⁹

$$\frac{F_0}{F} = 1 + K_{SV}[S] = 1 + k_q\tau_0[S] \quad (\text{Eq 3.5})$$

where F_0 and F are the peak fluorescence intensities without and with the addition of the surfactants, K_{SV} is the SV quenching constant, $[S]$ is the concentration of added surfactant, k_q is the bimolecular quenching rate constant and τ_0 is the average fluorescence lifetime of the BSA in the absence surfactants. Taking into account that the value of τ_0 for BSA is 6×10^{-9} s,^{40, 41} the resulting k_q values may be calculated. Should these calculated values exceed the largest possible value of $\sim 1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$,³⁸ then the binding may be considered to involve static quenching, which is expected due to several studies in the literature reporting a static quenching mechanism for the binding of various ionic and nonionic amphiphiles to

serum albumins.⁴⁰⁻⁴² The effective binding constant can then be determined by the following equation:^{42, 43}

$$\log \frac{F_0 - F}{F} = \log K_b + n \log [S] \quad (\text{Eq 3.6})$$

where n is the number of different binding site types.

3.4.4 Quartz Crystal Microbalance-Dissipation

QCM-D experiments were performed to monitor the adsorption behavior of serum albumin proteins onto a silica coated sensor surface in real time. Its measurement principle is based on the application of an alternating current on the piezoelectric quartz crystal inducing oscillations at its resonance frequency.⁴⁴ Changes in the resonance frequency (ΔF) for each overtone, n , are recorded and corresponds to mass changes at the sensor surface according to the Sauerbrey equation:⁴⁵

$$\Delta F_n = -C n m_a \quad (\text{Eq 3.7})$$

where m_a is the adsorbate mass (per unit area) and C is the mass sensitivity constant which is defined by:

$$C = \frac{t_q \times \rho_q}{f_o} \quad (\text{Eq 3.8})$$

where t_q is the thickness of the quartz crystal, ρ_q is the density of quartz and f_o is the resonance frequency of the quartz crystal. For AT-cut quartz crystals, the value of C is - 17.7 ng cm⁻² Hz. Importantly, the Sauerbrey equation only holds for the deposition of thin, rigid, homogeneous films, and the attachment of non-rigid films such as proteins and other biomolecules such as lipid vesicles deviate from the Sauerbrey equation. Such films exhibit viscoelastic properties which drove the development of the simultaneous characterization of ΔF as well as measuring energy dissipation changes (ΔD),⁴⁶ the latter of which

corresponds to the viscoelasticity of the film. Indeed, this is done by measuring the amount of energy loss from the system when the oscillating drive to the crystal is cut and is defined by the following equation:⁴⁷

$$D = \frac{1}{\pi f \tau} \quad (\text{Eq 3.9})$$

where f is the resonant frequency and τ is the time decay constant. A soft or highly viscoelastic film would greatly damp the oscillation resulting in high ΔD whereas a rigid film would be less dampening and result in low ΔD (**Figure 3.4**). Another important feature of the QCM-D is its ability to provide information on the mass of adsorbed proteins as well as any hydrodynamically coupled solvent; the relative contributions from these two masses towards the QCM-D ΔF signal can then be discriminated when coupled together with information from complementary sensing techniques.^{48, 49} Such information was highly useful in this thesis when investigating the structure of the different protein adlayers.

Figure 3.4 QCM-D measurement principle. **a**, An AC voltage is applied across the quartz crystal sensor, producing a resonance frequency that decreases upon any addition of mass on the sensor surface. **b**, When the AC power is cut off, the sensor's oscillation decays, and rigidly attached adsorbates on the sensor surface result in longer decay patterns, while **c**, viscoelastic adsorbates result in shorter decay curves. Adapted with permission from ref. 50. Copyright 2021, Nanoscience Instruments.

A Q-Sense E4 instrument (Biolin Scientific AB, Stockholm, Sweden) was used together with AT-cut quartz crystal sensor chips with a 5 MHz fundamental frequency that has been sputter coated with a 50-nm-thick layer of silica (QSX 303, Biolin Scientific). Prior to each experiment, the sensor chips were successively rinsed with 1% SDS, water and ethanol, then dried under a gentle stream of nitrogen gas, followed by treatment with oxygen plasma (PDC-002, Harrick Plasma, Ithaca, NY, USA) for 3 min at maximum radiofrequency. A peristaltic pump was used to inject liquid samples into the measurement chamber at a constant flow rate of 100 $\mu\text{L}/\text{min}$. A stable baseline signal was established in buffer

solution before serum albumin proteins were introduced into the measurement chamber for 30 min followed by buffer rinsing. Time-dependent shifts in ΔF and ΔD were recorded at multiple odd overtones and the normalized data at the fifth overtone are reported. All QCM-D data were collected by the QSoft (Biolin Scientific) software package.

The QCM-D technique was also employed to determine the passivation efficiency of BSA coatings against serum biofouling. For these experiments, 100 μM BSA solution was first added to a bare silica surface under continuous flow for 60 min, followed by a 40 min buffer washing step. Undiluted fetal bovine serum (FBS) was then introduced under continuous flow for 80 min, followed by a final buffer washing step for 30 min. The passivation efficiency percentage was calculated by comparing the absolute difference between the frequency shift due to BSA adsorption alone (after the first buffer washing step) and the subsequent frequency shift due to FBS biofouling (after the second buffer washing step), which is denoted as $\Delta F_{\text{FBS-BSA}}$. A control experiment without BSA coating was also conducted in order to measure the absolute frequency shift due to FBS biofouling alone (after the buffer washing step), which is denoted as $\Delta F_{\text{Control}}$. For each BSA coating, the passivation efficiency percentage was calculated by the following equation:

$$\text{Passivation efficiency (\%)} = \left(1 - \frac{\Delta F_{\text{FBS-BSA}}}{\Delta F_{\text{Control}}}\right) \times 100\% \quad (\text{Eq 3.10})$$

Of note, the QCM-D is only capable of ensemble-averaged measurements and does not provide information on single molecules. Therefore, the observed positive adsorption kinetics should be interpreted as the net adsorption from the combined adsorption and desorption of many individual molecules rather than a system with only molecular adsorption.

3.4.5 Localized Surface Plasmon Resonance Sensing

The LSPR sensing platform was used to complement QCM-D data in monitoring serum albumin adsorption onto a silica surface. The sensing principle of LSPR makes use of the optically-based surface plasmon resonance phenomenon confined or localized to a

nanoparticle surface. Localized surface plasmons are collective oscillations of free electrons that are confined to a nano-sized metal surface during the interaction with light. These collective oscillations, when in resonance with the incident light, give rise to surface plasmon resonance and a peak in optical extinction at the resonance wavelength (λ_{max}). The λ_{max} is highly sensitive to changes in the refractive index in the surrounding dielectric medium with a short sensing depth or decay length of about 10 to 30 nm.⁵¹ An increase in the refractive index due to the binding of biomolecules within the sensing depth will cause an increase in the resonance wavelength (red-shift), which can be easily monitored by measuring the shift in the wavelength of the optical extinction peak ($\Delta\lambda_{\text{max}}$) (**Figure 3.5**).

Figure 3.5 Schematic of the LSPR measurement setup. Optical extinction is recorded in transmission mode and the shifts in wavelength of the maximum optical extinction due to the presence of biomolecules are monitored. Adapted with permission from ref. 52. Copyright 2017, Royal Society of Chemistry.

Furthermore, due to the sharply decaying evanescent electromagnetic wave from the sensor surface, the LSPR signal readout is sensitive to the spatial proximity of the bound molecule

to the sensor surface. That is, molecules closer to the sensor surface give rise to greater magnitudes of $\Delta\lambda$.^{28, 53-55} This concept has been highly useful in this thesis as differences in $\Delta\lambda$ can be correlated to different extents of protein deformation upon adsorption, which is described according to the following relation:²⁸

$$\Delta\lambda \propto \int_0^h \frac{C\pi\rho^2(z)}{(R_* + z)^6} dz \quad (\text{Eq 3.11})$$

where z is the coordinate perpendicular to the sensor surface, h is the height of the protein molecule in this direction, C is the concentration of proteins on the surface, $\rho(z)$ is the cross-sectional radius of a circle on the sensor surface made by the adsorbed protein in contact with the sensor surface, and R_* is the effective radius from the gold nanodisk transducers on the LSPR sensor corresponding to the regions that mainly contribute to the LSPR signal. Experimentally, this can be verified by comparing the derivative of $\Delta\lambda$ with respect to time ($d\Delta\lambda/dt$) during the initial stages of adsorption with the diffusion-limited transport rate of the protein molecules. This is because the magnitude of $d\Delta\lambda/dt$ is contributed by (1) the diffusion-limited transport rate of protein molecules to the sensor surface, and (2) the extent of deformation of the protein molecules. The diffusion-limited transport kinetics can be described by the following equations:

$$\Delta\lambda \propto D^{2/3} \quad (\text{Eq 3.12})$$

$$D = \frac{k_B T}{6\pi\eta r} \quad (\text{Eq 3.13})$$

where D is the diffusion coefficient, k_B is the Boltzmann constant, T is temperature, η is the solvent viscosity, and r is the hydrodynamic radius of the adsorbing protein, which may be obtained from DLS measurements. Therefore, when the experimentally obtained $d\Delta\lambda/dt$ values are compared to their corresponding theoretically derived diffusion-limited values, any discrepancy between these values can be attributed to the extent of adsorption-related protein deformation.

Ensemble-averaged LSPR measurements in optical transmission mode were conducted using an Insplorion XNano instrument (Insplorion, Gothenburg, Sweden). A white light beam ($\sim 4 \text{ mm}^2$) is transmitted through a sensor chip (Insplorion) consisting of well-separated gold nanodisk arrays ($\sim 8\%$ surface coverage) deposited by hole-mask colloidal lithography⁵⁶ on a fused silica substrate. The nanodisks have an average height and diameter of 20 and 120 nm respectively and the surface of the sensor chip was coated with a silicon nitride film ($\sim 10 \text{ nm}$ thick). The light was then passed through a quartz glass window and collected by a spectrophotometer. Prior to each experiment, the sensor chip was sequentially rinsed with 1% SDS aqueous solution, water and ethanol, then dried under a gentle stream of nitrogen gas. The chip was then treated with oxygen plasma for 2.5 min at maximum radiofrequency which forms a layer of silica on the sensor surface. After this, the sensor chip was loaded into the measurement chamber and liquid samples were introduced by a peristaltic pump at a flow rate of $100 \mu\text{L}/\text{min}$. A stable baseline signal was established in buffer solution before serum albumin proteins were introduced into the measurement chamber for 30 min followed by buffer rinsing. The Insplorion (Insplorion) software package was used to record the LSPR extinction spectra with a time resolution of 1 Hz and to determine the centroid (peak) position in the extinction spectrum using a high-order polynomial fitting⁵⁷. Rate of change of LSPR extinction peak shifts were calculated using OriginPro 2016 (OriginLab) software package and smoothed by adjacent-average method to 30 points.

The optically-based LSPR sensor used herein is based on the indirect nanoplasmonic sensing platform in which a thin dielectric layer (*i.e.*, silicon nitride) is deposited over the gold nano-transducers on an optically transparent solid support.⁵⁸ This makes it more versatile than conventional SPR sensors as while biomolecular interaction (*i.e.*, protein adsorption and conformation changes) can only be studied on a gold surface for SPR, interaction studies on a variety of dielectric substrates is possible for LSPR sensing. In this case, upon oxygen plasma treatment of the silicon nitride surface, protein adsorption onto silica surface can be studied. Due to it being optically-based, the LSPR sensor gives information only on the bound protein molecules and does not take into consideration any

coupled solvent effects. Taken together with the QCM-D data, this allows the mass contributions between adsorbed proteins and coupled solvent to be resolved. However, just like the QCM-D, the LSPR technique only provides ensemble-averaged measurements and therefore net adsorption rates must be taken into consideration.

3.4.6 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy

ATR-FTIR experiments were conducted to further investigate the conformational changes that occur upon BSA adsorption and support the LSPR data. Data interpretation is based on the same principle as FTIR in which infrared radiation excites molecules into vibration and information is obtained based on the relationship between the absorbance wavenumber (inverse of wavelength) and the nature of the molecular bond. In the context of studying protein conformation, the amide I vibration band, located near the wavenumber 1650 cm^{-1} is often analyzed for its information on protein secondary structure and is contributed mainly by C=O stretching vibrations with minor contributions from out-of-phase C-N stretching vibrations.⁵⁹ In ATR-FTIR, samples are placed on an ATR crystal (also referred to as the internal reflection element). When the infrared light is focused and incident on one of the side faces of the ATR crystal and hits the interface between the crystal and the sample at an angle greater than the critical angle, then the light will totally internally reflect (**Figure 3.6**). The critical angle (θ_{crit}) is calculated as

$$\theta_{\text{crit}} = \sin^{-1} \frac{\text{refractive index of the sample}}{\text{refractive index of the ATR crystal}} \quad (\text{Eq 3.14})$$

For example, for a protein solution sample of refractive index (RI) of 1.5 at 1550 cm^{-1} and a ZnSe ATR crystal with an RI of 2.4, the θ_{crit} will be about 36.7° . Where there is total internal reflection at the interface between the ATR crystal and the sample, an evanescent wave is formed on the exterior surface of the crystal that penetrates into and is absorbed by the protein sample. The intensity (I) of the evanescent wave exponentially decays with distance (z) from the ATR crystal surface by:

$$I = I_0 e^{-z/d_p} \quad (\text{Eq 3.15})$$

where d_p is the penetration depth, which is the distance at which the intensity of the evanescent wave drops to $1/e$ of the surface. d_p can be calculated by:⁶⁰

$$d_p = \frac{\lambda}{2\pi n_1 \sqrt{(\sin^2 \theta - (n_2/n_1)^2)}} \quad (\text{Eq 3.16})$$

Where λ is the wavelength of the incident infrared light, n_1 is the RI of the ATR crystal and n_2 is the RI of the sample and θ is the angle of incidence of infrared light on the ATR crystal. For an incident angle of 45° on a ZnSe ATR crystal with a typical protein sample and the study in the amide I region, the d_p is about $1.22 \mu\text{m}$. The infrared light is attenuated by the absorbing sample on the ATR crystal and this information is collected by the detector. The sensitivity is increased with increasing number of internal reflections.⁶⁰

Figure 3.6 Schematic of ATR-FTIR optical setup. An infrared beam is totally internally reflected at the interface between the ATR crystal and the sample, which covers the top of the crystal. An evanescent wave is created and is absorbed by the sample at areas of total internal reflectance. Information on the absorbance by the sample is collected by the detector. Adapted with permission from ref. 61. Copyright 2015, IOS Press.

The surface sensitivity of the ATR-FTIR makes it particularly useful for studying adsorbed protein films. A protein film may be prepared by drying an aqueous protein solution on the ATR crystal surface and consists of multiple layers of proteins where the first layer in contact with the ATR surface has undergone surface-induced denaturation while subsequent layers further away from the surface remain hydrated and in their apparent native state. The near absence of water in this case is beneficial as the FTIR spectrum of

water shows intense absorption between 1620 and 1680 cm^{-1} , which overlaps with the amide I region centered around 1650 cm^{-1} , and which is often circumvented by a subtraction of the water spectrum.⁶² However, questions remain on whether protein structural information obtained in dried protein films can be taken to be representative of the protein structure in the solution phase.⁶³

This thesis requires the study of proteins in both their solution and adsorbed state, and while the use of the dry film technique to obtain information on the native protein structure would be a simple method, there is no appropriate corresponding method to obtain information on their surface-induced denaturation state. Furthermore, drying also increases the concentration of the Tris in the FTIR signal which shows significant absorbance at 1600 cm^{-1} associated with N-H bending and would consequently interfere with amide I analysis. Although the problem of the interference of water may be circumvented by replacing water with deuterium oxide (D_2O), concerns on whether such a deuterated environment affects protein properties remain, and water is still the preferred solvent.⁶⁴ Therefore, the ATR-FTIR spectra of proteins in this thesis were obtained from aqueous protein solutions placed on the ATR crystal with an appropriate subtraction of buffer spectra, of which a simple method is to subtract the protein sample spectra with an appropriate fraction of the buffer spectra until the region from 1900 to 1740 cm^{-1} is flat.^{60, 65, 66} Information on the proteins conformation in solution can be obtained by recording the spectra immediately after the protein solution has been deposited to minimize information interference with adsorbed proteins. The penetration depth of the evanescent wave (1-2 μm) is larger than the thickness of adsorbed BSAs (3-5 nm) by more than two orders of magnitude, therefore any contribution from surface-denatured BSA proteins in this case are negligible. Information on proteins that have been irreversibly adsorbed can then be obtained by rinsing the sample with buffer solution after a period of incubation to remove non-adsorbing or weakly adsorbed proteins.^{63, 67, 68}

ATR-FTIR spectroscopy experiments in this project were conducted using a Bruker Vertex 70 FTIR spectrometer with a liquid-nitrogen-cooled mercury cadmium telluride photodetector (Bruker, Baden-Württemberg, Germany) equipped with a MIRacle ATR

accessory containing a three-reflection ZnSe ATR crystal (PIKE Technologies, WI, USA). A 30 μL aliquot of 100 μM serum albumin protein solution was deposited onto the ATR crystal and the first absorbance spectra was immediately recorded to characterize the secondary structure of serum albumins in solution. The serum albumin was then incubated with the ATR crystal surface at room temperature for 30 min, followed by a series of ten rinsing steps conducted in succession to remove non-adsorbed and weakly adsorbed proteins. During each washing step, 30 μL of extra buffer solution (without protein) was added to the sample droplet, followed by aspiration of a 30 μL volume from the droplet. After the washing steps were completed, then a second absorbance spectrum was recorded to characterize the secondary structure of BSA proteins in the adsorbed state. Ten rinsing steps were sufficient as further rinsing did not cause much more significant changes in the amide I absorbance peak values, indicating that only strongly bound proteins remain. During measurements, the sample compartment was purged continuously with nitrogen gas to minimize ambient moisture. Using the OPUS 6.5 (Bruker) software, spectra were collected at a 4 cm^{-1} resolution and averaged over 128 double-sided forward-backward scans. Blackman-Harris 3-term apodization was used. All recorded absorbance spectra were normalized by subtracting background spectra from buffer and water vapor alone to eliminate water and water vapor absorbance artefacts, respectively.

The relative contribution of different secondary structure elements was determined for all samples by curve fitting the amide I bands of the absorbance spectra (1720 – 1580 cm^{-1}) to Gaussian components based on the peaks identified in the second derivative plot of the absorbance spectra and from literature references.^{69, 70} All curve fitting analyses were conducted using the Peak Analyzer function in the OriginPro 2019b software package. The absorbance spectrum obtained from experiment was assumed to be a linear combination of multiple constituent Gaussian peaks, each centered around a given wavenumber value and representing a protein secondary structure element. The secondary structure percentage values were determined by calculating the area under the curve-fitted peaks that had been assigned to each secondary structure element as a fraction of the total area under the fitted curve. Peaks centered at 1682-1678 cm^{-1} were assigned to β -turn structures, 1656-1654 cm^{-1}

¹ to α -helix, 1649-1648 cm^{-1} to random coil, 1639-1624 cm^{-1} to extended chains, and 1618-1612 cm^{-1} to intermolecular β -sheets.

ATR-FTIR is a powerful tool as it is capable of determining the secondary structure of the serum albumin in both solution phase and upon adsorption. It is used in this project to complement the solution-based secondary structure information obtained by CD measurements while supporting observations from the surface-sensitive QCM-D and LSPR techniques. Although potential errors may arise from the various manual processes such as the subtraction of the water and water vapor spectra, it has been reported that even when the water spectra was deliberately over or under subtracted, the positions and relative intensities of each peak components, and therefore the secondary structural information are maintained.⁷¹ Errors were further reduced by conducting independent measurements and data processing of samples in triplicate.

3.4.7 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot

The Western blot technique is a commonly used immunoassay to determine the presence of a particular protein in a sample.⁷² This is done by first separating the protein samples by molecular weight through gel electrophoresis. The separated proteins are then electrophoretically transferred onto a hydrophobic blotting membrane. A ‘blocking’ protocol is then conducted in which the membrane is incubated in a ‘blocking’ solution whereby exposed membrane surfaces are blocked by adsorption onto any bare membrane surfaces which prevents subsequent nonspecific adsorption of any antibodies onto the membrane surface. The protein of interest is then detected by first incubating the membrane in a monoclonal antibody (primary antibody) solution which specifically binds to the protein of interest, followed by incubation with a secondary antibody that specifically binds to the primary antibody and which is conjugated with a reporter enzyme, such as horseradish peroxidase (HRP), which allows detection by enhanced chemiluminescence (Figure 3.7).

Figure 3.7 Schematic of Western blot process flow. Proteins from a sample are separated by SDS-PAGE, and then transferred to a nitrocellulose membrane. After blocking with BSA blocking solution, membranes are incubated with primary followed by HRP-conjugated secondary antibodies. Upon addition of a chemiluminescent substrate, which reacts with the HRP, Western blot bands can be imaged. Adapted with permission from ref. 73. Copyright 2019, Elsevier.

For experiments in this thesis, 10% polyacrylamide resolving gels at pH 8.8 and 5% stacking gels at pH 6.8 with fifteen sample loading wells per gel were prepared before experiments. For sample preparation, normal human serum (NHS) was thawed and then immediately diluted to 1% (vol/vol) in Tris buffer, followed by incubation at 37 °C for 30

min. A 180 μ L aliquot of diluted NHS was then mixed with 54 μ L of 4 $\times$ Laemmli buffer and 6 μ L of 2-mercaptoethanol. The sample mixture was then heated to 95 $^{\circ}$ C for 5 min for complete denaturation of serum proteins, before cooling back to room temperature. A 10 μ L aliquot of protein standards was loaded into the first lane of the polyacrylamide gel and the next two lanes were each loaded with 10 μ L of the sample mixture; this sequence was repeated five times in total to fill all lanes within the gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then conducted at 50 V for 15 min, followed by 100 V for 90 min in running buffer.

The protein bands in the gel were then transferred to a nitrocellulose membrane at 300 mA for 2 h in transfer buffer, and ice packs were used to keep the transfer buffer cool during the transfer process. After the transfer step, the membranes were cut into strips, each one containing the three lanes of protein standards (lane 1) and serum samples (lanes 2-3). Surface passivation (“blocking”) solutions were prepared using BSA in TBST [(cf. Sample Preparation); 3% (wt/vol)] and each strip was incubated with 5 mL of the appropriate BSA blocking solution at room temperature for 1 h. The membranes were then incubated with complement C3b monoclonal antibody as the primary antibody (1:500 dilution in blocking solution) overnight. Each membrane was then washed with TBST for 10 min. After three washing steps, the membranes were incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (1:2000 dilution in blocking solution) for 1 h, followed by four subsequent washing steps with TBST. The membranes were then incubated with Clarity Max Western ECL substrate for 5 min in order to enhance chemiluminescence properties. All incubation and washing steps were conducted on a rocking platform. The protein bands were subsequently imaged using an Amersham Imager 600 instrument and its accompanying software (v0.9.8) (GE Healthcare, Chicago, IL, USA) with a 1 s exposure time.

In Western blot experiments, the blocking step prior to antibody incubation is a critical part of the protocol to minimize nonspecific adsorption of primary and/or secondary antibodies onto the blot membrane, which can lead to false signals and poor blot resolution. To this end, BSA is the gold-standard blocking reagent for surface passivation although there is no

discussion in the scientific literature about the blocking performance of differently processed BSA proteins. Therefore, in this thesis, Western blot experiments were conducted using normal human serum (NHS) as the protein sample against a C3b monoclonal primary antibody, which produces two specific bands corresponding to C3b (104 kDa) and iC3b (40 kDa) proteins. The appearance of any additional bands was therefore taken to arise from nonspecific adsorption and the ‘blocking’ effectiveness of the BSA was evaluated based on the intensity of these bands. A previous study conducted a similar test to compare the blocking efficiency of native and heat-denatured BSA.²⁷ It was reported that heat-denatured BSA performed better as a blocking agent by producing blots with lower intensity nonspecific bands (**Figure 3.8**)

Figure 3.8 Comparison of native and heat-denatured BSA proteins as blocking agents. **a**, Schematic illustration of Western blot blocking protocol using a blocking solution made from either native or heat-denatured BSA. Gels were loaded with SiNP-treated serum (lanes 1-4), untreated serum (lane 5) and buffer solution (lane 6) and after transfer to nitrocellulose membrane, were blocked with **b**, native BSA monomers or **c**, heat-denatured BSA oligomers/aggregates prior to antibody incubation. The differences in band intensity indicate the superior blocking performance of denatured BSA aggregates Adapted with permission from ref. 27. Copyright 2018, American Chemical Society.

In order to conduct a quantitative analysis on the blocking performance of the BSA proteins tested in this thesis, the Fiji/ImageJ software package⁷⁴ was used to quantify the intensity of the noise bands that were located near the 250, 75, and 50 kDa molecular weight markers. The digital image of the blots was first set to 8-bit greyscale, and then an intensity profile from each selected lane was plotted using the Analyze Gels function. The peaks from non-specific bands of interest were identified and a baseline was created by drawing a straight line between the two minima points on either side of each peak of interest. The Wand tool was then used to determine the area bounded by the peak and the baseline. These values were defined as the intensity values for each selected non-specific band.

3.4.8 Silica Nanoparticle Preparation and Enzyme-Linked Immunosorbent Assay for Complement Activation

The surface passivation performance of BSA proteins onto nanomaterials were also evaluated based on their adsorption onto SiNP and their subsequent extent of protection rendered against SiNP-induced complement activation upon incubation in NHS. BSA-coated silica nanoparticle samples were prepared by mixing equal volumes of the appropriate 1 mg mL⁻¹ BSA solution with 2 mg mL⁻¹ silica nanoparticles in Tris buffer. The mixtures were then incubated at 37 °C for 2 h, followed by centrifugation at 16,000×g for 30 min. The supernatants were removed, and the nanoparticles were resuspended with fresh Tris buffer. Another round of centrifugation and resuspension was conducted to yield 1 mg mL⁻¹ BSA-coated silica nanoparticle samples. A 10 µL aliquot of each BSA-coated silica nanoparticle sample was then mixed with a 40 µL aliquot of freshly thawed normal human serum (NHS). A 50 µL aliquot of NHS by itself was used as the negative control (lowest level of complement activation) and 10 µL of 1 mg mL⁻¹ uncoated silica nanoparticles plus 40 µL NHS was used as the positive control (highest level of complement activation). All test samples were incubated at 37 °C for 30 min.

The extent of complement activation was measured based on the concentration of the solubilized membrane attack complex sC5b-9 which is formed by the assembly of

complement proteins of C5b through C9 and typically marks the end of the classical, alternative or lectin pathway of complement activation.⁷⁵ Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of sC5b-9 generated by SiNP-incubated NHS. ELISA is a commonly employed biomolecular technique for determining the presence and concentration of a particular analyte. The ELISA setup typically consists of an immobilized antigen or antibody on a solid surface which specifically binds to and captures a target analyte within a sample solution.⁷⁶ Once the other proteins are removed by washing, an enzyme-labeled antibody is added which binds to the target analyte and a further addition of an enzymatic substrate produces an optical signal such as a color change which can be correlated to the concentration of the target analyte. Quantification is done by comparing a sample's optical signal intensity with that produced by a standard curve.

The MicroVue SC5b-9 Plus Enzyme Immunoassay kit (catalog no. A020; Quidel, San Diego, CA, USA) was used for ELISA experiments and the assay was performed using the 96-well plates that were provided in the ELISA kit according to the manufacturer's instructions. All serum samples, including controls, were diluted 40-times by using the provided sample diluent solution before addition to the wells. Standard solutions were added without dilution. The absorbance values from each well were determined by an Infinite Pro 200 plate reader (Tecan, Männedorf, Switzerland). Standard curves were calculated by correlating the known concentrations of the standard solutions to the respective absorbance values. The SC5b-9 protein concentration in each sample was then computed. The degree of protection afforded by each BSA coating was calculated by treating the SC5b-9 protein concentrations from the negative and positive control samples as 100% and 0% protection, respectively.

References

1. Sigma-Aldrich Bovine Serum Albumins.
<https://www.sigmaaldrich.com/catalog/substance/bovineserumalbumin12345904846811?lang=en®ion=SG> (accessed Apr 2021).

2. Cohn, E. J.; Strong, L. E.; Hughes, W.; Mulford, D.; Ashworth, J.; Melin, M.; Taylor, H., Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.* **1946**, *68* (3), 459-475.
3. Reid, A. F. Method of purifying albumin. 2,705,230, 1955.
4. Schneider, W.; Lefevre, H.; Fiedler, H.; McCarty, L. J., An alternative method of large scale plasma fractionation for the isolation of serum albumin. *Blut* **1975**, *30* (2), 121-134.
5. Gemini Bio BSA: Cohn Cold EtOH vs. Heat-Shock. <https://www.gembio.com/post/bsa-cohn-cold-etoh-vs-heat-shock> (accessed Apr 2021).
6. Equitech-Bio Bovine Serum Albumin. <https://www.equitech-bio.com/product-tag/bovine-serum-albumin/> (accessed Apr 2021).
7. Burnouf, T., Modern plasma fractionation. *Transfus. Med. Rev.* **2007**, *21* (2), 101-117.
8. Hao, Y. I., A simple method for the preparation of human serum albumin 1. *Vox Sang.* **1979**, *36* (5), 313-320.
9. Tanaka, K.; Shigueoka, E.; Sawatani, E.; Dias, G.; Arashiro, F.; Campos, T.; Nakao, H., Purification of human albumin by the combination of the method of Cohn with liquid chromatography. *Braz. J. Med. Biol. Res.* **1998**, *31* (11), 1383-1388.
10. Rocky Mountain Biologicals Bovine Serum Albumin Standard Heat Shock - BSA. <https://www.rmbio.com/bovine-serum-albumin-heat-shock> (accessed Apr 2021).
11. Bovogen Biologicals Purified Proteins Bovine Serum Albumin Bovostar. <https://bovogen.com/our-products/purified-proteins/bovine-serum-albumin/> (accessed Apr 2021).
12. Mannuzza, F. J.; Montalto, J. G., Is bovine albumin too complex to be just a commodity? *BioProcess Int.* **2010**, *8* (4), 42-49.
13. Denizli, A., Plasma fractionation: conventional and chromatographic methods for albumin purification. *Hacettepe J. Biol. Chem.* **2011**, *39* (4), 315-341.

14. Chen, R. F., Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **1967**, *242* (2), 173-181.
15. Ballou, G. A.; Boyer, P. D.; Luck, J. M.; Lum, F. G., The heat coagulation of human serum albumin. *J. Biol. Chem.* **1944**, *153* (2), 589-605.
16. Thumser, A. E.; Buckland, A. G.; Wilton, D. C., Monoacylglycerol binding to human serum albumin: evidence that monooleoylglycerol binds at the dansylsarcosine site. *J. Lipid Res.* **1998**, *39* (5), 1033-1038.
17. Duff, S. M.; Kalambur, S.; Boyle-Roden, E., Serum albumin binds β - and α -monoolein in vitro. *J. Nutr.* **2001**, *131* (3), 774-778.
18. Arvidsson, E.; Belfrage, P., Monoglyceride-protein interaction. The binding of monoolein to native human serum albumin. *Acta Chem. Scand.* **1969**, *23* (1), 232-236.
19. Spector, A. A.; John, K.; Fletcher, J. E., Binding of long-chain fatty acids to bovine serum albumin. *J. Lipid Res.* **1969**, *10* (1), 56-67.
20. Morrisett, J. D.; Pownall, H.; Gotto, A., Bovine serum albumin. Study of the fatty acid and steroid binding sites using spin-labeled lipids. *J. Biol. Chem.* **1975**, *250* (7), 2487-2494.
21. Aitken, A.; Learmonth, M. P., Protein determination by UV absorption. In *The Protein Protocols Handbook*, Springer: 2009; pp 3-6.
22. Li, Z.; Barnes, J. C.; Bosoy, A.; Stoddart, J. F.; Zink, J. I., Mesoporous silica nanoparticles in biomedical applications. *Chem. Soc. Rev.* **2012**, *41* (7), 2590-2605.
23. Giacomelli, C. E.; Norde, W., The adsorption-desorption cycle. Reversibility of the BSA-silica system. *J. Colloid Interface Sci.* **2001**, *233* (2), 234-240.
24. Kurrat, R.; Prenosil, J.; Ramsden, J., Kinetics of human and bovine serum albumin adsorption at silica-titania surfaces. *J. Colloid Interface Sci.* **1997**, *185* (1), 1-8.
25. Park, J. H.; Sut, T. N.; Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Cho, N.-J., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19* (13), 8854-8865.

26. Ferhan, A. R.; Jackman, J. A.; Sut, T. N.; Cho, N.-J., Quantitative comparison of protein adsorption and conformational changes on dielectric-coated nanoplasmonic sensing arrays. *Sensors* **2018**, *18* (4).
27. Park, J. H.; Jackman, J. A.; Ferhan, A. R.; Ma, G. J.; Yoon, B. K.; Cho, N.-J., Temperature-induced denaturation of BSA protein molecules for improved surface passivation coatings. *ACS Appl. Mater. Interfaces* **2018**, *10* (38), 32047-32057.
28. Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Park, J. H.; Zhdanov, V. P.; Cho, N.-J., Indirect nanoplasmonic sensing platform for monitoring temperature-dependent protein adsorption. *Anal. Chem.* **2017**, *89* (23), 12976-12983.
29. Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T., How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **1995**, *4* (11), 2411-2423.
30. 3P Instruments Dynamic Light Scattering. <https://www.3p-instruments.com/measurement-methods/dynamic-light-scattering/> (accessed Apr 2021).
31. Rodger, A., Circular dichroism spectroscopy: units. In *Encyclopedia of Biophysics*, Springer: 2013; pp 316-317.
32. Sreerama, N.; Woody, R. W., Computation and analysis of protein circular dichroism spectra. In *Methods in Enzymology*, Elsevier: 2004; Vol. 383, pp 318-351.
33. Holzwarth, G.; Doty, P., The ultraviolet circular dichroism of polypeptides1. *J. Am. Chem. Soc.* **1965**, *87* (2), 218-228.
34. Greenfield, N. J., Using circular dichroism spectra to estimate protein secondary structure. *Nat. Protoc.* **2006**, *1* (6), 2876.
35. Wei, Y.; Thyparambil, A. A.; Latour, R. A., Protein helical structure determination using CD spectroscopy for solutions with strong background absorbance from 190 to 230 nm. *Biochim. Biophys. Acta, Proteins Proteomics* **2014**, *1844* (12), 2331-2337.
36. Morrisett, J. D.; David, J. S.; Pownall, H. J.; Gotto Jr, A. M., Interaction of an apolipoprotein (apoLP-alanine) with phosphatidylcholine. *Biochemistry* **1973**, *12* (7), 1290-1299.

37. Spector, A. A.; John, K. M., Effects of free fatty acid on the fluorescence of bovine serum albumin. *Arch. Biochem. Biophys.* **1968**, *127*, 65-71.
38. Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*. Springer Science & Business Media: 2013.
39. Stern, O.; Volmer, M., Über die abklingzeit der fluoreszenz. *phys. Z* **1919**, *20*, 183-188.
40. Ghosh, S.; Dey, J., Binding of fatty acid amide amphiphiles to bovine serum albumin: role of amide hydrogen bonding. *J. Phys. Chem. B* **2015**, *119* (25), 7804-7815.
41. Iovescu, A.; Băran, A.; Stîngă, G.; Cantemir-Leontieș, A. R.; Maxim, M. E.; Anghel, D. F., A combined binding mechanism of nonionic ethoxylated surfactants to bovine serum albumin revealed by fluorescence and circular dichroism. *J. Photochem. Photobiol., B* **2015**, *153*, 198-205.
42. Zhu, T.-T.; Zhang, Y.; Luo, X.-A.; Wang, S.-Z.; Jia, M.-Q.; Chen, Z.-X., Difference in binding of long-and medium-chain fatty acids with serum albumin: The role of macromolecular crowding effect. *J. Agric. Food Chem.* **2018**, *66* (5), 1242-1250.
43. Min, J.; Meng-Xia, X.; Dong, Z.; Yuan, L.; Xiao-Yu, L.; Xing, C., Spectroscopic studies on the interaction of cinnamic acid and its hydroxyl derivatives with human serum albumin. *J. Mol. Struct.* **2004**, *692* (1-3), 71-80.
44. Reviakine, I.; Johannsmann, D.; Richter, R. P., Hearing what you cannot see and visualizing what you hear: interpreting quartz crystal microbalance data from solvated interfaces. *Anal. Chem.* **2011**, *83* (23), 8838-8848.
45. Sauerbrey, G., The use of quartz oscillators for weighing thin layers and for microweighing. *Z. Phys.* **1959**, *155*, 206-222.
46. Rodahl, M.; Höök, F.; Fredriksson, C.; Keller, C. A.; Krozer, A.; Brzezinski, P.; Voinova, M.; Kasemo, B., Simultaneous frequency and dissipation factor QCM measurements of biomolecular adsorption and cell adhesion. *Faraday Discuss.* **1997**, *107*, 229-246.
47. Rodahl, M.; Kasemo, B., On the measurement of thin liquid overlayers with the quartz-crystal microbalance. *Sens. Actuators, A* **1996**, *54* (1), 448-456.

48. Höök, F.; Vörös, J.; Rodahl, M.; Kurrat, R.; Böni, P.; Ramsden, J.; Textor, M.; Spencer, N.; Tengvall, P.; Gold, J., A comparative study of protein adsorption on titanium oxide surfaces using in situ ellipsometry, optical waveguide lightmode spectroscopy, and quartz crystal microbalance/dissipation. *Colloids Surf. B* **2002**, 24 (2), 155-170.
49. Höök, F.; Kasemo, B.; Nylander, T.; Fant, C.; Sott, K.; Elwing, H., Variations in coupled water, viscoelastic properties, and film thickness of a Mefp-1 protein film during adsorption and cross-linking: a quartz crystal microbalance with dissipation monitoring, ellipsometry, and surface plasmon resonance study. *Anal. Chem.* **2001**, 73 (24), 5796-5804.
50. Nanoscience Instruments Quartz Crystal Microbalance (QCM). <https://www.nanoscience.com/techniques/quartz-crystal-microbalance/> (accessed Apr 2021).
51. Mazzotta, F.; Johnson, T. W.; Dahlin, A. B.; Shaver, J.; Oh, S.-H.; Höök, F., Influence of the evanescent field decay length on the sensitivity of plasmonic nanodisks and nanoholes. *ACS Photonics* **2015**, 2 (2), 256-262.
52. Jackman, J. A.; Ferhan, A. R.; Cho, N.-J., Nanoplasmonic sensors for biointerfacial science. *Chem. Soc. Rev.* **2017**, 46 (12), 3615-3660.
53. Jackman, J. A.; Špačková, B.; Linardy, E.; Kim, M. C.; Yoon, B. K.; Homola, J.; Cho, N.-J., Nanoplasmonic ruler to measure lipid vesicle deformation. *Chem. Commun.* **2016**, 52 (1), 76-79.
54. Ferhan, A. R.; Jackman, J. A.; Cho, N.-J., Probing spatial proximity of supported lipid bilayers to silica surfaces by localized surface plasmon resonance sensing. *Anal. Chem.* **2017**, 89 (7), 4301-4308.
55. Ferhan, A. R.; Spackova, B.; Jackman, J. A.; Ma, G. J.; Sut, T. N.; Homola, J.; Cho, N.-J., Nanoplasmonic ruler for measuring separation distance between supported lipid bilayers and oxide surfaces. *Anal. Chem.* **2018**, 90 (21), 12503-12511.
56. Fredriksson, H.; Alaverdyan, Y.; Dmitriev, A.; Langhammer, C.; Sutherland, D. S.; Zäch, M.; Kasemo, B., Hole-mask colloidal lithography. *Adv. Mater.* **2007**, 19 (23), 4297-4302.

57. Dahlin, A. B.; Tegenfeldt, J. O.; Höök, F., Improving the instrumental resolution of sensors based on localized surface plasmon resonance. *Anal. Chem.* **2006**, *78* (13), 4416-4423.
58. Langhammer, C.; Larsson, E. M.; Kasemo, B.; Zoric, I., Indirect nanoplasmonic sensing: ultrasensitive experimental platform for nanomaterials science and optical nanocalorimetry. *Nano Lett.* **2010**, *10* (9), 3529-3538.
59. Krimm, S.; Bandekar, J., Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. In *Advances in Protein Chemistry*, Elsevier: 1986; Vol. 38, pp 181-364.
60. Chittur, K. K., FTIR/ATR for protein adsorption to biomaterial surfaces. *Biomaterials* **1998**, *19* (4-5), 357-369.
61. Ausili, A.; Sánchez, M.; Gómez-Fernández, J. C., Attenuated total reflectance infrared spectroscopy: A powerful method for the simultaneous study of structure and spatial orientation of lipids and membrane proteins. *Biomed. Spectrosc. Imaging* **2015**, *4* (2), 159-170.
62. Powell, J.; Wasacz, F.; Jakobsen, R., An algorithm for the reproducible spectral subtraction of water from the FT-IR spectra of proteins in dilute solutions and adsorbed monolayers. *Appl. Spectrosc.* **1986**, *40* (3), 339-344.
63. Oberg, K. A.; Fink, A. L., A new attenuated total reflectance Fourier transform infrared spectroscopy method for the study of proteins in solution. *Anal. Biochem.* **1998**, *256* (1), 92-106.
64. Kong, J.; Yu, S., Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim. Biophys. Sin.* **2007**, *39* (8), 549-559.
65. Bouhekka, A.; Bürgi, T., In situ ATR-IR spectroscopy study of adsorbed protein: Visible light denaturation of bovine serum albumin on TiO₂. *Appl. Surf. Sci.* **2012**, *261*, 369-374.
66. Dong, A.; Huang, P.; Caughey, W. S., Protein secondary structures in water from second-derivative amide I infrared spectra. *Biochemistry* **1990**, *29* (13), 3303-3308.
67. Zeng, H.; Chittur, K. K.; Lacefield, W. R., Analysis of bovine serum albumin adsorption on calcium phosphate and titanium surfaces. *Biomaterials* **1999**, *20* (4), 377-384.

68. Goldberg, M. E.; Chaffotte, A. F., Undistorted structural analysis of soluble proteins by attenuated total reflectance infrared spectroscopy. *Protein Sci.* **2005**, *14* (11), 2781-2792.
69. Barth, A., Infrared spectroscopy of proteins. *Biochim. Biophys. Acta, Bioenerg.* **2007**, *1767* (9), 1073-1101.
70. Givens, B. E.; Xu, Z.; Fiegel, J.; Grassian, V. H., Bovine serum albumin adsorption on SiO₂ and TiO₂ nanoparticle surfaces at circumneutral and acidic pH: a tale of two nano-bio surface interactions. *J. Colloid Interface Sci.* **2017**, *493*, 334-341.
71. Holloway, P. W.; Mantsch, H. H., Structure of cytochrome b5 in solution by Fourier-transform infrared spectroscopy. *Biochemistry* **1989**, *28* (3), 931-935.
72. Towbin, H.; Staehelin, T.; Gordon, J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* **1979**, *76* (9), 4350-4354.
73. Roy, J.; Jain, N.; Singh, G.; Das, B.; Mallick, B., Chapter 5 - small RNA proteome as disease biomarker: an incognito treasure of clinical utility. In *AGO-Driven Non-Coding RNAs*, Mallick, B., Ed. Academic Press: 2019; pp 101-136.
74. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B., Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **2012**, *9* (7), 676.
75. Muller-Eberhard, H. J., The membrane attack complex of complement. *Annu. Rev. Immunol.* **1986**, *4* (1), 503-528.
76. Avrameas, S.; Guilbert, B., Enzyme-immunoassay for the measurement of antigens using peroxidase conjugates. *Biochimie* **1972**, *54* (7), 837-842.

Chapter 4*

Understanding How Natural Sequence Variation in Serum Albumin Proteins Affects Conformational Stability and Protein Adsorption

Serum albumins are evolutionary conserved proteins that are found in many animal species, and are widely used in biotechnology applications, such as components within surface passivation coatings and drug delivery systems. As such, there is interest in studying how serum albumins adsorb onto solid supports, although existing studies are limited to one or two species. Herein, three serum albumins of bovine (BSA), human (HSA), and rat (RSA) origin were comprehensively investigated and differences in their conformational stability and adsorption properties were discovered. Bioinformatics analysis and solution-phase measurements revealed that BSA and HSA existed as individual monomers while RSA formed multimers, and each protein exhibited sequence-dependent variations in conformational stability. Adsorption measurements showed that BSA and HSA proteins adsorb to form well-packed adlayers that depended on their unique conformational stabilities while RSA adsorption formed sparse adlayers and appreciably less spreading of the adsorbed multimers.

*This chapter is published substantially as G.J Ma, A.R. Ferhan, T.N. Sut, J.A. Jackman, N.-J. Cho. Understanding how natural sequence variation in serum albumin proteins affects conformational stability and protein adsorption. *Colloids and Surfaces B: Biointerfaces* **2020**, 194, 111194. DOI: 10.1016/j.colsurfb.2020.111194. (No written permission from Elsevier is necessary for thesis purposes)

4.1 Introduction

There is broad interest in studying protein adsorption at solid-liquid interfaces,¹ which is important for fundamental biointerfacial science as well as for applications such as medical implants,² biosensors,³ and bionanotechnology tools.^{4, 5} Over the years, a wide range of model protein systems have been studied and perhaps the most important one is the class of serum albumin proteins that are found in the vertebrate blood of various mammals, are naturally abundant, and are well-characterized in terms of fractionation methods for extraction.^{6, 7} Certain species are also widely used as surface passivation (“blocking”) agents in molecular biology and biotechnology applications.⁸⁻¹⁰

One important factor affecting the adsorption behavior of serum albumins, and proteins in general, is the conformational stability of a protein in the solution phase. A protein will naturally fold into the most energetically stable structure that is kinetically accessible, and a protein with greater conformational stability (*i.e.*, due to stronger intramolecular forces) will prefer to remain in that state and adsorption can be less favorable in such cases. On the other hand, proteins with lower conformational stability typically have weaker intramolecular forces that hold together the folded protein structure, leaving protein molecules more prone to unfold and denature upon adsorption due to protein-surface interactions.^{11, 12} A key factor influencing conformational stability is the primary amino acid sequence of a protein and corresponding effects on the folded structure. To date, this factor has been explored using engineered proteins with rationally inserted amino acid mutations to either increase or decrease conformational stability and to evaluate the effects on protein adsorption accordingly. Karlsson *et al.* first demonstrated the relationship between protein structure, conformational stability, and adsorption behavior by using several engineered versions of the carbonic anhydrase II protein.¹³ The results showed greater adsorption irreversibility for engineered proteins with lower conformational stability in the solution phase.

While protein engineering enables the systematic investigation of specific amino acid mutations, it is also interesting to profile the conformational stability of naturally occurring,

structurally related proteins in order to determine such effects on adsorption properties, and serum albumins from different species provide an ideal option. Indeed, serum albumins from different species share certain physicochemical features^{14, 15} and similar three-dimensional crystal structures,¹⁶⁻¹⁸ but there are also differences in solution properties,¹⁹⁻²¹ which provides motivation to investigate how structural variations in serum albumin proteins can affect their conformational stability and adsorption behavior. So far, there have been reports that serum albumins from bovine, human, rat, dog, rabbit, and pig species exhibit unique characteristics related to solution-phase conformational stability, such as thermal denaturation profile.²²⁻²⁵ However, a comparative evaluation of the adsorption behavior of different serum albumin species and how such properties are linked with conformational stability is lacking and the adsorption properties of bovine and human serum albumins have been the only ones tested.²⁶⁻³²

Herein, bovine serum albumin (BSA), human serum albumin (HSA), and rat serum albumin (RSA) were experimentally investigated to understand the interplay between naturally occurring sequence variations and corresponding effects on adsorption. Using a bioinformatics approach, amino acid sequence comparisons were first conducted followed by experimental characterization of each protein's solution-phase conformational stability, real-time adsorption behavior, and adsorption-related conformational changes. Collectively, these findings build a mechanistic picture of how sequence variations in BSA, HSA, and RSA proteins affect adsorption behavior and the resulting insights can also guide the engineering of proteins with modulated conformational stabilities for fabricating adsorbed protein layers as biointerfaces.

4.2 Materials and Methods

4.2.1 Reagents

Bovine serum albumin (BSA, A7030, lot no. SLBT4132), human serum albumin (HSA, A3782, lot no. SLBN5035V), rat serum albumin (RSA, A6414, lot no. SLBQ0209V), sodium dodecyl sulfate (SDS, L4390), and sodium chloride (NaCl, 746398) were

purchased from Sigma-Aldrich (USA). Tris(hydroxymethyl)aminomethane (Tris, 0497) was purchased from Amresco (USA). Ethanol (95%) was purchased from Aik Moh (Singapore), and hydrochloric acid (100317) was purchased from Merck (USA).

4.2.2 Sample Preparation

Buffer solutions were prepared to 10 mM Tris, 150 mM NaCl, and pH 7.5 in Milli-Q-treated water (resistivity of $>18.2 \text{ M}\Omega\cdot\text{cm}$ at 25°C) and filtered through a $0.2 \mu\text{m}$ filter (595-4520 Thermo Scientific, USA). All protein solutions were prepared by dissolving lyophilized powders in buffer solution and then filtering through a $0.2 \mu\text{m}$ syringe filter (PN-4612, Pall Corporation, USA). The molar concentrations of BSA, HSA, and RSA proteins in aqueous buffer solution were determined by ultraviolet light absorbance measurements at 280 nm using molar extinction coefficient values of 42,925, 34,445, and $38,915 \text{ M}^{-1} \text{ cm}^{-1}$ respectively.³³

4.2.3 Structural Modeling

The 3D structures of BSA, HSA and RSA proteins were modeled using the SWISS-MODEL server³⁴ by aligning their amino acid sequences with BSA (PDB ID: 4F5S¹⁷), HSA (PDB ID: 1BM0¹⁶), and leporine serum albumin (PDB ID: 6OCK³⁵) template structures, respectively. This alignment was conducted using the ProMod3 modeling engine. Briefly, the models were built by extracting structural information from the template structures, and then resolving amino acid discrepancies (if any) with the target sequence, and the final model was regularized by using a force field.³⁶ The models were visualized using Pymol software.³⁷

4.2.4 Multiple Sequence Alignment

Sequences for BSA, HSA, and RSA proteins were obtained from UniProt with accession numbers P02769, P02768, and P02770 respectively.³⁸ Viewing and editing of the multiple sequence alignments were performed using the Jalview software.³⁹ A gap was added to the

BSA sequence after the L115 position to maximize sequence similarity with HSA and RSA. Coloring of non-conserved amino acids was based on the Zappo coloring scheme,⁴⁰ which qualitatively assigns colors to amino acids based on their physicochemical properties, categorized herein as hydrophobic, polar, positive, negative, aromatic and conformationally special.

4.2.5 Dynamic Light Scattering

Intensity-weighted size distributions of 150 μM BSA, HSA, and RSA proteins were determined by dynamic light scattering (DLS) measurements (ZetaPALS, Brookhaven Instruments, USA) and analyzed by the Particle Solutions software (Brookhaven Instruments). By controlling the temperature in the measurement chamber, temperature-dependent measurements were first recorded at 25 $^{\circ}\text{C}$, and then from 50 to 75 $^{\circ}\text{C}$ in 5 $^{\circ}\text{C}$ increments. A 5-min equilibration was conducted after each temperature increment. Time-dependent measurements were conducted by maintaining the temperature at 60 $^{\circ}\text{C}$ and taking size measurements every 10 min for 200 min. All reported values were obtained from 5 measurements. The standard deviation (s.d.) related to the size distribution of the protein samples was obtained by dividing the full-width-at-half-maximum (FWHM) of the Gaussian size distribution from DLS measurements by $2\sqrt{2 \ln 2} \approx 2.355$.

4.2.6 Circular Dichroism Spectroscopy

An AVIV Model 420 circular dichroism (CD) spectrometer (AVIV Biomedical, USA) was used to measure CD spectra of the protein samples with 1 nm bandwidth, 0.5 nm step size, 0.1 s averaging, and a 200 to 260 nm spectral range. 2.5 μM samples were measured in a 1 mm path length quartz cuvette (110 QS, Hellma, Germany). Temperature-dependent CD spectroscopy measurements were conducted in a similar format to DLS experiments. Measurements were taken in triplicate and all the sample spectra were subtracted by a background buffer spectrum. CD spectra were presented in molar residue ellipticity ($[\theta]$) units based on the following equation:

$$[\theta](\text{degree cm}^2\text{dmol}^{-1}) = \frac{\text{millidegrees}}{n \times c \times l \times 10} \quad (\text{Eq 4.1})$$

where n is the number of amino acid residues, c is the protein molar concentration, and l is the cuvette path length in cm. All CD spectra were smoothed by using the Savitzky-Golay smoothing function⁴¹ with a smoothing window of 20 points and a polynomial order of 2 in the OriginPro 2019b (v9.6.5.169) software package (OriginLab, Northampton, MA, USA). The α -helical percentage of each BSA protein was calculated from the $[\theta]$ data at 222 nm ($[\theta]_{222}$) based on the following equation:⁴²

$$\% \text{ Helicity} = \frac{([\theta]_{222} - 3000)}{(-36000 - 3000)} \times 100 \quad (\text{Eq 4.2})$$

4.2.7 Quartz Crystal Microbalance-Dissipation

The adsorption kinetics of BSA, HSA, and RSA proteins on silica-coated sensor surfaces were characterized by quartz crystal microbalance-dissipation (QCM-D) experiments conducted using the QSense E4 instrument (Biolin Scientific AB, Sweden) with silica-coated quartz crystal sensor chips (QSX 303, Biolin Scientific). Before each experiment, the sensor chips were washed by sequentially rinsing with 1% (wt/vol) aqueous SDS solution, water, and ethanol. After drying under a gentle stream of nitrogen gas, the chips were subjected to oxygen plasma treatment (PDC-002, Harrick Plasma, USA) for 3 min before assembling into the measurement chamber. For adsorption experiments, liquid samples were injected into the measurement chamber at a flow rate of 100 $\mu\text{L}/\text{min}$. Buffer solution was first injected to establish a stable baseline, and then 50 μM protein samples were injected for 30 min, followed by a buffer washing step. All measurements were conducted at 25 °C. The corresponding resonance frequency (ΔF) and energy dissipation (ΔD) shifts were recorded in real-time at multiple odd overtones, as previously described.⁴³ Data collected at the fifth overtone are reported. Measurement operations were controlled by the QSoft 401 (Biolin Scientific) software package.

4.2.8 Localized Surface Plasmon Resonance Sensing

Ensemble-averaged localized surface plasmon resonance (LSPR) experiments were conducted to characterize the adsorption behavior of BSA, HSA, and RSA protein samples onto silica-coated sensor chips containing gold nanodisk arrays using an Insplorion XNano instrument (Insplorion AB, Sweden), as previously described.⁴⁴ Sensor chip washing and LSPR adsorption experiments were conducted in a similar format to QCM-D experiments. The Insplorion software package (Insplorion AB) was used to record shifts in the LSPR centroid (peak) position ($\Delta\lambda$) from the extinction spectrum. The rate of change of the $\Delta\lambda$ shifts was determined by calculating the first derivative with respect to time ($d\Delta\lambda/dt$) (see ref. 45) by using the OriginPro 2019b software package.

4.2.9 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy experiments were conducted using a Bruker Vertex 70 FTIR spectrometer with a liquid-nitrogen-cooled mercury cadmium telluride photodetector (Bruker, Billerica, MA, USA) equipped with a MIRacle three-reflection ZnSe ATR accessory (PIKE Technologies, Madison, WI, USA). 100 μM of each protein solution was pipetted onto the ATR crystal and the absorbance spectrum was immediately recorded to characterize the proteins in their solution-phase. After incubation for 30 min, ten buffer washing steps were performed to remove proteins in solution and weakly adsorbed proteins, and then a second absorbance spectrum was recorded to obtain information on the proteins in the adsorbed state. In each washing step, 30 μL of extra buffer solution (without protein) was added to the sample droplet, followed by aspiration of 30 μL volume from the droplet. The sample compartment was continuously purged with nitrogen gas to minimize ambient moisture during the entire experiment. All spectra were recorded with 4 cm^{-1} resolution and averaged over 128 scans. Spectra of the protein samples were individually subtracted by both background spectra of buffer and water vapor. All measurement operations and spectra subtractions were conducted using the OPUS 6.5 software package (Bruker). The relative contribution of

different secondary structure elements was determined for all samples by curve fitting the amide I bands of the absorbance spectra ($1720 - 1580 \text{ cm}^{-1}$) to Gaussian components based on the peaks identified in the second derivative plot of the absorbance spectra and from literature references.⁴⁶⁻⁵⁰ The secondary structure percentage values were determined by calculating the area under the curve-fitted components that had each been assigned to a secondary structure element as a fraction of the total area under the fitted curve. Peaks centered at $1681\text{-}1678 \text{ cm}^{-1}$ were assigned to β -turn structures, $1656\text{-}1653 \text{ cm}^{-1}$ to α -helix, $1646\text{-}1643 \text{ cm}^{-1}$ to random coil, $1638\text{-}1621 \text{ cm}^{-1}$ to extended chains, and $1618\text{-}1611 \text{ cm}^{-1}$ to intermolecular β -sheets. All curve fitting analyses were conducted using the Peak Analyzer function in the OriginPro 2019b software package.

4.2.10 Statistical Analysis

Statistical analyses were conducted using the GraphPad Prism (v8.0.1) software package. One-way analysis of variance (ANOVA) with multiple comparisons test on the DLS, QCM-D, and LSPR data as well as two-way ANOVA with multiple comparisons test on the ATR-FTIR spectroscopy data were conducted to compute multiplicity-adjusted P values. $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$ indicate the respective levels of statistical significance, as indicated by *, **, ***, and ****. Non-significance is indicated by NS.

4.3 Results and Discussion

4.3.1 Comparing the Amino Acid Sequences Between Serum Albumin Species

The amino acid sequences of BSA, HSA, and RSA proteins were first compared to identify regions of variation that might impact protein conformational stability, followed by experimental characterization of protein conformational stability and adsorption behavior (**Figure 4.1a**). BSA, HSA, and RSA were selected as naturally occurring structural variants of serum albumin proteins. From their 3D model structures, it can be observed that certain similarities exist between the three proteins along with subtle distinctions (**Figure 4.1b**).

Possible sources of distinction would be the different hydrophobicity of the substituted amino acids as well as the different numbers of positively [arginine (R) & lysine (K)] and negatively [aspartic acid (D) & glutamic acid (E)] charged residues, amounting to grand average hydropathicity values of -0.475, -0.395 and -0.432, as well as predicted net charges of -17, -15, and -12 for BSA, HSA, and RSA proteins, respectively, which could affect protein conformational stability.⁵¹

Figure 4.1 **a**, Experimental strategy to characterize how structural variations between serum albumin proteins affect conformational stability and adsorption behavior. **b**, Modeled 3D structures of BSA, HSA, and RSA. **c**, Multiple sequence alignment of BSA, HSA, and RSA. Amino acids are

classified based on their physicochemical properties according to the Zappo color scheme. **d**, List of different physicochemical property pairs and respective number of amino acid substitutions between BSA, HSA, and RSA. Substitutions involving changes in hydrophobic interactions and electrostatic interactions were designated as Type 1 and Type 2, respectively.

To further analyze their sequence differences, a multiple sequence alignment for BSA, HSA, and RSA proteins was plotted (**Figure 4.1c**). The Zappo color scheme helps to identify sequence variations with different physicochemical properties. Such variations could indicate different extents of intramolecular interactions (hydrophobic, electrostatic, and hydrogen bonding) which directly affect protein conformational stability.^{51, 52} Since certain substitutions occur between residues under the same physicochemical classification, different physicochemical property pairs and their respective number of substitutions were listed and in doing so, two types of substitutions that were most likely to affect protein conformational stability were identified: (1) hydrophobic/polar and hydrophobic/charged substitutions that lead to changes in hydrophobic interaction propensity; and (2) neutral/charged substitutions and substitutions between oppositely charged residues that lead to changes in electrostatic interactions (**Figure 4.1d**). Type 1 substitutions comprise almost 40% of all substitutions while Type 2 substitutions were around 60%. These analyses indicate that the three proteins likely have different conformational stabilities on account of variations in amino acid sequence that influence hydrophobic and electrostatic interactions vis-à-vis protein-surface and protein-protein interactions.

4.3.2 Solution-Phase Conformational Stability of Serum Albumin Proteins

The solution-phase conformational stability of each serum albumin was then measured by conducting temperature-dependent dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy experiments. The onset temperature of oligomerization/aggregation was determined by recording the lowest temperature at which an appreciable increase in protein size was observed. CD spectroscopy measurements were conducted to determine the extent of thermal unfolding as indicated by changes in secondary structure.

Figure 4.2 DLS characterization of BSA, HSA, and RSA protein size distributions. DLS measurements of intensity-weighted size distributions for **a**, BSA, **b**, HSA, and **c**, RSA proteins at 25 °C (n=5). The mean hydrodynamic diameter (labeled as D) and polydispersity index (labeled as PDI) are indicated.

The measured sizes of BSA, HSA, and RSA at 25 °C were 9.4, 8.1, and 28.7 nm, respectively, indicating that BSA and HSA exist as monomers while the larger size of RSA suggests that it is present either as irreversibly denatured aggregates^{32, 53} or as non-denatured multimers⁵⁴ (**Figure 4.2 & 4.3**). In all cases, no temperature-induced aggregation was observed at 50 and 55 °C as the sizes of the three serum albumins remained constant up to this temperature range. By contrast, at 60 °C, the sizes of BSA, HSA, and RSA proteins were 12.8, 8.2, and 34.1 nm, respectively, while at 65 °C, their sizes were 23.8, 12.4, and 33.2 nm, respectively. These data indicate that BSA aggregation occurred at 60 °C, while HSA aggregation occurred at a higher temperature of 65 °C. This finding supports that HSA is more stable against temperature-induced aggregation than BSA. Time-dependent experiments further verified that BSA underwent more extensive aggregation than HSA (**Figure 4.4**). Conversely, RSA displayed temperature-induced aggregation behavior at 70 °C, reaching 184.6 nm. In both temperature-dependent and time-dependent DLS measurements, RSA exhibited a slight decrease in size prior to aggregation. This is likely due to temperature-induced unfolding and reconfiguration of its constituent monomers to form more compact denatured oligomers or aggregates. Interestingly, at 70 °C, HSA achieved a larger mean size of 65.7 nm compared to 34.3 nm for BSA despite having a higher onset temperature of aggregation. At 75 °C, HSA and RSA protein aggregated to micron-scale sizes, which were about two orders of magnitude larger than BSA protein aggregates.

Figure 4.3 Hydrodynamic diameter of BSA, HSA, and RSA measured by dynamic light scattering (DLS) as a function of temperature. Data are presented as mean $\pm$ standard deviation (s.d.) [n=5, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (versus data at 25 °C)].

Figure 4.4 Time-dependent changes in the mean hydrodynamic diameters of BSA, HSA, and RSA proteins at 60 °C. Values are presented as mean $\pm$ s.d., where s.d. is defined as full-width-at-half-maximum (FWHM)/2.355 (n=5).

Next, CD spectroscopy measurements were conducted to determine the percentage of α -helical secondary structure in each protein as a function of temperature (**Figure 4.5 & 4.6**). CD spectroscopy measurements showed that, at 25 °C, BSA, HSA, and RSA had helicity percentage values of 62.3, 61.8, and 60.2% respectively, indicating that all serum albumins have high levels of secondary structure and were not denatured.^{32, 53, 55} Taking this together with the DLS measurements at 25 °C, which showed that RSA possessed a hydrodynamic diameter approximately three times larger than BSA and HSA, the overall data support that RSA's larger size is not due to the denaturation and subsequent aggregation of RSA monomers, but due to the formation of a multimeric complex by several non-denatured RSA monomers, i.e., a multimer. The multimeric configuration of RSA proteins might relate to its lower net charge than BSA and HSA as mentioned above, which could facilitate more extensive protein-protein interactions.

Figure 4.5 Temperature-dependent circular dichroism (CD) spectra of BSA, HSA, and RSA proteins. CD spectra of **a**, BSA, **b**, HSA, and **c**, RSA proteins were measured at 25, 50, 55, 60, 65, 70, and 75 °C and are reported in molar residue ellipticity units $[\theta]$.

Figure 4.6 α -helical percentage of BSA, HSA, and RSA as a function of temperature from circular dichroism (CD) spectroscopy experiments. Data are reported as mean $\pm$ s.d. ($n=3$). Mean values are presented on top of each column.

At higher temperatures, all three proteins underwent thermally induced unfolding, which

is observed by a decrease in α -helical percentage at each temperature increment. Serum albumins underwent reversible unfolding at lower temperatures while the folding became irreversible at higher temperatures.²⁴ In order to take into account the latter, which is usually related to temperature-induced aggregation, the mean α -helical percentage values at 65 °C were compared, which showed that BSA had the lowest mean α -helical percentage value of 48.8%, followed by RSA with 49.0% and HSA with 51.9%. This trend continued at 70 °C with BSA, RSA, and HSA having values of 41.8%, 42.3%, and 43.5%, respectively, which indicates that BSA underwent the most extensive thermally induced unfolding, followed by RSA and then HSA. At 75 °C, all three serum albumins had similar helicity values despite HSA and RSA displaying more extensive aggregation than BSA, as indicated by the DLS experiments. Therefore, the DLS and CD spectroscopy data indicate that, while BSA had the lowest onset temperature of aggregation and most extensive thermal unfolding, BSA also formed the smallest protein aggregates. These data support that BSA has lower conformational stability but greater colloidal stability than HSA and RSA. In other words, HSA and RSA have higher onset temperatures of aggregation, but form larger aggregates than BSA once unfolded. Collectively, both DLS and CD experiments show that BSA has the lowest solution-phase conformational stability while HSA has the highest, which agrees with the DLS data and other methods.²²⁻²⁴

4.3.3 Comparing the Adsorption Behavior of Serum Albumin Proteins

The adsorption behavior of the serum albumin proteins on hydrophilic silica surfaces were next characterized by using the quartz crystal microbalance-dissipation (QCM-D) technique. The QCM-D resonance frequency shift (ΔF) corresponds to the mass of the adsorbed protein molecules, including hydrodynamically-coupled solvent, while the energy dissipation shift (ΔD) corresponds to the viscoelasticity of the adsorbed film. Larger ΔF shifts typically indicate more adsorption uptake. At adsorption saturation, the adsorption uptake of BSA, HSA, and RSA in terms of mean absolute final frequency shift ($|\Delta F_{\max}|$) was 38.0, 45.0, and 53.4 Hz, respectively (**Figure 4.7a, b**). The corresponding mean dissipation shifts ($\Delta D_{\max}$) were 2.6, 2.4, and 4.5×10^{-6} , respectively (**Figure 4.7c, d**). The higher $|\Delta F_{\max}|$ of HSA vs. BSA supports that more HSA monomers adsorbed onto the

silica surface while similar $\Delta D_{\max}$ values were also recorded. Conversely, the larger $|\Delta F_{\max}|$ and $\Delta D_{\max}$ values from the adsorption of multimeric RSA proteins indicates the formation of a thicker and more viscoelastic layer on the silica surface. Further analysis of time-independent F-D curves showed that the BSA and HSA curves have similar gradients and thus form similarly rigid adlayers (**Figure 4.8**). By contrast, RSA's F-D curve yielded a steeper gradient (greater increase in energy dissipation per change in frequency), supporting that RSA multimers form less rigid adlayers than BSA and HSA monomers.

Figure 4.7 **a**, Time-resolved QCM-D ΔF shifts and **b**, corresponding $|\Delta F_{\max}|$ values at adsorption saturation. **c**, Time-resolved QCM-D ΔD shifts and **d**, corresponding $\Delta D_{\max}$ values at adsorption saturation. Data in **b** and **d** are presented as mean $\pm$ s.d. ($n=3$, one-way ANOVA with Tukey's multiple comparisons test). Test results are reported for HSA and RSA (versus BSA). Dots represent individual data points.

Figure 4.8 QCM-D frequency-energy dissipation curves for comparing the adsorption profiles of each protein onto silica surface. Time-independent frequency-energy dissipation (F-D) curves are derived from QCM-D frequency and energy dissipation shifts for BSA, HSA, and RSA protein adsorption onto silica surfaces at 25 °C.

To corroborate the QCM-D adsorption results, localized surface plasmon resonance (LSPR) experiments were next conducted to track protein adsorption onto silica-coated gold nanodisk arrays. Unlike the acoustic-based QCM-D technique, the LSPR platform is optical-based and does not take into account hydrodynamically-coupled solvent mass, only the protein mass itself.⁵⁶⁻⁵⁸ LSPR measurements also have greater surface sensitivity because of the technique's much shorter sensing depth of <20 nm compared to ~200 nm in QCM-D measurements,⁵⁹ and therefore yields a higher signal response when an adsorbed protein undergoes more extensive deformation-related spreading and is thus, on average, closer to the sensor surface.⁵⁷ LSPR extinction peak shifts ($\Delta\lambda$) were recorded in real time to track the adsorption of the three serum albumins (**Figure 4.9a**). At adsorption saturation,

BSA, HSA, and RSA yielded mean $\Delta\lambda_{\max}$ shifts of 1.59, 1.44, and 1.28 nm, respectively (**Figure 4.9b**). Interestingly, this trend is directly opposite of the $|\Delta F_{\max}|$ trend obtained from the QCM-D experiments and provides insight into the relative degree of protein denaturation on the silica surface.

Figure 4.9 **a**, Time-resolved LSPR $\Delta\lambda$ shifts and **b**, corresponding $\Delta\lambda_{\max}$ shifts at adsorption saturation. Data in **b**, are presented as mean $\pm$ s.d. ($n=3$, one-way ANOVA with Tukey's multiple comparisons test). Test results are reported for HSA and RSA (versus BSA). Dots represent individual data points.

The findings from both QCM-D and LSPR experiments support that the BSA proteins are, on average, closer to the sensor surface, undergo more surface-induced spreading, and possess a larger adsorption footprint, thereby reducing the total number of adsorbed BSA molecules on the sensor surface compared to the HSA case. Regarding RSA adsorption behavior, the comparatively smaller LSPR $\Delta\lambda$ shift indicates that the entire RSA multimer is detected by the QCM-D platform but only partially detected by the LSPR platform's shorter sensing depth, and that adsorbed RSA molecules are, on average, farther away from the sensor surface and have less dense packing, due to larger-sized RSA multimers having a lower probability of adsorbing between gaps in the pre-adsorbed layer, according to the random sequential adsorption model.⁶⁰ Also, the QCM-D platform detects hydrodynamically-coupled solvent in void gaps, while the LSPR platform does not.⁶¹

Taken together, the combination of QCM-D and LSPR experiments revealed key mechanistic differences between the adsorption behavior and resulting adsorbed film properties of each protein. Particularly, BSA underwent greater spreading than HSA upon adsorption to silica surfaces while RSA multimers were more sparsely adsorbed.

4.3.4 Adsorption-Related Protein Conformational Changes

The adsorption-related conformational changes of each protein were next investigated by further analyzing the LSPR data. The rate of change of LSPR peak shift ($d\Delta\lambda/dt$) was calculated which correlates with the relative extent of surface-induced protein conformational changes (**Figure 4.10a**).⁵⁶⁻⁵⁸ The maximum rate of change ($(d\Delta\lambda/dt)_{\max}$) during the initial stage of adsorption was computed and the mean $(d\Delta\lambda/dt)_{\max}$ values for BSA, HSA, and RSA adsorption were 0.42, 0.42, and 0.16 nm/min, respectively (**Figure 4.10b**). It should be noted here that the experimentally measured rate reflects both the diffusion-limited adsorption and the extent of surface-induced denaturation.^{57, 58} The parameters that affect diffusion is described by the following equation:

$$D = \frac{k_B T}{6\pi\eta r} \quad (\text{Eq 4.3})$$

where D is the diffusion coefficient, k_B is the Boltzmann constant, T is temperature, η is viscosity, and r is the hydrodynamic radius of the adsorbing protein. Since all experiments were conducted at constant room temperature and in identical solution conditions, diffusion-limited adsorption and therefore the theoretical LSPR signal would only be affected by the size of the protein samples according to the following equation:⁵⁷

$$\Delta\lambda \propto D^{2/3} \text{ or } \Delta\lambda \propto \left(\frac{1}{r}\right)^{2/3} \quad (\text{Eq 4.4})$$

To resolve the effects of protein size and surface-induced denaturation on the LSPR rate, the theoretical size-dependent diffusion-limited adsorption rate was compared according

to Eq 4.4 with the experimentally measured rate, and differences between the two trends can be attributed to differences in surface-induced deformation. Upon normalizing to the BSA case, the rates were plotted in **Figure 4.10c** and the diffusion-limited rates for the HSA and RSA were higher than their measured rates, suggesting that HSA and RSA undergo less surface-induced denaturation than BSA during the initial stage of adsorption.

Figure 4.10 **a**, Rate of change of LSPR peak shift during the initial stage of adsorption ($d\Delta\lambda/dt$) as a function of time and **b**, corresponding maximum rate of change of LSPR peak shift during the initial stage of adsorption $(d\Delta\lambda/dt)_{max}$. **c**, Comparison between the rate of size-related diffusion-limited adsorption and the maximum rate of change in LSPR peak shift data from **b**. Rates are normalized to the BSA case. **d**, α -helical percentage of BSA, HSA, and RSA proteins in solution and upon adsorption from ATR-FTIR spectroscopy experiments. Data in **b**, **c**, and **d** are presented as mean $\pm$ s.d. [$n=3$, one-way ANOVA in **b** and two-way ANOVA in **d** with Tukey's multiple comparisons test]. Test results in **b** are reported for HSA and RSA (versus BSA). Test results in **d** are reported for HSA and RSA (versus BSA) in solution and are separately reported for HSA and RSA (versus BSA) in the adsorbed state. Dots in **b** and **d** represent individual data points.

To corroborate these results, attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy experiments were conducted to determine the secondary structure of the three serum albumins in the solution state and in the adsorbed state (**Figure 4.10d** & **Figure 4.11**). The mean α -helical percentage of BSA, HSA, and RSA were 61.2, 63.0, and 63.0%, respectively, in the solution state, and 46.1, 51.9, and 53.8%, respectively, in the adsorbed state. These results show that BSA experienced a greater loss in α -helical percentage than HSA, supporting the adsorption data that BSA underwent more extensive surface-induced denaturation upon adsorption and are more spread out than HSA. Conversely, RSA proteins incurred the least extensive loss in α -helicity. Notably, the ATR-FTIR penetration depth is $\sim 1.2\ \mu\text{m}$,⁶² indicating that the data reflects the average secondary structure of all the molecules within the RSA multimer and supporting that the molecules in contact with the surface denature while other molecules in the multimer predominately remain in their native state, thereby preventing RSA multimers from deforming as much as BSA and HSA monomers. This supports evidence from the adsorption studies that RSA multimers adsorb with relatively larger interparticle spacing due to its larger size and also due to its restricted ability to spread on the surface.

Figure 4.11 Quantification of adsorption-related changes in secondary structure for BSA, HSA, and RSA proteins by ATR-FTIR measurements. Amide I regions from ATR-FTIR spectrograms of **a**, BSA, **b**, HSA, and **c**, RSA proteins in solution (left panels) and in the adsorbed state (right panels). Experimentally obtained absorbance spectra were resolved into individual component

curves, which were each assigned to a specific secondary structure element.

4.3.5 Mechanistic Picture of BSA, HSA and RSA Adsorption Behavior

To summarize the data collected thus far, an overall mechanistic picture of the adsorption behavior for each serum albumin was presented, including their relationship to solution-phase conformational stability. BSA and HSA in solution exist as individual molecules in their native state (monomers), while RSA in solution exist as composites of native monomers (multimers) (**Figure 4.12a**). Compared to HSA, BSA has lower conformational stability in solution, as observed by BSA having a lower onset of temperature-induced aggregation and undergoing more extensive temperature-induced conformational changes. Adsorbed BSA proteins have more extensive surface-induced denaturation than HSA and thus undergo more spreading on the surface as well (**Figure 4.12b**). Taken together, these findings demonstrate how natural sequence variations between serum albumins give rise to variations in conformational stability, and surprisingly colloidal organization (monomers vs. multimers) as well, that directly impact the specific adsorption behavior of each protein. In general, the data support that BSA forms tightly packed adlayers consisting of well-spread protein molecules while HSA does so to a lesser extent. Due to its larger size, RSA forms sparser adlayers which are likely less effective as surface passivation coatings.

Figure 4.12 Schematic illustration of BSA, HSA, and RSA proteins in solution and conformational stability-related adsorption behavior trends. **a**, BSA and HSA proteins exist as monomers while RSA proteins are assembled as multimers in solution. **b**, BSA, HSA, and RSA display different adsorption behaviors. BSA's lower conformational stability than HSA leads to greater surface-induced denaturation and larger footprint size. Adsorption of larger RSA multimers results in denaturation of only the molecules in contact with the surface (darker colored) while other molecules remain in their native state.

4.4 Conclusion

Herein, the conformational stability and adsorption behavior of three different serum albumins, BSA, HSA, and RSA, were compared to unravel how naturally occurring, evolutionary conserved variations in amino acid sequence affect protein structure and consequently influence protein adsorption behaviors. It was found that BSA and HSA are monomers in solution, while RSA exists as multimers consisting of helical protein molecules, and the two types of macromolecular assemblies have distinct adsorption behaviors. BSA and HSA adsorbed onto silica surfaces to form a single layer of adsorbed protein molecules and the extent of protein adsorption uptake and spreading depended on

their conformational stability. Conversely, RSA adsorption resulted in less-dense adlayers because the adsorbing species were multimers and there was less spreading of adsorbed protein molecules within the adlayer because only some protein molecules within the multimers contacted the surface and were subjected to protein-surface interactions directly. Thus, even with the range of evolutionary conserved serum albumins with high sequence and structural similarity, key differences in conformational stability and adsorption behavior were observed. These findings not only offer fundamental insight into protein adsorption but also provide mechanistic support to explain why, among the serum albumins, BSA is so useful to form surface passivation (“blocking”) coatings on solid supports. The combination of solution-phase and surface-sensitive experiments along with bioinformatics provides an avenue to also engineer serum albumin derivatives and other proteins with programmed conformational stabilities and other physiochemical characteristics (*e.g.*, net charge) to modulate adsorption behaviors and potentially control the formation of protein layers with enhanced application possibilities.

References

1. Rabe, M.; Verdes, D.; Seeger, S., Understanding protein adsorption phenomena at solid surfaces. *Adv. Colloid Interface Sci.* **2011**, *162* (1-2), 87-106.
2. Meyers, S. R.; Grinstaff, M. W., Biocompatible and bioactive surface modifications for prolonged in vivo efficacy. *Chem. Rev.* **2011**, *112* (3), 1615-1632.
3. del Río, J. S.; Henry, O. Y.; Jolly, P.; Ingber, D. E., An antifouling coating that enables affinity-based electrochemical biosensing in complex biological fluids. *Nat. Nanotechnol.* **2019**, *14* (12), 1143-1149.
4. Walkey, C. D.; Chan, W. C., Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. *Chem. Soc. Rev.* **2012**, *41* (7), 2780-2799.
5. Lu, X.; Xu, P.; Ding, H.-M.; Yu, Y.-S.; Huo, D.; Ma, Y.-Q., Tailoring the component of protein corona via simple chemistry. *Nat. Commun.* **2019**, *10* (1), 1-14.

6. Cohn, E. J.; Strong, L. E.; Hughes, W.; Mulford, D.; Ashworth, J.; Melin, M.; Taylor, H., Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.* **1946**, 68 (3), 459-475.
7. Schneider, W.; Lefevre, H.; Fiedler, H.; McCarty, L. J., An alternative method of large scale plasma fractionation for the isolation of serum albumin. *Blut* **1975**, 30 (2), 121-134.
8. Sweryda-Krawiec, B.; Devaraj, H.; Jacob, G.; Hickman, J. J., A new interpretation of serum albumin surface passivation. *Langmuir* **2004**, 20 (6), 2054-2056.
9. Xiao, Y.; Isaacs, S. N., Enzyme-linked immunosorbent assay (ELISA) and blocking with bovine serum albumin (BSA)—not all BSAs are alike. *J. Immunol. Methods* **2012**, 384 (1-2), 148-151.
10. Belling, J. N.; Jackman, J. A.; Yorulmaz Avsar, S.; Park, J. H.; Wang, Y.; Potroz, M. G.; Ferhan, A. R.; Weiss, P. S.; Cho, N.-J., Stealth immune properties of graphene oxide enabled by surface-bound complement Factor H. *ACS Nano* **2016**, 10 (11), 10161-10172.
11. Tanford, C., Protein denaturation. In *Advances in Protein Chemistry*, Elsevier: 1968; Vol. 23, pp 121-282.
12. Norde, W., Adsorption of proteins from solution at the solid-liquid interface. *Adv. Colloid Interface Sci.* **1986**, 25, 267-340.
13. Karlsson, M.; Ekeröth, J.; Elwing, H.; Carlsson, U., Reduction of irreversible protein adsorption on solid surfaces by protein engineering for increased stability. *J. Biol. Chem.* **2005**, 280 (27), 25558-25564.
14. Peters Jr, T., Serum albumin. In *Adv. Protein Chem.*, Elsevier: 1985; Vol. 37, pp 161-245.
15. Carter, D. C.; Ho, J. X., Structure of serum albumin. In *Advances in Protein Chemistry*, Elsevier: 1994; Vol. 45, pp 153-203.
16. Sugio, S.; Kashima, A.; Mochizuki, S.; Noda, M.; Kobayashi, K., Crystal structure of human serum albumin at 2.5 Å resolution. *Protein Eng.* **1999**, 12 (6), 439-446.
17. Bujacz, A., Structures of bovine, equine and leporine serum albumin. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2012**, 68 (10), 1278-1289.

18. Bujacz, A.; Talaj, J. A.; Zielinski, K.; Pietrzyk-Brzezinska, A. J.; Neumann, P., Crystal structures of serum albumins from domesticated ruminants and their complexes with 3, 5-diiodosalicylic acid. *Acta Crystallogr., Sect. D: Struct. Biol.* **2017**, *73* (11), 896-909.
19. Kosa, T.; Maruyama, T.; Otagiri, M., Species differences of serum albumins: I. Drug binding sites. *Pharm. Res.* **1997**, *14* (11), 1607-1612.
20. Pistolozzi, M.; Bertucci, C., Species-dependent stereoselective drug binding to albumin: a circular dichroism study. *Chirality* **2008**, *20* (3-4), 552-558.
21. Poór, M.; Li, Y.; Matisz, G.; Kiss, L.; Kunsági-Máté, S.; Kőszegi, T., Quantitation of species differences in albumin–ligand interactions for bovine, human and rat serum albumins using fluorescence spectroscopy: a test case with some Sudlow's site I ligands. *J. Lumin.* **2014**, *145*, 767-773.
22. Kosa, T.; Maruyama, T.; Otagiri, M., Species differences of serum albumins: II. Chemical and thermal stability. *Pharm. Res.* **1998**, *15* (3), 449-454.
23. Bleustein, C. B.; Sennett, M.; Kung, R. T.; Felsen, D.; Poppas, D. P.; Stewart, R. B., Differential scanning calorimetry of albumin solders: interspecies differences and fatty acid binding effects on protein denaturation. *Lasers Surg. Med.* **2000**, *27* (5), 465-470.
24. Michnik, A.; Michalik, K.; Kluczevska, A.; Drzazga, Z., Comparative DSC study of human and bovine serum albumin. *J. Therm. Anal. Calorim.* **2005**, *84* (1), 113-117.
25. Ahmad, E.; Sen, P.; Khan, R. H., Structural stability as a probe for molecular evolution of homologous albumins studied by spectroscopy and bioinformatics. *Cell Biochem. Biophys.* **2011**, *61* (2), 313-325.
26. Kurrat, R.; Prenosil, J.; Ramsden, J., Kinetics of human and bovine serum albumin adsorption at silica–titania surfaces. *J. Colloid Interface Sci.* **1997**, *185* (1), 1-8.
27. Mao, Y.; Wei, W.; Peng, H.; Zhang, J., Monitoring for adsorption of human serum albumin and bovine serum albumin onto bare and polystyrene-modified silver electrodes by quartz crystal impedance analysis. *J. Biotechnol.* **2001**, *89* (1), 1-10.

28. Iafisco, M.; Sabatino, P.; Lesci, I. G.; Prat, M.; Rimondini, L.; Roveri, N., Conformational modifications of serum albumins adsorbed on different kinds of biomimetic hydroxyapatite nanocrystals. *Colloids Surf., B* **2010**, *81* (1), 274-284.
29. Voicescu, M.; Ionescu, S.; Angelescu, D. G., Spectroscopic and coarse-grained simulation studies of the BSA and HSA protein adsorption on silver nanoparticles. *J. Nanopart. Res.* **2012**, *14* (10), 1174.
30. Bekale, L.; Agudelo, D.; Tajmir-Riahi, H., Effect of polymer molecular weight on chitosan–protein interaction. *Colloids Surf., B* **2015**, *125*, 309-317.
31. Cristian, R. E.; Mohammad, I. J.; Mernea, M.; Sbarcea, B. G.; Trica, B.; Stan, M. S.; Dinischiotu, A., Analyzing the interaction between two different types of nanoparticles and serum albumin. *Materials* **2019**, *12* (19), 3183.
32. Park, J. H.; Ferhan, A. R.; Jackman, J. A.; Cho, N.-J., Modulating conformational stability of human serum albumin and implications for surface passivation applications. *Colloids Surf., B* **2019**, *180*, 306-312.
33. Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T., How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **1995**, *4* (11), 2411-2423.
34. Waterhouse, A.; Bertoni, M.; Bienert, S.; Studer, G.; Tauriello, G.; Gumienny, R.; Heer, F. T.; de Beer, T. A. P.; Rempfer, C.; Bordoli, L., SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* **2018**, *46* (W1), W296-W303.
35. Zielinski, K.; Sekula, B.; Bujacz, A.; Szymczak, I., Structural investigations of stereoselective profen binding by equine and leporine serum albumins. *Chirality* **2020**.
36. Mackerell Jr, A. D.; Feig, M.; Brooks III, C. L., Extending the treatment of backbone energetics in protein force fields: limitations of gas-phase quantum mechanics in reproducing protein conformational distributions in molecular dynamics simulations. *J. Comput. Chem.* **2004**, *25* (11), 1400-1415.
37. DeLano, W. L., PyMOL. 2002.
38. Consortium, U., UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res.* **2019**, *47* (D1), D506-D515.

39. Waterhouse, A. M.; Procter, J. B.; Martin, D. M.; Clamp, M.; Barton, G. J., Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **2009**, *25* (9), 1189-1191.
40. Procter, J. B.; Thompson, J.; Letunic, I.; Creevey, C.; Jossinet, F.; Barton, G. J., Visualization of multiple alignments, phylogenies and gene family evolution. *Nat. Methods* **2010**, *7* (3), S16-S25.
41. Savitzky, A.; Golay, M. J., Smoothing and differentiation of data by simplified least squares procedures. *Anal. Chem.* **1964**, *36* (8), 1627-1639.
42. Morrisett, J. D.; David, J. S.; Pownall, H. J.; Gotto Jr, A. M., Interaction of an apolipoprotein (apoLP-alanine) with phosphatidylcholine. *Biochemistry* **1973**, *12* (7), 1290-1299.
43. Cho, N.-J.; Frank, C. W.; Kasemo, B.; Höök, F., Quartz crystal microbalance with dissipation monitoring of supported lipid bilayers on various substrates. *Nat. Protoc.* **2010**, *5* (6), 1096.
44. Jackman, J. A.; Zhdanov, V. P.; Cho, N.-J., Nanoplasmonic biosensing for soft matter adsorption: kinetics of lipid vesicle attachment and shape deformation. *Langmuir* **2014**, *30* (31), 9494-9503.
45. Zan, G. H.; Jackman, J. A.; Kim, S. O.; Cho, N. J., Controlling lipid membrane architecture for tunable nanoplasmonic biosensing. *Small* **2014**, *10* (23), 4828-4832.
46. Murayama, K.; Tomida, M., Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy. *Biochemistry* **2004**, *43* (36), 11526-11532.
47. Kong, J.; Yu, S., Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim. Biophys. Sin.* **2007**, *39* (8), 549-559.
48. Barth, A., Infrared spectroscopy of proteins. *Biochim. Biophys. Acta, Bioenerg.* **2007**, *1767* (9), 1073-1101.
49. Srour, B.; Bruechert, S.; Andrade, S. L.; Hellwig, P., Secondary Structure Determination by Means of ATR-FTIR Spectroscopy. In *Membrane Protein Structure and Function Characterization*, Springer: 2017; pp 195-203.

50. Givens, B. E.; Xu, Z.; Fiegel, J.; Grassian, V. H., Bovine serum albumin adsorption on SiO₂ and TiO₂ nanoparticle surfaces at circumneutral and acidic pH: a tale of two nano-bio surface interactions. *J. Colloid Interface Sci.* **2017**, *493*, 334-341.
51. Strickler, S. S.; Gribenko, A. V.; Gribenko, A. V.; Keiffer, T. R.; Tomlinson, J.; Reihle, T.; Loladze, V. V.; Makhatadze, G. I., Protein stability and surface electrostatics: a charged relationship. *Biochemistry* **2006**, *45* (9), 2761-2766.
52. Kragh-Hansen, U.; Saito, S.; Nishi, K.; Anraku, M.; Otagiri, M., Effect of genetic variation on the thermal stability of human serum albumin. *Biochim. Biophys. Acta, Proteins Proteomics* **2005**, *1747* (1), 81-88.
53. Park, J. H.; Jackman, J. A.; Ferhan, A. R.; Ma, G. J.; Yoon, B. K.; Cho, N.-J., Temperature-induced denaturation of BSA protein molecules for improved surface passivation coatings. *ACS Appl. Mater. Interfaces* **2018**, *10* (38), 32047-32057.
54. Mannuzza, F. J.; Montalto, J. G., Is bovine albumin too complex to be just a commodity? *BioProcess Int.* **2010**, *8* (4), 42-49.
55. Zazeri, G.; Povinelli, A. P. R.; Lima, M. d. F.; Cornélio, M. L., Experimental Approaches and Computational Modeling of Rat Serum Albumin and Its Interaction with Piperine. *Int. J. Mol. Sci.* **2019**, *20* (12), 2856.
56. Jackman, J. A.; Ferhan, A. R.; Cho, N.-J., Nanoplasmonic sensors for biointerfacial science. *Chem. Soc. Rev.* **2017**, *46* (12), 3615-3660.
57. Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Park, J. H.; Zhdanov, V. P.; Cho, N.-J., Indirect nanoplasmonic sensing platform for monitoring temperature-dependent protein adsorption. *Anal. Chem.* **2017**, *89* (23), 12976-12983.
58. Ferhan, A. R.; Jackman, J. A.; Sut, T. N.; Cho, N.-J., Quantitative comparison of protein adsorption and conformational changes on dielectric-coated nanoplasmonic sensing arrays. *Sensors* **2018**, *18* (4).
59. Jackman, J. A.; Yorulmaz Avsar, S.; Ferhan, A. R.; Li, D.; Park, J. H.; Zhdanov, V. P.; Cho, N.-J., Quantitative profiling of nanoscale liposome deformation by a localized surface plasmon resonance sensor. *Anal. Chem.* **2017**, *89* (2), 1102-1109.
60. Nord, R.; Evans, J. W., Irreversible immobile random adsorption of dimers, trimers,... on 2 D lattices. *J. Chem. Phys.* **1985**, *82* (6), 2795-2810.

61. Gillissen, J. J.; Jackman, J. A.; Tabaei, S. R.; Cho, N.-J., A numerical study on the effect of particle surface coverage on the quartz crystal microbalance response. *Anal. Chem.* **2018**, *90* (3), 2238-2245.
62. Chittur, K. K., FTIR/ATR for protein adsorption to biomaterial surfaces. *Biomaterials* **1998**, *19* (4-5), 357-369.

Chapter 5*

Quantitative Assessment of Bovine Serum Albumin Proteins from Different Purification and Processing Strategies

Bovine serum albumin (BSA) is one of the most widely used protein reagents in the scientific community, especially for surface passivation (“blocking”) applications in various bioassays. Numerous BSA protein options are commercially available, however, there is scarce information about which ones are preferable for blocking applications. Herein, various biophysical measurements were conducted to quantitatively compare the conformational and adsorption properties of BSA protein reagents that were obtained through six purification methods. Depending on the method, there were significant differences in the conformational and adsorption properties of BSA proteins, mainly due to the presence of fatty acid stabilizers. The mechanistic factors behind these performance variations were critically discussed and these findings offer a practical framework to guide BSA selection for surface passivation applications.

*Results in this chapter are published substantially as G.J Ma, A.R. Ferhan, J.A. Jackman, N.-J. Cho. Conformational flexibility of fatty acid-free bovine serum albumin proteins enables superior antifouling coatings. *Communications Materials* **2020**, *1*, 45. DOI: 10.1038/s43246-020-0047-9 (Under the terms of the CC BY 4.0 license, no permission is required to reuse this article).

Results in this chapter were also submitted as a preprint as G.J Ma, A.R. Ferhan, J.A. Jackman, N.-J. Cho. Quantitative assessment of bovine serum albumin proteins for blocking applications. *bioRxiv* **2019**, 869677. DOI: 10.1101/869677. Reprinted with permission. Copyright 2019, the author.

5.1 Introduction

A common element of bioassays is the need to minimize nonspecific binding of biomolecules in order to maximize assay specificity and sensitivity.^{1, 2} To meet this objective, a wide range of reagents, ranging from crude materials such as milk proteins to synthetic nanomaterials such as finely tuned polymers, have been developed to coat target surfaces and to minimize nonspecific binding events—a surface passivation function that is commonly referred to as “blocking”.³⁻⁷ Among the various options, bovine serum albumin (BSA) is the most widely used protein reagent for blocking applications and has several practical advantages such as natural abundance, low cost, well-established purification methods, and broad availability. BSA is regarded as a gold-standard blocking reagent in numerous bioassays, including enzyme-linked immunosorbent assays (ELISA),^{8, 9} blots,^{10, 11} immunohistochemistry,^{12, 13} cell culture,¹⁴ and biosensors.^{15, 16} It is used as a coating to improve nanoparticle stability and biocompatibility,^{17, 18} and is also commonly incorporated into buffer solutions to prevent the loss of precious materials such as nucleic acids, peptides, and enzymes.^{19, 20}

Within this scope, an often-overlooked aspect of BSA as a blocking reagent is that there are many types of commercially available BSA that differ in terms of how the protein was isolated from bovine plasma, which is its principal natural source.²¹ These processing differences relate to the choice of fractionation method and the use of chemical stabilizers, and such differences can cause significant variations in blocking performance in bioassays, even when comparing BSA reagents from the same company.²² However, there is essentially no discussion in the scientific literature about what type(s) of BSA reagents are useful to pick for blocking applications, especially from a mechanistic perspective. Most blocking-related studies that investigate mechanistic details have only evaluated a single type of BSA and compare BSA adsorption and surface-induced conformational changes on different surfaces^{23, 24} or investigate the effects of ionic strength²⁵ or BSA pretreatment.²⁶ However, the comparison of BSA proteins obtained through different purification methods remains unexplored despite being the greatest source of variability in BSA reagent options on the market. Furthermore, there is limited reporting of the type of

BSA reagent that is used in most scientific publications; the supplier company is sometimes listed, but the catalog number of the reagent is rarely provided. As such, BSA reagent selection for blocking applications largely remains a matter of trial-and-error and laboratory precedent, and establishing an evidence-backed practical framework to guide BSA reagent selection is an outstanding need.

Herein, the objective of this study was to investigate the conformational and adsorption properties of commercially available BSA reagents that were obtained through six purification methods, and to develop a practical framework to guide BSA reagent selection for blocking applications. These findings provide mechanistic insights to explain why certain types of BSA reagents are favorable over other options, and also outline key functional criteria to develop improved versions of BSA and other protein reagents for blocking applications.

5.2 Materials and Methods

5.2.1 Bovine Serum Albumin Proteins

The six types of bovine serum albumin (BSA) used in this study were selected based on three different fractionation methods without or with a fatty acid removal step, and were procured from Sigma-Aldrich (St. Louis, MO, USA). Lyophilized powders of fatty acid-containing BSA proteins purified by cold ethanol fractionation, heat-shock fractionation, or cold ethanol followed by heat-shock fractionation were selected and have catalog nos. A2153, A3059, and A7638, respectively. These three fatty BSA proteins were labeled BSA 1, 2, 3, respectively. The corresponding fatty acid-free versions were also obtained and have catalog nos. A6003, A7030 and A0281, respectively. These three defatted BSA proteins were labeled BSA 4, 5, 6, respectively.

5.2.2 Reagents

Sodium dodecyl sulfate (SDS, catalog no. L4390), sodium chloride (NaCl, catalog no.

746398), sodium hydroxide (catalog no. S5881) and octanoic acid (caprylic acid, catalog no. C2875) were also purchased from Sigma-Aldrich while Tris(hydroxymethyl)aminomethane (Tris, catalog no. 0497) was purchased from Amresco (Solon, OH, USA). Ethanol (95%) was purchased from Aik Moh (Singapore), hydrochloric acid (HCl, catalog no. 100317) was purchased from Merck (Burlington, MA, USA).

5.2.3 Sample Preparation

An aqueous buffer solution of 10 mM Tris, 150 mM NaCl, and pH 7.5 was prepared with Milli-Q-treated water (resistivity of $>18.2 \text{ M}\Omega\cdot\text{cm}$ at 25°C) and filtered through a $0.2 \mu\text{m}$ polyethersulfone (PES) membrane filter (Thermo Fisher Scientific, catalog no. 595-4520). BSA solutions were prepared by dissolving lyophilized BSA powder in this Tris buffer and then filtering through a $0.2 \mu\text{m}$ syringe filter (catalog no. PN-4612; Pall Corporation, Port Washington, NY, USA). The molar concentrations of BSA proteins in aqueous buffer solution were determined by ultraviolet (UV) light absorption measurements at 280 nm and with a molar extinction coefficient value of $43,824 \text{ M}^{-1} \text{ cm}^{-1}$. Where applicable, the addition of caprylic acid to BSA samples was conducted by first dissolving caprylic acid and sodium hydroxide into the Tris buffer in order to make a 50 mM caprylic acid solution at pH 7.5. The caprylic acid solution was then added to appropriate amounts of BSA solution in order to yield caprylic acid-doped BSA sample with a molar ratio of 10:1 caprylic acid:BSA.

5.2.4 Dynamic Light Scattering

Dynamic light scattering (DLS) was employed to measure the average hydrodynamic diameter and polydispersity of BSA protein molecules in solution using a particle size analyzer (ZetaPALS, Brookhaven Instruments, Holtsville, NY, USA) that is equipped with a 658.0 nm monochromatic laser. Measurements were taken at a 90° scattering angle and the BIC Particle Sizing software (v5.27; Brookhaven Instruments) was used to analyze the intensity autocorrelation function in order to obtain the intensity-weighted Gaussian size distribution. The temperature in the measurement chamber was controlled with a feedback

loop and measurements of a protein sample were first recorded at 25 °C, followed by heating and increasing the temperature from 50 °C to 75 °C in 5 °C increments. After each temperature step increase, the measurement chamber was equilibrated for 5 min before the DLS measurement was performed. In select time-dependent measurements, the temperature in the measurement chamber was first raised from 50 °C to 55 °C before maintaining a constant temperature at 60 °C and measuring protein size every 10 min for 200 min. All reported values were obtained from 5 measurements.

5.2.5 Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy experiments were conducted using an AVIV Model 420 CD spectrometer with the AVIV CDS software package (v3.36 MX) (AVIV Biomedical, Lakewood, NJ, USA). CD spectroscopy measurements were conducted using 400 µL solutions of 2.5 µM BSA samples in a 1 mm path length cuvette with a polytetrafluoroethylene (PTFE) stopper (catalog no. 110-QS; Hellma, Müllheim, Germany). The measurements were recorded with a 1 nm spectral bandwidth, 0.5 nm step size, and an averaging time of 0.1 s. Elevated temperature experiments were conducted by increasing the temperature in the measurement chamber in 5 °C increments from 50 to 75 °C and recording the spectra at every temperature point after a 5 min equilibration time. All resulting spectra were processed by subtracting a background spectra of equivalent solution conditions without BSA protein and the data were presented in molar residue molar ellipticity ($[\theta]$) units based on the following equation:

$$[\theta](\text{degree cm}^2\text{dmol}^{-1}) = \frac{\text{millidegrees}}{n \times c \times l \times 10} \quad (\text{Eq 5.1})$$

where n is the number of amino acid residues, c is the protein molar concentration, and l is the cuvette path length in cm. All CD spectra were smoothed by using the Savitzky-Golay²⁷ smoothing function with a smoothing window of 20 points and a polynomial order of 2 in the OriginPro 2019b (v9.6.5.169) software package (OriginLab, Northampton, MA, USA). The α -helical percentage of each BSA protein was calculated from the $[\theta]$ data at 222 nm

($[\theta]_{222}$) based on the following equation:²⁸

$$\% \text{ Helicity} = \frac{([\theta]_{222} - 3000)}{(-36000 - 3000)} \times 100 \quad (\text{Eq 5.2})$$

5.2.6 Quartz Crystal Microbalance-Dissipation

Quartz crystal microbalance-dissipation (QCM-D) measurements were conducted using a QSense E4 instrument (Biolin Scientific AB, Stockholm, Sweden). A silica-coated AT-cut quartz crystal sensor chip with a fundamental frequency of 5 MHz (QSX 303, Biolin Scientific) was used to characterize protein adsorption onto silica surfaces. The temperature was set to 25 °C for all experiments, and the measurement operation was controlled by the QSoft 401 (v2.5.13.664) (Biolin Scientific) software package. Prior to each experiment, the sensor chips were sequentially rinsed with 1% (wt/vol) aqueous SDS solution, water, and ethanol, and then dried under a gentle stream of nitrogen gas, followed by treatment with oxygen plasma (PDC-002, Harrick Plasma, Ithaca, NY, USA) for 3 min. A peristaltic pump was used to inject liquid samples into the measurement chamber at a volumetric flow rate of 100 $\mu\text{L min}^{-1}$. A stable baseline signal was first established in Tris buffer solution before a 100 μM BSA protein was introduced into the measurement chamber for 30 min, followed by a buffer washing step. The resonance frequency (ΔF) and energy dissipation (ΔD) shifts were recorded in real-time at multiple odd overtones, as previously described,²⁹ and the normalized data at the fifth overtone are reported.

5.2.7 Localized Surface Plasmon Resonance Sensing

Ensemble-averaged localized surface plasmon resonance (LSPR) measurements were conducted in optical transmission mode using an Insplorion XNano instrument (Insplorion AB, Gothenburg, Sweden), as previously described.³⁰ A white light beam illuminating a circular area of $\sim 4 \text{ mm}^2$ is transmitted through a silica-coated sensor chip (Insplorion) consisting of well-separated gold nanodisk arrays ($\sim 8\%$ surface coverage) deposited by hole-mask colloidal lithography³¹ on a transparent glass substrate. The nanodisks had an

average height and diameter of 20 and 120 nm, respectively, and the surface of the sensor chip was conformally coated with a silica film (~10 nm thick). The light was then passed through a quartz glass window and collected by a spectrophotometer. Prior to each experiment, the sensor chip was sequentially rinsed with 1% (wt/vol) aqueous SDS solution, water and ethanol, then dried under a gentle stream of nitrogen gas. The chip was then treated with oxygen plasma for 2.5 min. The sensor chip was then loaded into the measurement chamber and liquid samples were introduced by a peristaltic pump at a volumetric flow rate of $100 \mu\text{L min}^{-1}$. A stable baseline signal was established in Tris buffer solution before $100 \mu\text{M}$ BSA was introduced into the measurement chamber under continuous flow for 30 min, followed by a buffer washing step. The Insploer software package (Insploerion AB) was used to record the LSPR extinction spectra with a time resolution of 1 Hz and the centroid (peak) position (λ) in the extinction spectrum at each time point was calculated by using a high-order polynomial fitting.³² Thus, it was possible to determine the time-resolved $\Delta\lambda$ shift due to protein adsorption and the time-derivative plot of the $\Delta\lambda$ shift was also calculated using the OriginPro 2019b software package.

5.2.8 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy experiments were conducted using a Bruker Vertex 70 FTIR spectrometer with a liquid-nitrogen-cooled mercury cadmium telluride (MCT) photodetector (Bruker, Baden-Württemberg, Germany) that was equipped with a MIRacle ATR accessory containing a three-reflection ZnSe ATR crystal (PIKE Technologies, Fitchburg, WI, USA). A $30 \mu\text{L}$ aliquot of $100 \mu\text{M}$ BSA solution was pipetted onto the ATR crystal to form a droplet, and an absorbance spectrum was immediately recorded to characterize the secondary structure of solution-phase BSA proteins. The BSA was then incubated with the ATR crystal surface at room temperature for 30 min, followed by a series of ten buffer washing steps to remove weakly adsorbed proteins. During each washing step, $30 \mu\text{L}$ of extra buffer solution (without protein) was added to the sample droplet, followed by aspiration of a $30 \mu\text{L}$ volume from the droplet. After the washing steps were completed, then a second absorbance spectrum was recorded to characterize the secondary structure of BSA proteins

in the adsorbed state. During measurements, the sample compartment was purged continuously with nitrogen gas to minimize ambient moisture. The OPUS 6.5 software package (Bruker) was used to collect the ATR-FTIR absorbance spectra at a 4 cm^{-1} resolution and averaged over 128 scans. All recorded spectra were normalized by subtracting background spectra from buffer and water vapor alone in order to remove water and ambient water vapor absorbance artefacts, respectively.

The relative contribution of different secondary structure elements was determined for all samples by curve fitting the amide I bands of the absorbance spectra ($1720 - 1580\text{ cm}^{-1}$) to Gaussian components based on the peaks identified in the second derivative plot of the absorbance spectra and from literature references.³³⁻³⁷ All curve fitting analyses were conducted using the Peak Analyzer function in the OriginPro 2019b software package. The absorbance spectrum obtained from experiment was assumed to be a linear combination of multiple constituent Gaussian peaks, each centered around a given wavenumber value and representing a protein secondary structure element. The secondary structure percentage values were determined by calculating the area under the curve-fitted peaks that had been assigned to each secondary structure element as a fraction of the total area under the fitted curve. Peaks centered at $1682\text{-}1678\text{ cm}^{-1}$ were assigned to β -turn structures, $1656\text{-}1654\text{ cm}^{-1}$ to α -helix, $1649\text{-}1648\text{ cm}^{-1}$ to random coil, $1639\text{-}1624\text{ cm}^{-1}$ to extended chains, and $1618\text{-}1612\text{ cm}^{-1}$ to intermolecular β -sheets.

5.2.9 Statistical Analysis

Statistical analyses were conducted using the GraphPad Prism (v8.0.1) software package from GraphPad Software (La Jolla, CA, USA). One-way or two-way analysis of variance (ANOVA) with the appropriate multiple comparisons test and the unpaired t-test were used to compute the statistical significance of experimental data as appropriate. All statistical analysis involved two-tailed tests. Unpaired t-tests results are reported as *P* values while multiple comparisons test results are reported as multiplicity-adjusted *P* values. $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$ indicate the levels of statistical significance.

5.3 Results

5.3.1 Evaluation Strategy

BSA reagents are widely used to coat target surfaces in order to form surface passivation coatings, which minimize the nonspecific binding of interfering biomolecules that can affect bioassay readouts. The target surface can be a bulk surface such as a glass coverslip or polymeric membrane, or a nanostructured surface such as an inorganic nanoparticle. In practice, an experimentalist will incubate the target surface with an aqueous BSA solution, whereby protein molecules noncovalently adsorb and undergo a conformational change (“denature”) on the surface to form a thin film protein coating (**Figure 5.1a**).

Based on this coating process, the evaluation strategy is focused on characterizing the conformational, adsorption, and blocking properties of six commercial BSA reagents that were purified by different methods (**Figure 5.1b**). A wide range of solution-phase and surface-sensitive biophysical measurement tools were employed to characterize the BSA reagents in this Chapter while surface- and nanoparticle-based bioassays were employed to evaluate the BSA reagents for blocking applications in Chapter 7. This approach allowed not only for direct comparison of the functional performance of different BSA reagents in blocking applications, but also to obtain key mechanistic insights into how subtle differences in the conformational properties of proteins – as influenced by the processes involved in each purification method – affect protein adsorption, surface-induced denaturation, and resulting blocking performance.

Figure 5.1 Evaluation of BSA protein reagents for blocking applications. **a**, Overview of basic steps in BSA blocking protocols. (i) A target surface is identified to coat with BSA for surface passivation (“blocking”). (ii) BSA proteins in aqueous solution are incubated with the target surface. (iii) BSA proteins adsorb and denature on the target surface. (iv) A washing step is performed to remove weakly bound BSA protein molecules. (v) A firmly attached monolayer of adsorbed BSA protein molecules is formed on the surface. **b**, Overall experimental strategy to analyze BSA reagent properties. A series of BSA protein reagents, which were purified by different methods, were selected and their conformational, adsorption, and blocking properties were evaluated. A series of solution-phase and surface-sensitive biophysical measurement approaches were employed along with surface- and nanoparticle-based bioassays. **c**, Outline of purification routes to isolate BSA protein molecules from bovine plasma. Fatty acid-containing BSA proteins (referred to as “fatted”) were prepared by (1) cold ethanol fractionation, (2) heat-shock fractionation, and (3) cold ethanol fractionation followed by heat-shock fractionation and these reagents are designated as BSA 1, 2, 3, respectively. Fatty acid-free versions (referred to as “defatted”) of BSA 1, 2, 3 were also prepared and are designated as BSA 4, 5, 6, respectively.

Six commercial BSA reagents were selected that were purified through three different fractionation routes, without or with a fatty acid removal step (**Figure 5.1c** & **Table 5.1**).

The fractionation step is necessary in order to separate BSA from other serum components and there are two main options: cold ethanol fractionation and heat-shock fractionation. Briefly, cold ethanol fractionation involves reducing the sample temperature to approximately -5 °C followed by the addition of ethanol and adjusting the solution pH and ionic strength in order to isolate BSA protein.³⁸⁻⁴⁰ By contrast, heat-shock fractionation involves heating the sample (typically to ~60 °C or slightly higher) in order to isolate BSA protein and is conducted in the presence of a fatty acid stabilizer (caprylic acid).⁴¹⁻⁴³ These two fractionation processes can be completed alone or sequentially (cold ethanol, then heat-shock).

Table 5.1 Summary of BSA protein reagents. The albumin content percentage by agarose electrophoresis and the percentage of free fatty acids were obtained from the manufacturer's certificates of analysis. The amount of free fatty acid in BSA 1-3 were not reported because they were “fatted” reagents while the free fatty acid percentage for BSAs 4-6 verifies that they were “defatted” reagents.

BSA name	Catalogue no.	Lot no.	Purification method	Albumin by agarose electrophoresis (%)	Free fatty acid (%)
BSA 1	A2153	SLBW1397	Cold ethanol fractionation	100	NA
BSA 2	A3059	SLBS4332	Heat shock fractionation	100	NA
BSA 3	A7638	SLBW3326	Cold ethanol and heat shock fractionation	100	NA
BSA 4	A6003	SLBR4436V	Cold ethanol fractionation	100	0.00
BSA 5	A7030	SLBK3074V	Heat shock fractionation	99	0.00
BSA 6	A0281	021M7403V	Cold ethanol and heat shock fractionation	100	0.01

After the fractionation step(s) is completed, purified BSA reagents are obtained. Notably, at this stage, all of the BSA reagents still contain fatty acids that were either naturally bound or were added as a stabilizer.⁴⁴ Fatty acid-containing BSA reagents (referred to as “fatted”) that were prepared by (1) cold ethanol fractionation, (2) heat-shock fractionation, and (3) cold ethanol fractionation followed by heat-shock fractionation were tested in this study and were designated as BSA 1, 2, 3, respectively. To remove fatty acids, an extra processing step is required, which involves the addition of activated charcoal followed by adjusting solution pH and temperature⁴⁵ and they were designated as BSA 4, 5, 6,

respectively. In total, six different BSA reagents were tested consisting of three fatty acid-containing (1-3) and three fatty acid-free (4-6) versions.

ATR-FTIR spectra of BSA proteins were analyzed for spectral features to verify that the fattened BSA proteins (1-3) have more bound fatty acids compared to the defatted BSA proteins (4-6). As such, the peak absorbance intensity ratio of the CH_2 asymmetric stretch ($\nu_{\text{as}}(\text{CH}_2)$) to the CH_3 asymmetric stretch ($\nu_{\text{as}}(\text{CH}_3)$) in the ATR-FTIR spectra, which arises from the methylene chain and terminal methyl group of fatty acid tails, respectively, was quantified (**Figure 5.2**). $\nu_{\text{as}}(\text{CH}_2)$ peaks were centered around the wavenumber value of 2936 cm^{-1} while $\nu_{\text{as}}(\text{CH}_3)$ peaks were centered around 2961 cm^{-1} and a higher $\nu_{\text{as}}(\text{CH}_2)/\nu_{\text{as}}(\text{CH}_3)$ ratio indicates a greater amount of fatty acids present in BSA proteins.^{46,}
⁴⁷ The $\nu_{\text{as}}(\text{CH}_2)/\nu_{\text{as}}(\text{CH}_3)$ values for fattened BSA 1-3 were 0.86, 0.98 and 1.06, respectively, while the values for defatted BSA 4-6 were 0.84, 0.79, and 0.81, respectively. Another spectral feature is the asymmetric stretch of the COO^- functional group, which is a peak centered around the wavenumber value of 1570 cm^{-1} and which coincides with the amide II band (1600 to 1500 cm^{-1}).⁴⁸ Fattened BSA proteins have greater amide II peak heights relative to amide I (1700 to 1600 cm^{-1}) compared to defatted BSA proteins and, when taken together with the $\nu_{\text{as}}(\text{CH}_2)/\nu_{\text{as}}(\text{CH}_3)$ values, provide strong evidence that fattened BSA proteins have more bound fatty acid molecules than defatted BSA proteins.

Figure 5.2 Quantification of ATR-FTIR spectral features sensitive to the presence of bound fatty acids in BSA proteins. ATR-FTIR spectrograms of (a-f) BSA proteins 1-6 in solution. The peak absorbance intensity ratio of the asymmetric stretch of methylene groups ($\nu_{as}(\text{CH}_2)$, 2936 cm^{-1}) to the asymmetric stretch of methyl groups ($\nu_{as}(\text{CH}_3)$, 2961 cm^{-1}) was quantified as an indication of the amount of bound fatty acids for each BSA protein. Insets show an expanded view of the region enclosed within the dashed box where the peaks are located.

5.3.2 Thermal Stability of Fatted and Defatted BSA Proteins

Like other globular proteins, BSA folds in aqueous solution in order to minimize its conformational free energy. The folding pathway is dictated by the net balance of stabilizing and destabilizing forces, and the resulting folded structure is typically only a few kcal mol⁻¹ lower in free energy than the unfolded amino acid chain.⁴⁹ Thus, subtle differences in the purification method – such as the fractionation route, thermal history, and fatty acid content – can have important consequences on the conformational free energy of BSA protein molecules. Such effects can manifest themselves by influencing the thermal stability of proteins in the solution phase as well as by modulating adsorption and surface-induced denaturation profiles on a target surface.

As a first step, the thermal stability of the different BSA reagents was investigated by conducting dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy experiments. The experiments were conducted in temperature-controlled measurement chambers, starting at 25 °C and the temperature was gradually raised in 5 °C increments. The DLS technique measures the size distribution of protein molecules in bulk solution and could detect the onset of temperature-induced protein aggregation, as indicated by a marked increase in the average hydrodynamic diameter. The size increase occurs when thermally denatured protein monomers re-assemble into oligomeric/aggregated structures.⁵⁰ A higher onset temperature indicates greater thermal stability and vice-versa. On the other hand, the CD spectroscopy technique identifies the secondary structure characteristics of protein molecules in bulk solution, and the decrease in α -helical character with increasing temperature due to protein unfolding was tracked.

Figure 5.3 DLS characterization of BSA protein size distribution. **a-f**, DLS measurements of BSA proteins 1-6 at 25 °C ($n=5$). The mean hydrodynamic diameter (D) and polydispersity index (PDI) are indicated in each panel.

Figure 5.4 Hydrodynamic diameter of BSA protein molecules as a function of temperature, as measured in DLS experiments. Data are reported as mean $\pm$ standard deviation (s.d.), where s.d. is defined as the full-width-at-half-maximum (FWHM)/2.355 [$n=5$, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (versus data at 25 °C)]. Dots represent individual data points.

Across the temperature range of 25 to 55 °C, the DLS data showed that all six BSA proteins have ~8-nm diameter, which agrees well with the expected size of BSA monomers (**Figure 5.3 & 5.4**).^{25, 26, 51, 52} At 60 °C, a marked increase in the sizes of defatted BSA proteins (4-6) was detected, with average diameters around 20 nm that indicated the onset of protein aggregation. By contrast, the sizes of fatted BSA proteins (1-3) remained stable and did not increase at 60 °C. At 65 °C, the sizes of the defatted BSA proteins (4-6) increased to around 45 nm diameter and the onset of protein aggregation occurred for one of the fatted BSA proteins (1), with an average diameter around 35 nm. By contrast, the onset temperatures for the other fatted BSA proteins (2) and (3) were 70 and 75 °C, respectively. By extending the incubation time to 2 hours and fixing the temperature at 60 °C, it was verified that defatted BSA proteins are prone to more rapid and extensive aggregation while fatted BSA proteins are more thermally stable (**Figure 5.5**).

Figure 5.5 Time-dependent changes in protein size at 60 °C incubation temperature. Time-dependent changes in the mean hydrodynamic diameters of BSA proteins 1-6 at 60 °C. Values are presented as mean $\pm$ s.d. ($n=5$) where s.d. is defined as full-width-at-half-maximum (FWHM)/2.355.

Thermally induced aggregation of BSA molecules is triggered by irreversible conformational changes in the protein structure once there is a critical degree of protein

unfolding and corresponding loss of α -helical character. The CD spectroscopy data showed that all six BSA proteins exhibited around 60-63% α -helical character at 25 °C, followed by a progressive decrease in α -helical character with increasing temperature (**Figure 5.6** and **Figure 5.7**).^{25, 26, 53, 54} Both reversible conformational changes (below the onset temperature of aggregation) and irreversible conformational changes (at and above the onset temperature) contribute to protein unfolding so the helicity data at 65 °C was compared. All three defatted BSA proteins sample (4-6) had helical percentages below 50% on average, while all three fatted BSA protein samples (1-3) had helical percentages above 51% on average. More pronounced differences in helicity were observed at 70 °C, and mirroring the DLS data, the findings also support that, among the fatted BSA samples, BSA protein (1) had the lowest helical percentage and hence, lowest thermal stability. The relatively low thermal stability of BSA protein (1) is consistent with its processing by cold ethanol fractionation alone, and thus only natural fatty acids are bound in the absence of additional fatty acid stabilizer (as used in heat-shock fractionation). Collectively, the DLS and CD spectroscopy findings demonstrate that fatted BSA samples have greater thermal stability than defatted BSA samples, supporting that the conformational free energy of fatted BSA samples is lower than defatted BSA samples.

Figure 5.6 CD spectroscopy characterization of BSA proteins. **a**, Circular dichroism (CD) spectra of BSA proteins 1-6 at 25 °C are reported in molar residue ellipticity units $[\theta]$. **b-g**, CD spectra of BSA proteins 1-6 at 25, 50, 55, 60, 65, 70 and 75 °C are reported in molar residue ellipticity units $[\theta]$. All elevated temperature measurements were recorded after a 5-min temperature equilibration period. All measurements were conducted in triplicate.

Figure 5.7 Fractional percentage of α -helicity in BSA protein molecules as a function of temperature, as measured in CD spectroscopy experiments. Mean values are presented on top of each column. Values were computed from molar residue ellipticity data and data are reported as mean $\pm$ s.d. ($n=3$). Dots represent individual data points. The grey horizontal line in each graph corresponds to 50% helicity.

5.3.3 Adsorption Behavior of Fatted and Defatted BSA Proteins

The adsorption properties of BSA proteins on hydrophilic silica surfaces were next investigated. When a protein with higher conformational energy adsorbs onto a solid surface, it is expected to undergo greater unfolding and spreading on the surface due to entropic gains while other factors such as protein-surface and protein-protein interactions also affect the outcome.^{55, 56} Quartz crystal microbalance-dissipation (QCM-D) experiments were conducted in order to track the adsorption kinetics and corresponding mass and viscoelastic properties of adsorbed BSA protein molecules. The QCM-D technique measures real-time changes in the resonance frequency (ΔF) and energy dissipation (ΔD) properties of a silica-coated sensor surface as a function of time, which provide insight into the hydrodynamically-coupled mass and viscoelastic properties of adsorbed BSA protein molecules on the silica surface, respectively. A larger $|\Delta F_{\max}|$ shift indicates greater protein adsorption, while a larger $|\Delta F/\Delta D|$ shift reflects greater rigidity (less viscoelasticity) within the adsorbed protein layer. The experiments were conducted using a BSA protein concentration of 100 μM ($\sim 6.6 \text{ mg mL}^{-1}$) in an aqueous buffer (10 mM Tris [pH 7.5] with 150 mM NaCl) which is representative of the typical solution conditions used in BSA coating protocols.^{11, 24, 57}

Figure 5.8 QCM-D experiments were conducted to measure frequency (ΔF) and energy dissipation (ΔD) signals related to the protein adsorption process. **a**, Time-resolved QCM-D ΔF shifts and **b**, corresponding $|\Delta F_{\max}|$ shifts at saturation. **c**, Time-resolved QCM-D ΔD shifts. **d**, $|\Delta F_{\max}/\Delta D_{\max}|$ ratios obtained from saturation data in panels **a** and **c**. A buffer washing step is performed at around $t=35$ min and is indicated by an arrow in panels **a** and **c**. Data in **b** and **d** are reported as mean $\pm$ s.d. ($n=3$, one-way ANOVA with Tukey's multiple comparisons test). P values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method). Dots represent individual data points.

All six BSA proteins adsorbed onto the silica surface and the QCM-D frequency signals indicated monotonic adsorption kinetics (**Figure 5.8a**). Most adsorbed protein molecules were bound irreversibly as judged by a buffer washing step. In general, there were larger $|\Delta F_{\max}|$ shifts of around 35 to 45 Hz for defatted BSA proteins (4-6), indicating greater adsorption (**Figure 5.8b**). By contrast, there was less adsorption of fatted BSA proteins (1-3), as indicated by $|\Delta F_{\max}|$ shifts around 15 to 35 Hz. The corresponding QCM-D energy

dissipation signals also indicated monotonic protein adsorption and some degree of viscoelastic character within the adsorbed protein layer (**Figure 5.8c**). After a buffer washing step, the remaining bound protein molecules exhibited a more rigid arrangement. Further analysis of the $|\Delta F_{\max}/\Delta D_{\max}|$ ratio revealed that adsorbed protein layers from defatted BSA proteins (4-6) were more tightly bound to the silica surface, which is consistent with lower conformational stability (in solution) and greater surface-induced denaturation (**Figure 5.8d & 5.9**). On the other hand, fatty BSA proteins (1-3) exhibited greater viscoelastic character indicative of less surface-induced denaturation.

Figure 5.9 Comparison of QCM-D frequency-energy dissipation curves for BSA adsorption onto silica surfaces. Time-independent frequency-energy dissipation (F-D) curves derived from QCM-D frequency and energy dissipation shifts related to the adsorption of BSA proteins 1-6 onto silica surfaces at 25°C.

To corroborate the QCM-D results, localized surface plasmon resonance (LSPR) experiments were also performed to measure BSA protein adsorption onto silica-coated surfaces. While the QCM-D technique is sensitive to adsorbed protein mass and

hydrodynamically-coupled solvent mass, the LSPR technique detects the wavelength shift ($\Delta\lambda$) that is associated with changes in local refractive index near the sensor surface and the measurement signal is only sensitive to the adsorbed protein mass (not coupled solvent).⁵² In LSPR experiments, the $\Delta\lambda$ shift is tracked as a function of time, whereby a larger $\Delta\lambda$ shift indicates greater protein adsorption. For all six BSA proteins, a positive $\Delta\lambda$ shift occurred and monotonic adsorption kinetics were observed (**Figure 5.10a**). After a buffer washing step, most protein molecules remained bound. Importantly, the $\Delta\lambda_{\max}$ shifts showed that the defatted BSA proteins exhibited greater adsorption uptake than the fatty BSA proteins ($\sim 1.2 \pm 0.2$ nm vs. $\sim 0.6 \pm 0.2$ nm), which agrees well with the QCM-D data (**Figure 5.10b**). Thus, defatted BSA proteins exhibited higher adsorption uptake and tighter adlayer packing, whereas fatty BSA proteins had lower adsorption uptake and weaker adlayer packing.

Figure 5.10 LSPR experiments were conducted to measure $\Delta\lambda_{\max}$ signals related to the protein adsorption process. **a**, Time-resolved LSPR wavelength shifts ($\Delta\lambda$) and **b**, corresponding $\Delta\lambda_{\max}$ shifts at saturation. Data in **b** are reported as mean $\pm$ s.d. ($n=3$, one-way ANOVA with Tukey's multiple comparisons test). P values are reported for defatted BSA proteins (versus fatty BSA proteins from the same fractionation method). Dots represent individual data points.

5.3.4 Adsorption-Related Protein Conformational Changes

To further compare the extent of surface-induced protein denaturation for each BSA protein, the LSPR measurement data were analyzed by computing the initial rate of adsorption uptake, which is denoted as $(d\Delta\lambda/dt)_{\max}$ (**Figure 5.11**). The LSPR technique is a highly surface-sensitive measurement approach and it is possible to relate the initial adsorption rate to the relative extent of surface-induced protein denaturation, whereby a larger initial rate is indicative of greater denaturation.^{54, 58} Consequently, the LSPR data showed that defatted BSA proteins undergo more extensive surface-induced denaturation, as indicated by initial adsorption rates around 0.6-0.7 nm min⁻¹ (**Figure 5.12a**). On the other hand, the initial adsorption rates for fatted BSA proteins were only around 0.2-0.3 nm min⁻¹, which indicate less surface-induced denaturation.

Figure 5.11 Time-derivative plot of LSPR-tracked BSA adsorption kinetics. Time-derivative of wavelength shifts ($d\Delta\lambda/dt$) corresponding to the LSPR-tracked adsorption of BSA proteins 1-6 onto silica-coated sensor surfaces.

Figure 5.12 Evaluation of adsorption-related conformational changes in BSA protein structure.

a, Maximum rate of change in the LSPR wavelength shift ($d\Delta\lambda/dt$)_{max} during the initial adsorption stage. Values are computed from data in Figure 5.10. Data are reported as mean $\pm$ s.d. ($n=3$, one-way ANOVA with Tukey's multiple comparisons test). **b**, Fractional percentage of α -helicity in BSA protein molecules in solution and in the adsorbed state, as determined in ATR-FTIR spectroscopy experiments. Data are reported as mean $\pm$ s.d. ($n=3$, two-way ANOVA with Tukey's multiple comparisons test). P values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method) in **a**, and are separately reported for defatted BSA proteins in solution (versus fatted BSA proteins in solution from the same fractionation method) and for defatted BSA proteins in the adsorbed state (versus fatted BSA proteins in the adsorbed state from the same fractionation method) in **b**. Dots represent individual data points.

In addition, attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy measurements were performed to measure the change in BSA protein structure due to adsorption-related conformational changes. In general, in the solution phase, fatted BSA molecules had α -helicities around 63% while defatted ones had α -helicities around 60%, which agree with literature values (**Figure 5.12b**, **Figure 5.13** & **Table 5.2**).^{37, 59} Upon adsorption, fatted BSA molecules underwent surface-induced denaturation and the fractional helicities decreased to around 53%, indicating a net helical loss of $\sim 10\%$. By contrast, upon adsorption, defatted BSA molecules underwent greater surface-induced denaturation and the resulting fractional helicities were around 45%, which corresponds to a net helical loss of $\sim 15\%$. At the same time, the analysis showed that defatted BSA proteins had a greater increase in random coil fraction of $\sim 12\%$

compared to an increase of ~9% for fatted BSA proteins upon adsorption. These findings agree with previous studies that reported how the loss in α -helical structure due to adsorption is largely balanced by an increase in random coil fraction.^{37, 60, 61} Hence, fatty acid stabilization appears to directly influence the adsorption-related transition from α -helical structure to random coil. On the other hand, both fatted and defatted BSA proteins underwent similar net increases in β -turn and intermolecular β -sheet fractions as well as similar net decreases in extended chain fractions, indicating that fatty acid stabilization mainly conferred protection to α -helical regions. Thus, multiple lines of experimental data support that defatted BSA molecules exhibit greater surface-induced denaturation than fatted BSA molecules. This finding is consistent with the lower conformational stability exhibited by defatted BSA molecules in solution and supports that a decreased conformational stability of a solution-phase protein translates into more pronounced denaturation in the adsorbed state.

Figure 5.13 Quantification of adsorption-related protein conformational changes in the

secondary structure of BSA proteins based on ATR-FTIR measurements. Amide I regions from attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectrograms of **a-f**, BSA proteins 1-6 in solution (left panels) and in the adsorbed state (right panels). Experimentally obtained absorbance spectra were resolved into individual component curves, which were each assigned to a secondary structure element.

Table 5.2 Secondary structure percentage of BSA proteins 1-6 in solution and in the adsorbed state from ATR-FTIR spectroscopy measurements. Data are reported as mean $\pm$ s.d. ($n=3$).

Assigned Secondary Structure	BSA 1 (%)		BSA 2 (%)		BSA 3 (%)	
	Solution	Adsorbed	Solution	Adsorbed	Solution	Adsorbed
β -turn	6.0 $\pm$ 0.0	9.3 $\pm$ 0.6	6.7 $\pm$ 0.6	8.7 $\pm$ 1.5	6.0 $\pm$ 1.0	8.3 $\pm$ 1.5
α -helix	63.7 $\pm$ 1.5	52.7 $\pm$ 3.1	65.3 $\pm$ 0.6	53.7 $\pm$ 2.1	65.3 $\pm$ 1.5	54.0 $\pm$ 2.7
Random coil	-	8.3 $\pm$ 2.1	-	10.3 $\pm$ 1.2	-	7.0 $\pm$ 1.7
Extended chains	23.3 $\pm$ 1.5	21.3 $\pm$ 3.1	21.3 $\pm$ 1.2	19.3 $\pm$ 3.1	21.3 $\pm$ 2.3	22.3 $\pm$ 3.2
Intermolecular β -sheet	7.0 $\pm$ 1.0	8.3 $\pm$ 1.5	6.7 $\pm$ 1.2	8.0 $\pm$ 3.5	7.3 $\pm$ 2.9	8.3 $\pm$ 3.2
Assigned Secondary Structure	BSA 4 (%)		BSA 5 (%)		BSA 6 (%)	
	Solution	Adsorbed	Solution	Adsorbed	Solution	Adsorbed
β -turn	7.7 $\pm$ 1.2	10.7 $\pm$ 1.5	7.3 $\pm$ 1.2	9.0 $\pm$ 1.0	7.7 $\pm$ 1.5	10.0 $\pm$ 1.0
α -helix	61.7 $\pm$ 1.2	45.7 $\pm$ 3.5	62.0 $\pm$ 2.0	46.0 $\pm$ 3.6	59.7 $\pm$ 1.5	44.7 $\pm$ 3.2
Random coil	-	11.0 $\pm$ 1.0	-	13.0 $\pm$ 1.0	-	13.3 $\pm$ 2.5
Extended chains	23.0 $\pm$ 2.0	21.7 $\pm$ 2.1	24.3 $\pm$ 4.0	22.7 $\pm$ 2.1	27.0 $\pm$ 1.7	23.0 $\pm$ 1.0
Intermolecular β -sheet	7.7 $\pm$ 1.2	11.0 $\pm$ 2.6	6.3 $\pm$ 2.1	9.3 $\pm$ 1.5	5.7 $\pm$ 1.5	9.0 $\pm$ 2.6

5.3.5 Addition of Caprylic Acid to Defatted and Fatted BSA Proteins

To verify that the observed differences in the conformational and adsorption properties of fatted and defatted BSA proteins are due to fatty acids, BSA 5 was treated with caprylic acid supplementation in order to convert defatted BSA 5 into fatted CA-BSA 5 (10:1 molar ratio of caprylic acid to BSA 5 protein). Caprylic acid is the most widely used fatty acid in the BSA fractionation process in order to stabilize protein molecules against temperature-induced denaturation.⁶²⁻⁶⁴ In order to quantify the amount of caprylic acid bound to BSA, it is noted that the binding constant and stoichiometry for caprylic acid binding to BSA is $3.65 \times 10^4 \text{ M}^{-1}$ and 2, respectively, as previously determined by isothermal titration calorimetry.⁶⁵ By applying these parameters to the one-site binding model⁶⁶ and taking into account BSA and caprylic acid concentrations of 100 μM and 1000 μM , respectively, the fraction of sites in BSA occupied by caprylic acid was calculated to be ~ 0.97 . The solution-phase and adsorption data are presented in **Figure 5.14** and **Table 5.3**. Overall, the data support that fatty acid-containing CA-BSA 5 is more stable than fatty acid-free BSA 5, with respect to thermal denaturation and adsorption-related surface denaturation. This finding confirms the important role of fatty acids in modulating the conformational and adsorption properties of BSA proteins.

Figure 5.14 Verification of the role of fatty acid in causing differences in the conformational and adsorption properties between fatty and defatted BSAs via caprylic acid doping of defatted BSA 5. **a**, DLS measurements of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5) at 25 °C ($n=5$). The mean hydrodynamic diameter (D) and polydispersity index (PDI) are indicated in each panel.

b, Hydrodynamic diameter of BSA 5 and CA-BSA 5 as a function of temperature, as measured in DLS experiments. Data are reported as mean $\pm$ s.d. [$n=5$, one-way ANOVA with Dunnett's multiple comparisons test (versus data at 25 °C) for CA-BSA 5 data]. Dots represent individual data points. **c**, Circular dichroism (CD) spectra of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5) at 25 °C are reported in molar residue ellipticity units $[\theta]$ ($n=3$). **d**, CD spectra of BSA 5 and CA-BSA 5 at 25, 50, 55, 60, 65, 70 and 75 °C are reported in molar residue ellipticity units $[\theta]$ ($n=3$). All elevated temperature measurements were recorded after a 5-min temperature equilibration period. **e**, Fractional percentage of α -helicity in BSA 5 and CA-BSA 5 protein molecules as a function of temperature, as measured in CD spectroscopy experiments. Mean values are presented on top of each column. Values were computed from molar residue ellipticity data and data are reported as mean $\pm$ s.d. ($n=3$). Dots represent individual data points. **f**, Time-resolved QCM-D ΔF shifts and **g**, corresponding $|\Delta F_{\max}|$ shifts at saturation. **h**, Time-resolved QCM-D ΔD shifts. **i**, $|\Delta F_{\max}/\Delta D_{\max}|$ ratios obtained from saturation data in panels **f** and **h**. **j-k**. LSPR experiments were conducted to measure $\Delta\lambda_{\max}$ signals related to the protein adsorption process. **j**, Time-resolved LSPR wavelength shifts ($\Delta\lambda$) and **k**, corresponding $\Delta\lambda_{\max}$ shifts at saturation. Data in **g**, **i**, **k** are reported as mean $\pm$ s.d. ($n=3$, unpaired t-test). Dots represent individual data points. **l**, Time-independent frequency-energy dissipation (F-D) curves derived from QCM-D frequency and energy dissipation shifts related to the adsorption of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5) onto silica surfaces at 25°C. **m**, Time-derivative of wavelength shifts ($d\Delta\lambda/dt$) corresponding to the LSPR-tracked adsorption of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5) onto silica-coated sensor surfaces. **n-o**. Amide I regions from attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectrograms of **n**. BSA 5 and **o**. caprylic acid-doped BSA 5 (CA-BSA 5) in solution (left panels) and in the adsorbed state (right panels). Experimentally obtained absorbance spectra were resolved into individual component curves, which were each assigned to a secondary structure element. **p**, Maximum rate of change in the LSPR wavelength shift $(d\Delta\lambda/dt)_{\max}$ during the initial adsorption stage of BSA 5 and caprylic acid-doped BSA 5 (CA-BSA 5). Data are reported as mean $\pm$ s.d. ($n=3$, unpaired t-test). **q**, Fractional percentage of α -helicity in BSA 5 and CA-BSA 5 protein molecules in solution and in the adsorbed state, as determined in ATR-FTIR spectroscopy experiments. Data are reported as mean $\pm$ s.d. ($n=3$, two-way ANOVA with Sidak's multiple comparisons test). Dots represent individual data points.

Table 5.3 Secondary structure percentage of BSA 5 and CA-BSA 5 in solution and in the adsorbed state from ATR-FTIR spectroscopy measurements. Data are reported as mean $\pm$ s.d. ($n=3$).

Assigned Secondary Structure	BSA 5 (%)		CA-BSA 5 (%)	
	Solution	Adsorbed	Solution	Adsorbed
β -turn	7.3 ± 1.2	9.0 ± 1.0	6.3 ± 0.6	10.0 ± 1.0
α -helix	62.0 ± 2.0	46.0 ± 3.6	64.3 ± 2.1	53.0 ± 2.0
Random coil	-	13.0 ± 1.0	-	7.0 ± 1.0
Extended chains	24.3 ± 4.0	22.7 ± 2.1	23.3 ± 0.6	22.3 ± 1.5
Intermolecular β -sheet	6.3 ± 2.1	9.3 ± 1.5	6.0 ± 2.7	7.7 ± 1.2

While defatted BSA proteins performed quite similarly in all tested assays, fatted BSA proteins also showed some degree of variation in conformational stability depending on the assay. Among the fatted BSA proteins, BSA 3 typically showed the highest levels of conformational stability, as reflected across solution-phase and surface-sensitive biophysical measurements. This led to the suspicion that the fatted BSA proteins had different degrees of “fattening” since conformational stability is related to fatty acid-protein ratio⁶⁷ (higher fatty acid content yields greater stability). Therefore, the fatted BSA proteins 1-3 were supplemented with additional caprylic acid (10:1 molar ratio of caprylic acid to BSA proteins) and their effects on adsorption behavior was verified based on their QCM-D adsorption kinetics (**Figure 5.15**). Negligible changes in BSA 3 adsorption behavior were observed without or with caprylic acid doping, thereby confirming that the as-supplied BSA 3 was fully fatted. By contrast, there tended to be slight differences in the adsorption behavior of BSA 1 and 2 without and with caprylic acid doping, especially with respect to adlayer rigidity. Overall, the caprylic acid supplementation experiments verified that the fatted BSA proteins 1-3 all have high fatty acid contents.

Figure 5.15 Influence of caprylic acid doping on the adsorption of fatted BSA proteins 1-3, as measured by QCM-D experiments. **a**, Time-resolved QCM-D ΔF and **b**, ΔD shifts along with **c**, corresponding $|\Delta F_{\max}|$ shifts at saturation (from panel **a**) and **d**, $|\Delta F_{\max}/\Delta D_{\max}|$ ratios obtained from saturation data in panels **a** and **b** for BSA 1 without and with caprylic acid doping (labeled as BSA 1 and CA-BSA 1, respectively). **(e-h)** Equivalent data for BSA 2. **(i-l)** Equivalent data for BSA 3. Data in **c**, **d**, **g**, **h**, **k**, **l** are reported as mean $\pm$ s.d. ($n=3$, unpaired t-test). Dots represent individual data points.

5.4 Discussion

The experimental findings herein demonstrate that defatted BSA proteins exhibit distinct conformational and adsorption properties from fatted BSA proteins, and these differences lead to significant variations in solution-phase conformational stability and adsorption behavior. Overall, the data point to the central role of fatty acid molecules in affecting BSA structure and function. Mechanistically, the enhanced conformational stability of fatted BSA proteins can be understood through the insertion of caprylic acid molecules (via hydrophobic tails) into hydrophobic pockets on the molecular surface of BSA proteins, which confers a net stabilizing effect on protein structure.^{68, 69}

A less obvious but critically important effect of caprylic acid on BSA structure relates to the protein adsorption process itself (**Figure 5.16**). When a protein adsorbs onto a surface, the protein will typically undergo surface-induced denaturation and the extent of protein denaturation depends on a combination of factors, including the protein's intrinsic conformational stability and the magnitude of protein-surface interactions.⁵⁶ More denaturation causes greater protein spreading on the surface, which means that the contact surface area per adsorbing protein molecule is larger. As such, it is generally understood that greater spreading of individual protein molecules results in a smaller total number of adsorbed protein molecules on a target surface, irrespective of whether the greater spreading is due to conformational stability (see, *e.g.*, ref. ⁷⁰) or protein-surface interactions (see, *e.g.*, ref. ⁷¹). From this perspective, it is possible to rationalize how changing ionic strength affects BSA protein adsorption and spreading for a single type of BSA.²⁵

Figure 5.16 Effect of fatty acids on BSA conformational and adsorption properties and implications for blocking applications. **a**, Schematic illustration of fatted and defatted BSA protein molecules in solution. The presence of fatty acids leads to higher conformational stability in solution. **b**, Schematic illustration of fatted and defatted BSA protein molecules in the adsorbed state. Higher conformational stability of fatted BSA leads to less adsorption-induced denaturation, greater protein-protein repulsion, and thus less well-packed protein coatings. On the other hand, the lower conformational stability of defatted BSA leads to greater adsorption-induced denaturation, less protein-protein repulsion, and thus more well-packed protein coatings. As a result, defatted BSA proteins form more effective surface passivation coatings that could be more useful for blocking applications.

However, the situation is more nuanced when comparing defatted and fatted BSA proteins because caprylic acid molecules not only enhance conformational stability but the protruding carboxylic acid headgroups of caprylic acid molecules also increase the negative surface charge character of BSA protein molecules. Fatted BSA proteins have greater conformational stability and thus undergo less surface-induced denaturation in the adsorbed state. Nevertheless, while fatted BSA proteins have a smaller contact surface area per molecule, the maximum surface density of adsorbed protein molecules in this case is limited by repulsive electrostatic interactions between nearest-neighbor protein molecules. On the other hand, defatted BSA proteins have lower conformational stability and denature to a greater extent in the adsorbed state. Consequently, defatted BSA proteins undergo greater spreading and have a larger contact surface area per adsorbed molecule. However, the maximum surface density of adsorbed proteins in the defatted BSA case is still higher than in the fatted BSA case because there are less repulsive protein-protein interactions between adsorbed, defatted BSA molecules. Hence, defatted BSA proteins are likely to be more useful to form passivation coatings for two main reasons. First, they have lower conformational stability that permits greater denaturation and spreading in the adsorbed state. Second, the absence of fatty acid molecules in defatted BSA proteins permits more favorable protein-protein interactions that support the formation of close-packed, adsorbed protein layers on target surfaces. While another possible mechanism such as how fatty acids could cover zwitterionic amino acid pairs (i.e., EK and DK) on the fatted BSA proteins – which contribute to the BSA's antifouling capability⁷² – may also be considered

to explain why fatted BSA proteins have poorer surface passivation performance, it is not likely to be the dominant mechanism as the decreased adsorption uptake and surface-induced denaturation from the QCM-D, LSPR and ATR-FTIR experiments strongly support that fatted BSA proteins form significantly lower-density BSA coatings.

5.5 Conclusion

Taken together, these findings reveal that fatty acid-free BSA have lower solution-phase conformational stability and exhibit greater adsorption uptake compared to their fatty acid-containing counterpart, which suggests that it is preferable to use fatty acid-free BSA protein reagents for blocking applications. This trend is largely independent of the fractionation route that was used in the purification stage. Mechanistically, it was discussed how an additional negative charge conferred by bound fatty acids onto BSA proteins causes more pronounced intermolecular repulsion between adsorbed fatty acid-containing BSA protein molecules which resulted in the formation of a lower density BSA coating and which would likely not perform as well in surface passivation evaluations compared to fatty acid-free BSA proteins. Looking forward, the analytical approach presented herein outlines a series of important functional properties – solution-phase conformational stability, adsorption uptake, surface-induced denaturation, and molecular packing within the protein coating – that are important to consider when evaluating protein-based reagents for coating applications and can also guide the development of improved versions of BSA and other protein-based blocking reagents alone and as part of multi-component buffer solutions.

References

1. Nakanishi, K.; Sakiyama, T.; Imamura, K., On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. *J. Biosci. Bioeng.* **2001**, *91* (3), 233-244.
2. Gray, J. J., The interaction of proteins with solid surfaces. *Curr. Opin. Struct. Biol.* **2004**, *14* (1), 110-115.

3. Nuzzo, R. G., Biomaterials: stable antifouling surfaces. *Nat. Mater.* **2003**, 2 (4), 207.
4. Miller, R. T.; Kubier, P.; Reynolds, B.; Henry, T.; Turnbow, H., Blocking of endogenous avidin-binding activity in immunohistochemistry: the use of skim milk as an economical and effective substitute for commercial biotin solutions. *Appl. Immunohisto. M. M.* **1999**, 7 (1), 63-65.
5. Kirschner, C. M.; Brennan, A. B., Bio-inspired antifouling strategies. *Annu. Rev. Mater. Res.* **2012**, 42, 211-229.
6. Wei, Q.; Becherer, T.; Angioletti-Uberti, S.; Dzubiella, J.; Wischke, C.; Neffe, A. T.; Lendlein, A.; Ballauff, M.; Haag, R., Protein interactions with polymer coatings and biomaterials. *Angew. Chem. Int. Edit.* **2014**, 53 (31), 8004-8031.
7. Lowe, S.; O'Brien-Simpson, N. M.; Connal, L. A., Antibiofouling polymer interfaces: poly (ethylene glycol) and other promising candidates. *Polym. Chem.* **2015**, 6 (2), 198-212.
8. Avrameas, S.; Guilbert, B., Enzyme-immunoassay for the measurement of antigens using peroxidase conjugates. *Biochimie* **1972**, 54 (7), 837-842.
9. Jitsukawa, T.; Nakajima, S.; Sugawara, I.; Watanabe, H., Increased coating efficiency of antigens and preservation of original antigenic structure after coating in ELISA. *J. Immunol. Methods* **1989**, 116 (2), 251-257.
10. Towbin, H.; Staehelin, T.; Gordon, J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* **1979**, 76 (9), 4350-4354.
11. Mahmood, T.; Yang, P.-C., Western blot: technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* **2012**, 4 (9), 429.
12. Ramos-Vara, J., Technical aspects of immunohistochemistry. *Vet. Pathol.* **2005**, 42 (4), 405-426.
13. Buchwalow, I. B.; Böcker, W., Background Staining, Autofluorescence and Blocking Steps. In *Immunohistochemistry: Basics and Methods*, Springer Berlin Heidelberg: Berlin, Heidelberg, 2010; pp 41-46.
14. Francis, G. L., Albumin and mammalian cell culture: implications for biotechnology applications. *Cytotechnology* **2010**, 62 (1), 1-16.

15. Špačková, B.; Wrobel, P.; Bocková, M.; Homola, J., Optical biosensors based on plasmonic nanostructures: a review. *Proc. IEEE* **2016**, *104* (12), 2380-2408.
16. Ronkainen, N. J.; Halsall, H. B.; Heineman, W. R., Electrochemical biosensors. *Chem. Soc. Rev.* **2010**, *39* (5), 1747-1763.
17. Brewer, S. H.; Glomm, W. R.; Johnson, M. C.; Knag, M. K.; Franzen, S., Probing BSA binding to citrate-coated gold nanoparticles and surfaces. *Langmuir* **2005**, *21* (20), 9303-9307.
18. Mariam, J.; Sivakami, S.; Dongre, P. M., Albumin corona on nanoparticles—a strategic approach in drug delivery. *Drug Deliv.* **2016**, *23* (8), 2668-2676.
19. Kreader, C. A., Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.* **1996**, *62* (3), 1102-1106.
20. Goebel-Stengel, M.; Stengel, A.; Taché, Y.; Reeve Jr, J. R., The importance of using the optimal plasticware and glassware in studies involving peptides. *Anal. Biochem.* **2011**, *414* (1), 38-46.
21. Mannuzza, F. J.; Montalto, J. G., Is bovine albumin too complex to be just a commodity? *Bioprocess Int.* **2010**.
22. Xiao, Y.; Isaacs, S. N., Enzyme-linked immunosorbent assay (ELISA) and blocking with bovine serum albumin (BSA)—not all BSAs are alike. *J. Immunol. Methods* **2012**, *384* (1-2), 148-151.
23. Sweryda-Krawiec, B.; Devaraj, H.; Jacob, G.; Hickman, J. J., A new interpretation of serum albumin surface passivation. *Langmuir* **2004**, *20* (6), 2054-2056.
24. Jeyachandran, Y.; Mielczarski, J.; Mielczarski, E.; Rai, B., Efficiency of blocking of non-specific interaction of different proteins by BSA adsorbed on hydrophobic and hydrophilic surfaces. *J. Colloid Interface Sci.* **2010**, *341* (1), 136-142.
25. Park, J. H.; Sut, T. N.; Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Cho, N.-J., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19* (13), 8854-8865.
26. Park, J. H.; Jackman, J. A.; Ferhan, A. R.; Ma, G. J.; Yoon, B. K.; Cho, N.-J., Temperature-induced denaturation of BSA protein molecules for improved surface passivation coatings. *ACS Appl. Mater. Interfaces* **2018**, *10* (38), 32047-32057.

27. Savitzky, A.; Golay, M. J., Smoothing and differentiation of data by simplified least squares procedures. *Anal. Chem.* **1964**, 36 (8), 1627-1639.
28. Morrisett, J. D.; David, J. S.; Pownall, H. J.; Gotto Jr, A. M., Interaction of an apolipoprotein (apoLP-alanine) with phosphatidylcholine. *Biochemistry* **1973**, 12 (7), 1290-1299.
29. Cho, N.-J.; Frank, C. W.; Kasemo, B.; Höök, F., Quartz crystal microbalance with dissipation monitoring of supported lipid bilayers on various substrates. *Nat. Protoc.* **2010**, 5 (6), 1096.
30. Jackman, J. A.; Zhdanov, V. P.; Cho, N.-J., Nanoplasmonic biosensing for soft matter adsorption: kinetics of lipid vesicle attachment and shape deformation. *Langmuir* **2014**, 30 (31), 9494-9503.
31. Fredriksson, H.; Alaverdyan, Y.; Dmitriev, A.; Langhammer, C.; Sutherland, D. S.; Zäch, M.; Kasemo, B., Hole-mask colloidal lithography. *Adv. Mater.* **2007**, 19 (23), 4297-4302.
32. Dahlin, A. B.; Tegenfeldt, J. O.; Höök, F., Improving the instrumental resolution of sensors based on localized surface plasmon resonance. *Anal. Chem.* **2006**, 78 (13), 4416-4423.
33. Murayama, K.; Tomida, M., Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy. *Biochemistry* **2004**, 43 (36), 11526-11532.
34. Kong, J.; Yu, S., Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim. Biophys. Sin.* **2007**, 39 (8), 549-559.
35. Barth, A., Infrared spectroscopy of proteins. *BBA-Bioenergetics* **2007**, 1767 (9), 1073-1101.
36. Srour, B.; Bruechert, S.; Andrade, S. L.; Hellwig, P., Secondary Structure Determination by Means of ATR-FTIR Spectroscopy. In *Membrane Protein Structure and Function Characterization*, Springer: 2017; pp 195-203.
37. Givens, B. E.; Xu, Z.; Fiegel, J.; Grassian, V. H., Bovine serum albumin adsorption on SiO₂ and TiO₂ nanoparticle surfaces at circumneutral and acidic pH: a tale of two nano-bio surface interactions. *J. Colloid Interface Sci.* **2017**, 493, 334-341.

38. Cohn, E. J.; Strong, L. E.; Hughes, W.; Mulford, D.; Ashworth, J.; Melin, M. e.; Taylor, H., Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.* **1946**, *68* (3), 459-475.
39. Kistler, P.; Nitschmann, H., Large scale production of human plasma fractions: eight years experience with the alcohol fractionation procedure of Nitschmann, Kistler and Lergier. *Vox Sang.* **1962**, *7* (4), 414-424.
40. Hao, Y. I., A simple method for the preparation of human serum albumin. *Vox Sang.* **1979**, *36* (5), 313-320.
41. Hoch, H.; Chanutin, A., Albumin from heated human plasma. I. Preparation and electrophoretic properties. *Arch. Biochem. Biophys.* **1954**, *51* (1), 271-276.
42. Reid, A. F., Method of purifying albumin. Google Patents: 1955.
43. Schneider, W.; Lefevre, H.; Fiedler, H.; McCarty, L. J., An alternative method of large scale plasma fractionation for the Isolation of serum albumin. *Blut* **1975**, *30* (2), 121-134.
44. Spector, A. A., Fatty acid binding to plasma albumin. *J. Lipid Res.* **1975**, *16* (3), 165-179.
45. Chen, R. F., Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **1967**, *242* (2), 173-181.
46. Yu, P.; Damiran, D., Heat-induced changes to lipid molecular structure in Vimy flaxseed: Spectral intensity and molecular clustering. *Spectrochim. Acta, Part A* **2011**, *79* (1), 51-59.
47. Oleszko, A.; Hartwich, J.; Gąsior-Głogowska, M.; Olsztyńska-Janus, S., Changes of albumin secondary structure after palmitic acid binding: FT-IR spectroscopic study. *Acta Bioeng. Biomech.* **2018**, *20* (1).
48. Grdadolnik, J.; Maréchal, Y., Bovine serum albumin observed by infrared spectrometry. I. methodology, structural investigation, and water uptake. *Biopolymers* **2001**, *62* (1), 40-53.
49. Dobson, C. M.; Šali, A.; Karplus, M., Protein folding: a perspective from theory and experiment. *Angew. Chem. Int. Edit.* **1998**, *37* (7), 868-893.

50. Militello, V.; Casarino, C.; Emanuele, A.; Giostra, A.; Pullara, F.; Leone, M., Aggregation kinetics of bovine serum albumin studied by FTIR spectroscopy and light scattering. *Biophys. Chem.* **2004**, *107* (2), 175-187.
51. Jachimska, B.; Wasilewska, M.; Adamczyk, Z., Characterization of globular protein solutions by dynamic light scattering, electrophoretic mobility, and viscosity measurements. *Langmuir* **2008**, *24* (13), 6866-6872.
52. Jackman, J. A.; Ferhan, A. R.; Cho, N.-J., Nanoplasmonic sensors for biointerfacial science. *Chem. Soc. Rev.* **2017**, *46* (12), 3615-3660.
53. Moriyama, Y.; Watanabe, E.; Kobayashi, K.; Harano, H.; Inui, E.; Takeda, K., Secondary structural change of bovine serum albumin in thermal denaturation up to 130 C and protective effect of sodium dodecyl sulfate on the change. *J. Phys. Chem. B* **2008**, *112* (51), 16585-16589.
54. Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Park, J. H.; Zhdanov, V. P.; Cho, N.-J., Indirect nanoplasmonic sensing platform for monitoring temperature-dependent protein adsorption. *Anal. Chem.* **2017**, *89* (23), 12976-12983.
55. Norde, W. In *Driving forces for protein adsorption at solid surfaces*, Macromol. Symp., Wiley Online Library: 1996; pp 5-18.
56. Rabe, M.; Verdes, D.; Seeger, S., Understanding protein adsorption phenomena at solid surfaces. *Adv. Colloid Interface Sci.* **2011**, *162* (1-2), 87-106.
57. Jeyachandran, Y.; Mielczarski, E.; Rai, B.; Mielczarski, J., Quantitative and qualitative evaluation of adsorption/desorption of bovine serum albumin on hydrophilic and hydrophobic surfaces. *Langmuir* **2009**, *25* (19), 11614-11620.
58. Jackman, J. A.; Špačková, B.; Linardy, E.; Kim, M. C.; Yoon, B. K.; Homola, J.; Cho, N.-J., Nanoplasmonic ruler to measure lipid vesicle deformation. *Chem. Commun.* **2016**, *52* (1), 76-79.
59. Kossovsky, N.; Nguyen, A.; Sukiassians, K.; Festekjian, A.; Gelman, A.; Sponsler, E., Secondary structure of albumin acquired rapidly by modified conventional ATR-FTIR is comparable to CD spectral data. *J. Colloid interface Sci.* **1994**, *166* (2), 350-355.

60. Zeng, H.; Chittur, K. K.; Lacefield, W. R., Analysis of bovine serum albumin adsorption on calcium phosphate and titanium surfaces. *Biomaterials* **1999**, 20 (4), 377-384.
61. Roach, P.; Farrar, D.; Perry, C. C., Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. *J. Am. Chem. Soc.* **2006**, 128 (12), 3939-3945.
62. Arakawa, T.; Kita, Y., Stabilizing effects of caprylate and acetyltryptophanate on heat-induced aggregation of bovine serum albumin. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2000**, 1479 (1-2), 32-36.
63. Anraku, M.; Tsurusaki, Y.; Watanabe, H.; Maruyama, T.; Kragh-Hansen, U.; Otagiri, M., Stabilizing mechanisms in commercial albumin preparations: octanoate and N-acetyl-L-tryptophanate protect human serum albumin against heat and oxidative stress. *Biochim. Biophys. Acta, Proteins Proteomics* **2004**, 1702 (1), 9-17.
64. Mannuzza, F. J.; Montalto, J. G., Is bovine albumin too complex to be just a commodity? *BioProcess Int.* **2010**, 8 (4), 42-49.
65. Zhu, T.-T.; Zhang, Y.; Luo, X.-A.; Wang, S.-Z.; Jia, M.-Q.; Chen, Z.-X., Difference in binding of long-and medium-chain fatty acids with serum albumin: The role of macromolecular crowding effect. *J. Agric. Food Chem.* **2018**, 66 (5), 1242-1250.
66. Freyer, M. W.; Lewis, E. A., Isothermal titration calorimetry: experimental design, data analysis, and probing macromolecule/ligand binding and kinetic interactions. *Methods Cell Biol.* **2008**, 84, 79-113.
67. Boyer, P. D.; Lum, F. G.; Ballou, G. A.; Luck, J. M.; Rice, R. G., The combination of fatty acids and related compounds with serum albumin I. stabilization against heat denaturation. *J. Biol. Chem.* **1946**, 162 (2), 181-198.
68. Curry, S.; Mandelkow, H.; Brick, P.; Franks, N., Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat. Struct. Mol. Biol.* **1998**, 5 (9), 827.
69. Curry, S., Plasma albumin as a fatty acid carrier. In *Advances in Molecular and Cell biology*, 2003; Vol. 33, pp 29-46.

70. Karlsson, M.; Ekeröth, J.; Elwing, H.; Carlsson, U., Reduction of irreversible protein adsorption on solid surfaces by protein engineering for increased stability. *J. Biol. Chem.* **2005**, 280 (27), 25558-25564.
71. Ramsden, J.; Prenosil, J., Effect of ionic strength on protein adsorption kinetics. *J. Phys. Chem.* **1994**, 98 (20), 5376-5381.
72. Chen, S.; Cao, Z.; Jiang, S., Ultra-low fouling peptide surfaces derived from natural amino acids. *Biomaterials* **2009**, 30 (29), 5892-5896.

Chapter 6*

Elucidating How Different Amphipathic Stabilizers Affect BSA Protein Conformational Properties and Adsorption Behavior

There have been extensive studies investigating how fatty acids and related amphiphiles affect solution-phase BSA conformational properties while it is far less understood how amphipathic stabilizers might influence noncovalent BSA adsorption onto solid supports, which is practically relevant to form surface coatings. Herein, the binding interactions between BSA proteins and different molar ratios of caprylic acid (CA), monocaprylin (MC), and methyl caprylate (ME) amphiphiles – all of which have 8-carbon long, saturated hydrocarbon chains with distinct headgroups – and resulting effects on BSA adsorption behavior on silica surfaces were systematically investigated. It was revealed that CA had the greatest binding affinity to BSA, causing greater solution-phase conformational stability, reduced adsorption-related conformational changes and lower packing densities in fabricated BSA adlayers. By contrast, nonionic MC had moderate binding affinity to BSA and stabilized BSA conformational properties while also enabling BSA adlayers to form with higher packing densities. Physicochemical factors that contribute to these performance differences were discussed.

*This chapter is published substantially as G.J. Ma, A.R. Ferhan, J.A. Jackman, N.-J. Cho. Elucidating how different amphipathic stabilizers affect BSA protein conformational properties and adsorption behavior. *Langmuir* **2020**, 36, 35, 10606-10614. DOI: 10.1021/acs.langmuir.0c02048. Reprinted with permission. Copyright 2020, American Chemical Society.

6.1 Introduction

The phenomenon of noncovalent protein adsorption at solid-liquid interfaces is closely linked with a wide range of medical and biotechnology applications, including implantable medical devices, biosensors, and drug delivery systems.¹⁻⁵ Mechanistically, the protein adsorption process is governed by a combination of protein-surface interactions and lateral protein-protein interactions between adsorbed proteins.⁶⁻⁸ Importantly, both protein-surface and protein-protein interactions can be modulated depending on key molecular properties of the adsorbing protein, which opens the door to controlling protein adsorption processes based on molecular design strategies in line with the nanoarchitectonics concept.^{9, 10}

Towards this objective, a popular strategy involves tuning the thermodynamic stability of the folded protein structure in bulk solution – termed conformational stability – as a means to modulate protein adsorption behavior. For example, Karlsson *et al.* showed experimentally that certain synthetic mutants of the carbonic anhydrase II protein, which were engineered (via amino acid substitution) to have lower conformational stability, underwent more extensive surface-induced denaturation, and exhibited greater adsorption irreversibility on various solid supports.¹¹ The conformational stability of natural proteins can also be modulated by adjusting environmental conditions, such as temperature, solution pH, and solvent conditions, which in turn affect protein adsorption behavior. This latter approach has been applied to bovine serum albumin (BSA), which is a widely studied protein that is isolated from bovine plasma and commonly used as a reagent for blocking and surface passivation applications.¹²⁻¹⁷ For example, Park *et al.* reported that heat pretreatment converts BSA protein monomers into oligomers/aggregates, which adsorb onto solid surfaces to a greater extent than monomers at room temperature due to distinct conformational properties and consequently exhibit superior surface passivation properties.¹³ It is also important to consider how the interactions of BSA with other types of molecules affect protein structure and function.

As part of processing methods to isolate BSA proteins from crude protein mixtures, fatty acids, most often caprylic acid (CA), are widely used as dopants to endow BSA proteins with greater stability during high-temperature extraction steps. Fatty acids and related amphipathic molecules with hydrophilic headgroups and hydrophobic chains are known to bind to BSA proteins through hydrophobic, electrostatic, and hydrogen bonding interactions.¹⁸⁻²⁰ There have been extensive studies looking at how factors such as amphiphile chain length²¹⁻²³ and headgroup properties²⁴⁻²⁸ affect the binding of amphipathic molecules to BSA proteins along with the extent of solution-phase conformational stability in response to chemical and thermal denaturants.²⁹⁻³¹ On the other hand, it was only recently observed that fatty acid doping also significantly affects protein adsorption and resulting surface passivation performance.³² In particular, fatty acid-doped BSA proteins exhibit less adsorption uptake and adsorption-related denaturation on silica surfaces as well as reduced surface passivation performance, as compared to fatty acid-free BSA proteins. These effects are related to (1) greater conformational stability and (2) more repulsive protein-protein interactions of fatty acid-doped BSA molecules, the latter of which originates from the anionic character of bound fatty acids and results in a lower surface coverage of adsorbed proteins. At the same time, there remains an outstanding need to more broadly understand how different molecular features of amphipathic molecules, including fatty acids and related compounds, affect BSA adsorption behavior.

It was investigated herein how the binding interactions of CA fatty acid and its monoglyceride and methyl ester derivatives termed monocaprylin (MC) and methyl caprylate (ME), respectively, with BSA proteins affect BSA adsorption behavior on silica surfaces by utilizing a combination of biophysical solution-phase and surface-sensitive measurement techniques. The overall experimental strategy is presented in **Figure 6.1**. All three compounds have an 8-carbon long saturated hydrocarbon chain along with distinct headgroup properties. The anionic CA headgroup consists of a carboxylic acid functional group while the nonionic MC headgroup is formed from the conjugation of a hydrophilic glycerol molecule to CA and the nonionic ME headgroup is formed by the addition of a hydrophobic methyl group to the carboxylic acid of CA. Particular attention was focused on scrutinizing how CA, MC, and ME affect solution-phase BSA conformational stability

in the broader context of tracking protein adsorption uptake and kinetics along with related conformational changes in the adsorbed state.

Figure 6.1 Overview of experimental strategy. The binding interactions of BSA with different amphipathic molecules, namely CA, MC, and ME, were quantitatively characterized by fluorescence spectroscopy along with dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy measurements to evaluate solution-phase conformational stability. Quartz crystal microbalance-dissipation (QCM-D), localized surface plasmon resonance (LSPR), and attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy experiments were conducted to determine how binding interactions with the tested amphipathic molecules affected BSA protein adsorption behavior in terms of adsorption uptake and kinetics along with related conformational changes.

6.2 Materials and Methods

6.2.1 Reagents

Fatty acid-free BSA (A7030, lot no. SLBT4132), octanoic acid (caprylic acid, C2875), 1-monocapryloyl-rac-glycerol (monocaprylin, M2265), methyl octanoate (methyl caprylate, 260673), sodium dodecyl sulfate (SDS, L4390), sodium chloride (NaCl, 746398), and sodium hydroxide (S5881), were purchased from Sigma-Aldrich (USA). Tris(hydroxymethyl)aminomethane (Tris, 0497) was purchased from Amresco (USA). Ethanol (95%) was purchased from Aik Moh (Singapore) and hydrochloric acid (HCl, 100317) was purchased from Merck (USA).

6.2.2 Sample Preparation

An aqueous buffer solution of 10 mM Tris, 150 mM NaCl, and pH 7.5 was prepared with Milli-Q-treated water (resistivity of $>18.2 \text{ M}\Omega\cdot\text{cm}$ at 25°C) and filtered through a $0.2 \mu\text{m}$ polyethersulfone (PES) membrane filter (Thermo Fisher Scientific, 595-4520). BSA solutions were prepared by dissolving lyophilized BSA powder in the buffer solution and then filtering the BSA suspension through a syringe filter with $0.2\text{-}\mu\text{m}$ diameter pores (PN-4612; Pall Corporation, USA). The molar concentrations of BSA protein in aqueous buffer solution was determined by ultraviolet (UV) light absorbance measurements at 280 nm and the molar extinction coefficient value of BSA was taken to be $43824 \text{ M}^{-1} \text{ cm}^{-1}$. To prepare CA-doped BSA, CA was dissolved in protein-free Tris buffer, followed by NaOH titration, to make a 50 mM CA solution at pH 7.5. The CA solution was then added to appropriate amounts of BSA solution in order to yield CA-doped BSA samples with a CA:BSA molar ratio of 10:1 (CA 10) or 100:1 (CA 100). MC and ME solutions were made by dissolving appropriate amounts in equivalent buffer solution to make 20 mM MC or ME solutions, followed by heating to 70°C for 30 min to aid dissolution. The samples were then cooled down to room temperature before adding the appropriate amounts of BSA solution in order to yield MC or ME-doped BSA samples with MC:BSA and ME:BSA molar ratios of 10:1 (MC 10 and ME 10) or 100:1 (MC 100 and ME 100).

6.2.3 Fluorescence Spectroscopy

The intrinsic fluorescence of BSA protein samples was characterized by fluorescence spectroscopy measurements using a Tecan Spark plate reader (Tecan, Switzerland) with the temperature set to 25 °C, an excitation wavelength of 280 nm, and the emission spectra recorded from 325 to 450 nm. For each sample, the BSA concentration was fixed at 10 μ M while CA, MC, and ME concentrations were varied. All spectra of BSA samples were recorded in triplicate and subtracted by the background spectra of equivalent test compound concentrations without BSA protein to remove any possible contribution from the CA, MC, or ME background signal alone. The emission maxima for all BSA samples were located at 340 nm and fluorescence intensity values at this wavelength were used for Stern-Volmer and binding analyses.

6.2.4 Dynamic Light Scattering

The intensity-weighted size distributions of 150 μ M BSA protein samples were determined by the DLS technique using a particle size analyzer (ZetaPALS, Brookhaven Instruments, USA). The BIC Particle Sizing software package (v5.27; Brookhaven Instruments) was used for data collection and analysis. Measurements were first recorded at 25 °C, followed by increasing the temperature from 50 °C to 75 °C in 5 °C increments, as previously described.³² Samples were equilibrated for 5 min after each temperature adjustment before measurements were performed. For time-dependent measurements, samples were equilibrated at 50 °C and then 55 °C for 10 min each before maintaining a constant temperature at 60 °C and measuring protein size every 10 min for 200 min.

6.2.5 Circular Dichroism Spectroscopy

CD spectroscopy experiments were conducted using an AVIV Model 420 CD spectrometer with the AVIV CDS software package (v3.36 MX) (AVIV Biomedical, Lakewood, NJ, USA). 2.5 μ M BSA protein samples were measured in a 1 mm path length cuvette with a polytetrafluoroethylene (PTFE) stopper (110-QS; Hellma, Germany). Spectra were

recorded with a 1 nm spectral bandwidth, 0.5 nm step size, and an averaging time of 0.1 s from 200 to 260 nm. CD data are presented in molar residue ellipticity ($[\theta]$) units based on the following equation:

$$[\theta](\text{degree cm}^2\text{dmol}^{-1}) = \frac{\text{millidegrees}}{n \times c \times l \times 10} \quad (\text{Eq 6.1})$$

where n is the number of amino acid residues, c is the protein molar concentration, and l is the cuvette path length in cm. All CD spectra were smoothed by using the Savitzky-Golay³³ smoothing function with a smoothing window of 20 points and a polynomial order of 2 in the OriginPro 2019b (v9.6.5.169) software package (OriginLab, Northampton, MA, USA). The α -helical percentage of each BSA protein was calculated from the $[\theta]$ data at 222 nm ($[\theta]_{222}$) based on the following equation:³⁴

$$\% \text{ Helicity} = \frac{([\theta]_{222} - 3000)}{(-36000 - 3000)} \times 100 \quad (\text{Eq 6.2})$$

Temperature-dependent measurements were conducted in a similar format to the DLS experiments as described above. All spectra of BSA samples were subtracted by background spectra of equivalent conditions, including appropriate CA, MC, or ME concentration, without BSA protein.

6.2.6 Quartz Crystal Microbalance-Dissipation

The real-time adsorption of BSA protein samples onto silica surfaces was characterized by QCM-D measurements that were conducted by using a QSense E4 instrument (Biolin Scientific AB, Stockholm, Sweden) with silica-coated AT-cut quartz crystal sensor chips with a fundamental frequency of 5 MHz (QX 303, Biolin Scientific, Sweden). Before every experiment, the sensor chips were sequentially rinsed with 1% (wt/vol) aqueous SDS solution, water, and ethanol, and then dried under a gentle stream of nitrogen gas, followed by oxygen plasma treatment (PDC-002, Harrick Plasma, USA) for 3 min. A peristaltic pump was used to inject liquid samples into the measurement chamber at a flow rate of 100

$\mu\text{L}/\text{min}$. A stable baseline signal was first established in Tris buffer solution before 100 μM BSA protein was introduced into the measurement chamber for 30 min, followed by a buffer washing step. The resonance frequency (ΔF) and energy dissipation (ΔD) shifts were recorded in real-time at multiple odd overtones, as previously described.³⁵ All experiments were conducted at 25 °C and all measurement operations were controlled by the QSoft 401 (v2.5.13.664) (Biolin Scientific) software package. The normalized data at the fifth overtone are reported.

6.2.7 Localized Surface Plasmon Resonance Sensing

BSA protein adsorption onto silica-coated gold nanodisk arrays was characterized by ensemble-averaged localized surface plasmon resonance (LSPR) measurements, which were conducted in optical transmission mode by using an Insplorion XNano instrument (Insplorion AB, Gothenburg, Sweden), as previously described.³⁶ The sensor chip preparation and LSPR adsorption experiments were conducted in a similar format to the QCM-D experiments described above. The Insplorer software package (Insplorion AB, Sweden) was used to record the LSPR extinction spectra with a time resolution of 1 Hz and the centroid (peak) position ($\Delta\lambda$) in the extinction spectrum at each time point was recorded in real-time. The rates of change of the $\Delta\lambda$ shifts were also determined by calculating the first derivative with respect to time ($d\Delta\lambda/dt$) (see ref. 37) by using the OriginPro 2019b software package.

6.2.8 Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy

ATR-FTIR spectroscopy experiments were conducted by using a Bruker Vertex 70 FTIR spectrometer with a liquid-nitrogen-cooled mercury cadmium telluride (MCT) photodetector (Bruker, Germany) and equipped with a MIRacle ATR accessory containing a three-reflection ZnSe ATR crystal (PIKE Technologies, USA). The secondary structures of 100 μM BSA proteins in solution and in the adsorbed state were determined by ATR-FTIR spectroscopy experiments, as previously described.³² A 30 μL aliquot of a 100 μM BSA sample solution was pipetted onto the ATR crystal and the absorbance spectrum was

recorded to obtain the secondary structure of solution-phase BSA proteins. The BSA solution was then incubated with the ATR crystal surface at room temperature for 30 min, followed by a series of ten buffer washing steps – by pipetting an additional 30 μL of fresh buffer to the aliquot and drawing 30 μL of the mixed sample ten consecutive times – to remove weakly adsorbed proteins. A second absorbance spectrum was recorded to obtain the secondary structure of BSA proteins in the adsorbed state. All measurements were conducted in triplicate. During the experiment, the sample compartment was purged continuously with nitrogen gas to minimize ambient moisture. The OPUS 6.5 software package (Bruker) was used to collect the ATR-FTIR absorbance spectra at a 4 cm^{-1} resolution and averaged over 128 scans. All recorded spectra were normalized by subtracting background spectra from buffer and water vapor alone in order to remove water and ambient water vapor absorbance artefacts, respectively. The relative contribution of different secondary structure elements was determined for all samples by curve fitting the amide I bands of the absorbance spectra (1720 – 1580 cm^{-1}) to Gaussian components based on the peaks identified in the second derivative plot of the absorbance spectra and from literature references.³⁸⁻⁴² The secondary structure percentage values were determined by calculating the area under the curve-fitted components that had each been assigned to a secondary structure element as a fraction of the total area under the fitted curve. Peaks centered at 1682-1678 cm^{-1} were assigned to β -turn structures, 1656-1654 cm^{-1} to α -helix, 1643-1647 cm^{-1} to random coil, 1639-1622 cm^{-1} to extended chains, and 1619-1611 cm^{-1} to intermolecular β -sheets. All curve fitting analyses were conducted using the Peak Analyzer function in the OriginPro 2019b software package.

6.2.9 Statistical Analysis

The tests were conducted using the GraphPad Prism (v8.0.1) software package from GraphPad Software (USA). One-way and two-way analysis of variance (ANOVA) with the appropriate multiple comparisons test were conducted to compute multiplicity-adjusted *P* values to determine the statistical significance of measurement data as appropriate. All statistical analysis involved two-tailed tests. $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$ indicate the levels of statistical significance and NS is “not significant”.

6.3 Results and Discussion

6.3.1 Binding Interaction Analysis

The binding affinities of CA, MC, and ME to solution-phase BSA proteins were first measured by steady-state fluorescence spectroscopy (**Figure 6.2** and **Table 6.1**). Like how fatty acids can insert into hydrophobic pockets on the BSA protein surface, it is known that monoglycerides⁴³⁻⁴⁵ and fatty acid methyl esters^{46, 47} can bind similarly to serum albumin proteins in some cases. The hydrophobic pocket is near one of two Trp residues in BSA (Trp-212) compared to the other Trp residue (Trp-134) which is near the surface of the BSA.^{27, 48, 49} The fluorescence emission spectra corresponding to the intrinsic fluorescence of tryptophan residues in BSA alone and in BSA together with varying amounts of CA, MC, or ME were evaluated (**Figures 6.2a-c**). The excitation wavelength was 280 nm and the maximum-intensity emission wavelength was 340 nm in all cases. At higher CA and MC concentrations, there was greater quenching, which resulted in a lower maximum intensity at the 340 nm wavelength, while ME had negligible effect on the intensity value.

Figure 6.2 Evaluation of CA, MC, and ME binding interactions with BSA proteins. Intrinsic fluorescence emission spectra of 10 μM BSA in the absence and presence of **a**, CA, **b**, MC, and **c**, ME amphiphiles. **d**, Stern-Volmer plots corresponding to the data in panels **a-c**. Data are presented as mean $\pm$ standard deviation (s.d.). Linear fits and corresponding K_{SV} values of CA, MC, and ME binding to BSA are presented for each data set. **e**, Plot of $\log((F_0-F)/F)$ versus $\log[S]$. Linear fits and corresponding K_b values of CA and MC binding to BSA are presented for each data set.

Table 6.1 Summary of binding interaction data from fluorescence spectroscopy experiments. Stern-Volmer constant (K_{SV}), bimolecular quenching rate constant (k_q), effective binding constant (K_b), and number of different binding site types (n) for the amphiphile-doped BSA samples are shown. Calculations of K_b and n were omitted for ME samples due to insignificant quenching effects related to negligible binding. K_{SV} values are presented as fitted slope value $\pm$ standard error of the slope, while the values and uncertainty for k_q were derived from K_{SV} based on Eq 6.3.

	K_{SV} (M^{-1})	$k_q \times 10^{10}$ ($\text{M}^{-1}\text{s}^{-1}$)	K_b (M^{-1})	n
CA	466 ± 17	7.77 ± 0.28	390	0.97
MC	243 ± 22	4.10 ± 0.37	210	0.97

ME	16 ± 10	0.27 ± 0.17	-	-
----	---------	-------------	---	---

This reduction in fluorescence intensity (quenching) due to the addition of CA and MC amphiphiles to BSA in solution can occur by static quenching, which is due to the formation of a non-fluorescent complex between the quencher (CA or MC) and the fluorophore (BSA) in the ground state, or by dynamic quenching, which is due to the interaction between the quencher and the excited state of the fluorophore. To distinguish between these two types of quenching, the Stern-Volmer (SV) equation was used to analyze the experimental data:⁵⁰

$$\frac{F_0}{F} = 1 + K_{SV}[S] = 1 + k_q\tau_0[S] \quad (\text{Eq 6.3})$$

where F_0 and F are the experimentally determined maximum-intensity values of BSA alone and in the presence of CA, MC, or ME at different concentrations, respectively, K_{SV} is the SV quenching constant, $[S]$ is the concentration of amphiphile in the bulk solution, k_q is the bimolecular quenching rate constant, and τ_0 is the average fluorescence lifetime of BSA alone. Taking into account that the value of τ_0 for BSA is 6×10^{-9} s,^{27, 49} the resulting k_q values for CA and MC are around 7.77×10^{10} and $4.10 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, respectively, which exceed the largest possible value of k_q for a dynamic quenching process ($\sim 1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$).⁵¹ Therefore, the binding of CA and MC to BSA can be considered to involve static quenching, which agrees well with literature reports for the binding of various ionic and nonionic amphiphiles to serum albumins.^{27, 48, 49} The ME case was excluded from the analysis due to its negligible binding interaction with BSA. Next, the effective binding constant (K_b) values for CA and MC was determined according to the following equation:^{48,}

52

$$\log \frac{F_0 - F}{F} = \log K_b + n \log [S] \quad (\text{Eq 6.4})$$

where n is the number of different binding site types, and the corresponding log plot is

presented in **Figure 6.2e**. The average K_b values were determined to be 390 M^{-1} and 210 M^{-1} for the BSA complexes with CA and MC, respectively, while n was calculated to be 0.97 for both cases. The binding interaction data are summarized in **Table 6.1**. The results indicate that anionic CA has the strongest binding affinity with BSA proteins, followed by the polar and nonionic MC. By contrast, the nonionic ME, with the least polar methanol ester headgroup, had the lowest binding affinity to BSA. The calculated n value agrees well with previous works for CA binding⁴⁸ and indicates that both amphiphiles bind to a single type of binding site, which consists of hydrophobic pockets on the BSA protein surface. In other words, each amphiphile displayed one mode of binding to BSA. Collectively, the data support that the carboxylic acid headgroup of CA has stronger interactions with BSA, likely through electrostatic interactions and/or hydrogen bonding, while the glycerol ester headgroup of MC forms more extensive hydrogen bonds with BSA as compared to the methanol ester headgroup of ME. As such, these findings demonstrate that headgroup polarity strongly influences the binding interaction of amphipathic molecules with BSA proteins even when the different amphiphiles have identical hydrocarbon chains.

6.3.2 Solution-Phase Conformational Stability

The effects of CA, MC, and ME doping (10:1 and 100:1 molar ratios of amphiphile:BSA) on the solution-phase conformational stability of BSA were then compared by conducting temperature-dependent DLS and CD spectroscopy experiments. A defatted BSA sample without CA, MC, or ME was also tested as a control.

In the DLS measurements, the onset temperature of aggregation was determined by recording the lowest temperature at which a marked increase in the mean hydrodynamic diameter of BSA protein samples occurred. BSA samples with increased conformational stability tend to have a higher onset temperature of aggregation.³² From 25 to 55 °C, all BSA samples had similar mean hydrodynamic diameters of ~9 nm (**Figures 6.3 and 6.4**), which is around the expected size of BSA monomers.^{12, 13, 53} At 60 °C, the diameters of defatted BSA and ME samples increased to ~11 nm, indicating the onset of aggregation. MC 10 and MC 100 samples started aggregating at 65 °C and reached ~16 and ~13 nm in

diameter, respectively, while CA 10 samples started aggregating at 70 °C and reached ~15 nm in diameter. Interestingly, at 70 °C, MC 100 aggregated to a larger size than defatted BSA, MC 10, and both ME samples and was about twice the size of these other samples at 75 °C. By contrast, the CA 100:1 sample did not display any evidence of protein aggregation across the tested temperature range. Together, these data indicate that CA doping endows BSA proteins with the highest conformational and/or colloidal stability, followed by MC, whereas ME was essentially ineffective and ME-doped samples behaved almost equivalently to the defatted BSA control.

Figure 6.3 DLS-measured size distributions of BSA proteins in the absence and presence of the three tested amphiphiles. Mean hydrodynamic diameter (labeled as D) and polydispersity index (labeled as PDI) of **a**, defatted BSA, **b**, CA 10, **c**, CA 100, **d**, MC 10, **e**, MC 100, **f**, ME 10, and **g**, ME 100 samples were measured at 25 °C and are indicated in each panel.

Figure 6.4 Temperature-dependent DLS and CD spectroscopy measurements to evaluate solution-phase conformational stability of amphiphile-doped BSA proteins. **a**, Hydrodynamic diameter of BSA proteins alone and in the presence of CA, MC, and ME at amphiphile:BSA molar ratios of 10:1 and 100:1 as a function of temperature. Data are presented as mean $\pm$ standard

deviation (s.d.), where s.d. is defined as the full-width-at-half-maximum (FWHM)/2.355 [$n=5$, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (versus data at 25 °C)]. Dots represent individual data points.

The data also revealed that CA and MC amphiphiles more effectively decreased the onset temperature of aggregation of BSA proteins with higher bulk concentrations of amphiphile, which would maximize binding site occupancy. Time-dependent size measurements at a constant temperature of 60 °C further confirmed these trends (**Figure 6.5**). Notably, MC 100 samples in time-dependent experiments did not aggregate as extensively as in temperature-dependent experiments, indicating that this behavior is only triggered at higher temperatures (>65 °C) upon more extensive thermal unfolding.

Figure 6.5 Time-dependent changes in BSA protein size in the absence and presence of the three tested amphiphiles at 60 °C incubation temperature. Data were obtained by DLS measurements and are presented as mean $\pm$ s.d. ($n=5$) where s.d. is defined as full-width-at-half-maximum (FWHM)/2.355.

To corroborate the DLS data, CD spectroscopy measurements were conducted to monitor temperature-induced unfolding of each BSA sample with respect to the loss of α -helical

secondary structure at higher temperatures. At 25 °C, all BSA samples had similar degrees of α -helicity with an average of ~62% and the percentage value decreased with increasing temperature due to thermally induced protein unfolding (**Figures 6.6 and 6.7**).^{13, 53} While reversible protein unfolding occurs with relatively small temperature increases, irreversible protein unfolding occurs above the onset temperature of aggregation in the range of 60 °C or higher depending on the sample conditions. Thus, the degree of α -helicity at 65 °C was compared in order to scrutinize differences in conformational stabilities between the various amphiphile-doped BSA samples. At 65 °C, defatted BSA had the lowest degree of α -helicity with a percentage value around 46.8%, followed by the ME samples with ~47.7%, and then MC 10 and MC 100 with around 47.9% and 48.9%, respectively. On the other hand, the CA 10 and CA 100 samples maintained a higher degree of α -helicity, with percentage values around 51.9% and 53.5%, respectively. This trend agrees with the DLS data and supports that CA is most effective at increasing BSA conformational stability, followed by MC and then ME.

Figure 6.6 Temperature-dependent CD spectra of BSA proteins in the absence and presence of the three tested amphiphiles. CD spectra of **a**, defatted BSA, **b**, CA 10, **c**, CA 100, **d**, MC 10, **e**, MC 100, **f**, ME 10, and **g**, ME 100 samples were measured at 25, 50, 55, 60, 65, 70, and 75 °C and are reported in molar residue ellipticity units $[\theta]$.

Figure 6.7 Degree of α -helical secondary structure, expressed in percentage values, for BSA alone and in the presence of CA, MC, and ME at amphiphile:BSA molar ratios of 10:1 and 100:1 as a function of temperature obtained from CD spectroscopy experiments. Data were computed from molar residue ellipticity values and are reported as mean $\pm$ s.d. ($n=3$). Dots represent

individual data points. Mean values are presented on top of each column.

Upon further increasing the temperature to 70 °C, the CA 10 and CA 100 samples had α -helical values of 47.7% and 51.9% respectively, while the degree of α -helicity for MC 100 had decreased to 42.1% and all other BSA samples had values around ~41%. Likewise, at 75 °C, the CA 100 sample had 49.8% α -helicity while all other samples had similar degrees of α -helicity around ~37%. Together with the DLS data, these results support that MC 100 has greater conformational stability but lower colloidal stability than defatted BSA, as indicated by its higher onset temperature of aggregation and formation of larger protein aggregates upon sufficiently extensive thermal unfolding. Collectively, the DLS and CD spectroscopy measurements both showed that the stabilizing effectiveness of the amphiphiles was in the order of CA>MC>ME, and that the extent of amphiphile-induced protein stability in the solution phase was greater at higher bulk amphiphile concentrations in general.

6.3.3 Nature of Amphiphile Headgroup-BSA Interactions

The molecular structures of the amphiphiles directly affect their binding to BSA since binding involves hydrophobic interactions between the hydrocarbon tails of each amphiphile and hydrophobic pockets on the BSA protein surface, as well as polar interactions between the amphiphile headgroup and polar residues on the BSA protein surface.^{54, 55} As the three amphiphiles have the same tail length, the observed trend in their binding affinities to BSA and stabilizing effectiveness (CA>MC>ME) can be explained in terms of their distinct headgroups. CA is an anionic amphiphile with a highly polar, negatively charged carboxylic acid headgroup that is capable of both electrostatic interactions and hydrogen bonding. On the other hand, the headgroup of nonionic MC consists of a glycerol molecule ester that is bonded to a carboxylic acid group, which is capable of hydrogen bonding with its two hydroxyl groups and one glycerol ester group. By contrast, the headgroup of ME consists of a methanol molecule that is ester-bonded to the carboxylic acid group and only has one methyl ester group available for hydrogen bonding.

Figure 6.8 Temperature-dependent DLS measurements of amphiphile-doped BSA samples in different ionic strength conditions. Hydrodynamic diameter of **a**, CA 10, **b**, MC 10, and **c**, ME 10 samples in 1.5, 150, and 500 mM NaCl Tris buffer solutions as a function of temperature. Data are presented as mean $\pm$ s.d. ($n=5$) where s.d. is defined as full-width-at-half-maximum (FWHM)/2.355.

Given these molecular differences, the thermal stability of CA 10, MC 10, and ME 10 samples in Tris buffer solutions with different ionic strength conditions of 1.5 mM, 150 mM, and 500 mM NaCl were compared by temperature-dependent DLS measurements (**Figure 6.8**). It was hypothesized that reducing the ionic strength might reduce the charge shielding effect, thereby increasing electrostatic attraction between CA and BSA molecules and in turn enhance stabilizing effectiveness. Hence, the onset temperature of aggregation for CA 10 was expected to increase with decreasing ionic strength and vice versa. At 1.5 mM NaCl, the extent of aggregation for the CA 10 sample was greatly reduced, indicating that charge shielding in the system was decreased as electrostatic repulsion between net negatively charged BSA protein molecules was enhanced⁵⁶ while both 150 mM and 500 mM NaCl conditions yielded similar extents of aggregation. Importantly, the onset temperature of aggregation for CA 10 samples remained at 70 °C across all tested ionic strength conditions. Control experiments were also conducted on MC 10 and ME 10 samples, and the variation in ionic strength similarly reduced the extent of aggregation at

1.5 mM NaCl while the onset temperatures of aggregation remained unaffected across all ionic strength conditions. These results support that amphiphile-protein electrostatic interactions do not play a dominant role in determining the difference in binding strength and stabilizing effectiveness between the tested amphiphiles. Rather, the experimentally observed difference in binding affinities between BSA and the different amphiphiles is likely due to the strength and extensiveness of hydrogen bonding interactions between the amphiphiles and BSA protein molecules.^{27, 49, 57} The carboxylic acid headgroup of CA is the most polar of the three and would form the strongest hydrogen bonds, while the glycerol ester headgroup of MC would be able to form more hydrogen bonds than the methyl ester headgroup of ME.

6.3.4 Real-Time Adsorption Behavior

Next, the effect of CA, MC, and ME on BSA protein adsorption onto a hydrophilic silica surface was investigated by conducting quartz crystal microbalance-dissipation (QCM-D) experiments. The isoelectric point (IEP) of silica is around 3.9,⁵⁸ while the IEP of BSA protein is around 5.1.⁵⁹ As such, in the pH 7.5 buffer solution, both the silica surface and BSA protein surface are expected to have net negative charges and hence possibly some degree of electrostatic repulsion between them. Nevertheless, BSA proteins can still adsorb onto the silica surface because positively charged regions on the BSA molecular surface can contact the silica surface, yielding attractive electrostatic interactions while hydrogen bonding and van der Waals interactions can also influence BSA adsorption.⁶⁰ The amount and charge of bound amphiphiles are also expected to influence the extent of protein adsorption.

Figure 6.9 QCM-D and LSPR measurements for tracking real-time adsorption of amphiphile-doped BSA proteins onto silica surfaces. **a**, QCM-D frequency (ΔF) shifts as a function of time and **b**, corresponding $|\Delta F_{\max}|$ shifts at adsorption saturation. **c**, QCM-D energy dissipation (ΔD) shifts as a function of time. **d**, $|\Delta F_{\max}/\Delta D_{\max}|$ values obtained from the data in panels **a** and **c** at adsorption saturation. Data in panels **b** and **d** are presented as mean $\pm$ s.d. [$n=3$ biological replicates, one-way ANOVA with Dunnett's multiple comparisons test (versus defatted BSA)]. Dots represent individual data points.

Experimentally, the QCM-D frequency (ΔF) and energy dissipation (ΔD) shifts correspond to the hydrodynamically-coupled mass and viscoelastic properties of adsorbed BSA protein molecules, respectively,^{35, 61, 62} and were recorded as a function of time. The time-resolved ΔF shift data indicated monotonic adsorption of BSA proteins in all cases and most adsorbed proteins remained irreversibly attached, as indicated by a subsequent buffer

washing step without protein (**Figure 6.9a**). The ΔF shift at adsorption saturation (denoted as $|\Delta F_{\max}|$) was analyzed for the different BSA samples and a larger $|\Delta F_{\max}|$ indicates greater adsorption uptake (**Figure 6.9b**). The adsorption of defatted BSA proteins yielded an average $|\Delta F_{\max}|$ value of 45.5 Hz, while CA 10 and CA 100 samples tended to have smaller average $|\Delta F_{\max}|$ values of 44.1 and 39.9 Hz, respectively. By contrast, MC 10 and MC 100 had average $|\Delta F_{\max}|$ values around 45.5 and 47.1 Hz, respectively, whereas the ME 10 and ME 100 samples had average $|\Delta F_{\max}|$ values of 44.3 and 44.2 Hz, respectively. Together, these data support that increasing amounts of CA doping tended to decrease BSA adsorption uptake while MC and ME doping had nearly negligible effects.

The time-resolved ΔD shift mirrored the kinetic profiles observed in the ΔF shift data and the $|\Delta F_{\max}/\Delta D_{\max}|$ ratio were further analyzed, whereby a larger ratio indicates a more tightly coupled BSA adlayer and vice versa (**Figures 6.9c,d**). Accordingly, it was observed that defatted BSA had an average $|\Delta F_{\max}/\Delta D_{\max}|$ value of $12.2 \text{ Hz} \cdot 10^6$, while CA 10 and CA 100 samples had smaller average $|\Delta F_{\max}/\Delta D_{\max}|$ values around 11.2 and $9.1 \text{ Hz} \cdot 10^6$, respectively. On the other hand, MC 10 and MC 100 samples had average $|\Delta F_{\max}/\Delta D_{\max}|$ values of 12.2 and $10.9 \text{ Hz} \cdot 10^6$, respectively, and ME 10 and ME 100 samples had average $|\Delta F_{\max}/\Delta D_{\max}|$ values of 13.6 and $14.8 \text{ Hz} \cdot 10^6$, respectively. Together, these results show that CA addition tended to reduce BSA adsorption uptake. Notably, the CA 100 sample had the lowest adsorption uptake and formed the least rigid protein adlayer, which support that high levels of CA doping endow the BSA proteins with greater conformational stability. By contrast, the defatted BSA and MC 10 samples had similar levels of adsorption uptake and adlayer rigidity, while the MC 100 sample tended to result in greater adsorption uptake and less adlayer rigidity. In addition, the ME 10 and ME 100 samples had similar adsorption properties to the defatted BSA control sample. As such, the overall findings indicate that both the type and amount of amphiphile influences BSA adlayer properties.

In addition to QCM-D measurements, localized surface plasmon resonance (LSPR) sensing experiments were also conducted as a complementary approach to monitor the adsorption of BSA proteins onto silica-coated gold nanodisk arrays. While QCM-D measurements are sensitive to the mass of adsorbed protein molecules and

hydrodynamically-coupled solvent molecules, *i.e.*, “wet mass”, LSPR measurements are sensitive only to the “dry mass” corresponding to adsorbed protein molecules.⁶³ Furthermore, the LSPR measurement approach has a shorter penetration depth of ~20 nm or less compared to 100-300 nm for the QCM-D technique.⁶³ Hence, protein adsorption events would occur within a larger fraction of the LSPR-tracked probing volume, which can potentially yield higher surface sensitivity to subtle variations in adsorption behavior and adsorption-related conformational properties. As presented in **Figure 6.10a**, BSA protein adsorption was monitored in real-time by tracking wavelength shifts ($\Delta\lambda$) of the LSPR extinction peak position, which arise due to the adsorption and spreading of BSA proteins on the sensor surface.⁵³ Larger $\Delta\lambda$ shifts correspond to greater adsorption uptake and monotonic $\Delta\lambda$ shift increases were observed in all cases. Most attached BSA molecules were irreversibly adsorbed, as indicated by a buffer washing step.

Figure 6.10 **a**, LSPR wavelength ($\Delta\lambda$) shifts as a function of time and **b**, corresponding $\Delta\lambda_{\max}$ shifts at adsorption saturation. Data in panel **b** are presented as mean $\pm$ s.d. [$n=3$ biological replicates, one-way ANOVA with Dunnett’s multiple comparisons test (versus defatted BSA)]. Dots represent individual data points.

To compare the different BSA samples, the $\Delta\lambda$ shifts corresponding to adsorption at saturation ($\Delta\lambda_{\max}$) were recorded (**Figure 6.10**). For defatted BSA, the average $\Delta\lambda_{\max}$ shift was around 1.72 nm, while the CA 10 and CA 100 samples had average $\Delta\lambda_{\max}$ shifts around

1.61 and 1.25 nm, respectively. On the other hand, the MC 10 and MC 100 samples had average $\Delta\lambda_{\max}$ shifts around 1.76 and 1.96 nm, respectively, while the ME 10 and ME 100 samples had average $\Delta\lambda_{\max}$ shifts around 1.83 and 1.91 nm, respectively. Collectively, the results agree well with the trends observed in the QCM-D measurements while the LSPR measurement signal proved more sensitive to distinguish between the adsorption profiles of different BSA samples. Overall, the data indicate that CA addition reduced BSA adsorption uptake, MC addition increased adsorption uptake only at adsorption saturation when added at high concentrations, and ME addition tended to increase adsorption uptake only slightly. Furthermore, taking into account that there is negligible binding between the ME amphiphile and BSA protein, the slight increase in adsorption uptake for ME-doped BSA samples in the QCM-D and LSPR experiments suggest that free ME can adsorb onto the silica surface.⁶⁴

6.3.5 Adsorption-Related Protein Conformational Changes

To further analyze the adsorption behavior of the different BSA samples, the extent of adsorption-related protein conformational changes was evaluated by calculating the maximum rate of change in the LSPR $\Delta\lambda$ shift during the initial stage of adsorption $(d\Delta\lambda/dt)_{\max}$ (**Figures 6.11 & 6.12a**).^{53, 63, 65} A larger $(d\Delta\lambda/dt)_{\max}$ value indicates more extensive adsorption-related conformational changes, especially protein spreading whereby the adsorbed protein mass is closer, on average, to the sensor surface. The average $(d\Delta\lambda/dt)_{\max}$ value for defatted BSA samples was around 0.74 nm/min, while the CA 10 and CA 100 samples had average $(d\Delta\lambda/dt)_{\max}$ values of 0.54 and 0.39 nm/min, respectively. Likewise, the MC 10 and MC 100 samples had average $(d\Delta\lambda/dt)_{\max}$ values of 0.66 and 0.52 nm/min, respectively, whereas the ME 10 and ME 100 samples had average $(d\Delta\lambda/dt)_{\max}$ values around 0.75 and 0.90 nm/min, respectively. The findings indicate that both CA and MC doping reduced the extent of adsorption-related BSA conformational changes. In this respect, CA was more effective than MC in maintaining protein conformational stability. On the other hand, the ME 10 and ME 100 sample underwent protein spreading in the adsorbed state to similar extents as the defatted BSA control.

Figure 6.11 Rates of change of LSPR peak shift ($d\Delta\lambda/dt$) corresponding to the adsorption of amphiphile-doped BSA proteins onto silica surfaces as a function of time. The rates of change were recorded during the initial stage of adsorption.

Figure 6.12 Evaluation of adsorption-related protein conformational changes for amphiphile-doped BSA proteins. **a**, Maximum rate of change in the $\Delta\lambda$ shift during the initial stage of adsorption $(d\Delta\lambda/dt)_{\max}$. **b**, Fractional percentage of α -helical secondary structure for BSA proteins in solution and in the adsorbed state, as measured by ATR-FTIR spectroscopy experiments. Data

in panels **a** and **b** are presented as mean $\pm$ s.d. [$n=3$ biological replicates, one-way ANOVA with Dunnett's multiple comparisons test (versus defatted BSA) in panel **a** and two-way ANOVA with Dunnett's multiple comparisons test for all samples in solution (versus defatted BSA in solution) and separately for all samples in the adsorbed state (versus defatted BSA in the adsorbed state) in panel **b**]. Dots represent individual data points.

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy experiments were also performed to quantitatively determine the fractional percentage of α -helical secondary structure for the BSA protein samples in solution and in the adsorbed state (**Figures 6.12b** and **6.13**). In solution, all BSA samples had similar degrees of α -helicity, with values around ~62.8%. By contrast, in the adsorbed state, defatted BSA samples underwent partial unfolding and the percentage of α -helical character decreased to around 45.1%. In marked contrast, the CA 10, CA 100, MC 10, and MC 100 samples maintained α -helical character to a greater extent and the corresponding values in the adsorbed state were 52.4%, 55.0%, 48.6%, and 52.7%, respectively. This trend agrees well with the LSPR measurement data and provides additional evidence that both CA and MC stabilize BSA to reduce the extent of adsorption-related protein conformational changes. Furthermore, the ATR-FTIR spectroscopy results also show that CA confers greater conformational stability to BSA proteins than MC. On the other hand, adsorption of the ME 10 and ME 100 samples caused similar losses in α -helicity that mirrored the defatted BSA case, with average α -helicity values of 46.0% and 46.4%, respectively, in the adsorbed state. As such, the data indicate that ME doping does not endow BSA proteins with greater conformational stability.

Figure 6.13 Quantification of adsorption-related protein conformational changes of amphiphile-doped BSA proteins by ATR-FTIR spectroscopy measurements. Amide I regions from ATR-FTIR spectrograms of **a**, defatted BSA, **b**, CA 10, **c**, CA 100, **d**, MC 10, **e**, MC 100, **f**, ME 10, and **g**, ME 100 samples in solution (left panels) and in the adsorbed state (right panels). Experimentally obtained absorbance spectra were resolved into individual component curves, which were each assigned to a secondary structure element.

6.3.6 Schematic Overview of Amphiphile Effects

Figure 6.14 presents a schematic illustration that summarizes the main experimental findings and describes how CA, MC, and ME amphiphiles affect BSA protein adsorption behavior on a case-by-case basis. ME had the least effect on BSA adsorption, which is

consistent with the fluorescence spectroscopy data that indicated a weak binding interaction between BSA and ME. As such, ME-doped BSA samples had similar solution-phase conformational stability and adsorption behavior to the defatted BSA control sample. On the other hand, CA had the highest binding affinity to BSA and CA-doped BSA samples exhibited the greatest solution-phase conformational stability. Moreover, while less surface-induced denaturation typically leads to greater total adsorption uptake due to a smaller contact area per molecule, CA-doped BSA proteins also had the lowest total adsorption uptake and consequently lowest packing density of adsorbed protein molecules.³² This trend occurred because the carboxylic acid functional groups of CA are negatively charged and thus CA-doped BSA proteins have intermolecular repulsion, which limits the surface coverage of adsorbed protein molecules.

Figure 6.14 Schematic illustration summarizing how CA, MC, and ME amphiphiles affect the solution-phase conformational stability and adsorption properties of BSA proteins. ME had low binding affinity to BSA proteins and hence largely negligible effects. MC moderately enhanced conformational stability, which reduced adsorption-related denaturation while the nonionic character of MC allowed MC-doped BSA protein adlayers to have relatively higher packing densities. Likewise, CA strongly enhanced conformational stability, which also reduced

adsorption-related denaturation. However, the anionic character of CA created more repulsive interactions between CA-doped BSA proteins, which led to CA-doped BSA protein adlayers with relatively lower packing densities.

Between these two cases, interesting results were also observed with the MC amphiphile, whereby it moderately enhanced solution-phase protein conformational stability and led to the formation of well-packed protein adlayers at adsorption saturation. MC is nonionic so there was appreciably less intermolecular repulsion between MC-doped BSA proteins, which enabled higher total adsorption uptake due to greater packing density within the BSA adlayer. While CA had notable effects even at a 10:1 CA:BSA molar ratio, the effects of MC on BSA adsorption properties were most clearly observed at a 100:1 MC:BSA molar ratio because MC has a lower binding affinity to BSA than that of CA. Of note, while MC-doped BSA displayed greatest adsorption uptake at adsorption saturation, it also displayed slightly more extensive desorption upon buffer wash, resulting in similar final adsorption uptake levels as defatted BSA. This is likely due to the increased conformational stability of MC-doped BSA resulting in smaller adsorption footprint and thus, a comparatively weaker protein-surface interaction that led to greater desorption upon buffer wash. However, this may potentially be overcome by adding a cross-linking agent such as glutaraldehyde at adsorption saturation in order to stabilize the well-packed adlayer formed by MC-doped BSA. Indeed, a similar strategy was previously done for BSA adsorption in low ionic strength conditions.⁶⁶ Together, these data support that amphiphile doping can affect BSA adsorption properties by two main effects, including enhancing conformational stability and modulating protein-protein interactions in the adsorbed state. Rational selection of the amphiphile type and amount used to stabilize BSA proteins can therefore enable control over BSA adlayer properties in terms of adsorption-related denaturation and packing density.

6.4 Conclusion

In this study, a wide range of biophysical and surface-sensitive measurement techniques were employed to characterize the effects of CA, MC, and ME amphiphiles on BSA

conformational stability and adsorption behavior on silica surfaces. While each amphiphile had an 8-carbon long, saturated hydrocarbon chain, marked differences in the binding affinity of each amphiphile to BSA proteins were observed, which indicates that the amphiphile headgroup also plays an important role in binding interactions. Both CA and MC demonstrated binding to BSA proteins and improved solution-phase conformational stability along with reducing surface-induced denaturation in the adsorbed state. Due to difference in binding affinity, CA was generally effective at both 10:1 and 100:1 amphiphile:BSA molar ratios, while MC was more effective at the 100:1 molar ratio. Notably, the anionic character of CA fatty acids led to reduced packing densities within CA-doped BSA protein adlayers, whereas the nonionic character of MC monoglycerides enabled higher packing densities within MC-doped BSA protein adlayers. These findings also agree well with the temperature-dependent protein aggregation data, indicating that CA modulates both conformational and colloidal stabilities whereas MC mainly influences conformational stability. While CA is typically used to dope BSA proteins as part of purification processes, these findings point to the potential significance of doping BSA proteins with MC in order to create purified BSA proteins with improved properties for BSA adlayer-based surface passivation applications.

References

1. Gray, J. J., The interaction of proteins with solid surfaces. *Curr. Opin. Struct. Biol.* **2004**, *14* (1), 110-115.
2. Meyers, S. R.; Grinstaff, M. W., Biocompatible and bioactive surface modifications for prolonged in vivo efficacy. *Chem. Rev.* **2011**, *112* (3), 1615-1632.
3. Bhakta, S. A.; Evans, E.; Benavidez, T. E.; Garcia, C. D., Protein adsorption onto nanomaterials for the development of biosensors and analytical devices: A review. *Anal. Chim. Acta* **2015**, *872*, 7-25.
4. Pinholt, C.; Hartvig, R. A.; Medlicott, N. J.; Jorgensen, L., The importance of interfaces in protein drug delivery – why is protein adsorption of interest in pharmaceutical formulations? *Expert Opin. Drug Delivery* **2011**, *8* (7), 949-964.

5. Schöttler, S.; Becker, G.; Winzen, S.; Steinbach, T.; Mohr, K.; Landfester, K.; Mailänder, V.; Wurm, F. R., Protein adsorption is required for stealth effect of poly (ethylene glycol)-and poly (phosphoester)-coated nanocarriers. *Nat. Nanotechnol.* **2016**, *11* (4), 372.
6. Rabe, M.; Verdes, D.; Seeger, S., Understanding protein adsorption phenomena at solid surfaces. *Adv. Colloid Interface Sci.* **2011**, *162* (1-2), 87-106.
7. Tsapikouni, T. S.; Missirlis, Y. F., Protein–material interactions: from micro-to-nano scale. *Mater. Sci. Eng., B* **2008**, *152* (1-3), 2-7.
8. Yano, Y. F., Kinetics of protein unfolding at interfaces. *J. Phys.: Condens. Matter* **2012**, *24* (50), 503101.
9. Ariga, K.; Ji, Q.; Hill, J. P.; Bando, Y.; Aono, M., Forming nanomaterials as layered functional structures toward materials nanoarchitectonics. *NPG Asia Mater.* **2012**, *4* (5), e17.
10. Ariga, K.; Ji, Q.; Nakanishi, W.; Hill, J. P.; Aono, M., Nanoarchitectonics: a new materials horizon for nanotechnology. *Mater. Horiz.* **2015**, *2* (4), 406-413.
11. Karlsson, M.; Ekeröth, J.; Elwing, H.; Carlsson, U., Reduction of irreversible protein adsorption on solid surfaces by protein engineering for increased stability. *J. Biol. Chem.* **2005**, *280* (27), 25558-25564.
12. Park, J. H.; Sut, T. N.; Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Cho, N.-J., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19* (13), 8854-8865.
13. Park, J. H.; Jackman, J. A.; Ferhan, A. R.; Ma, G. J.; Yoon, B. K.; Cho, N.-J., Temperature-induced denaturation of BSA protein molecules for improved surface passivation coatings. *ACS Appl. Mater. Interfaces* **2018**, *10* (38), 32047-32057.
14. Peng, Q.; Zhang, S.; Yang, Q.; Zhang, T.; Wei, X.-Q.; Jiang, L.; Zhang, C.-L.; Chen, Q.-M.; Zhang, Z.-R.; Lin, Y.-F., Preformed albumin corona, a protective coating for nanoparticles based drug delivery system. *Biomaterials* **2013**, *34* (33), 8521-8530.

15. del Río, J. S.; Henry, O. Y.; Jolly, P.; Ingber, D. E., An antifouling coating that enables affinity-based electrochemical biosensing in complex biological fluids. *Nat. Nanotechnol.* **2019**, *14* (12), 1143-1149.
16. Tan, J. Y. B.; Yoon, B. K.; Ma, G. J.; Sut, T. N.; Cho, N.-J.; Jackman, J. A., Unraveling how ethanol-induced conformational changes affect BSA protein adsorption onto silica surfaces. *Langmuir* **2020**.
17. Ma, G. J.; Ferhan, A. R.; Sut, T. N.; Jackman, J. A.; Cho, N.-J., Understanding how natural sequence variation in serum albumin proteins affects conformational stability and protein adsorption. *Colloids Surf., B* **2020**, *194*, 111194.
18. Peters, T., Serum albumin. In *Adv. Protein Chem.*, Elsevier: 1985; Vol. 37, pp 161-245.
19. Kelley, D.; McClements, D., Interactions of bovine serum albumin with ionic surfactants in aqueous solutions. *Food Hydrocolloids* **2003**, *17* (1), 73-85.
20. Hamilton, J. A., NMR reveals molecular interactions and dynamics of fatty acid binding to albumin. *Biochim. Biophys. Acta, Gen. Subj.* **2013**, *1830* (12), 5418-5426.
21. Wasylewski, Z.; Kozik, A., Protein—non-ionic detergent interaction: interaction of bovine serum albumin with alkyl glucosides studied by equilibrium dialysis and infrared spectroscopy. *Eur. J. Biochem.* **1979**, *95* (1), 121-126.
22. Kragh-Hansen, U.; Watanabe, H.; Nakajou, K.; Iwao, Y.; Otagiri, M., Chain length-dependent binding of fatty acid anions to human serum albumin studied by site-directed mutagenesis. *J. Mol. Biol.* **2006**, *363* (3), 702-712.
23. Ojha, B.; Das, G., Role of hydrophobic and polar interactions for BSA–amphiphile composites. *Chem. Phys. Lipids* **2011**, *164* (2), 144-150.
24. Gelamo, E. L.; Tabak, M., Spectroscopic studies on the interaction of bovine (BSA) and human (HSA) serum albumins with ionic surfactants. *Spectrochim. Acta, Part A* **2000**, *56* (11), 2255-2271.
25. Nielsen, A. D.; Borch, K.; Westh, P., Thermochemistry of the specific binding of C12 surfactants to bovine serum albumin. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2000**, *1479* (1-2), 321-331.

26. Chakraborty, T.; Chakraborty, I.; Moulik, S. P.; Ghosh, S., Physicochemical and conformational studies on BSA– surfactant interaction in aqueous medium. *Langmuir* **2009**, 25 (5), 3062-3074.
27. Ghosh, S.; Dey, J., Binding of fatty acid amide amphiphiles to bovine serum albumin: role of amide hydrogen bonding. *J. Phys. Chem. B* **2015**, 119 (25), 7804-7815.
28. De, S.; Girigoswami, A.; Das, S., Fluorescence probing of albumin–surfactant interaction. *J. Colloid Interface Sci.* **2005**, 285 (2), 562-573.
29. Boyer, P. D.; Lum, F. G.; Ballou, G. A.; Luck, J. M.; Rice, R. G., The combination of fatty acids and related compounds with serum albumin I. stabilization against heat denaturation. *J. Biol. Chem.* **1946**, 162 (2), 181-198.
30. Boyer, P. D.; Ballou, G. A.; Luck, J. M., The combination of fatty acids and related compounds with serum albumin II. Stabilization against urea and guanidine denaturation. *J. Biol. Chem.* **1946**, 162 (2), 199-208.
31. Deep, S.; Ahluwalia, J. C., Interaction of bovine serum albumin with anionic surfactants. *Phys. Chem. Chem. Phys.* **2001**, 3 (20), 4583-4591.
32. Ma, G. J.; Ferhan, A. R.; Jackman, J. A.; Cho, N.-J., Conformational flexibility of fatty acid-free bovine serum albumin proteins enables superior antifouling coatings. *Commun. Mater.* **2020**, 1 (1), 45.
33. Savitzky, A.; Golay, M. J., Smoothing and differentiation of data by simplified least squares procedures. *Anal. Chem.* **1964**, 36 (8), 1627-1639.
34. Morrisett, J. D.; David, J. S.; Pownall, H. J.; Gotto Jr, A. M., Interaction of an apolipoprotein (apoLP-alanine) with phosphatidylcholine. *Biochemistry* **1973**, 12 (7), 1290-1299.
35. Cho, N.-J.; Frank, C. W.; Kasemo, B.; Höök, F., Quartz crystal microbalance with dissipation monitoring of supported lipid bilayers on various substrates. *Nat. Protoc.* **2010**, 5 (6), 1096.
36. Jackman, J. A.; Zhdanov, V. P.; Cho, N.-J., Nanoplasmonic biosensing for soft matter adsorption: kinetics of lipid vesicle attachment and shape deformation. *Langmuir* **2014**, 30 (31), 9494-9503.

37. Zan, G. H.; Jackman, J. A.; Kim, S. O.; Cho, N. J., Controlling lipid membrane architecture for tunable nanoplasmonic biosensing. *Small* **2014**, *10* (23), 4828-4832.
38. Murayama, K.; Tomida, M., Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy. *Biochemistry* **2004**, *43* (36), 11526-11532.
39. Kong, J.; Yu, S., Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim. Biophys. Sin.* **2007**, *39* (8), 549-559.
40. Barth, A., Infrared spectroscopy of proteins. *Biochim. Biophys. Acta, Bioenerg.* **2007**, *1767* (9), 1073-1101.
41. Srour, B.; Bruechert, S.; Andrade, S. L.; Hellwig, P., Secondary Structure Determination by Means of ATR-FTIR Spectroscopy. In *Membrane Protein Structure and Function Characterization*, Springer: 2017; pp 195-203.
42. Givens, B. E.; Xu, Z.; Fiegel, J.; Grassian, V. H., Bovine serum albumin adsorption on SiO₂ and TiO₂ nanoparticle surfaces at circumneutral and acidic pH: a tale of two nano-bio surface interactions. *J. Colloid Interface Sci.* **2017**, *493*, 334-341.
43. Thumser, A. E.; Buckland, A. G.; Wilton, D. C., Monoacylglycerol binding to human serum albumin: evidence that monooleoylglycerol binds at the dansylsarcosine site. *J. Lipid Res.* **1998**, *39* (5), 1033-1038.
44. Duff, S. M.; Kalambur, S.; Boyle-Roden, E., Serum albumin binds β - and α -monoolein in vitro. *J. Nutr.* **2001**, *131* (3), 774-778.
45. Arvidsson, E.; Belfrage, P., Monoglyceride-protein interaction. The binding of monoolein to native human serum albumin. *Acta Chem. Scand.* **1969**, *23* (1), 232-236.
46. Spector, A. A.; John, K.; Fletcher, J. E., Binding of long-chain fatty acids to bovine serum albumin. *J. Lipid Res.* **1969**, *10* (1), 56-67.
47. Morrisett, J. D.; Pownall, H.; Gotto, A., Bovine serum albumin. Study of the fatty acid and steroid binding sites using spin-labeled lipids. *J. Biol. Chem.* **1975**, *250* (7), 2487-2494.
48. Zhu, T.-T.; Zhang, Y.; Luo, X.-A.; Wang, S.-Z.; Jia, M.-Q.; Chen, Z.-X., Difference in binding of long- and medium-chain fatty acids with serum albumin:

- The role of macromolecular crowding effect. *J. Agric. Food Chem.* **2018**, 66 (5), 1242-1250.
49. Iovescu, A.; Băran, A.; Stîngă, G.; Cantemir-Leontieș, A. R.; Maxim, M. E.; Anghel, D. F., A combined binding mechanism of nonionic ethoxylated surfactants to bovine serum albumin revealed by fluorescence and circular dichroism. *J. Photochem. Photobiol., B* **2015**, 153, 198-205.
50. Stern, O.; Volmer, M., Über die abklingzeit der fluoreszenz. *phys. Z* **1919**, 20, 183-188.
51. Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*. Springer Science & Business Media: 2013.
52. Min, J.; Meng-Xia, X.; Dong, Z.; Yuan, L.; Xiao-Yu, L.; Xing, C., Spectroscopic studies on the interaction of cinnamic acid and its hydroxyl derivatives with human serum albumin. *J. Mol. Struct.* **2004**, 692 (1-3), 71-80.
53. Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Park, J. H.; Zhdanov, V. P.; Cho, N.-J., Indirect nanoplasmonic sensing platform for monitoring temperature-dependent protein adsorption. *Anal. Chem.* **2017**, 89 (23), 12976-12983.
54. Peters Jr, T., Serum albumin. In *Adv. Protein Chem.*, Elsevier: 1985; Vol. 37, pp 161-245.
55. Curry, S., Plasma albumin as a fatty acid carrier. *Adv. Mol. Cell Biol.* **2003**, 33, 29-46.
56. Yohannes, G.; Wiedmer, S. K.; Elomaa, M.; Jussila, M.; Aseyev, V.; Riekkola, M.-L., Thermal aggregation of bovine serum albumin studied by asymmetrical flow field-flow fractionation. *Anal. Chim. Acta* **2010**, 675 (2), 191-198.
57. Nnyigide, O. S.; Lee, S.-G.; Hyun, K., In silico characterization of the binding modes of surfactants with bovine serum albumin. *Sci. Rep.* **2019**, 9 (1), 1-16.
58. Cuddy, M. F.; Poda, A. R.; Brantley, L. N., Determination of isoelectric points and the role of pH for common quartz crystal microbalance sensors. *ACS Appl. Mater. Interfaces* **2013**, 5 (9), 3514-3518.
59. Jachimska, B.; Pajor, A., Physico-chemical characterization of bovine serum albumin in solution and as deposited on surfaces. *Bioelectrochemistry* **2012**, 87, 138-146.

60. Kubiak-Ossowska, K.; Tokarczyk, K.; Jachimska, B.; Mulheran, P. A., Bovine serum albumin adsorption at a silica surface explored by simulation and experiment. *J. Phys. Chem. B* **2017**, *121* (16), 3975-3986.
61. Peeters, M.; Jiménez-Monroy, K.; Libert, C.; Eurlings, Y.; Cuypers, W.; Wackers, G.; Duchateau, S.; Robaey, P.; Nesládek, M.; Van Grinsven, B., Real-time monitoring of aptamer functionalization and detection of Ara H1 by electrochemical impedance spectroscopy and dissipation-mode quartz crystal microbalance. *J. Biosens. Bioelectron.* **2014**, *5* (3).
62. Neupane, S.; De Smet, Y.; Renner, F. U.; Losada-Pérez, P., Quartz crystal microbalance with dissipation monitoring: A versatile tool to monitor phase transitions in biomimetic membranes. *Front. Mater.* **2018**, *5*, 46.
63. Jackman, J. A.; Ferhan, A. R.; Cho, N.-J., Nanoplasmonic sensors for biointerfacial science. *Chem. Soc. Rev.* **2017**, *46* (12), 3615-3660.
64. Slawson, V.; Mead, J. F., Stability of unsaturated methyl esters of fatty acids on surfaces. *J. Lipid Res.* **1972**, *13* (1), 143-146.
65. Ferhan, A. R.; Jackman, J. A.; Sut, T. N.; Cho, N.-J., Quantitative comparison of protein adsorption and conformational changes on dielectric-coated nanoplasmonic sensing arrays. *Sensors* **2018**, *18* (4).
66. Park, J.; Sut, T.; Jackman, J.; Ferhan, A.; Yoon, B.; Cho, N., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19*.

Chapter 7*

Evaluating the Surface Passivation Performance of BSA Proteins for Antifouling Applications

Bovine serum albumin (BSA) protein is widely used to fabricate antifouling coatings for nanobiotechnology applications. Numerous BSA protein options are commercially available, however, there has been no guidance on which ones are preferable for antifouling coatings. While fatty acid-free BSA proteins exhibit greater conformational flexibility and less charge repulsion which allow them to form more tightly packed coatings, a direct correlation between their adsorption behavior and their surface passivation performance has yet to be established. Herein, the surface passivation properties of fatty acid-containing and fatty acid-free BSA proteins on flat and nanoparticle surfaces were evaluated using a combination of biophysical and biological assays, such as quartz crystal microbalance-dissipation, Western blot and enzyme-linked immunosorbent assay. Fatty acid-free BSA proteins were found to have superior antifouling applications performance on both flat and nanoparticle surfaces.

*Results in this chapter are published substantially as G.J Ma, A.R. Ferhan, J.A. Jackman, N.-J. Cho. Conformational flexibility of fatty acid-free bovine serum albumin proteins enables superior antifouling coatings. *Communications Materials* **2020**, *1*, 45. DOI: 10.1038/s43246-020-0047-9 (Under the terms of the CC BY 4.0 license, no permission is required to reuse this article).

Results in this chapter were also submitted as a preprint as G.J Ma, A.R. Ferhan, J.A. Jackman, N.-J. Cho. Quantitative assessment of bovine serum albumin proteins for blocking applications. *bioRxiv* **2019**, 869677. DOI: 10.1101/869677. Reprinted with permission. Copyright 2019, the author.

7.1 Introduction

Ultrathin film coatings are widely used to coat flat, nanostructured, and nanoparticle surfaces in order to confer advantageous properties, such as antifouling or stealth immune profile features.^{1, 2} Many types of coating materials, ranging from natural proteins to synthetic nanomaterials such as polymers with finely tuned architectures, have been explored³⁻⁵ and one of the most popular options for antifouling applications is bovine serum albumin (BSA) protein.⁶ BSA has advantageous properties such as natural abundance, simple coating procedure, low cost, well-established purification methods, and broad availability. Indeed, BSA coatings – often in the form of a single protein monolayer – have long been used in numerous biological assays, including enzyme-linked immunosorbent assays (ELISA)⁷, blots,⁸ immunohistochemistry,⁹ and polymerase chain reaction,¹⁰ in order to minimize nonspecific binding events and thus enhance assay performance. In recent years, BSA has also found extensive usage as an antifouling coating material in biosensing¹¹ and nanomedicine¹² applications. For example, the simplicity of forming BSA-based coatings has inspired the development of advanced antifouling coatings that enable high-performance electrochemical biosensing in complex biological fluids.¹³ In addition, BSA coatings are used to improve nanoparticle stability and biocompatibility,^{14, 15} and to passivate surfaces to inhibit nonspecific adsorption of bioanalytes.⁴

An often-overlooked aspect of BSA is that there are many commercially available versions which differ in how they were isolated from bovine plasma,¹⁶ even for those from the same company.¹⁷ Guidelines to predict which BSA version would be most suitable for antifouling applications are lacking despite broad interest in developing a nanodescriptor index to predict protein adsorption at nano-bio interfaces.¹⁸ Indeed, the selection of BSA protein type has been largely a matter of trial-and-error and laboratory precedent, and there is oftentimes limited reporting on which particular BSA was used in scientific publications. To address this knowledge gap, a systematic quantitative assessment of differently processed BSA proteins was conducted and reported in Chapter 5 of this thesis. It was revealed that fatty acid-free BSA possessed greater conformational flexibility and less intermolecular charge repulsion which led to the formation of more tightly packed films.

While such fundamental studies are important to establish trends in adsorption behavior, further studies are required to unravel how such trends relate to antifouling application performance.

Herein, BSA-based antifouling coatings were fabricated from BSA proteins obtained through different purification methods, and their antifouling properties on flat and nanoparticle surfaces were scrutinized using both biophysical and biological assays such as quartz crystal microbalance-dissipation (QCM-D), Western blot and enzyme-linked immunosorbent assay (ELISA) and key mechanistic factors that drive the formation of high-performance ultrathin film coatings with superior antifouling properties were discussed.

7.2 Materials and Methods

7.2.1 Reagents

Six types of BSA used in this study were selected based on three different fractionation methods without or with a fatty acid removal step, and were procured from Sigma-Aldrich (St. Louis, MO, USA). Lyophilized powders of fatty acid-containing BSA proteins purified by cold ethanol fractionation, heat-shock fractionation, or cold ethanol followed by heat-shock fractionation were selected and have catalog nos. A2153, A3059, and A7638, respectively. These three fatty BSA proteins were labeled BSA 1, 2, 3, respectively. The corresponding fatty acid-free versions were also obtained and have catalog nos. A6003, A7030 and A0281, respectively. These three defatted BSA proteins were labeled BSA 4, 5, 6, respectively. Sodium dodecyl sulfate (SDS, L4390), and sodium chloride (NaCl, 746398) were purchased from Sigma-Aldrich (USA). Tris(hydroxymethyl)aminomethane (Tris, 0497) was purchased from Amresco (USA). Ethanol (95%) was purchased from Aik Moh (Singapore), and hydrochloric acid (100317) was purchased from Merck (USA).

In addition, fetal bovine serum (FBS, catalog no. SV30160.03, lot no. RC35960) was purchased from HyClone Laboratories (Logan, UT, USA) and stored at -20 °C until

experiment. Normal human serum (catalog no. NHS, lot no. 38) was obtained from Complement Technology (Tyler, TX, USA) and stored at -80 °C until experiment. 30% Acrylamide/Bis Solution 29:1 (catalog no. 1610156), ammonium persulfate (catalog no. 1610700), tetramethylethylenediamine (TEMED, catalog no. 1610800), 4× Laemmli sample buffer (catalog no. 1610747), 2-mercaptoethanol (catalog no. 1610710), 10× Tris buffered saline (TBS, catalog no. 1706435), 10× Tris/glycine buffer (catalog no. 1610734), 10× Tris/glycine/SDS buffer (catalog no. 1610732), Tween 20 (catalog no. 1706531), nitrocellulose membranes (catalog no. 1620112), Precision Plus Protein Standards (catalog no. 1610375), Clarity Max Western enhanced chemiluminescent (ECL) substrate (catalog no. 1705062), and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (catalog no. 1721011, batch no. 64109318) were all purchased from Bio-Rad Laboratories (Hercules, CA, USA). Complement C3b monoclonal antibody (catalog no. MA1-40155, lot no. SH2428445) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Methanol (catalog no. M/4000/17) was obtained from Fisher Scientific (Loughborough, UK) and 100-nm diameter silica nanoparticles (catalog no. SISN100) were obtained from nanoComposix (San Diego, CA, USA).

7.2.2 Sample Preparation

An aqueous buffer solution of 10 mM Tris, 150 mM NaCl, and pH 7.5 was prepared with Milli-Q-treated water (resistivity of >18.2 MΩ.cm at 25 °C) and filtered through a 0.2 μm polyethersulfone (PES) membrane filter (Thermo Fisher Scientific, 595-4520). BSA solutions were prepared by dissolving lyophilized BSA powder in the buffer solution and then filtering the BSA suspension through a syringe filter with 0.2-μm diameter pores (PN-4612; Pall Corporation, USA). The molar concentrations of BSA protein in aqueous buffer solution was determined by ultraviolet (UV) light absorbance measurements at 280 nm and the molar extinction coefficient value of BSA was taken to be 43824 M⁻¹ cm⁻¹. The Western blot running buffer was prepared by diluting 10× Tris/glycine/SDS buffer with water to 1× concentration, and the transfer buffer was prepared by diluting the 10× Tris/glycine buffer with water and methanol to 1× concentration and 20% (vol/vol) of methanol. The BSA-

containing surface passivation (“blocking”) solutions for Western blot experiments were prepared by first diluting 10× TBS with water, followed by the addition of Tween 20 to make a 1× TBS solution with 0.1% (vol/vol) Tween 20 (TBST). BSA was then dissolved in TBST to make a 3% (wt/vol) BSA blocking solution.

7.2.3 Quartz Crystal Microbalance-Dissipation

QCM-D measurements were conducted using a QSense E4 instrument (Biolin Scientific AB, Stockholm, Sweden) and a silica-coated AT-cut quartz crystal sensor chip with a fundamental frequency of 5 MHz (QSX 303, Biolin Scientific). The temperature was set to 25 °C for all experiments, and the measurement operation was controlled by the QSoft 401 (v2.5.13.664) (Biolin Scientific) software package. Prior to each experiment, the sensor chips were sequentially rinsed with 1% (wt/vol) aqueous SDS solution, water, and ethanol, and then dried under a gentle stream of nitrogen gas, followed by treatment with oxygen plasma (PDC-002, Harrick Plasma, Ithaca, NY, USA) for 3 min. A peristaltic pump was used to inject liquid samples into the measurement chamber at a volumetric flow rate of 100 $\mu\text{L min}^{-1}$. To determine the passivation efficiency of BSA coatings against serum biofouling, 100 μM BSA solution was first added to a bare silica surface under continuous flow for 60 min, followed by a 40 min buffer washing step. Undiluted fetal bovine serum (FBS) was then introduced under continuous flow for 80 min, followed by a final buffer washing step for 30 min. The passivation efficiency percentage was calculated by comparing the absolute difference between the frequency shift due to BSA adsorption alone (after first buffer washing step) and the subsequent frequency shift due to FBS biofouling (after second buffer washing step), which is denoted as $\Delta F_{\text{FBS-BSA}}$. A control experiment without BSA coating was also conducted in order to measure the absolute frequency shift due to FBS biofouling alone (after buffer washing step), which is denoted as $\Delta F_{\text{Control}}$. For each BSA coating, the passivation efficiency percentage was calculated by the following equation:

$$\text{Passivation efficiency (\%)} = \left(1 - \frac{\Delta F_{\text{FBS-BSA}}}{\Delta F_{\text{Control}}}\right) \times 100\% \quad (\text{Eq 7.1})$$

7.2.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot

10% polyacrylamide resolving gels at pH 8.8 and 5% stacking gels at pH 6.8 with fifteen sample loading wells per gel were prepared before experiments. For sample preparation, normal human serum (NHS) was thawed and then immediately diluted to 1% (vol/vol) in Tris buffer, followed by incubation at 37 °C for 30 min. A 180 μ L aliquot of diluted NHS was then mixed with 54 μ L of 4 $\times$ Laemmli buffer and 6 μ L of 2-mercaptoethanol. The sample mixture was then heated to 95 °C for 5 min for complete denaturation of serum proteins, before cooling back to room temperature. A 10 μ L aliquot of protein standards was loaded into the first lane of the polyacrylamide gel and the next two lanes were each loaded with 10 μ L of the sample mixture; this sequence was repeated five times in total to fill all lanes within the gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then conducted at 50 V for 15 min, followed by 100 V for 90 min in running buffer.

The protein bands in the gel were then transferred to a nitrocellulose membrane at 300 mA for 2 h in transfer buffer, and ice packs were used to keep the transfer buffer cool during the transfer process. After the transfer step, the membranes were cut into strips, each one containing the three lanes of protein standards (lane 1) and serum samples (lanes 2-3). Surface passivation (“blocking”) solutions were prepared using fatted or defatted BSA [3% (wt/vol) BSA in TBST; BSA 1 or 5] and each strip was incubated with 5 mL of the appropriate BSA blocking solution at room temperature for 1 h. The membranes were then incubated with complement C3b monoclonal antibody as the primary antibody (1:500 dilution in blocking solution) overnight. Each membrane was then washed with TBST for 10 min. After three washing steps, the membranes were incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (1:2000 dilution in blocking solution) for 1 h, followed by four subsequent washing steps with TBST.

The membranes were then incubated with Clarity Max Western ECL substrate for 5 min in order to enhance chemiluminescence properties. All incubation and washing steps were conducted on a rocking platform. The protein bands were subsequently imaged using an

Amersham Imager 600 instrument and its accompanying software (v0.9.8) (GE Healthcare, Chicago, IL, USA) with a 1 s exposure time. The Fiji/ImageJ software package¹⁹ was then used to quantify the intensity of the noise bands that were located near the 250, 75, and 50 kDa molecular weight markers. The digital image of the blots was first set to 8-bit greyscale, and then an intensity profile from each selected lane was plotted using the Analyze Gels function. The peaks from non-specific bands of interest were identified and a baseline was created by drawing a straight line between the two minima points on either side of each peak of interest. The Wand tool was then used to determine the area bounded by the peak and the baseline. These values were defined as the intensity values for each selected non-specific band.

7.2.5 Silica Nanoparticle Preparation and Enzyme-Linked Immunosorbent Assay for Complement Activation

The MicroVue SC5b-9 Plus Enzyme Immunoassay kit (catalog no. A020; Quidel, San Diego, CA, USA) was used for enzyme-linked immunosorbent assay (ELISA) experiments. BSA-coated silica nanoparticle samples were prepared by mixing equal volumes of the appropriate 1 mg mL⁻¹ BSA solution with 2 mg mL⁻¹ silica nanoparticles in Tris buffer. The mixtures were then incubated at 37 °C for 2 h, followed by centrifugation at 16,000×g for 30 min. The supernatants were removed, and the nanoparticles were resuspended with fresh Tris buffer. Another round of centrifugation and resuspension was conducted to yield 1 mg mL⁻¹ BSA-coated silica nanoparticle samples. A 10 µL aliquot of each BSA-coated silica nanoparticle sample was then mixed with a 40 µL aliquot of freshly thawed normal human serum (NHS). A 50 µL aliquot of NHS by itself was used as the negative control (lowest level of complement activation) and 10 µL of 1 mg mL⁻¹ uncoated silica nanoparticles plus 40 µL NHS was used as the positive control (highest level of complement activation). All test samples were incubated at 37 °C for 30 min.

The assay was then performed using the 96-well plates that were provided in the ELISA kit according to the manufacturer's instructions. All serum samples, including controls, were diluted 40-times by using the provided sample diluent solution before addition to the

wells. Standard solutions were added without dilution. The absorbance values from each well were determined by an Infinite Pro 200 plate reader (Tecan, Männedorf, Switzerland). Standard curves were calculated by correlating the known concentrations of the standard solutions to the respective absorbance values. The SC5b-9 protein concentration in each sample was then computed. The degree of protection afforded by each BSA coating was calculated by treating the SC5b-9 protein concentrations from the negative and positive control samples as 100% and 0% protection, respectively.

7.2.6 Statistical Analysis

Statistical analyses were conducted using the GraphPad Prism (v8.0.1) software package from GraphPad Software (La Jolla, CA, USA). One-way analysis of variance (ANOVA) with the appropriate multiple comparisons test and the unpaired t-test were used to compute the statistical significance of experimental data as appropriate. All statistical analysis involved two-tailed tests. Unpaired t-tests results are reported as *P* values while multiple comparisons test results are reported as multiplicity-adjusted *P* values. $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$ indicate the levels of statistical significance.

7.3 Results and Discussion

7.3.1 Inhibiting Serum Biofouling

Since numerous applications of BSA protein molecules are linked to their role as surface coatings, the application performance of the different BSA types were first tested for their performance in inhibiting serum biofouling by FBS on a silica surface measured by QCM-D. Indeed, the QCM-D has previously been employed to monitor the frequency changes (ΔF) associated with the mass of the adsorbed fouling species.²⁰⁻²² This passivation strategy employed herein is illustrated in **Figure 7.1**. First, 100 μM of BSA solution was introduced into the sensor chamber for 60 min followed by a buffer wash which forms an ultrathin BSA coating on the silica surface. Undiluted FBS is then introduced for 80 min followed by a washing step. The obtained ΔF is compared to that obtained from the introduction of

FBS on an uncoated silica surface in order to calculate the passivation efficiency. Examples of the QCM-D experimental runs conducted for BSA proteins 1 and 5 are shown in **Figure 7.2**.

Figure 7.1 Schematic illustration of serum biofouling assay. Pristine and BSA-coated silica surfaces were incubated in 100% fetal bovine serum (FBS) followed by a buffer washing step, and the amount of serum biofouling was measured. The passivation efficiency of the BSA coatings was calculated relative to the uncoated surface.

Figure 7.2 Quantification of BSA surface passivation performance against serum biofouling based on QCM-D measurements. Time-resolved QCM-D frequency shifts (ΔF) evaluating the passivation efficiency of BSA protein coatings against fetal bovine serum (FBS) fouling of silica surfaces for **a**, BSA protein 1 and **b**, BSA protein 5. The protocol steps involved (1) 100 μM BSA addition, (2) buffer washing step, (3) addition of undiluted FBS, and (4) a buffer washing step. Bare surface control refers to a control experiment whereby FBS was added to an uncoated silica surface (no BSA coating). The difference in ΔF values due to BSA adsorption alone (post-washing) and after FBS incubation (post-washing) was computed ($\Delta F_{\text{FBS-BSA}}$, indicated by dashed lines) and the passivation efficiency percentage was calculated from this value compared to the equivalent value computed in the control experiment without BSA coating.

Based on this, defatted BSA proteins was found to possess superior passivation efficiency over fatty BSA proteins (**Figure 7.3**). Defatted BSA 5 and 6 exhibited a passivation efficiency of around 90%. By contrast, fatty BSA 1 and 3 demonstrated less than 40% passivation efficiency. In addition, it was found that BSA 4, the lowest performing of the defatted BSA proteins, outperformed BSA 2, the highest performing of the fatty BSA proteins based on their passivation efficiencies of around 66% and 56%, respectively. These findings are consistent with greater adsorption uptake and higher packing density of

adsorbed, defatted BSA protein molecules and support that these adlayer properties contribute to defatted BSA proteins' superior antifouling performance.

Figure 7.3 Passivation efficiencies of BSA coatings to inhibit serum biofouling ($n=3$ biological replicates). Data are reported as mean $\pm$ standard error of the mean (s.e.m.) (one-way ANOVA with Tukey's multiple comparisons test). P values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method). Dots represent individual data points.

7.3.2 Inhibiting Nonspecific Adsorption in Western Blots

While the superior antifouling capabilities of defatted BSA have been demonstrated on silica, which is a popular hydrophilic surface used in various biological and biotechnology assays, there is also interest in assessing their performance capabilities on a hydrophobic surface in application-relevant contexts. Therefore, Western blot experiments were

conducted, which involved the use of hydrophobic nitrocellulose membrane surfaces, to evaluate the surface passivation performance of fatted vs defatted BSA proteins.

In Western blot experiments, the blocking step prior to antibody incubation is a critical part of the protocol to minimize nonspecific adsorption of primary and/or secondary antibodies onto the blot membrane, which can lead to false signals and poor blot resolution. To this end, BSA is the gold-standard blocking reagent for surface passivation although there is no discussion in the scientific literature about the blocking performance of fatted versus defatted BSA proteins.

Therefore, Western blot experiments were conducted and normal human serum (NHS) was used as the protein sample against a C3b monoclonal primary antibody, which produces two specific bands corresponding to C3b (104 kDa) and iC3b (40 kDa) proteins. While these two bands are clearly visible in all Western blots, a few additional nonspecific bands near the 250, 75, and 50 kDa molecular weight markers were also identified. The experimental results demonstrated that defatted BSA significantly decreased the intensity of these nonspecific bands, as compared to fatted BSA (**Figure 7.4**).

Figure 7.4 Blocking performance of BSA protein reagents in Western blot experiments. **a**, Schematic illustration of the BSA blocking step in a Western blot experiment. **b**, Three Western blot lanes consisted of a colored molecular weight marker (lane 1) and normal human serum (NHS) samples (lanes 2-3). Each membrane was individually blocked with BSA 1 (fatted) or BSA 5 (defatted) prior to C3b antibody incubation. Red arrows indicate the position of nonspecific bands located near the 250, 75, and 50 kDa molecular weight markers. Black arrows indicate the position of specific bands corresponding to C3b (104 kDa) and iC3b (40 kDa). The objective of the blocking step is to minimize signal noise coming from nonspecific bands in order to focus analysis on specific bands.

This observation was confirmed by quantitative evaluation of the chemiluminescence intensity values of the nonspecific bands, as determined by Fiji/ImageJ software.¹⁹ The method used to quantify the band intensities by Fiji/ImageJ software is illustrated in **Figure 7.5** and the quantitative comparison of the nonspecific band intensities are presented in **Figure 7.6**. The results indicate that the membranes “blocked” by defatted BSA proteins produced lower intensity nonspecific bands, supporting that the higher packing density of adsorbed defatted BSA proteins more effectively prevented nonspecific interactions

between the primary and/or secondary antibodies with the membrane surface. Taken together with the serum biofouling results, these data support that defatted BSA proteins exhibit superior surface passivation performance on hydrophilic and hydrophobic surfaces.

Figure 7.5 Quantification of Western blot band intensities. **a**, Greyscale digital images of Western blots with the three lanes selected for the extraction of lane intensity profiles. Lane 1 is from molecular weight markers while lanes 2 and 3 are from NHS samples. Left and right panels show membranes blocked by BSA 1 and BSA 5 proteins, respectively. **b**, Intensity profiles from lanes 1, 2, and 3 are shown in the top, middle, and bottom rows, respectively. Bands in the digital image appear as peaks in the intensity profile. Baselines were established by connecting the two minima on both sides of a selected peak. The intensity values were obtained based on the area under the curves bounded by the established baseline. Left and right panels show membranes blocked by BSA proteins 1 and 5, respectively. Arrows indicate the position of nonspecific bands located near the 250, 75, and 50 kDa molecular weight markers.

Figure 7.6 Comparison of nonspecific band intensities resulting from the use of fatted and defatted BSA proteins in Western blot experiments. Intensity values of the nonspecific bands that are located near the 250, 75, and 50 kDa molecular weight markers from membranes that were blocked with a blocking solution that included BSA 1 (fatted) or BSA 5 (defatted) ($n=8$ biological replicates, unpaired t-test). Data were obtained from Fiji/ImageJ software program and are reported as mean $\pm$ standard error of the mean (s.e.m.). Dots represent individual data points and intensities are in arbitrary units.

7.3.3 Inhibiting Nanoparticle-Induced Complement Activation

Next, the blocking performance of BSA coatings were tested on silica nanoparticles to inhibit nanoparticle-induced complement activation, which is an acute immune response that can occur in human serum (**Figure 7.7**).^{23, 24} Silica nanoparticles were first coated with the different BSA proteins and then incubated in normal human serum (NHS) in order to trigger complement activation.

Figure 7.7 Schematic illustration of nanoparticle-triggered complement activation assay. Pristine and BSA-coated silica nanoparticles were incubated in 100% normal human serum (NHS), followed by ELISA measurements to determine the extent of nanoparticle-triggered complement activation. The degree of protection for BSA-coated nanoparticles was calculated relative to uncoated nanoparticles.

Using an enzyme-linked immunosorbent assay (ELISA), the degree of protection which BSA coatings conferred against nanoparticle-induced complement activation was measured, as judged by the amount of SC5b-9 protein biomarker in solution (**Figure 7.8**). Greater SC5b-9 protein concentrations indicate more extensive complement activation and conversely, nanoparticles with more protective coatings would trigger lower SC5b-9 protein concentration levels compared to bare nanoparticles. A positive control sample (highest complement activation) was also included whereby a bare, uncoated silica

nanoparticle was incubated in NHS and which yielded the highest levels of SC5b-9. These results confirm that silica nanoparticles do induce complement activation and supports that the BSA coatings on silica nanoparticles confer protection against complement activation, but to various degrees.

Figure 7.8 Reduction in silica nanoparticle-induced complement activation due to BSA coatings, as quantified by normalized SC5b-9 levels. The level of complement activation for each sample was determined by measuring SC5b-9 concentrations by enzyme-linked immunosorbent assay (ELISA) and normalized against SC5b-9 levels in NHS without nanoparticles [$n=5$ biological replicates, one-way ANOVA with Dunnett's multiple comparisons test (versus uncoated nanoparticles)]. Data are reported as mean $\pm$ s.e.m. and dots represent individual data points.

In order to more directly quantify the extent of protection conferred by the different BSA coatings, the fractional protection against nanoparticle-induced complement activation was determined by comparing the SC5b-9 levels induced by the BSA-coated silica nanoparticles with the positive control as well as a negative control which consists of measuring the SC5b-9 levels of NHS alone (**Figure 7.9**). Experimentally, it was

determined that defatted BSA coatings inhibited more than 60% of SC5b-9 generation, whereas the fatty BSA coatings exhibited 40% or less protection efficiency. This finding supports that defatted BSA coatings are superior to fatty BSA coatings to protect against nanoparticle-induced complement activation. Thus, across the surface- and nanoparticle-based assays, defatted BSA proteins exhibited better functional performance than fatty BSA proteins.

Figure 7.9 Protection efficiencies of BSA coatings to inhibit nanoparticle-induced complement activation ($n=5$ biological replicates). Data are reported as mean $\pm$ standard error of the mean (s.e.m.) (one-way ANOVA with Tukey's multiple comparisons test). P values are reported for defatted BSA proteins (versus fatty BSA proteins from the same fractionation method). Dots represent individual data points.

7.4 Conclusion

Taken together, these findings support that it is preferable to use fatty acid-free BSA proteins to form antifouling coatings on flat and nanostructured surfaces. In general, fatty

acid-free BSA proteins outperformed fatty acid-stabilized BSA proteins regardless of the fractionation route used in the purification stage. Mechanistically, defatted BSA proteins are superior because they have lower solution-phase conformational stability and reduced charge repulsion in the adsorbed state, which translates into greater surface-induced denaturation and adsorption uptake, resulting in tighter adlayer packing that yields superior passivation coatings. Looking forward, these findings will be relevant for studying other fatty acid-binding proteins such as human serum albumin across various applications, including protein corona formation, and can also be further explored in the context of nanoparticle properties (*i.e.*, surface chemistry, shape, and size)²⁵⁻²⁸. It is anticipated that the rational selection of BSA protein options without or with fatty acid stabilizers can enable the fabrication of superior antifouling coatings for a wide range of applications, which can be readily implemented by researchers across different fields of materials science and nanobiotechnology.

References

1. Hucknall, A.; Rangarajan, S.; Chilkoti, A., In pursuit of zero: polymer brushes that resist the adsorption of proteins. *Adv. Mater.* **2009**, *21* (23), 2441-2446.
2. Schöttler, S.; Becker, G.; Winzen, S.; Steinbach, T.; Mohr, K.; Landfester, K.; Mailänder, V.; Wurm, F. R., Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers. *Nat. Nanotechnol.* **2016**, *11* (4), 372.
3. Sapsford, K. E.; Algar, W. R.; Berti, L.; Gemmill, K. B.; Casey, B. J.; Oh, E.; Stewart, M. H.; Medintz, I. L., Functionalizing nanoparticles with biological molecules: developing chemistries that facilitate nanotechnology. *Chem. Rev.* **2013**, *113* (3), 1904-2074.
4. Wei, Q.; Becherer, T.; Angioletti-Uberti, S.; Dzubiella, J.; Wischke, C.; Neffe, A. T.; Lendlein, A.; Ballauff, M.; Haag, R., Protein interactions with polymer coatings and biomaterials. *Angew. Chem. Int. Edit.* **2014**, *53* (31), 8004-8031.

5. Luo, N.; Weber, J. K.; Wang, S.; Luan, B.; Yue, H.; Xi, X.; Du, J.; Yang, Z.; Wei, W.; Zhou, R., PEGylated graphene oxide elicits strong immunological responses despite surface passivation. *Nat. Commun.* **2017**, 8 (1), 1-10.
6. Sweryda-Krawiec, B.; Devaraj, H.; Jacob, G.; Hickman, J. J., A new interpretation of serum albumin surface passivation. *Langmuir* **2004**, 20 (6), 2054-2056.
7. Steinitz, M., Quantitation of the blocking effect of tween 20 and bovine serum albumin in ELISA microwells. *Anal. Biochem.* **2000**, 282 (2), 232-238.
8. Mahmood, T.; Yang, P.-C., Western blot: technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* **2012**, 4 (9), 429.
9. Buchwalow, I. B.; Böcker, W., Background Staining, Autofluorescence and Blocking Steps. In *Immunohistochemistry: Basics and Methods*, Springer Berlin Heidelberg: Berlin, Heidelberg, 2010; pp 41-46.
10. Besecker, J.; Cornell, K. A.; Hampikian, G., Dynamic passivation with BSA overcomes LTCC mediated inhibition of PCR. *Sens. Actuators, B* **2013**, 176, 118-123.
11. Convert, L.; Chabot, V.; Zermatten, P.-J.; Hamel Jr, R.; Cloarec, J.-P.; Lecomte, R.; Aimez, V.; Charette, P. G., Passivation of KMPPR microfluidic channels with bovine serum albumin (BSA) for improved hemocompatibility characterized with metal-clad waveguides. *Sens. Actuators, B* **2012**, 173, 447-454.
12. Van Hong Nguyen, B.-J. L., Protein corona: a new approach for nanomedicine design. *Int. J. Nanomed.* **2017**, 12, 3137.
13. del Río, J. S.; Henry, O. Y.; Jolly, P.; Ingber, D. E., An antifouling coating that enables affinity-based electrochemical biosensing in complex biological fluids. *Nat. Nanotechnol.* **2019**, 14 (12), 1143-1149.
14. Peng, Q.; Zhang, S.; Yang, Q.; Zhang, T.; Wei, X.-Q.; Jiang, L.; Zhang, C.-L.; Chen, Q.-M.; Zhang, Z.-R.; Lin, Y.-F., Preformed albumin corona, a protective coating for nanoparticles based drug delivery system. *Biomaterials* **2013**, 34 (33), 8521-8530.
15. Mariam, J.; Sivakami, S.; Dongre, P. M., Albumin corona on nanoparticles—a strategic approach in drug delivery. *Drug Deliv.* **2016**, 23 (8), 2668-2676.

16. Mannuzza, F. J.; Montalto, J. G., Is bovine albumin too complex to be just a commodity? *BioProcess Int.* **2010**, 8 (4), 42-49.
17. Xiao, Y.; Isaacs, S. N., Enzyme-linked immunosorbent assay (ELISA) and blocking with bovine serum albumin (BSA)—not all BSAs are alike. *J. Immunol. Methods* **2012**, 384 (1-2), 148-151.
18. Xia, X.-R.; Monteiro-Riviere, N. A.; Riviere, J. E., An index for characterization of nanomaterials in biological systems. *Nat. Nanotechnol.* **2010**, 5 (9), 671-675.
19. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B., Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **2012**, 9 (7), 676.
20. Inoue, Y.; Ishihara, K., Reduction of protein adsorption on well-characterized polymer brush layers with varying chemical structures. *Colloids and Surfaces B: Biointerfaces* **2010**, 81 (1), 350-357.
21. Park, J. H.; Sut, T. N.; Jackman, J. A.; Ferhan, A. R.; Yoon, B. K.; Cho, N.-J., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, 19 (13), 8854-8865.
22. Park, J. H.; Jackman, J. A.; Ferhan, A. R.; Ma, G. J.; Yoon, B. K.; Cho, N.-J., Temperature-induced denaturation of BSA protein molecules for improved surface passivation coatings. *ACS Appl. Mater. Interfaces* **2018**, 10 (38), 32047-32057.
23. Ricklin, D.; Hajishengallis, G.; Yang, K.; Lambris, J. D., Complement: a key system for immune surveillance and homeostasis. *Nat. Immunol.* **2010**, 11 (9), 785.
24. Szebeni, J., Complement activation-related pseudoallergy: a stress reaction in blood triggered by nanomedicines and biologicals. *Mol. Immunol.* **2014**, 61 (2), 163-173.
25. Rial, R.; Tichnell, B.; Latimer, B.; Liu, Z.; Messina, P. V.; Ruso, J. M., Structural and kinetic visualization of the protein corona on bioceramic nanoparticles. *Langmuir* **2018**, 34 (7), 2471-2480.
26. Galdino, F. E.; Picco, A. S.; Sforca, M. L.; Cardoso, M. B.; Loh, W., Effect of particle functionalization and solution properties on the adsorption of bovine serum albumin and lysozyme onto silica nanoparticles. *Colloids Surf., B* **2020**, 186, 110677.

27. Givens, B. E.; Xu, Z.; Fiegel, J.; Grassian, V. H., Bovine serum albumin adsorption on SiO₂ and TiO₂ nanoparticle surfaces at circumneutral and acidic pH: a tale of two nano-bio surface interactions. *J. Colloid Interface Sci.* **2017**, *493*, 334-341.
28. Roach, P.; Farrar, D.; Perry, C. C., Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. *J. Am. Chem. Soc.* **2006**, *128* (12), 3939-3945.

Chapter 8

Conclusions and Recommendations

In this chapter, the significant findings from the previous chapters are summarized and critical conclusions are drawn with respect to how they support the overall hypothesis of this thesis, and how they contribute to the development of the analytical framework for characterizing protein conformational stability, adsorption behavior and coating performance. Based on these findings, follow-up studies are introduced that expand on the analytical framework by characterizing how protein conformational stability modulations by the addition of other amphiphilic molecules or solvents can influence adsorption behavior and coating performance, as well as by studying alternative proteins with bioactive functional properties. Looking forward, the experimental approaches and capabilities developed in this thesis will be broadly applicable in studying other classes of proteins that will be useful in fabricating functional protein coatings for a wide range of applications ranging from nanobiotechnology to food science technology that will benefit human health.

8.1 Discussion and Conclusion

In this thesis, an experimental approach was developed to understand how rationally tuning the conformational stabilities of protein molecules based on materials science concepts can affect their adsorption behavior and how the insights gained from this can be used to engineer improved protein coating applications. The scope of study was focused on serum albumin proteins, particularly, whether natural variations in the amino acid sequence originating from different species as well as processing variations originating from different purification steps – which respectively correspond to the Structure and Processing tenets of the materials science paradigm – have any modulatory effects on its conformational stability, adsorption behavior and its surface passivation performance. To this end, a comprehensive analytical framework was developed to i) determine the serum albumins' solution-phase conformational stability in terms of the onset temperature of thermal aggregation and the extent of temperature-induced secondary structural loss, ii) to characterize the serum albumins' adsorption properties in terms of adsorption uptake, adsorption kinetics, and the extent of surface-induced denaturation and to iii) evaluate the surface passivation performance of the serum albumins in terms of antifouling capability as well as in mitigating nanoparticle-induced complement activation and correlating how different conformational stabilities influence surface passivation performance. Along this line, four specific aims were established to address the hypothesis of this thesis.

The first aim focused on determining whether natural sequence variation of serum albumin proteins from different species can affect protein conformational stability and adsorption behavior and was presented in Chapter 4. Specific attention was placed on fatty acid-free bovine serum albumin (BSA), human serum albumin (HSA) and rat serum albumin (RSA). Bioinformatics analysis using sequence alignment together with the classification of amino acids according to their physicochemical properties revealed a significant amount of amino acid substitutions that either lead to changes in hydrophobic interaction propensity or changes in electrostatic interaction between the three serum albumins and provided a strong case for differences in their conformational properties. Experimental characterization confirmed this, and it was discovered that BSA and HSA are monomeric while RSA is

multimeric, and that BSA and HSA had the lowest and highest solution-phase conformational stability, respectively. Adsorption experiments further revealed that BSA monomers underwent more extensive adsorption-related deformation than HSA monomers and formed a more tightly packed adlayer on the surface while RSA multimers adsorbed to form a less densely packed adlayer on the surface. These findings provided an empirical basis for why BSA remains a popular and effective surface passivation agent for various biotechnological applications and supports that a protein with lower conformational stability adsorbs to form a more tightly packed adlayer, which would be a more effective passivating layer for coating applications.

The second aim focused on determining whether differently processed serum albumins possess different conformational stabilities and adsorption behavior. Specifically, whether BSA proteins that have been purified using vastly different thermal conditions (*i.e.*, cold ethanol fractionation *vs.* heat-shock fractionation) as well as whether fatty acid addition, which has been reported to improve solution-phase conformational stability, would influence BSA adsorption behavior. These findings are reported in Chapter 5, and it was revealed that the presence of fatty acids play a more dominant role in modulating BSA conformational stability and adsorption behavior compared to the purification route alone. In particular, the fatty acid-free BSA proteins, regardless of purification route, all possessed significantly lower conformational stability (higher conformational flexibility) and also exhibited increased adsorption uptake and more extensive surface-induced denaturation compared to fatty acid-containing BSA proteins. Importantly, these findings provide, for the first time, an analytical and empirical basis for why certain types of BSA proteins *i.e.*, fatty acid-free BSA proteins are more effective surface passivation agents than others, which would greatly aid the scientific community. These findings further support the approach that proteins with increased conformational flexibility form adlayers with greater surface coverage due to more extensive deformation upon adsorption.

The third aim is presented in Chapter 6 and expands upon the findings from the second aim in the previous chapter and is focused on understanding how other amphipathic stabilizers – specifically two other fatty acid derivatives with different head group chemistries:

monoglyceride and fatty acid methyl ester – influence BSA conformational stability and adsorption behavior in comparison to fatty acid. The binding affinities of caprylic acid (CA), monocaprin (MC) and methyl caprylate (ME) were characterized and it was revealed that CA had the highest binding affinity as well as stabilizing effect on the BSA protein's solution-phase conformational stability followed by MC and ME. Since the amphiphiles all have the same length in their carbon tail group, the differences in binding affinities were largely attributed to differences in the degrees of hydrogen bonding between the available functional groups on the amphiphile head group and the BSA. In adsorption experiments, the additional negative charge conferred to BSA proteins by the anionic CA caused intermolecular repulsion between adsorbed BSA molecules which resulted in reduced adsorption uptake. On the other hand, the charge-neutral MC doping allowed for relatively closer packing between adsorbed BSA molecules, which, when coupled together with its stabilizing effect, formed an adlayer consisting of adsorbed BSA proteins which are less extensively deformed and therefore, resulted in a higher adsorption uptake compared to pristine BSA proteins. By contrast, ME doping did not exhibit significant binding or modulatory effects towards BSA. The experimental approach applied in this chapter will be advantageous in the exploration of other additives that either increase or decrease BSA conformational stability which can be useful in tuning specific adsorption behaviors for fabricating functional protein coatings.

The fourth and final aim is presented in Chapter 7 and is focused on evaluating the surface passivation performance of BSA proteins with varying conformational stabilities in order to correlate protein conformational stability with surface passivation performance. An integrative approach was adopted, involving various biophysical and biological methods of evaluation including the use of various surfaces with different surface chemistries (hydrophilic *vs.* hydrophobic) and topographies (flat *vs.* nanoparticle). BSA proteins with poorer conformational stability, which exhibited greater surface-induced denaturation and adsorption uptake, also displayed superior surface passivation performances in terms of preventing serum biofouling, giving rise to more highly resolved Western blot bands as well as more effectively mitigating nanoparticle-induced complement activation when

coated onto flat and nanoparticles surfaces on account of their ability to form more tightly packed adlayers.

The findings in this thesis demonstrate how protein conformational stability may be modulated through the Processing and Structure aspects of the material science tetrahedron. While both the Processing and Structure aspects have been shown to be effective modulators of conformational stability, there is greater potential in exploring the Processing aspect because more widely available proteins and their respective coating applications may be improved upon, similar to how the Processing aspect was used to determine how fatty acid-free BSA proteins do not have additional negative charge, have increased conformational flexibility, generally exhibit increased surface-induced denaturation and therefore form more tightly packed adlayers which translate to more effective surface passivation coatings. Moreover, it was then shown through further exploration of the Processing aspect that although MC-doped BSA had increased conformational stability and a smaller adsorption footprint, it produced films with higher packing density which could potentially translate to more effective surface passivation coatings as well. These results highlight the different ways in which modulations in protein stability can potentially give rise to different coating application performances.

Together with a comprehensive suite of biophysical and biological characterization methods, this materials science framework, with particular emphasis on the Processing aspect, can be applied to other protein systems to improve their respective coating applications. This motivates several future research directions which will be discussed in the next section of this chapter.

8.2 Future Directions

8.2.1 Alternative Methods for Modulating Protein Conformational Properties

The discovery in this thesis that amphipathic molecules can modulate the conformational and adsorption properties of BSA proteins opens up numerous research opportunities in

terms of exploring how other types of additives affect protein solution-phase conformational stability and adsorption behavior. For example, monocaprylin's (MC) ability to increase adsorption uptake warrants further study on the adsorption behavior of other monoglyceride-BSA complexes. In a direct follow-up to the conclusions from Chapter 6, it may be hypothesized that longer chain length monoglycerides such as monocaprin and monolaurin (10- and 12-carbon chain monoglycerides, respectively) have increased binding affinity compared to MC due to increased hydrophobic interactions. Indeed, longer amphiphile chain lengths have been correlated to increased binding affinity to BSA proteins.¹⁻³ Such amphiphiles with greater binding affinity are expected to confer greater conformational stability to BSA, causing less extensive adsorption-related deformation, and would therefore result in the formation of a more densely packed BSA adlayer. Of note, based on the MC data in Chapter 6, the formation of a more densely packed adlayer appears to be most pronounced at adsorption saturation (*i.e.*, before the buffer washing step). Therefore, future studies may also be conducted which explore how adlayer stabilization may be conducted, for example by adding a cross-linking agent such as glutaraldehyde before buffer washing,⁴ and how such processing steps improve the antifouling properties of the adlayer.

In another approach, the solution-phase conformational stability and adsorption behavior of proteins may be modulated by altering solvent conditions, for example through the use of water-miscible organic solvents.⁵ In a study on the effects of ethanol on BSA conformation, Yoshikawa *et al.* reported that BSA proteins denatured and underwent a loss in secondary structure from 0-50% (vol/vol) ethanol, but recovered its secondary structure from 50-100% ethanol.⁶ This behavior was attributed to ethanol interfering with long-range intramolecular hydrophobic interactions and hydrogen bonds at lower ethanol fractions (0-50% ethanol), followed by more extensive solvation of hydrophobic side chains by ethanol at higher ethanol fractions (above 60% ethanol) making up for the loss in long-range intramolecular hydrophobic interactions and hydrogen bonds.⁷ This trend indicates a corresponding ethanol-induced modulation in BSA conformational stability which would be expected to give rise to different adsorption behaviors.

In order to rationalize how ethanol affects BSA solution-phase conformational stability and, in turn, influence protein adsorption, a study was conducted in which BSA proteins were dissolved in water-ethanol solvents containing 0-60% ethanol in 10% ethanol increments, and their adsorption behavior on silica surface was studied using quartz crystal microbalance-dissipation (QCM-D) and localized surface plasmon resonance (LSPR) measurements.⁸ The QCM-D adsorption kinetics are presented in **Figure 8.1** while the LSPR adsorption kinetics are presented in **Figure 8.2**. The data from both measurement techniques showed a generally increasing trend in adsorption uptake with increasing ethanol content. Indeed, ethanol-induced solvation of more hydrophobic regions of the BSA protein molecule that are typically buried in the interior regions of the protein's folded structure in fully aqueous environments would result in a more unfolded protein conformation, greater conformational flexibility and more extensive adsorption-related deformation. Although more extensive deformation typically results in a larger surface area occupied per adsorbed BSA molecule and thus, lower adsorption uptake, a larger adsorption uptake was observed, which indicates that the resulting adlayer consists of a larger amount of BSA molecules which are densely packed, likely due to ethanol-enhanced protein-protein interactions.

Figure 8.1 QCM-D measurement of 100 μM BSA protein adsorption onto silica surfaces in water-ethanol mixtures. **a**, Frequency (ΔF) and **b**, energy dissipation (ΔD) shifts are reported as a function of time. The baseline was recorded in the appropriate water-ethanol mixture and then BSA in the equivalent solvent mixture was added at $t = 5$ min followed by a washing step in the same

solvent mixture (without protein) at $t = 35$ min. The insets in panel **a**, represent normalized data for the washing step and the baselines correspond to the adsorbed protein layers before washing. Reproduced with permission from ref. 8. Copyright 2020, American Chemical Society.

Figure 8.2 LSPR measurement of 100 μ M BSA protein adsorption onto silica-coated gold nanodisk arrays in water-ethanol mixtures. **a**, LSPR peak shifts ($\Delta\lambda$) signal are reported as a function of time. The baseline was recorded in the appropriate water-ethanol mixture and then BSA in the equivalent solvent mixture was added at $t = 5$ min followed by a washing step in the same solvent mixture (without protein) at $t = 35$ min. **b**, Magnified view of normalized $\Delta\lambda$ data from panel **a** for the washing step and the baselines correspond to the adsorbed protein layers before washing. Reproduced with permission from ref. 8. Copyright 2020, American Chemical Society.

Interestingly, an increase in signal response upon washing with water-ethanol solvents after BSA adsorption at higher ethanol fractions was observed, and the increase in LSPR $\Delta\lambda$ for 50-60% ethanol fractions was especially large. This behavior indicates that, contrary to the desorption behavior typically observed for protein adsorption experiments conducted in fully aqueous environments, the BSA adlayers in ethanolic conditions underwent densification and also became, on average, closer to the sensor surface upon solvent washing. It is likely that this adlayer densification is facilitated by BSA proteins that were weakly adsorbed onto the BSA adlayer due to ethanol-enhanced protein-protein interactions being washed away by the solvent washing step. Of note, subsequent washing of the BSA adlayers fabricated in ethanolic conditions with an aqueous salt buffer led to

significant desorption of the BSA molecules from the surface, whereas adlayers remained mostly intact upon washing with deionized water.⁹ This indicates the importance of electrostatics in maintaining the integrity of the adlayer, and also serves as guidelines on the optimal environmental conditions for adlayer stability.

Taken together, these findings support the overall hypothesis of this thesis by revealing that the introduction of organic solvents such as ethanol leads to an increase in BSA conformational flexibility, an increase in BSA adsorption uptake and to the formation of more dense protein adlayers. Looking ahead, this experimental approach of utilizing water-organic solvent mixtures to fabricate highly dense protein adlayer opens up numerous fundamental and translational research opportunities such as how the use of other organic solvents may also cause adlayer densification, how such densified adlayers may be further stabilized for example by glutaraldehyde-mediated crosslinking strategies,⁴ and how such an approach may be applied to other protein systems. This latter point is expanded upon in the following section.

8.2.2 Application of Experimental Framework to Alternative Proteins

In this thesis, a robust experimental framework was developed to study how conformational stability modulations influence protein adsorption with a focus on serum albumin proteins as a model. Along this line, there is a great opportunity to expand upon the scope of study by including other functional proteins. One promising candidate is lysozyme protein, which has antimicrobial properties due to its enzymatic activity in breaking glycosidic bonds within bacterial cell walls. Its involvement in the immune system has also been implicated based on its lectin-like ability to bind to carbohydrates.¹⁰ Such properties has therefore generated interest in controlling lysozyme adsorption to fabricate antimicrobial coatings on flat and nanoparticle surfaces.¹¹⁻¹⁶ Furthermore, as opposed to BSA protein which is generally considered “soft” (low conformational stability) and is negatively charged at physiological pH, lysozyme protein is considered a “hard” protein and is positively charged at physiological pH.^{17, 18} These physicochemical distinctions from serum albumin, taken

together with its antimicrobial properties make lysozyme an interesting alternative protein to study from a fundamental scientific perspective.

In addition, the approach of studying how modulating protein conformational stability by solvent conditions affect protein adsorption behavior, as introduced in the previous section, can be applied to lysozyme as well. Indeed, organic solvents such as ethanol have been shown to have modulatory effects on lysozyme conformational stability.^{19,20} A particularly interesting observation as a result of such conformational modulations was made in which monomeric lysozyme protein molecules exhibited oscillatory size trends whereby its hydrodynamic diameter fluctuated from 2.6 to 5.8 nm as a function of ethanol fraction in water-ethanol solvents.²¹ Such an oscillatory trend is distinct from that of BSA proteins which exhibit monotonic size increases as a function of ethanol fraction and, when taken together with lysozyme's antimicrobial activity, warrants further investigation into how ethanol-induced modulations in lysozyme conformational properties influence adsorption behavior.

Lysozyme proteins were dissolved in water-ethanol solvents with ethanol fractions ranging from 0 to 40% (vol/vol) in 10% increments and their adsorption behavior onto silica surface was characterized using QCM-D and LSPR techniques.²² The QCM-D adsorption kinetics are presented in **Figure 8.3** while the LSPR adsorption kinetics are presented in **Figure 8.4**. The QCM-D signal responses displayed an oscillatory trend as a function of ethanol fraction similar to the hydrodynamic size trends in different ethanol fractions, and minimal changes to its final adsorption uptake. By contrast, the LSPR signal response showed a generally increasing trend in $\Delta\lambda$ shifts as a function of ethanol fraction. These findings highlight how ethanol-induced conformational changes to lysozyme proteins in solution significantly impact the resulting protein adlayers. Similar to the BSA case, a washing step in the respective water-ethanol solvents resulted in increases in both QCM-D and LSPR signal responses, with relatively larger LSPR $\Delta\lambda$ shifts compared to QCM-D ΔF shifts. This indicates densification of the lysozyme adlayers.

Figure 8.3 QCM-D tracking of lysozyme adsorption in water-ethanol mixtures. **a**, Time-resolved ΔF shifts for lysozyme adsorption onto a silica surface in different water-ethanol mixtures as a function of ethanol fraction. The baseline was recorded in the appropriate water-ethanol mixture, followed by 50 μM lysozyme addition from $t = 5$ min onwards and then a washing step in the equivalent water-ethanol mixture (without protein) from $t = 25$ min. The inset shows a magnified view of the normalized Δf shifts due to solvent washing. **b**, Corresponding data for the time-resolved ΔD shifts that were measured simultaneously in the QCM-D measurements. Reproduced from ref. 22.

Figure 8.4 LSPR tracking of lysozyme adsorption in water-ethanol mixtures. **a**, Time-resolved $\Delta\lambda$ shifts for lysozyme adsorption onto a silica-coated nanodisk surface in different water-ethanol mixtures as a function of ethanol fraction. The baseline was recorded in the appropriate water-ethanol mixture, followed by 50 μM lysozyme addition from $t = 5$ min onwards and then a washing step in the equivalent water-ethanol mixture (without protein) from $t = 25$ min. **b**, Magnified view of the normalized $\Delta\lambda$ shifts due to solvent washing. Reproduced from ref. 22.

These results indicate that, under ethanolic conditions, the amino acid residues of lysozyme proteins were solvated and their hydrophobic interactions were disrupted, leading to enhanced conformational flexibility.^{21, 23, 24} This causes the lysozyme proteins to undergo more extensive surface-induced denaturation upon adsorption, exhibit greater adsorption irreversibility and ultimately form adlayers with greater packing density. These results further underscore the usefulness of utilizing the analytical framework developed in this thesis to fundamentally and systematically characterize how modulations in conformational flexibility led to different adlayer properties.

Following these findings, future studies pertaining to the characterization of the functional properties of such protein adlayers are warranted. In the case of lysozyme proteins, the enzymatic and antimicrobial activity of lysozyme-functionalized surfaces may be quantified according to the methods described in refs. ^{13, 14} and references therein, and correlated to ethanol-induced modulations in solution-phase conformational flexibility and adsorption uptake. Such studies pave the way for engineering and design strategies to fabricate improved antibacterial protein-based coatings based on tuning the conformational flexibility of lysozyme proteins.

Other future studies may also be conducted with the goal of being able to tune the adsorption behavior of specific classes of proteins in order to engineer functional protein coatings for specific applications. For example, ovalbumin protein, which, like lysozyme protein, can be found in egg white, has often been used as a model antigen protein for vaccine and adjuvant development.²⁵⁻²⁸ Studying the adsorption behavior of ovalbumin on adjuvant nanomaterials can therefore provide fundamental insights that can guide the development of improved adjuvants and vaccines. In another example, alpha lactalbumin and beta lactoglobulin are two other proteins belonging to the class of whey proteins which are being investigated for their anti-cancer properties upon binding with fatty acids.²⁹⁻³³ Their ability to bind various other bioactive molecules as well have spurred several studies on nanoparticle functionalization strategies for drug delivery and nutraceutical applications.³⁴⁻³⁷ Studying the conformational changes of alpha lactalbumin and beta lactoglobulin upon binding with relevant bioactive molecules together with their adsorption

behavior on various surfaces can therefore provide clues to develop improved nanoparticle-based strategies that can improve human health. Looking forward, the results obtained from the application of the analytical framework developed in this thesis to study the conformational stability, adsorption behavior and coating performance of different protein systems highlight its utility in advancing fundamental science as well as the research opportunities with translational potential that arise across several aspects of nanobiotechnology and with potential implications in food science technology.

References

1. Wasylewski, Z.; Kozik, A., Protein—non-ionic detergent interaction: interaction of bovine serum albumin with alkyl glucosides studied by equilibrium dialysis and infrared spectroscopy. *Eur. J. Biochem.* **1979**, *95* (1), 121-126.
2. Kragh-Hansen, U.; Watanabe, H.; Nakajou, K.; Iwao, Y.; Otagiri, M., Chain length-dependent binding of fatty acid anions to human serum albumin studied by site-directed mutagenesis. *J. Mol. Biol.* **2006**, *363* (3), 702-712.
3. Ojha, B.; Das, G., Role of hydrophobic and polar interactions for BSA—amphiphile composites. *Chem. Phys. Lipids* **2011**, *164* (2), 144-150.
4. Park, J.; Sut, T.; Jackman, J.; Ferhan, A.; Yoon, B.; Cho, N., Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. *Phys. Chem. Chem. Phys.* **2017**, *19*.
5. Griebenow, K.; Klibanov, A. M., On protein denaturation in aqueous—organic mixtures but not in pure organic solvents. *J. Am. Chem. Soc.* **1996**, *118* (47), 11695-11700.
6. Yoshikawa, H.; Hirano, A.; Arakawa, T.; Shiraki, K., Effects of alcohol on the solubility and structure of native and disulfide-modified bovine serum albumin. *Int. J. Biol. Macromol.* **2012**, *50* (5), 1286-1291.
7. Nozaki, Y.; Tanford, C., The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions: Establishment of a hydrophobicity scale. *J. Biol. Chem.* **1971**, *246* (7), 2211-2217.

8. Tan, J. Y. B.; Yoon, B. K.; Ma, G. J.; Sut, T. N.; Cho, N.-J.; Jackman, J. A., Unraveling how ethanol-induced conformational changes affect BSA protein adsorption onto silica surfaces. *Langmuir* **2020**, *36* (31), 9215-9224.
9. Park, H.; Ma, G. J.; Yoon, B. K.; Cho, N.-J.; Jackman, J. A., Comparing protein adsorption onto alumina and silica nanomaterial surfaces: clues for vaccine adjuvant development. *Langmuir* **2021**, *37* (3), 1306-1314.
10. Zhang, R.; Wu, L.; Eckert, T.; Burg-Roderfeld, M.; Rojas-Macias, M. A.; Lütkeke, T.; Krylov, V. B.; Argunov, D. A.; Datta, A.; Markart, P.; Guenther, A.; Norden, B.; Schauer, R.; Bhunia, A.; Enani, M. A.; Billeter, M.; Scheidig, A. J.; Nifantiev, N. E.; Siebert, H.-C., Lysozyme's lectin-like characteristics facilitates its immune defense function. *Q. Rev. Biophys.* **2017**, *50*, e9.
11. Derde, M.; Vié, V.; Walrant, A.; Sagan, S.; Lechevalier, V.; Guérin-Dubiard, C.; Pezennec, S.; Cochet, M. F.; Paboeuf, G.; Pasco, M., Antimicrobial activity of lysozyme isoforms: key molecular features. *Biopolymers* **2017**, *107* (12), e23040.
12. Beaussart, A.; Retourney, C.; Quilès, F.; Morais, R. D. S.; Gaiani, C.; Fiérobe, H.-P.; El-Kirat-Chatel, S., Supported lysozyme for improved antimicrobial surface protection. *J. Colloid Interface Sci.* **2021**, *582*, 764-772.
13. Bayazidi, P.; Almasi, H.; Asl, A. K., Immobilization of lysozyme on bacterial cellulose nanofibers: characteristics, antimicrobial activity and morphological properties. *Int. J. Biol. Macromol.* **2018**, *107*, 2544-2551.
14. Buruaga-Ramiro, C.; Valenzuela, S. V.; Valls, C.; Roncero, M. B.; Pastor, F. J.; Díaz, P.; Martinez, J., Development of an antimicrobial bioactive paper made from bacterial cellulose. *Int. J. Biol. Macromol.* **2020**, *158*, 587-594.
15. Sarkar, S.; Gulati, K.; Mishra, A.; Poluri, K. M., Protein nanocomposites: special inferences to lysozyme based nanomaterials. *Int. J. Biol. Macromol.* **2020**, *151*, 467-482.
16. Das, S.; Pabba, M.; Dhushyandhun, M. E.; Patra, C. R., Theranostic Applications of Lysozyme-Based Nanoparticles. In *Nanoparticles in Medicine*, Shukla, A. K., Ed. Springer Singapore: Singapore, 2020; pp 1-23.

17. Billsten, P.; Wahlgren, M.; Arnebrant, T.; McGuire, J.; Elwing, H., Structural changes of T4 lysozyme upon adsorption to silica nanoparticles measured by circular dichroism. *J. Colloid Interface Sci.* **1995**, *175* (1), 77-82.
18. Norde, W.; Favier, J. P., Structure of adsorbed and desorbed proteins. *Colloids Surf.* **1992**, *64* (1), 87-93.
19. Sassi, P.; Onori, G.; Giugliarelli, A.; Paolantoni, M.; Cinelli, S.; Morresi, A., Conformational changes in the unfolding process of lysozyme in water and ethanol/water solutions. *J. Mol. Liq.* **2011**, *159* (1), 112-116.
20. Magsumov, T.; Ziyang, L.; Sedov, I., Comparative study of the protein denaturing ability of different organic cosolvents. *Int. J. Biol. Macromol.* **2020**, *160*, 880-888.
21. Chatteraj, S.; Mandal, A. K.; Bhattacharyya, K., Effect of ethanol-water mixture on the structure and dynamics of lysozyme: A fluorescence correlation spectroscopy study. *J. Chem. Phys.* **2014**, *140* (11), 115105.
22. Yoon, B. K.; Ma, G. J.; Park, H.; Ferhan, A. R.; Cho, N.-J.; Jackman, J. A., Solvent-induced conformational tuning of lysozyme protein adlayers on silica surfaces: A QCM-D and LSPR study. *Int. J. Biol. Macromol.* **2021**, *182*, 1906-1914.
23. Sarkar, S.; Biswas, B.; Singh, P. C., Spectroscopic and Molecular Dynamics Simulation Study of Lysozyme in the Aqueous Mixture of Ethanol: Insights into the Nonmonotonic Change of the Structure of Lysozyme. *J. Phys. Chem. B* **2018**, *122* (32), 7811-7820.
24. Liu, W.; Bratko, D.; Prausnitz, J. M.; Blanch, H. W., Effect of alcohols on aqueous lysozyme-lysozyme interactions from static light-scattering measurements. *Biophys. Chem.* **2004**, *107* (3), 289-298.
25. Mahony, D.; Cavallaro, A. S.; Stahr, F.; Mahony, T. J.; Qiao, S. Z.; Mitter, N., Mesoporous silica nanoparticles act as a self-adjuvant for ovalbumin model antigen in mice. *Small* **2013**, *9* (18), 3138-3146.
26. van der Maaden, K.; Varypataki, E. M.; Romeijn, S.; Ossendorp, F.; Jiskoot, W.; Bouwstra, J., Ovalbumin-coated pH-sensitive microneedle arrays effectively induce ovalbumin-specific antibody and T-cell responses in mice. *European Journal of Pharmaceutics and Biopharmaceutics* **2014**, *88* (2), 310-315.

27. Morefield, G. L.; Jiang, D.; Romero-Mendez, I. Z.; Geahlen, R. L.; HogenEsch, H.; Hem, S. L., Effect of phosphorylation of ovalbumin on adsorption by aluminum-containing adjuvants and elution upon exposure to interstitial fluid. *Vaccine* **2005**, *23* (12), 1502-1506.
28. Sahdev, P.; Podaralla, S.; Kaushik, R. S.; Perumal, O., Calcium phosphate nanoparticles for transcutaneous vaccine delivery. *J. Biomed. Nanotechnol.* **2013**, *9* (1), 132-141.
29. Håkansson, A.; Zhivotovsky, B.; Orrenius, S.; Sabharwal, H.; Svanborg, C., Apoptosis induced by a human milk protein. *Proc. Natl. Acad. Sci.* **1995**, *92* (17), 8064-8068.
30. Aits, S.; Gustafsson, L.; Hallgren, O.; Brest, P.; Gustafsson, M.; Trulsson, M.; Mossberg, A.-K.; Simon, H.-U.; Mograbi, B.; Svanborg, C., HAMLET (human α -lactalbumin made lethal to tumor cells) triggers autophagic tumor cell death. *Int. J. Cancer* **2009**, *124* (5), 1008-1019.
31. Rammer, P.; Groth-Pedersen, L.; Kirkegaard, T.; Daugaard, M.; Rytter, A.; Szyniarowski, P.; Høyer-Hansen, M.; Povlsen, L. K.; Nylandsted, J.; Larsen, J. E., BAMLET activates a lysosomal cell death program in cancer cells. *Mol. Cancer Ther.* **2010**, *9* (1), 24-32.
32. Lišková, K.; Auty, M. A. E.; Chaurin, V.; Min, S.; Mok, K. H.; O'Brien, N.; Kelly, A. L.; Brodtkorb, A., Cytotoxic complexes of sodium oleate with β -lactoglobulin. *Eur. J. Lipid Sci. Technol.* **2011**, *113* (10), 1207-1218.
33. Fang, B.; Zhang, M.; Tian, M.; Ren, F. Z., Self-assembled β -lactoglobulin–oleic acid and β -lactoglobulin–linoleic acid complexes with antitumor activities. *J. Dairy Sci.* **2015**, *98* (5), 2898-2907.
34. Waghmare, M. N.; Qureshi, T. S.; Shaikh, A. N.; Khade, B. S.; Murali Krishna, C.; Dongre, P. M., Functionalized alpha-lactalbumin conjugated with gold nanoparticle for targeted drug delivery. *ChemistrySelect* **2020**, *5* (6), 2035-2049.
35. Yang, J.; Wang, T.; Zhao, L.; Rajasekhar, V. K.; Joshi, S.; Andreou, C.; Pal, S.; Hsu, H.-t.; Zhang, H.; Cohen, I. J.; Huang, R.; Hendrickson, R. C.; Miele, M. M.; Pei, W.; Brendel, M. B.; Healey, J. H.; Chiosis, G.; Kircher, M. F.,

- Gold/alpha-lactalbumin nanoprobe for the imaging and treatment of breast cancer. *Nat. Biomed. Eng.* **2020**, *4* (7), 686-703.
36. Guillet-Nicolas, R.; Popat, A.; Bridot, J.-L.; Monteith, G.; Qiao, S. Z.; Kleitz, F., pH-responsive nutraceutical–mesoporous silica nanoconjugates with enhanced colloidal stability. *Angew. Chem. Int. Edit.* **2013**, *52* (8), 2318-2322.
37. Vian, R.; Salehi, H.; Lapierre, M.; Cuisinier, F.; Cavaillès, V.; Balme, S., Adsorption of proteins on TiO₂ particles influences their aggregation and cell penetration. *Food Chem.* **2021**, *360*, 130003.

List of Publications

Directly relevant to this thesis:

1. **Ma G.J.**, Ferhan A.R., Sut T.N., Jackman J.A., and Cho N.-J., "Understanding How Natural Sequence Variation in Serum Albumin Proteins Affects Conformational Stability and Protein Adsorption." *Colloids and Surfaces B: Biointerfaces* 194, (2020): 111194.
2. **Ma G.J.**, Ferhan A.R., Jackman J.A., and Cho N.-J., "Conformational Flexibility of Fatty Acid-Free Bovine Serum Albumin Proteins Enables Superior Antifouling Coatings." *Communications Materials* 1, no. 1 (2020): 45.
3. **Ma G.J.**, Ferhan A.R., Jackman J.A., and Cho N.-J., "Elucidating How Different Amphipathic Stabilizers Affect BSA Protein Conformational Properties and Adsorption Behavior." *Langmuir* 36, no. 35 (2020): 10606–10614.
4. Tan J.Y.B., Yoon B.K., **Ma G.J.**, Sut T.N., Cho N.-J., and Jackman J.A., "Unraveling How Ethanol-Induced Conformational Changes Affect BSA Protein Adsorption onto Silica Surfaces." *Langmuir* 36, no. 31 (2020): 9215-9224.
5. Yoon B.K.*, **Ma G.J.***, Park H., Ferhan A.R., Cho N.-J., and Jackman, J.A., "Solvent-Induced Conformational Tuning of Lysozyme Protein Adlayers on Silica Surfaces: A QCM-D and LSPR Study" *International Journal of Biological Macromolecules* 182, (2021): 1906-1914 (* denotes equal first authors).

Others:

1. **Ma G.J.**, Yoon B.K., Sut T.N., Yoo K.Y., Lee S.H., Jeon W.Y., Jackman J.A., Ariga K., and Cho N.J., "Lipid Coating Technology: A Potential Solution to Address the Problem of Sticky Containers and Vanishing Drugs" *View*
2. Wynendaele E., **Ma G.J.**, Xu X., Cho N.-J., and De Spiegeleer B., "Conformational Stability as a Quality Attribute for the Cell Therapy Raw Material Human Serum Albumin." *RSC Advances* 11, no. 25 (2021): 15332-15339.

3. **Ma G.J.**, Zhdanov V.P., Park S., Sut T.N., and Cho N.-J., "Mechanistic Aspects of the Evolution of 3D Cholesterol Crystallites in a Supported Lipid Membrane via Quartz Crystal Microbalance with Dissipation Monitoring." *Langmuir* 37, no. 15 (2021): 4562–4570.
4. Park H., **Ma G.J.**, Yoon B.K., Cho N.-J., and Jackman J.A., "Comparing Protein Adsorption onto Alumina and Silica Nanomaterial Surfaces: Clues for Vaccine Adjuvant Development." *Langmuir* 37, no. 3 (2021): 1306-1314.
5. Park S., Sut T.N., **Ma G.J.**, Parikh A.N., and Cho N.-J., "Crystallization of Cholesterol in Phospholipid Membranes Follows Ostwald's Rule of Stages." *Journal of the American Chemical Society* 142, no 52 (2020): 21872-21882.
6. Yoon B.K., Park S., **Ma G.J.**, Kolahdouzan K., Zhdanov V.P., Jackman J.A., and Cho N.-J., "Competing Interactions of Fatty Acids and Monoglycerides Trigger Synergistic Phospholipid Membrane Remodeling." *The Journal of Physical Chemistry Letters* 11, no. 13 (2020): 4951–4957.
7. Ferracci G., Zhu M. Ibrahim M.S., **Ma G.J.**, Fan T.F., Lee B.H., and Cho N.-J., "Photocurable Albumin Methacryloyl Hydrogels as a Versatile Platform for Tissue Engineering." *ACS Applied Bio Materials* 3, no. 2 (2020): 920-934.
8. Sut T.N., Jackman J.A., Yoon B.K., Park S., Kolahdouzan K., **Ma G.J.**, Zhdanov V.P., and Cho N.-J., "Influence of NaCl Concentration on Bicelle-Mediated SLB Formation." *Langmuir* 35, no. 32 (2019): 10658-10666.
9. Ferhan A.R., Spackova B., Jackman J.A., **Ma G.J.**, Sut T.N., Homola J., and Cho N.-J., "Nanoplasmonic Ruler for Measuring Separation Distance Between Supported Lipid Bilayers and Oxide Surfaces." *Analytical Chemistry* 90, no. 21 (2018): 12503-12511.
10. Park J.H., Jackman J.A., Ferhan A.R., **Ma G.J.**, Yoon B.K., and Cho N.-J., "Temperature-Induced Denaturation of BSA Protein Molecules for Improved Surface Passivation Coatings." *ACS Applied Materials & Interfaces* 10, no. 38 (2018): 32047-32057.
11. Ferhan A.R.*, **Ma G.J.***, Jackman J.A., Sut T.N., Park J.H., and Cho N.-J., "Probing the Interaction of Dielectric Nanoparticles with Supported Lipid Membrane Coatings on Nanoplasmonic Arrays." *Sensors* 17, no. 7 (2017): 1484. (* denotes equal first authors).