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**NANYANG
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SINGAPORE

**INVESTIGATING PROTEIN ADSORPTION AT SOLID-LIQUID
INTERFACES TOWARDS THE DEVELOPMENT OF
FUNCTIONAL BIOMATERIAL COATINGS**

PARK JAE HYEON

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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FUNCTIONAL BIOMATERIAL COATINGS**

PARK JAE HYEON

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2018

Statement of Originality

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

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This thesis contains material from papers published in the following peer-reviewed journals or those that are in progress where I was the first and/or corresponding author.

Chapter 4 is published as J.H. Park, T. N. Sut, J. A. Jackman, A. R. Ferhan, B. K. Yoon, N. J. Cho. Controlling Adsorption and Passivation Properties of Bovine Serum Albumin on Silica Surfaces by Ionic Strength Modulation and Cross-Linking. *Physical Chemistry Chemical Physics* **19**, 8854-8865 (2017). DOI: 10.1021/ic502747p.

The contributions of the co-authors are as follows:

- Prof Cho provided the initial project direction and edited the manuscript drafts.
- Dr. Jackman, Dr. Ferhan and I co-designed the detailed experimental plans and outlined the goals and objectives for the project
- Mr. Sut and I prepared the manuscript drafts. The manuscript was revised by Dr. Jackman and Dr. Ferhan.
- I performed all DLS/NTA/CD experiments
- Mr. Sut, Dr. Yoon and I performed all the QCM-D experiments

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- I prepared the manuscript drafts. The manuscript was revised by Dr. Jackman.
- I performed all DLS/NTA/CD experiments
- Dr. Yoon performed all the QCM-D experiments and I analyzed the data.

- Mr. Ma carried out all LSPR experiments and analysis.
- AFM images were assisted by Mr. Jin and the I analyzed the data.

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- I prepared the manuscript drafts. The manuscript was revised by Dr. Ferhan.
- I performed all DLS, NTA, CD experiments
- Dr. Ferhan and I performed all the QCM-D experiments and Dr. Jackman assisted with the analysis.

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Abstract

Surface and interfacial science is an essential field of study in determining the structure-property design parameters of materials. The fundamental knowledge is especially important in modern days whereby many practical applications rely on the compatibility of organic and inorganic substances. Protein adsorption is one of such areas of research that has been considered highly necessary and relevant for various applications, yet much of its details remain under vigorous scrutiny due to its complexity.

Protein adsorption is the first step in the acute biological reaction to an exposed surface, in which the functionalities or properties of a material in query could possibly be altered or even contaminated depending on the biomaterials accumulated on the surface. The protein-surface interaction occurs spontaneously and almost at random but the detailed process can be broken down into step-by-step phases whereby a certain level of higher governance is embedded within the system that dictates its kinetics and trends. The major factors can be largely classified into extrinsic and intrinsic factors, which include many other subfactors that are cross-interdependent of one another. Therefore, even with highly sophisticated computerized simulations, the theoretical studies are limited without the inclusion of systematic studies emphasizing data-driven empirical analysis.

With the emphasis on the empirical establishment of fundamental insights, this dissertation dives in to the interfacial science of protein-surface interactions using carefully structured experimental designs and intuition-based analyses to form fresh arguments to explain protein adsorption. While most of the experimental layouts are in reference to past studies, the methods were reinforced with modern techniques, and speculations were minimized through data verification *via* complimentary pairing of analytical devices.

The project is presented as a three-part series starting from a broad macroscopic view of the protein adsorption in ensemble, which then gradually narrows down to the

sub-molecular level of protein-surface interactions. Thorough investigations revealed that protein adsorption is indeed reliant on the environmental conditions (through control of ionic strength), but more specifically by factors that directly intervene with the conformational stability of the proteins. However, it was further revealed that the extrinsic controls were insufficient in determining the final outcome, ultimately guided by the protein's potential entropic energy that is confined within the malleable structure.

Finally, based on the insights and the unexpected and/or expected observations from the series of studies, scientific and practical solutions were discussed for relevant applications, particularly in regards to achieving an efficient and optimized surface passivation protocol. Revealing the need for a disruptive solution condition for the effective tuning of stable protein layers was especially critical considering the implications that it may have on the many relevant surface passivation applications that were considered common practice. This is only one such example, and clearly there is more room for improvement as there is much to be uncovered from the study of protein adsorption.

Lay Summary

Surface and interfacial science is the study of the microscopic interactions that take place in the outermost surface of materials. Protein adsorption is one of such studies that discuss the spontaneous accumulation of biological proteins to artificial materials, and address the relevant issues at hand, from medical implants to contact lenses, that are affected by the natural phenomenon. With the advancement in medical science, investigations to better understand the underlining mechanisms of protein adsorption had been in high demand for several decades, yet much of its details remain unclear and highly scrutinized.

Protein adsorption is the very first biological response to materials when the material comes into contact with bodily fluid. The subsequent host response “sees” and reacts to the surface-bound proteins instead of the material itself, and therefore, any functional designs on the material can be lost, or in some cases, induce inflammation, contamination or other detrimental consequences. The protein-surface interaction happens spontaneously and almost at random, but the detailed process can be broken down into specific phases whereby a higher order of governance exists to drive the interaction forward. The major factors driving the interaction can be classified into environmental extrinsic factors and inherent intrinsic factors of which numerous subfactors from each classifications are cross interdependent of one another, hence the challenge in making clear-cut isolated characterizations.

In order to get past beyond the complexity, the project studies a simplified representative version of a protein adsorption event to extract elementary knowledge from carefully designed experimental platforms. The detailed experiments are in reference to many classical studies but with reinforcement using modern analytical techniques and evidence-based intuitive inductive reasoning. The dissertation is presented as a three-part series starting from an overall scanning of how each key component of the system (*i.e.*, protein, adsorbent surface, solution) interacts in accords to the provided conditions. Subsequent chapters harness the specific details that were particular

noteworthy or concerning. Thorough analysis of the experimental data revealed that a environmental conditions could control protein adsorption to a certain degree, but more specifically, through means that would engage with the proteins to affect its structural properties. In terms of guiding the establishment of a protein-barrier across the surface, the environmental controls were insufficient in overruling the inherent properties of the proteins, and further input was needed in order to stabilize the surface-bound proteins for an effective surface coating layer.

The dissertation urges an awareness of the gap between the fundamental works of protein adsorption and the relevant applications. Once a connection is established, there is definite opportunity for groundbreaking discoveries using simple straightforward solutions. The reports presented within this dissertation provides a simple versatile experimental platform that can be easily expanded out to host many other similar projects.

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Abbreviations

AFM	Atomic Force Microscopy
BSA	Bovine Serum Albumin
CCD	Charge-Coupled Device
CD	Circular Dichroism
dBSA	Denatured Bovine Serum Albumin
DLS	Dynamic Light Scattering
dHSA	Denatured Human Serum Albumin
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
HSA	Human Serum Albumin
IEP	Isoelectric Point
INPS	Indirect Nanoplasmonic Sensing
LSPR	Localized Surface Plasmon Resonance
MRE	Molar Residue Ellipticity
MSD	Mean Square Displacement
nBSA	Native Bovine Serum Albumin
nHSA	Native Human Serum Albumin
NNLS	Non-Negative Least Square
NTA	Nanoparticle Tracking Analysis
PEO	Polyethylene Oxide
PDI	Polydispersity Index
QCM-D	Quartz Crystal Microbalance with Dissipation
RSA	Random Sequential Order
SAM	Self-Assembled Monolayer
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SLB	Supported Lipid Bilayers
TBST	Tris-buffered Saline with Tween 20
Δf	Frequency measurement
ΔD	Energy Dissipation measurement

Chapter 1

Introduction

Biofouling has become a key parameter in determining long-term stability and performance of biomaterials in industrial and medical applications. Several strategies for materialistic designs at the solid-liquid interface have been proposed including nanolithography or chemical functionalization for antifouling properties, but a viable solution is yet to be discovered. Herein, the fundamental mechanism of protein adsorption is systematically investigated with a representative model and a top-down investigative approach whereby macroscale experiments are used to analyze phenomenological trends to predict the kinetic processes of protein-surface interactions. The project then expands on exploring how the protein adsorption phenomenon can be exploited to develop a functional biomaterial surface instead of devising a strategy for biomaterial-repellent surfaces. Towards the end of the dissertation, the potentials and versatilities of the methods of surface functionalization using proteins is briefly demonstrated with preliminary data. This section provides a short outline of what is to be expected in this dissertation.

1.1 Problem Statement

Figure 1.1 Time-resolved schematic of protein biofouling on a bare biomaterial surface (Image adopted from Grainger DW [1] with permission from Nature).

Interaction between surfaces and proteins is an essential component of life. Embryogenesis and metabolism is regulated by the attachment and detachment of specific proteins [2]; proteins interacting with the cellular membrane mediate cell-to-cell communication [3, 4]; and even the control of diseases or the repair of damages is initiated by protein adsorption [5]. Inevitably, the initial host response to biomedical implants is the adsorption of proteins from the interstitial biofluids immediately surrounding the material (Figure 1.1). The biological response is guided by the nonspecific adsorption of proteins which can be supplementary to the intended functions, such as in the case of fibroblast or osteoblast recruitment that are needed for wound [6], but at times problematic and even pathological. It is therefore imperative to design surfaces that are resistant to protein adsorption (or selectively favorable to desirable proteins) to promote biocompatibility while maximizing the functionality of biomaterials. Unfortunately, protein adsorption is an unavoidable and universal process and hence

chemical modifications of the surface have been explored as an effort to mitigate the effect of the biofouling[7-9]. In any case, protein adsorption is the first step in the acute biological response to all exposed surfaces, and therefore, it is of paramount importance to understand the fundamental mechanisms of protein-surface interactions when considering artificial materials to be exposed to biological milieu [10].

Unlike the adsorption of small rigid molecules, the biophysical mechanism of protein adsorption is not as straightforward having to take into consideration of all the different phases besides adsorption and desorption kinetics including structural rearrangements, change in surface affinities, positive cooperative effects, surface aggregation, and more [11-17]. For over six decades, numerous investigations were focused on identifying the details of protein adsorption, but due to its complexity, there is still limited consensus among the investigators and heavy debates even on the most basic principles of protein adsorption [18]. Since its first systematic studies, continued progress in the analytical methods and techniques has allowed increased precision and sophistication in the experimental data, and new insights are being revealed with the newfound capabilities [18, 19]. Hence, the intention of this dissertation is to revisit the past systematic studies with modern analytical methods to identify the missing links, if any, to potentially reiterate the fundamental concepts of protein adsorption. In particular, this thesis focuses on the structural stability of proteins as a distinguishing factor in determining the binding strength between proteins and surfaces. Specifically, proteins that readily deform to spread onto the surface upon contact would establish a stronger and more stable structure, which would lead to better antifouling properties.

1.2 Objectives and Scope

In this dissertation, the protein adsorption phenomenon is systematically investigated to decipher the outliers that mandate the outcome of the protein interactions; in particular, focusing on how the structural stability of proteins is influenced by intrinsic and extrinsic factors, and in turn how it affects the quality of the protein coating on a material surface. Starting from a simplified planar model using serum albumin and silica as the representative protein and substrate, respectively, the conformational and colloidal

stability of proteins relative to the ionic strengths are first configured, and the corresponding adsorption trends are investigated. The insights are extended with use of proteins that have been purposefully corrupted in terms of structural integrity to further support the initial hypotheses in regards to the underlying mechanisms of protein-surface interactions. The theoretical mechanisms that are proposed herewith, as referred to as *principle outcomes*, are then integrated into relative applications to demonstrate the validity of the findings through evidence-based conclusions. The inductive approach of this project emphasizes the importance of fundamental studies in reaching a practical solution, and the necessity to integrate empirical research into theoretical investigations. The dissertation concludes with the implications of this researched topic and future directions that can spur from this work.

1.3 Dissertation Overview

The thesis has been structured to best present how each study had been designed sequentially providing motivation and rationale for the next topic. The investigations starts from a hypothesis addressing a broad macro scale view of protein adsorption observing the entire system, then pinpoints down to a more miniscule scale to corroborate with more detailed specifics of the protein adsorption process. Below outlines what to be expected within this dissertation.

Chapter 1 introduces the main hypothesis of the dissertation; provides a rationale for the research; outlines the goals scope of the project; and summarizes the principle outcomes of the entire project.

Chapter 2 reviews the literature concerning protein adsorption and particularly highlights the inconsistencies in interpreting the biophysical interactions. The literature review outlines the research trends and presents the works of other researchers that have motivated this project.

Chapter 3 lays out the overall experimental plans and methodologies carried out for the project. The rationale behind the project design is explained in detail and the motivation for allocating the particular analytical tools for each relevant study is discussed, including a brief summary of the measurement principles for each analytical

device. Lastly, the protocols for the experiments as well as the interpretation methods of the resulting data are also illustrated for each technique.

Chapter 4 elaborates on the effect of solution ionic strengths in determining protein interactions using a one-protein system. As the first sequel of the three-part series, it introduces the general scope of discussion with a minimalistic approach relying only on observation, logic, and intuition. Eventually, it unravels the uncertainties and inconsistencies in this particular field of study and opens up several questions for future studies.

Chapter 5 pinpoints on the significance of the conformational stability of proteins as a major contributory factor in configuring protein-surface interactions. The structural integrity of proteins is purposefully altered *via* thermal denaturation and the adsorption properties are compared. The superiority of denatured proteins as a blocking agent is highlighted, while at the same time, indirectly proving the importance of surface-induced denaturation and relative adsorption stability for proteins in a physiological setting.

Chapter 6 integrates the previous two chapters to compare the adsorption trends of homologous serum albumins. The complexity of protein adsorption is well demonstrated and exemplifies the need for multi-facet studies even when exploring a simple one-protein system setup. The overall experimental designs provide a valid framework for future structure-property studies and will be a valid reference especially when advancing to more complicated systems.

Chapter 7 summarizes the implications and impacts of this dissertation and offers a glimpse of its future outlook. More specifically, the proven works of enhanced surface passivation techniques are transitioned from a 2D planar surface to 3D nanostructures. Preliminary studies of this future project are presented in this chapter with open-ended conclusions and opportunities.

1.4 Findings and Outcomes/Originality

This research led to several novel outcomes by:

1. Establishing a framework for future protein adsorption comparison studies
2. Reiterating conformational stability as a multi-faceted concept and correlatively

- reestablishing appropriate conditions to enhance surface passivation properties.
3. Proposing a streamlined protein coating protocol on material surfaces that shows up to 10-fold improvement than conventional methods
 4. Opening opportunities for the translation of its applications into the field of nanoparticles and nanomedicine

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Chapter 2

Literature Review

Protein adsorption is a spontaneous biological reaction to any exposed surface that enters a biological milieu. The adsorbed protein masks the surface and dictates the downstream biological interactions making it problematic for relative applications that are required to be functional within a biological surrounding. The process consists of a diffusion of proteins from the bulk solution to the liquid-solid interface followed by the actual adsorption via protein-surface interactions and a post-annealing process to stabilize the structure. Although conceptually simple, the process is described with multifaceted complexity and hence the efforts to resolve the fundamental mechanism are still vigorously scrutinized amongst the dedicated literature. This chapter reviews the basic concepts of protein adsorption and discusses the major factors that are considered key in configuring the issue at hand.

2.1 Protein Adsorption

The generally accepted schematic of protein adsorption describes a multi-phased event consisting of a diffusion-limited adsorption phase followed by a post-adsorption annealing process [1]. On a relative timescale, the earlier phase occurs instantaneously in comparison to the latter, but the bulk of the resulting protein layer is predetermined from this transportation. Therefore, the adsorption process is often times considered to be electrostatic in nature [2], as evident by the abundant reports of pH or ionic strength effects associated with protein adsorption [3-8]. Conversely, the post-adsorption phase is equally critical as it contributes to the molecular-scale evolution of the organic layer in effect of the short-ranged protein-surface interactions, which in turn dictates the properties of the resulting protein film and consequently the adhesiveness of subsequently adsorbing proteins [9].

2.1.1 Thermodynamics of Protein Adsorption

The protein-surface interaction have been fitted in the standards of Gibbs free energy ($\Delta G = \Delta H - T\Delta S$), and simplified in terms of changes in enthalpy (H) and entropy (S), whereby the Coulombic and van der Waals interactions between the protein and surface are considered enthalpic factors and conformational changes in the proteins would be considered entropic factors [10]. With observations of the Gibbs energy, proteins that are less thermally stable were found to better associate with an adsorbent surface being driven by entropic factors, especially when interacting to surfaces that possessed unfavorable attributes (*e.g.*, hydrophilic apolar surfaces or electrostatic repulsive surfaces) [11]. Relatedly, a distinction of hard/soft proteins had frequently followed in literature based on the conformational stabilities to predict, classify, and compare the adsorption kinetics of two or more different proteins [11-15]. However, the boundary conditions for the distinctions remained vague and ill defined with limitations in its applicability. For example, the conformational stability of bovine serum albumin, which is commonly recognized as a soft globular protein [16], could be stabilized with changes in pH, ionic strength or temperature to be less malleable on the surface [17]. In another example, the

“hard” lysozyme was capable of changing conformation with the surface upon specific alterations in the solution conditions [18, 19].

Along the surface of the adsorbent, individual proteins are able to diffuse laterally along the surface colliding with other proteins in the vicinity to trigger cooperative effects [20] or at times to displace other proteins from the surface [21]. As the surface reach saturation, the protein-surface interactions are further restricted mainly by steric factors, and a drastic change in protein behavior will be observed as it is affected by protein-surface interactions as well as protein-protein interactions [22]. Nevertheless, the various factors are within the paradigm of thermodynamics, albeit with multiple layers of complexity, in which the sequence of actions of a protein molecule are determined by the sum of enthalpic energy input from its surrounding to an entropic output to maintain its most energetically favorable form.

In essence, protein adsorption is a thermodynamically defined event driven by energetics. Over time, a steady state is observed within the protein-substrate complex in ensemble, but in consideration of the reversible nature of adsorbed proteins and the repetitive cycling of proteins coming on and off of the surface, it is argued that thermodynamic equilibrium is unattainable [23, 24]. Thus, the post-adsorption phase carries particular significance in modulating the outcome of protein adsorption, but there remains an inevitable limitation in its assessment and controversies in the methodologies that are being used for analysis. For example, when proteins are labeled with radioactive tags or dyes, a standard rinsing protocol is almost always included, and in some cases vacuum drying is applied [25, 26]. For such cases, it must be assumed that the proteins are irreversibly bound to the surface or inherently strong enough to persist the perturbation from rinsing, but at the same time, it would implicitly approve a state of thermodynamic equilibrium. Recent advances in technologies provide greater sophistication in its method of detection and are now better equipped to address the concerns of rinsing without overtly agitating the solid-liquid interface, which includes in-situ ellipsometry [27, 28], quartz-crystal microbalance with dissipation [29, 30], and localized surface plasmon resonance [31, 32].

Within the basis of thermodynamics, two different approaches are generally taken when investigating the protein adsorption phenomenon: (1) a bottom-up approach

whereby the molecular-scale theoretical models are configured to be fitted into a macroscale experiment for protein adsorption analysis [33, 34]; or (2) a top-down approach in which ensemble adsorption trends are investigated through systematic empirical studies as to prescribe a phenomenological model of protein adsorption [35-38].

2.1.2 Single Molecule Perspective

With fluorescently labeled proteins, the trajectory of a single protein molecule can be traced by techniques such as total internal reflection fluorescence microscopy (TIRFM) [34, 39, 40] or fluorescence correlation spectroscopy (FCS) [41, 42]. The adsorption and desorption rates are deduced from the fluctuations in the fluorescent intensity and molecular behaviors of proteins in response to the opposing surface are defined. Kastantin *et al.* reports of how the conformational changes and protein footprint can be observed through TIRFM [34], and Langowski reports of his work on determining protein-protein interactions with FCS [41]. As such, the fine control over the experimental properties makes it a powerful tool to study protein adsorption, and especially efficient when paralleled with computer simulations. The bottom-up approach to interpreting protein adsorption uses the molecular behavior of proteins to formulate a best-fit kinetic model which involves numerous reiterations through tightly controlled experiments for the ensemble protein adsorption. However, the fundamental techniques rely on the use of fluorescent tags which could arguable cause deviations in the fundamental protein-surface interactions [43]. Furthermore, it may fail to capture the grander scale of protein-surface interactions and result in oversimplifications by neglecting the surrounding environmental conditions.

In a more refined view of the molecular arrangements at the solid-liquid interface, it is understood that the protein displaces the surface bound water molecules to establish contact with the surface, and the protein progressively unfolds along the surface mainly through van der Waals and hydrophobic interactions to compensate for the energy cost of replacing the water molecules (figure 2.1) [44]. When the adsorbent is hydrophilic, the water molecules nearest to the surface forms a hydration layer that is distinct from the bulk solution and increases the energetic barrier for the protein to establish contact with the surface [45]. On the other hand, the hydration layer on a hydrophobic adsorbent is

indifferent from the bulk solution making it a more favorable surface for protein uptake [15]. Regardless, an adsorbing protein is able to overcome the energetic barrier for both situations [34], and hence, the net energetics of protein-surface interaction is considered to be larger than thermal energy [46].

Figure 2.1 Step-wise evolutionary process of protein unfolding on a hydrated surface in the post-adsorption phase of a protein adsorption event (Image adopted from Penna, *et al.* [1] with permission from ACS).

Based on such assumptions and the amphipathic nature of proteins, it is widely accepted that the protein molecules on average would denature on the surface to varying degrees depending on its conformational stability [47, 48]. Alternatively, when the structural stability is significant, individual protein molecules could shift its binding orientation on the surface from an “end-on” to a “side-on” orientation to gain net attractive forces [49], especially at high packing densities near the surface where the lateral protein-protein interactions partake in stabilizing the surface bound proteins [50].

In the perspective of the protein, surface-induced denaturation highly resembles that of the thermal denaturation process and had been interchangeably used in literature to characterize the conformational stability of proteins [51, 52]. Both are endothermic processes whereby a steady input of energy allows the intermolecular bonds to destabilize and the conformation to unfold as ordained by entropy. In the case for the thermal denaturation process, there is a critical threshold in which the denaturation of the protein

becomes irreversible [53, 54]. Likewise, a similar critical threshold is argued for surface-induced denaturation where the loss of structure would be permanent and the adsorption to the surface be irreversible [55]. Karlsson *et al.* demonstrated that it is possible to control irreversible protein adsorption and the net protein uptake on the surface by artificially enhancing the stability of proteins [56], and similar results were also reported when aided by ligands or sugar excipient for reinforcements in protein stability [17, 57]. However, the notion of irreversible protein adsorption is highly scrutinized as discussed in the previous section [58]. In the context of physiological relevance where materials are constantly challenged by the surrounding environment, the quantitative analysis of long-lived versus short-lived proteins on an adsorbent is more or less biologically irrelevant, whereas the accurate assessment of reversible/irreversible adsorption may bear more significance in understanding the fundamental mechanisms of protein adsorption.

2.1.3 Ensemble Perspective

When an adsorbent surface is saturated, a steady state adlayer can be characterized by its packing density and surface coverage rate, which is dependent on the electrostatic interactions [59, 60], the bulk protein concentration [61], and the binding orientation of individual protein molecules on the surface [62]. Naturally, a protein adsorption event is a composite result of various factors of which may or may not be obvious when focused on the single molecule perspective, which becomes more evident as the investigation shifts towards handling increasingly more complex protein cocktails. It is therefore, vital to evaluate the assembly and evolutionary process of the protein adlayer in its entirety even when studying a single protein system. The accumulation of proteins adsorbing on the surface can be measured by surface sensitive analytical devices which includes ellipsometry, quartz crystal microbalance with dissipation (QCM-D), X-ray photoelectron spectroscopy (XPS), localized surface plasmon resonance (LSPR), and atomic force microscopy (AFM) (Table 2.1). The label-free and real-time analysis of protein adsorption provides a broader assessment of the macroscopic events, albeit with limitations relying on assumptions of the microscopic protein interactions that take place, yet with systematic studies, a trend analysis of protein behavior can be used to derive fundamental insights into understanding the schematics of protein adsorption.

Table 2.1 Conventionally used analytical techniques for protein adsorption studies

Technique	Category	Measurements and output	Ref
TIRFM	Single molecule	<ul style="list-style-type: none"> ○ Microscopic detection of fluorescently labeled protein at single molecule resolution ○ Trajectory tracking to measure adsorption, desorption and residency time in between 	[39, 40]
FCS	Single molecule	<ul style="list-style-type: none"> ○ Measurement of the fluctuation in fluorescence to detect protein molecules ○ Correlation function to extrapolate dynamic protein behavior 	[41, 42]
Ellipsometry	Ensemble	<ul style="list-style-type: none"> ● Detection of the polarization of light upon reflection on sample. ● Refractive index is used to obtain layer thickness data 	[63, 64]
QCM-D	Ensemble	<ul style="list-style-type: none"> ○ Measures changes in acoustic resonance frequency and energy dissipation. ○ Change in frequency and energy dissipation is used to obtain mass and viscoelasticity of layer 	[65, 66]
LSPR	Ensemble	<ul style="list-style-type: none"> ● Shift in resonance due to local index of refraction. ● Refractive index is used to detect molecular binding events and to obtain information on thickness 	[31, 67]
XPS	Ensemble	<ul style="list-style-type: none"> ○ Measures the energy of photoelectron emissions. ○ Intensity of photoelectron energy is used to quantify and identify surface element 	[68, 69]
AFM	Ensemble	<ul style="list-style-type: none"> ● Measures the deflection of cantilever which is relative to the sample position ● Topography and thickness information and force-distance relationships of protein-surface interactions 	[70, 71]

The most simplified kinetics model for protein adsorption is the Langmuir adsorption model, which assumes first-ordered kinetics of proteins adsorbing to and desorbing from the surface to establish a homogenous monolayer of protein with a specific surface coverage level at any given time [72]. The protein surface coverage (θ) can be expressed as a function of time (t) such that,

$$\theta(t) = \frac{c}{c + k_d/k_a} (1 + e^{-(k_a c + k_d)t})$$

where, c is the protein concentration in bulk solution and k_a and k_d are the adsorption and desorption rate constants, respectively. Based on the equation, the steady-state adlayer, which is also referred to as the Langmuir isotherm, would eventually reach complete surface saturation (*i.e.*, $\theta \approx 1$) when the protein concentration is significantly increased (*i.e.*, $c \gg k_d/k_a$). However, due to the random placement of proteins simultaneously arriving on the surface and the inevitable disorderliness, there will be gaps between two adjacent proteins, in which at times would be spatially insufficient to fit another protein molecule as demonstrated in the packing example of disks in Figure 2.2. Therefore, the maximum surface coverage would be nowhere close to 1. A random sequential adsorption (RSA) model estimates that the maximum surface coverage would fall between 0.54 when assuming circular surface area of contact or 0.56 when modeled as squares and is referred to as a jamming limit [73]. Experimentally determined surface coverage has been reported to be greater than the jamming limit, but this is most likely due to post-adsorption rearrangement of proteins and lateral diffusion which would improve the packing efficiency [74]. Still, the RSA model has merit in recognizing an imperfect protein surface coverage, which could then be expanded from the Langmuir model with an additional factor, θ_{max} , as follows:

$$\theta(t) = \theta_{max} \frac{c}{c + k_d/k_a} (1 + e^{-(k_a c + k_d)t})$$

Figure 2.2 Simulation results of random sequential adsorption model using circular disks (Image adopted from Cieřla and Nowak [75] with permission from Elsevier).

Table 2.2 Kinetic models hypothesized for protein adsorption studies

Model	Description	Schematic	Ref
Langmuir	Fixed adsorption sites ○ Full coverage ○ Monolayer		[76]
RSA	Random adsorption sites ○ Jamming limit ○ Adsorption based on spatial availability		[77]
Two-phase	Conformational Change ○ Post-adsorption transition in protein conformation		[78]
Multi-phase	Protein spreading ○ Progressive unfolding of protein conformation		[79]
Rollover	End-on to side-on reorientation of protein binding orientation		[80]
Cooperative Piggyback	Cooperative adsorption to protein clusters		[81]
Cooperative Tracking	Protein-protein interaction to guide subsequently arriving proteins to open binding sites		[82]

With lateral interactions and surface-induced denaturation of proteins, the base assumptions for the Langmuir model would no longer be applicable and the parameters must be adjusted to accommodate the deviations. Based on the theorized mechanistic details of protein adsorption such as protein spreading, protein reorientation, or other lateral interactions, several variations in the adsorption kinetic models have emerged as seen in table 2.2 . However, despite the several decades of theoretical work on protein adsorption, the variable parameters are yet to be agreed upon for a universal kinetic model that would closely match experimental observations. As such, comprehensive and

systematic studies are imperative to further advancing our knowledge in theoretical or simulation approaches for verification and refinement of the kinetic models.

2.2 Extrinsic Factors

Within a confined experimental setting, the protein behavior can be preconditioned by controlling the environmental factors such as temperature, solution pH, and ionic strength [3, 83]. For example, a change in temperature would change the protein's diffusivity to the surface as well as the lateral diffusion at the solid-liquid interface in accordance with the Stokes-Einstein relationship defined as,

$$D = \frac{3k_B T}{16R(\eta_B + \eta_I)}$$

whereby, k_B is the Boltzmann constant, T is the absolute temperature, R is the hydrodynamic radius of the protein, and η_B and η_I are the viscosities of the bulk solution and the liquid-surface interface, respectively [33]. In addition, the input of thermal energy can affect the conformation of the protein, which can lead to increased diffusivity and greater affinity to the surface [51, 83].

The effect of solution pH is straightforward as it can conveniently tune the net charge of the protein molecules and the adsorbent surfaces, simultaneously, making it easy to modulate the electrostatic forces [60, 84-86]. In particular, the adsorbed protein mass can be maximized when the pH is near the isoelectric point, whereby the net charge on proteins is neutralized which minimizes the repulsion between proteins and promote protein-protein interactions near the surface [59, 60]. In this case, the protein adsorption kinetics may be slower, but with close packing of surface adsorbed proteins, the protein uptake eventually exceeds the accumulated amount observed when pH is set to maximize the charge difference between protein and the substrate [60].

Modulation of the solution's ionic strength is effective in mitigating the electrostatic interactions by supplying counterions to balance out the effect of surface charges on proteins and substrates. Otherwise known as the charge shielding effect, the Debye length of particulates of which the electrostatic effects persist, would effectively collapse with sufficient salt ions [87-90], bringing like-charged particles closer in proximity while neutralizing the attractive forces of oppositely charged pairs [3]. In a

protein adsorption event, the charge shielding effect would increase the packing density of proteins at the surface [91], and likewise would increase aggregation propensity in bulk solution when absent of an adsorbent [92].

In reality, the theoretical predictions have limitations because, for one, the contributions of the extrinsic factors are either synergistic or antagonistic, and second, the core principles of the formulated predictions are originated from polymer adsorption studies [93]. Although proteins are essentially biopolymers and the concept of adsorption is similar, there are intrinsic distinctions that make protein adsorption studies unique and rather more complex, which will be discussed further in the next section. Nonetheless, because of the intrinsic differences between protein species, the correlated influence of the extrinsic factors can vary.

2.3 Intrinsic Properties

2.3.1 Adsorbate Properties

Proteins are biopolymers and share similar attributes as synthetic polymers in terms of adsorption behavior. However, with submolecular complexity, the adsorption of proteins are not as straightforward as synthetic polymers and it becomes difficult to come up with a uniform law of order in regards to how proteins would interact with the surface. According to Andrade *et al.*, each protein possesses its own ‘molecular personality [12]’. The relevant ‘personalities’ in this context include size, structural rigidity, charge distribution, wettability, and etc. Generally, the size of proteins determines the diffusivity to the surface while the charge distribution affects the electrostatics and structural properties determine the adsorption orientation and packing efficiency on the surface. In terms of rigidity, proteins can be classified as ‘hard’ or ‘soft’ proteins depending on the intermolecular cohesion strength (*i.e.*, conformational stability). Hard proteins (*e.g.*, Lysozyme) generally undergo limited conformational changes upon adsorption and only adhere to polar surfaces only if electrostatically attracted, while soft proteins (*e.g.* albumin and Immunoglobulin) make extensive changes in conformation upon adsorption resulting in a net entropy gain that is significant enough to adsorb against disapproving circumstances [11, 94].

The structural denaturation of proteins is interchangeably referred to as the thermodynamic stability of proteins. The underlying principle is similar for all cases of thermodynamic protein denaturation events, whereby the process is carried out from the gain in entropy through a medium of energy input, and an offsetting balance by a collection of weak and/or strong interactive forces to maintain stability. For example, heat denatured proteins are counterbalanced with hydrogen bonds from the surrounding water [95-98], chemically denatured proteins (*i.e.*, urea-mediated) are stabilized by solubilizing the hydrophobic regions of the proteins [99, 100], whereas detergent-mediated denaturation (*i.e.*, sodium-dodecyl-sulfate) of proteins are maintained through extensive hydrophobic bonds [53, 101]. The denaturation of protein *via* surface adsorption is also stabilized by submolecular factors within the scope of protein-surface interactions that include hydrophobic bonds, hydration bonds, ionic bonds, and more. Therefore, the adsorption behavior becomes unique for each protein species depending on the amino acid sequence of the protein, the batches of functional domains, and the hierarchical secondary structures, which can be aligned and compared by a detailed analysis of the functional attributes such as hydrophilic or hydrophobic, charged or non-charged, polar or non-polar per geometric locale [102-106].

A substrate in a blend of proteins will be subject to a dynamic exchange of adsorbing proteins with each protein having a unique value input into the composition of the protein layer. With newly arriving or readsorbing proteins, there will be instantaneous shifting in affinity allowing the proteins in the near vicinity to repeat the cycle of adsorption and desorption until a stable protein coating is formed on the outermost surface of the sorbent. Initially, the adsorption behavior will be size dependent with smaller proteins with faster diffusivity to interact with the surface, which would then be replaced by larger proteins with higher binding affinity in accordance to the Vroman effect [107]. However, further complexity beyond the Vroman model is expected with structural rearrangements and conformation changes in proteins which could either expose or conceal the functional groups leading to other anomalies such as protein aggregation, shifts in affinity, and cooperative or competitive binding [22].

2.3.2 Adsorbent Properties

The submolecular details of a protein can dictate the electrostatic potential and structural stability in an adsorption event. However, the adsorbent properties as its counterpart are equally significant, as it is capable of extracting out very different behaviors even from the same protein molecule. Yet, before discussing the specific protein-surface interactions primarily in accordance to the chemical functional groups, it is also important to keep in mind that the proteins would have to first diffuse into a pseudo-2D interface consisting of water molecules lining the surface, from where the proteins would undergo an adsorption-dehydration process to replace the water molecules on the adsorbent to establish the initial protein-surface interactions [23, 108-110]. Therefore, when discussing the intrinsic properties of the adsorbent such as the surface chemistry, topographical features, and crystallinity, it is important to include how each property affect the wettability of the surface [111].

The surface functional groups determine charge, polarity, and ultimately the affinity to the proteins. Most importantly, by controlling the density of surface functional groups, the likelihood of water coupling on surface can be determined (*e.g.*, *via* density of hydrogen bonding), which is the core guideline to the classification of hydrophobic and hydrophilic materials. Hydrophobic surfaces are generally accepted to be the preferable adsorbent for adsorbing proteins because of the hydrophobic interactions between protein and surface. However, in more detail, this is also correlated to the perturbation of water molecules that align the surface, whereby a weaker water-surface coupling on hydrophobic surfaces makes it easier to be replaced by incoming proteins and hence is more energetically favorable [112]. In the opposing case, hydrophilic surfaces can repel protein adsorption to a certain extent with the greater hydration layer and can be significantly improved with additional surface treatments to achieve an antifouling surface. Polymer brushes using polyethylene oxide (PEO) materials are examples of antifouling agents that are popularly explored on surfaces providing extreme hydrophilicity and steric hindrance [113, 114]. Other methods include oxygen plasma or UV-ozone treatment [115], or self-assembled monolayers (SAM) of functional molecules [45, 102, 116], polymer grafting-to and grafting-from the surface [117], and surface coating with self-assembled lipid bilayers, or supported lipid bilayers (SLB) [118, 119].

Table 2.3 Surface modification methods and techniques

Method	Technique
Chemical Modulation	<ul style="list-style-type: none"> ○ Oxidation reaction ○ Chemical vapor deposition ○ Addition reactions (Acetylation, Chlorination)
Coating	<ul style="list-style-type: none"> ● Solvent coating ● Pulsed laser deposition ● Plasma spray ● Rapid prototyping
Grafting	<ul style="list-style-type: none"> ○ Plasma grafting ○ Photografting ○ Radiation grafting ○ Chemical covalent grafting ○ Ion beam sputter deposition
Patterning	<ul style="list-style-type: none"> ● Lithography ● Direct-wiring ● Self-assembly ● 3D patterning
Roughening	<ul style="list-style-type: none"> ○ Plasma Etching ○ Mechanical roughening ○ Ion milling ○ Corona discharge
Biomolecular films	<ul style="list-style-type: none"> ● Langmuir-Blodgett film deposition ● Layer-by-Layer precipitation ● Supported lipid bilayer

In general, proteins favor adhesion to surfaces which are non-polar, charged, and with high surface tension. This was confirmed through studies that reported the adhesion energy of proteins to surfaces via atomic force microscopy [120-122]. In relation to that, a general finding that proteins favor adsorption on hydrophobic surfaces could possibly arise from the tendency for non-polar surfaces which can destabilize the proteins that would strengthen protein-protein and protein-surface interactions [102].

2.4 Implications in Nanotechnology

The application of nanoparticles in medicine has opened a new paradigm of therapeutic approaches to human diseases [123, 124]. The nano-scale technology has focused on exploiting the newly gained capabilities of breaching unexplored terrains with

deeper penetrability and prolonged sustainability within the body [125, 126]. In spite of the success in quality control, nanoscale manipulability, and *in vitro* clinical efficacy [127, 128], however, the hype is met with lackluster results with only a handful of commercialized applications [129-131]. The difficulty in its commercial translations include the conventional issues relating to strict regulations, manufacturing issues, or financial barriers, but the more significant reason is due to the unexpected complications from *in vivo* clinical trials, much like the fatal phase I trial that took place in France [132]. Further investigations on the nanoparticles exposed to *in vivo* or *ex vivo* conditions revealed a cloud of proteins surrounding the nanoparticle, or “protein corona”, which appeared to be in part responsible for the triggering of subsequent biological interactions [133, 134]. Essentially, nanoparticles were found to gain new bioidentities with the protein corona decorating its surface overruling the intended functional designs of the nanoparticle as seen in figure 2.3 [135, 136]. Due to the apparent issue at hand, subject matters on protein corona has gained the attention of a wider scientific community in response to the needs for new perspectives in the application of nanoparticles and especially to answer questions regarding the safety of nanomedicine [137].

Figure 2.3 The biological identity of nanoparticles determined by the protein corona and the relevant physiological response (Image adopted from Walkey and Chan [138] with permission from RSC)

Past work on protein corona had mainly focused on the compositional information or the proteomics of the protein structure. The unraveling of the proteomics had initially met difficulties facing the dynamic nature of the complex protein assembly, however, with exhaustive work, a total of 125 unique plasma proteins were identified adsorbing on one or more of various nanomaterials when incubated in blood [138-141] as seen in figure 2.4. The structure of protein corona is believed to be in a bilayer-like system where proteins with high affinity ('hard corona') would form the primary layer directly interacting with the surface while a secondary layer of loosely bound and dynamically transitioning proteins ('soft corona') would exist either by sparsely interacting with the surface or by protein-protein interactions [142], which is not to be confused with hard and soft proteins that is coined for the proteins' conformational stabilities. Further, it has been suggested that the physiological properties exerted from the hard corona species may have a higher impact in determining the functionality of the nanoparticle-corona complex and the relevant biophysical events. Hence, engineering solutions to address protein corona issue had been fixated on controlling the hard corona binding profiles by means of anti-fouling [143] or camouflaging [144, 145] to mitigate the unnecessary protein accumulation or the excessive biological responses. Both approaches are rationally valid, but both seize to look beyond the behavior of nanoparticles. It is arguably important to understand and establish a profile for nanoparticles affected by the surrounding, but in a protein-surface interaction scheme, it would be incomplete without understanding the protein behavior affected by the nanoparticles.

Fundamental adsorption studies are necessary since the proteins undergo far more complex and interesting dynamics when in the proximity of substrates unlike other rigid molecules [22]. Changes in binding orientation or structural rearrangement of proteins can cause significant shifts in adsorption/desorption kinetics while conformation changes in protein structure can significantly alter binding affinity towards surfaces or towards other proteins [1]. The underlying fundamental mechanisms of protein adsorption must be sufficiently analyzed before addressing the complex nano-bio interactions in order to come up with a more practical and viable solutions for nanoparticle applications.

Figure 2.4 Example of protein corona composition categorized into seven subgroups based on biological role in host system (Image from Saha *et al.* [141] with permission from ACS)

2.5 Reflection on Literature

The investigation of protein adsorption on solid surfaces is a conceptually simple but realistically complex subject matter. Over several decades of continuous effort there

has been significant advance in knowledge but also an equivalent amount of controversy owing to the multifaceted nature of the study but also largely due to the lack of consensus within the scientific community involved in this particular project. Some examples include the concept of reversibility/irreversibility of protein adsorption[61, 74], the mechanism of the Vroman effect[146-148], the argument of protein layer being either monolayer or multilayer[36, 149, 150], or the applicability of thermodynamic models [44]. In a more practical perspective, the contradictions are understandable in consideration of the intricate dynamics of the finite experimental conditions and the potential of each deviation causing a snowball effect. Therefore, there must be an ongoing effort to increase the accuracy and reliability of experimental work. Nonetheless, with the development of measuring techniques, there are yet better opportunities that can be exploited within the havoc, but particular attention to detail and reliability will always be important.

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Chapter 3

Experimental Methodology

There are multiple levels of complexity when attempting to figure out the fundamental process of protein adsorption. Therefore it is important to take a holistic approach in addressing each experimental data with a systematically designed experimental plan and backed with thorough empirical analyses. The main contributive factors that determine the overall protein behavior include the solution conditions (e.g., ionic strength, temperature, pH, etc.), intrinsic physicochemical properties of proteins, and the materialistic properties of the substrate, all being interdependent of each other. As such, each protein-surface interaction is unique. The experimental methodologies proposed herein, focuses on the interaction of bovine serum albumin and silica to observe the intrinsic and extrinsic factors that determine the overall outcomes. In order to better understand the proteins' behaviors in accords to the surface, the proposed experimental designs encompass the protein behaviors in bulk solution and in liquid-surface interfaces, separately. The analytical devices for each conditional setting have been adequately appropriated with complimentary pairing, and each extracted sets of data had been thoroughly investigated in multiple perspectives and in different facets. In the end, the fragmented results were pieced together, to draw out holistic conclusions with sufficient evidences to support the main proposals presented in this project.

3.1 Rationale of Approach

The study of protein adsorption had been considered key in determining the parameters of biomaterial surface design in the context of biomedical and industrial applications. As material scientists, an evidence-based proposal must be in order in defining the surface structure of a biomaterial to establish a proper surface-property relationship for applications. However, due to the many inconsistencies in literature, a clear-cut guideline to the reoccurring protein adsorption related issues are yet to be made, and with obvious limitations, risks and inefficiencies when relying on trial-and-error based approaches. Practical solutions are founded on recognizing the fundamental biochemical interactions and mechanisms within protein adsorption. When it comes to its fundamental studies, there is still a large knowledge gap that is being largely dismissed either purposefully or out of ignorance. For example, bovine serum albumin is generally accepted as the golden standard for surface passivation and various biological assays (*e.g.*, Western blot or ELISA) even though with an intuitive understanding that the protein-surface interaction may be unfavorable for many substrates.

Herein, a holistic problem solving approach is emphasized recognizing the importance of understanding a protein adsorption event in its entirety, and with systematic characterizations for thorough analysis. The project starts from assessing the external factors, namely the solution conditions, and gradually connects with the intrinsic factors that dictate the adsorption behavior of proteins. Most importantly, the investigation takes into account the state of the proteins in solution prior to its surface uptake, separately, to include protein preconditioning within the scope of protein adsorption, but also to draw links between protein-protein interactions and protein-surface interactions. For simplicity, bovine serum albumin was chosen to be the model protein and silica to be the model substrate. The analytical devices used for the entire project is described in the following section.

3.2 Material Selection and Preparation

3.2.1 Protein

BSA had been selected as the primary model protein for the entire project. BSA had been the most conventionally used model protein for many past protein adsorption studies being one of the most abundant natural proteins and as the most commonly used surface passivation agents in molecular biology [1-3]. The physicochemical properties of the proteins are well cited with an isoelectric point of 4.7 giving it an overall negative charge distribution at physiological conditions (*i.e.*, pH 7.4), and as a relatively large heart-shaped globular protein with a molecular weight of 66.5 kDa and an approximate dimension of 14×4×4 nm [4-7]. HSA was also used in this project, which is homologous to BSA with near identical physicochemical properties and 76% match in terms of amino acid sequence alignment [7, 8]. The homologous protein was used to define the subtle differences in protein behavior depending on the intrinsic conformational stability factors of proteins as presented in Chapter 6.

Lyophilized proteins were obtained from Sigma-Aldrich (Singapore) and were freshly dissolved in aqueous buffer in desired concentrations immediately before use and the concentrations of the protein samples were verified using UV-Vis spectroscopy (S-220, Boeco, Germany) at 280 nm wavelength using molar extinction coefficient of 43,824 and 35,700 M⁻¹ cm⁻¹ for BSA and HSA, respectively [9]. Where applicable, heat-treated proteins were obtained by incubating stock aliquots of protein solutions in a water bath for 30 min, which were then cooled for 2 hours at room temperature. The protein solution stocks were stored at room temperature when required.

3.2.2 Substrate

Silica-coated sensor chips (QSX303, Biolin Scientific) were used for the quartz crystal microbalance-dissipation (QCM-D) experiments. The surface of the sensor was cleansed by sequential rinsing using 1% SDS, Milli-Q water and ethanol to remove any organic contaminants, and was dried under a gentle stream of nitrogen gas. The sensor chip was then treated with oxygen plasma (PDC-002, Harrick Plasma, Ithaca, NY, USA) at maximum radiofrequency power for 2 min to oxidize the silicon substrate, then immediately assembled into the measurement chamber to preserve the surface properties throughout the experiment.

For the localized surface plasmon resonance (LSPR) system, the sensor chips (Insplosion AB) consisted of well-separated (~8% surface coverage) gold nanodisk arrays on a fused silica substrate, as fabricated by hole-mask colloidal lithography [10]. The sensor surface was coated with a thin, conformal layer of silicon nitride (~10 nm thick). Immediately before experiment, the sensor chip was sequentially rinsed with Milli-Q water and ethanol, and dried under a gentle stream of nitrogen gas followed by treatment with oxygen plasma at maximum radiofrequency power for 2 min. The latter process results in the formation of a silica layer on the sensor surface.

For atomic scope microscopy (AFM), a silicon wafer (Latech, Singapore) was similarly prepared by the rinsing, drying, and oxidation process mentioned above to prepare bare silica substrates for protein coating.

3.2.3 Aqueous Buffer

Tris based aqueous buffer was used throughout the experiments in this project. Briefly, the buffer solutions were prepared by dissolving 10 mM tris(hydroxymethyl)-aminomethane (Tris) into deionized water that was filtered through a Milli-Q water purification system (18.2 M Ω ·cm resistivity at 24 °C) and with varying concentrations of NaCl (Sigma-Aldrich) to modulate the ionic strength conditions (*i.e.*, 0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM). All buffer solutions were titrated to pH 7.5 using 1 M HCl (Sigma-Aldrich), and filtered through 0.22 μ m membrane filter before use. Stock buffer solutions were stored in room temperature and concealed away from sunlight when not in use.

3.3 Solution-based Analytical Devices

3.3.1 Dynamic Light Scattering

DLS is used to characterize the size of proteins suspended in liquid. The method of detection initiates by collecting the scattering light intensity, $I(t)$, as a function of time and converts the fluctuating intensity, $I(t+\tau)$, into a correlation curve (g).

$$g^2(\tau) = \frac{\langle I(t) \cdot I(t + \tau) \rangle}{I(\tau)}$$

The correlation curve is fitted into exponential functions whereby the exponential decay factor (Γ) can be derived, which proportionally relates to the diffusion coefficient (D) depending on the wave vector (q).

$$\Gamma = q^2 D$$

The obtained diffusion coefficient can then be used within the Stokes-Einstein equation to obtain the hydrodynamic radius (R) as a function of viscosity (η) and Temperature (T) of the solvent.

$$D = \frac{k_B T}{6\pi\eta R}$$

The obtained hydrodynamic radius is defined as the size of a hypothetical hard sphere that diffuses in the same rate as the measured particle. The measurement, however, is inaccurate for many biomolecules since it measures the apparent size of the hydrated molecule, which includes the surrounding hydration layer around the particle of interest.

Figure 3.1 Depiction of the hydrodynamic radius (R_H) of proteins in comparison to a spherical object and the distinction from radius of rotation (R_r), radius of gyration (R_g), and mass radius (R_m)

NanoBrook 90 Plus particle size analyzer (Brookhaven Instruments, Holtsville, NY) was employed with a 658.0 nm monochromatic laser set at a scattering angle of 90° to avoid loss of signal due to reflection. Each sample was measured five times and the resulting average and standard deviation were reported for comparison ($n=5$). The measured intensity was fitted to the autocorrelation function to yield an intensity-weighted size distribution through a deconvolution process using a non-negative least squares (NNLS) method. The intensity-weighted detection is able to resolve particle sizes

that are in the range from merely a few nanometers up to a few microns. However, the reliability of the data is questionable with polydispersed samples due to the inherent bias towards larger particles in this particular method of detection [11]. To make note of the uniformity of the particle sizes, DLS reports a polydispersity index (PDI), which is the square of the standard deviation divided by the mean. In a simplified point-of-view, a PDI of 0.0 to 0.1 would classify as a monodispersed sample while samples with a PDI above 0.1 would be considered polydispersed. A PDI greater than 0.4 is classified as an extremely polydispersed sample, and the corresponding data would be disregarded due to unreliability.

3.3.2 Nanoparticle Tracking Analysis

NTA, like DLS, observes the Brownian motion of particles to acquire the size of the particles in solution. While DLS collects the ensemble average of the Brownian motions in the form of scattered light, NTA tracks the two-dimensional trajectory of individual particles within the pane of sight and reports the amplitude of the individual Brownian motion as a mean square displacement (MSD). From the recorded MSD of particles, the diffusion coefficient (D) can be extracted by a linear function of time (t),

$$MSD = 2nDt$$

where, n is dimensionality ($n=2$ for NTA), which can then be used to obtain the hydrodynamic radius using the same method as above (*i.e.*, Stokes-Einstein equation). NTA, indeed, demonstrates an advantage in accurately evaluating the polydispersed sample pool [12], which would be otherwise incompatible with DLS, but since the technique relies on the optical recognition of particles in the video images captured by the built-in CCD camera, the practical range in detectable size is narrower at 30 to 1000 nm. Nevertheless, a broad-spectrum of protein size distribution can be evaluated with the pairing of NTS with DLS.

Herein, a LM10HS (Nanosight Limited, Amesbury, UK) supplied with a sensitive scientific CCD camera, an optical microscope with 20X objective lens, a blue laser module (wavelength 405nm, LM10 version C) was used for the NTA measurements, and the NTA 3.0 software was used to resolve the recorded results. The measurement chamber was connected to a peristaltic pump for controlled injection and the chamber

was rinsed sequentially with 1% SDS and water to remove any visible contaminants. Prior to sample injection, aqueous buffer strained through a 0.22 μm membrane filter was injected into the channel to verify chamber stability and each sample was recorded in triplicates without flow in 30 s frames for detailed analysis.

3.3.3 Circular Dichroism

Circular Dichroism (CD) spectra of proteins provide information on the secondary structure of the protein, and to a limited extent, the tertiary structural information. The protein solutions were injected in a 1 mm path length glass/quartz cuvette and measured using AVIV Model 420 spectrometer (AVIV Biomedical, Lakewood, NJ, USA). The background spectrum was also measured using protein-free aqueous buffer solution, which was used to normalize the background noise values from each sample measurements. The CD spectra of the protein samples were recorded at room temperature of 24 °C across the spectral range of 190 to 260 nm in 0.5 nm intervals with a 4 s averaging time and 1.0 nm bandwidth. Each protein measurements were performed in triplicate and the average values were reported as final.

For the purpose of analysis, ellipticity information, originally expressed in units of millidegrees, was converted to Molar Residue Ellipticity (MRE) by the following equation:

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

where n represents the number of amino acid residues, c is the protein concentration, and l is the path length of the cuvette. The MRE values at 208 nm and 222 nm were used to estimate the α -helicity of BSA proteins using the Greenfield and Fasman method as shown below[13, 14]:

$$\alpha \text{ helicity}(\%) = \frac{[\theta]_{208} + 4000}{-33000 + 4000} \times 100$$

$$\alpha \text{ helicity}(\%) = \frac{[\theta]_{222} - 3000}{-36000 - 3000} \times 100$$

In addition, the ratio of MREs at 222 nm and 208 nm ($[\theta]_{222}/[\theta]_{208}$) were used to predict the tertiary structural orientation of the individual α -helices through a rough

categorization whereby a ratio of approximately 0.8 would represent stable single-stranded α -helices and ratios exceeding 1.0 would shift the tertiary structural tendencies to favor intramolecular or intermolecular coiled-coils [13, 15, 16].

3.4 Surface-based Analytical Devices

3.4.1 Quartz Crystal Microbalance with Dissipation (QCM-D)

To investigate the real-time protein adsorption kinetics on the substrate, Q-Sense E4 instrument (Biolin Scientific, Gothenburg, Sweden) was utilized for the QCM-measurements. The acoustic biosensor applies oscillation on the piezoelectric quartz crystal and the change in resonant frequency ($\Delta f = f_0 - f$) is measured at real-time. From the measured Δf , the accumulation of mass (Δm) on the crystal is computed by the Sauerbrey equation,

$$\Delta m = -\frac{C}{n} \Delta f$$

whereby C ($= 17.7 \text{ ng cm}^{-2} \text{ Hz}^{-1}$ at $f/n = 5 \text{ MHz}$) is the mass sensitivity constant and n is the overtone number of resonance frequency. However, the Sauerbrey equation assumes air or vacuum conditions, while the actual measurements are carried out in liquid conditions. The resonance of the sensor would be dampened due to the fluidic viscosity, which would then affect the mass estimation. Therefore, by pulsating the oscillation across the sensor, the absolute dampening effect can be calculated by measuring the dissipation of the shear wave. The dissipation factor per oscillation (D) in the system is obtained based on the following formula:

$$D = \frac{E_d}{2\pi E_s}$$

where E_d is the energy dissipation of one oscillation and E_s is the stored energy within the oscillating system. The change in energy dissipation ($\Delta D = D - D_0$) is recorded, simultaneously, with the Δf to track the real-time changes in viscoelastic properties of the adsorbed layer. In general, a larger ΔD value represents a soft elastic layer and a smaller ΔD value represents a more rigid layer. Using the commercial program Q-tools software (QSense AB, Sweden), Δf and ΔD information can be fitted into the Voigt-Voinova

viscoelasticity model to obtain the film density, viscoelastic properties, and film thickness, respectively.

For the actual measurements, all solutions were introduced into the measurement chamber through 0.76 mm inner diameter tube (Pharmed®, Ismatec SA, Switzerland) with peristaltic pump (ISM833C, Ismatec SA) operating at nominal flow rate of 0.1 mL/min. Protein solutions were introduced into the measurement chamber and the shifts in frequency and dissipation factors were recorded at several different overtones ($n = 3, 5, 7, 9$ and 11). The fifth overtones data (25 MHz) were presented in data, unless otherwise stated.

In general, the protein adsorption studies in the QCM-D system were carried out in predefined steps, which include the baseline formation step, protein injection step, followed by an aqueous buffer wash step. Any additional treatments (*e.g.*, serum fouling) were conducted in subsequent sequence only after the previous step came to full conclusion as presented by stabilization in signals.

For data analysis, the frequency shift as a function of time and the energy dissipation shifts as a function of time were displayed as default for conventional comparison, and the Voigt-Voinova modeling was implemented to obtain critical information on the protein film. Additionally, the time independent dissipation plots as a function of frequency was used extensively to analyze the post-adsorption events in the protein-surface interactions, and more specifically to categorize the various protein-surface interactions that may occur in the post-adsorption process. Altogether, QCM-D data provided a wealth of information on the protein adsorption process, post-adsorption events, protein desorption process, and on the characteristics of the protein layer at saturation and the final outcome after buffer wash.

3.4.2 Localized Surface Plasmon Resonance (LSPR)

As discussed above, the interpretation of QCM-D measurements can be complicated especially when considering the effect of the hydration layer. The Voigt-Voinova viscoelastic modeling is still imperfect since the calculated fitting is based on false assumptions (*e.g.*, uniform film thickness, uniform film density, Newtonian liquid). Considering the analytical limitations and uncertainties, LSPR is another highly surface

sensitive technique that offers an alternative approach to analyzing the protein adsorption process. LSPR is an optical measurement, which contrasts with the acoustic measurements of QCM-D, that tracks the changes in the local refractive index near the surface making it suitable for the recognition of structural orientations of molecules adsorbing onto the surface. As a complimentary technique to QCM-D, it provides confirmatory data of the surface-induced behaviors of proteins.

LSPR measurements were conducted via indirect nanoplasmonic sensing (INPS) in which the changes in the dielectric properties of the sensor coating are tracked. The sensor chips (Insplorion AB, Sweden) consisted of well-separated (~8% surface coverage) gold nanodisk arrays fused on a silica substrate fabricated by hole-mask colloidal lithography. The individual gold nanodisks were about 22 nm in height and 150 nm in diameter at average after being coated with a conformal layer of silicon nitride that needed to be sufficiently thin (~10 nm thick) to remain with the nanoplasmonic evanescent field. Preliminary tests demonstrated a bulk sensitivity of about 120 nm per refractive index unit for the Si_3N_4 -coated nanodisks. Measurements of an ensemble-average of the sample were taken in optical transmission mode using an Insplorion XNano instrument (Insplorion AB) where a white beam of light would enter the measurement chamber to pass through the sensor chip (approximately 4 mm²) and transmit out a quartz glass window to be collected by a spectrophotometer for analysis. Extinction spectra were recorded and analyzed at a time resolution of 1 Hz, and the spectral resolution of the plasmon resonance was determined by high-order polynomial fitting wherein the centroid position and the full width at half-maximum (fwhm) were calculated.

Immediately before the experiments, the sensor chip was sequentially rinsed and treated with Oxygen plasma to form the silica layer on the surface. After surface treatment, the sensor chips were enclosed within a microfluidic flow-through chamber and liquid samples were introduced *via* a peristaltic pump at a flow rate of 50 $\mu\text{L}\cdot\text{min}^{-1}$, which is equivalent to an average flow velocity of 25 $\text{mm}\cdot\text{min}^{-1}$. The baseline LSPR response was established with aqueous buffer solution. After signal stabilization, the buffer solution was replaced with the protein solutions and the adsorption process was measured as plasmon peak shifts ($\Delta\lambda_{\text{max}}$) monitored at real-time. Red shifts in the spectra

were typically observed with the adsorption of proteins because of the higher refractive index of proteins in comparison to water, and were considered a quantitative measure to interpret the peak shift plot.

3.4.3 Atomic Force Microscopy (AFM)

Atomic Force Microscopy (AFM) was employed to identify the morphological properties of the BSA protein adsorbed on a silica surface. A commercial AFM system (NX-Bio, Park Systems, Suwon, South Korea) equipped with an Eclipse Ti optical microscope (Nikon, Tokyo, Japan) was used to obtain the images of samples that were prepared by adsorbing either native or denature proteins on a Oxygen plasma pretreated Silicon wafer (Latech, Singapore). Aluminum reflex coated silicon cantilever, PPP-NCHR (Nanosensors, Switzerland) with a spring constant of ~42 N/m and a resonance frequency of 100~150 kHz in liquid condition, was used in the non-contact mode while the sample was submerged in aqueous buffer. The images were acquired with a 0.1-0.5 Hz scan rate in order to minimize damage in the samples and to mitigate the signal noise. To estimate the thickness of the protein layer, a square test was conducted to obtain the height contrast between the indentation and protein-covered surface.

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Chapter 4: Results Part I

Controlling Adsorption and Passivation Properties of Bovine Serum Albumin with Ionic Strength Conditioning

In this chapter, the passivation properties of bovine serum albumin (BSA) on silica surfaces and the role of varying ionic strengths are investigated as to represent a simplified protein adsorption model platform. The investigation extends beyond electrostatic interactions and focuses on the relevant protein stability and protein aggregation in solution as key factors in post-adsorption passivation capabilities. Results indicated a lower protein uptake with higher ionic strength, but a greater surface area per bound protein molecule according to a Voigt-Voinova model analysis. On the other hand, a higher adsorption rate was observed at lower ionic strength conditions, but the surface passivation properties of the resulting adlayer under subsequent physiologically remained unchanged. Nevertheless, supplemented with gluteraldehyde cross-linking agents, the passivation properties of the adlayer were significantly enhanced. Ultimately, this study became the cornerstone of the entire project placing spotlight on protein stability as a major factor in determining adsorption kinetics as well as post-adsorption modulations.

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4.1 Introduction

Understanding the nonspecific protein-substrate interaction is important because of its integrated role in fundamental biology (*e.g.*, clotting, immune recognition) and industrial applications (*e.g.*, surface passivation, fouling) [1-7]. Throughout several decades of research, the step-wise stages of protein adsorption have been under scrutiny, and while the detailed processes remain unclear, the withstanding consensus of its process includes an adsorption stage (reversible or irreversible) through diffusion, followed by denaturation of proteins on the surface, leading to protein adlayer stabilization [4, 8-11]. With increasingly sophisticated analytical and microscopic measurements, more evidences reveal significant alterations in protein adsorption behavior dependent on surface coverages, emphasizing the significance of protein-protein interactions and protein-surface interactions at higher protein concentrations near the surface [12-15]. It is already well established that the protein characteristics (*e.g.*, size, conformation, hydrophobicity, and structure) [16-21], substrate properties (*e.g.*, topography, composition, surface charge) [22-26], and environmental conditions (*e.g.* ionic strength, solution pH, temperature) [27-31] are key factors in influencing the adsorption process. How each or a combination of such factors determine the surface coverage may provide important insights in better understanding the mechanistic details of protein adsorption.

The environmental conditions can be easily altered by means of control over ionic strength. While the role of ionic strength had been highlighted on several occasions for different protein-surface models [20, 32-36], its broad spectrum of effects on protein structure, substrate chemistry, and the corresponding behavior of adsorbed proteins at solid-liquid interfaces merit its further exploration. In general, ions affect the adsorption kinetics and total surface uptake of proteins by modulating the electrostatic interactions *via* charge-shielding effects [33-35] and simultaneously by direct binding to the protein surface [37-40]. Intuitively, the adsorption trends with respect to ionic strength would strongly depend on the net charge of the protein and the protein adsorbing material as well as the charge distribution of the interacting species. Yet, the effects of ionic strength are nuanced, and arguments solely based on the influence of charge-shielding can

oftentimes underestimate or be entirely insufficient in analyzing the observed trends in experimental data.

In one classical case study, a negatively charged apotransferrin (isoelectric point: 6.1) was adsorbed on to a negatively charged Si(Ti)O₂ surface at different ionic strength wherein an increased adsorption rate was to be expected at increasing ionic strengths in accordance to an enhanced charge shielding effect. On the contrary, Ramsden and Prenosil observed an overall decrease in protein uptake with increasing ionic strength, which was then quantitatively delineated through rigorous theoretical calculations to eventually discover an increase in the surface coverage per bound protein molecule that would cause interparticle repulsion, and therefore, thwart the diffusion of protein molecules to the surface [32]. Furthermore, an optimum level of salt concentration was predicted at which the surface coverage per protein molecule would be maximal, but a monotonic increase with increasing ionic strength was observed instead. Conclusively, the authors reiterated the discrepancies by suggesting a salting-out effect that would lead to the formation of protein oligomers, opening further discussions of a possible role of protein aggregation in determining the protein adsorption kinetics.

Bovine serum albumin (BSA) has been a widely employed model for protein adsorption studies being one of the most abundant natural proteins [41-50] and one of the most common surface passivation agents in molecular biology [51-53]. The physicochemical properties of the proteins are well cited as a negatively charged (isoelectric point of 4.7), and relatively large globular protein [54]. Despite its prevalence, the effects of ionic strength on BSA adsorption kinetics have so far been largely dismissed with most BSA related studies rather focusing on the effect of pH variations [42, 50]; there is no consensus to incubation conditions (*e.g.*, protein concentration, salt concentration) that give the best passivation performance [51, 55-57]; and the few previous studies that do investigate the role of ionic strength show inconsistent trends and conflicting arguments [27, 58-65], some of which appearing to be counterintuitive [59-61]. Therefore, the effect of ionic strength on BSA adsorption remains an outstanding question in many respects. Using single-molecule tracking analysis, McUmbler *et al.* reported a two-step adsorption process where an increase in adsorption rate of individual BSA molecules to silica surfaces was observed at higher ionic strength and physiological

pH primarily under the influence of electrostatic interactions, which was soon dominated by non-electrostatic interactions upon adsorption to the surface [48]. Such studies motivate promising directions to explore in the context of adsorbed BSA layers at saturation coverages, both in terms of fundamental understanding of protein-substrate and protein-protein interactions as well as practical application for preparing well-coated BSA layers for passivation applications.

To systematically investigate the effect of ionic strength on the behavior of BSA protein on silica, the study was designed in two parts. First, the protein stability and aggregation propensity in solution were observed by employing dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and circular dichroism (CD) spectroscopy experiments; then the adsorption kinetics and BSA uptake on silica surfaces were measured by quartz crystal microbalance-dissipation (QCM-D) experiments. Considering the general importance of BSA for passivation applications, we explored these aims in the context of both storage time before experiment (*i.e.*, aggregation is a kinetically dependent process [66, 67]), and the capability to prepare well-coated protein adlayers that could prevent nonspecific adsorption of serum onto the silica substrate.

4.2 Materials and Methods

4.2.1 Sample Preparation.

Lyophilized bovine serum albumin (A2153) and sodium chloride (S7653, BioXtra) were obtained from Sigma-Aldrich (St. Louis, MO). The BSA was dissolved in the appropriate buffer solution and the final concentration of all BSA stock solutions was 50 μ M, as determined by UV absorbance measurements at 280 nm. The buffer solutions contained 10 mM tris(hydroxymethyl)aminomethane (Tris) and the NaCl concentration was varied between 0 and 250 mM in 50 mM increments. The solution pH was adjusted to 7.5. The freshly prepared BSA samples were stored at 4 °C until experiment.

4.2.2 Dynamic Light Scattering.

The effect of ionic strength on the size distribution of BSA protein molecules was investigated by using the DLS technique. A NanoBrook 90Plus particle size analyzer

(Brookhaven Instruments, Holtsville, NY) was employed to measure the average size and size distribution of BSA molecules in the protein sample. All measurements were performed with a 658.0 nm monochromatic laser and recorded at scattering angle of 90° to minimize the reflection effect. The intensity-weighted size distributions of the BSA molecules were recorded every day for up to one month, and the average diameter and standard deviation of protein samples are reported from n=5 measurements.

4.2.3 Nanoparticle Tracking Analysis.

The NanoSight LM10 instrument (Malvern Instruments, Malvern, UK) was employed to investigate the size distribution of protein aggregates in solution. The nanoparticle tracking analysis (NTA) was performed with a 405-nm laser and the transmitted light was recorded by a built-in sCMOS camera. Dilute protein samples were introduced into the sample chamber manually with a sterile disposable syringe until the solution reached the tip of the nozzle. All measurements were performed at room temperature and under ambient conditions. The laser beam experienced Rayleigh scattering when incident with the protein molecules and the scattered light from each protein molecule was visualized by optical microscope (20x magnification) and recorded by the sCMOS camera for a period of 3 min at a rate of 25 frames per second. The measurement process was monitored and analyzed using the NTA 3.1 Build 3.1.46 software package. The hydrodynamic diameter of the protein molecules was calculated using the Stokes-Einstein equation from the time-resolved Brownian motion of individual particles tracked by the camera.

4.2.4. Circular Dichroism Spectroscopy.

CD spectra of the protein solution (50 μ M BSA) were measured using an AVIV Model 420 spectrometer (AVIV Biomedical, Lakewood, NJ, USA) with a quartz cuvette that has a 1 mm path length. The background spectra were also measured in equivalent Tris buffer solution and then subtracted from the sample spectra. The CD spectra were recorded at room temperature of 24 °C across the spectral range of 190 to 260 nm in 0.5 nm intervals with a 4 s averaging time and 1.0 nm bandwidth. Each measurement was performed in

triplicate. The ellipticity, expressed in units of millidegrees, was converted to the Molar Residue Ellipticity (MRE) by the following equation:

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

where n represents the number of amino acid residues, c is the protein concentration, and l is the path length of the cuvette. The MRE at 222 nm was used to calculate the α -helicity of BSA protein by using the following equation[68]:

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

4.2.5 Quartz Crystal Microbalance-Dissipation (QCM-D).

A Q-Sense E4 instrument (Biolin Scientific, Stockholm, Sweden) was utilized to monitor BSA adsorption on silica-coated sensor chips (QSX303, Biolin Scientific). The surface of the sensor was cleaned by sequential rinsing with Milli-Q-treated water and ethanol, followed by drying with a stream of nitrogen gas. The surface of the sensor chip was then pre-treated with oxygen plasma (PDC-002, Harrick Plasma, Ithaca, NY, USA) for 2 min immediately before experiment. All solutions were introduced into the measurement chamber through tubing with a 0.76 mm inner diameter (Pharmed, Ismatec SA, Switzerland) by a peristaltic pump (ISM833C, Ismatec SA) that was operated at a nominal flow rate of 0.1 mL/min. The shifts in the QCM-D resonance frequency and energy dissipation signals were recorded at several different odd overtones as a function of time. Data collected at the fifth overtone (25 MHz) are reported.

4.3 Principle Outcomes

4.3.1 Colloidal Stability of BSA Protein.

Changing the solution conditions has a direct impact on the colloidal stability of proteins and may lead to formation of aggregates which could eventually affect the overall adsorption behavior [69]. Antibodies are one such example where its affinity to surface resin becomes greater with aggregation than in its original monomeric state [70]. To evaluate the colloidal stability of BSA, the temporal state of the protein size distribution was monitored over several batches of protein solutions, which were each

treated with differing ionic strengths. With DLS analysis, the ensemble-average of the particles in solution was measured. Although the detectability of the system was robust enough to measure down to a few nanometers and up to a few microns, the reliability of the data in terms of accuracy was found to be questionable when dealing with polydispersed samples due to the inherent bias towards larger particles in its method of detection [71]. NTA was employed as a complementary technique to address the shortfalls of DLS, being capable of tracking individual particles but only in the size distribution range of 30 nm to 1000 nm. As a standalone device, NTA allowed accurate measurements of polydispersed protein oligomers but not monomers [72]. With the two complementary analytical tools combined, both BSA protein monomers and oligomers could be thoroughly characterized to paint a complete picture of the colloidal stability of BSA.

Figure 4.1 DLS measurements of BSA as a function of ionic strength and storage time

Initial measurements of freshly prepared BSA proteins solubilized in aqueous solutions with different ionic strengths (0-250 mM NaCl) were taken with DLS, then subsequent measurements were made periodically over a span of a month while kept at room temperature. The initial measurements reported an average diameter around 8 nm for all protein samples, which agrees well with the expected size of BSA monomers [73]. Over the evaluated period, the major fraction of the BSA molecules maintained stability in its monomeric form while some protein aggregation was observed in minor fractions. The kinetics of protein aggregation displayed non-monotonic trends with respect to increasing ionic strength, being most reactive in the range of 100 mM to 200 mM NaCl, while slower aggregation kinetics were observed in higher or lower ionic strength conditions. After two weeks, DLS and NTA analysis revealed a distinct population of BSA oligomers with increasing ionic strength, aligning well with previous studies on BSA fibrillation [74]. Further characterization by NTA showed an increasing randomness in BSA aggregate size distribution within solutions of ionic strengths greater than 100 mM. The observation becomes valid when the propagation of BSA aggregation is seen

Figure 4.2 DLS and NTA measurements of protein size distribution after 2 weeks of storage in different ionic strength treated solution

to be a combination of two distinct mechanisms [75, 76], in which the initial seeding (“primary aggregation”) is driven by nucleation *via* reversible attachment of monomers, while the proceeding populations (“secondary aggregation”) are formed *via* diffusion and coalescence [76]. With an increase in ionic strength, aggregate-aggregate coalescence becomes just as favorable as monomer addition, since the charge shielding effect occurs around the aggregates as much as the monomers [77, 78]. Hence, once colloidal stability collapses, a seemingly disordered proliferation of BSA oligomers would be expected adding diversity to the overall size distribution. Concurrently, the aggregation trends were still non-monotonic being most pronounced in the range of 100 to 200 mM NaCl, which can be attributed to the influences of both salting-in and salting-out effects and the complex interplay of protein-ion and protein-protein interactions [79]. Yet, for all protein solutions, the main population of BSA molecules still consisted of monomers indicating that the primary nucleation event remained as the rate-limiting step for aggregation regardless of ionic strength.

Apart from its colloidal stability, the secondary structure of the BSA protein was investigated to obtain correlative information on conformational stability as a function of ionic strength. It has been reported that the binding of salt ions to protein surfaces can affect the structural integrity of proteins [80-82], which in turn could indirectly influence the protein adsorption process [18]. Through CD spectroscopy, it was identified that there was no dependence on ionic strength in regards to the BSA protein secondary structure.

Figure 4.3 CD spectra of BSA secondary structure in varying ionic strength conditions

All samples, including those that were measured after the one-month storage period, showed similar minima peaks at 208 nm and 222 nm which can be converted to a helical fraction of around 67%. The results were in agreement with reported values found in literature [83], and confirmed the conformational stability of BSA across the tested range of ionic strength conditions regardless of the aggregation state of the BSA populations. This further supports that the detected minor population of aggregated BSA likely occurred from colloidal aggregation supplemented with effective charge shielding, largely in a reversible fashion that would not require extensive conformational alterations.

4.3.2 Protein Adsorption onto Silica Surfaces.

Figure 4.4 QCM-D analysis of BSA Adsorption trends in varying ionic strength conditions presented as (a) frequency changes and (b) energy dissipation changes as a function of time

Using freshly prepared BSA protein solutions, the near-surface interaction of the protein molecules onto a silica surface have been investigated through QCM-D experiments. The frequency and energy dissipation were monitored at real-time to characterize the accumulated mass and viscoelastic properties of the adlayer, respectively [84]. Briefly, the measurements captured the initial lag phase where a baseline signal would be established using aqueous buffer, then an injection phase where 50 μ M of BSA protein would be added, and finally a washing phase to remove artifacts from the formed adlayer. Each run was conditioned with varying ionic strengths and the final frequency and dissipation shifts were compared for analysis. In sum, a strong dependence on ionic

strength was observed with a monotonic frequency shift trend starting at approximately -72 Hz at 0 mM NaCl salt concentration gradually reducing to a -11 Hz at 250 mM NaCl.

Figure 4.5 QCM-D analysis of BSA Adsorption trends in varying ionic strength conditions. Data representing energy dissipation shifts as a function of frequency shifts

Correspondingly, an energy dissipation shift of 4-to-6 $\times 10^{-6}$ in the lower ionic strengths (0 – 100 mM NaCl) was observed whereas a change of 2.3 $\times 10^{-6}$ was measured at 250 mM NaCl salt concentration, indicating a more rigid BSA adlayer with increasing ionic strength. Looking at in a different perspective, the change in energy dissipation shifts can be traced by the unit changes in frequency to take a closer look at the properties of the proteins adsorbing on to the surface. The most unique outcome came from the 0 mM NaCl case, for which a positive slope continued until around -40 Hz followed by only minor changes in the energy dissipation with subsequent protein uptake. This suggests an early establishment of the protein adlayer with no further advances in the overall structure despite additional proteins arriving onto the surface, and a modestly different protein adsorption pathway in the absence of NaCl salt. When under the influence of NaCl, a moderate decrease in the slope was observed with increasing ionic strength at higher coverage regimes. In addition, a notable feature was evident for the plots of 150 mM NaCl and higher ionic strength conditions with a significant pivot in the slopes indicating partial dehydration in the protein adlayer [85].

Figure 4.6 Statistical analysis of maximum frequency shifts upon BSA adsorption ($n=6$) measured after various storage times (0, 2, 4, 7, 14, 21, 28 days) in different ionic strength conditioned buffer solutions

Over the course of the storage time, the same series of experiments was repeated to see the effect of the minor protein aggregation, if any. The minor fraction of protein aggregation within solution showed negligible effect as similar adsorption behavior was observed for the various storage time durations. In part, this was most likely due to the molecules remaining as monomers, as previously discussed. Because the overall events of protein adsorption is diffusion-limited, it is reasonable to expect that the primary construct of the protein layers would be governed by protein monomers possessing a faster diffusion coefficient than that of protein oligomers [8], at least until surface-induced reconfiguration of the adlayer takes place. Collectively taken together, the QCM-D data support that total BSA adsorption decreases with increasing ionic strength but with greater rigidity in the final construct. Also, it is noteworthy to point out a particular influence of the NaCl salt that could be worthwhile to investigate in a future project.

With evident differences in the adlayer properties, further analysis was rendered using the Voigt-Voinova viscoelastic model to extract the effective thickness of the protein adlayer based on measured trends. At 0 mM NaCl, the effective thickness of the final BSA protein adlayer was 12.7 nm, which is within reason assuming an upright

orientation of the bound BSA molecules. With increasing ionic strength, the effective thickness decreased down to 4 nm in 200 to 250 mM NaCl conditions, which directly supports the increasing footprint of each bound protein molecule with increasing ionic strength and is consistent with a correlative lower protein uptake at higher ionic strengths. Although, it may be difficult to verify whether the increased footprint arose from a change in preferred binding orientation and/or substrate-induced conformational spreading, nonetheless, the QCM-D measurements and viscoelastic modeling provided direct evidence of greater protein spreading at higher ionic strengths. To further elaborate on the significance of this finding, it is important to recall the conventional expectation of a system consisting of a negatively charged protein adsorbing onto a negatively charged surface, for which a greater charge-shielding effect would be the primary governing factor to overcome the overall electrostatic repulsive barrier. However, the opposite is upheld herewith, which in fact agrees well with the quantitative model proposed by Ramsden and Prenosil as discussed in the Introduction section [32]. The decreasing thickness observed for BSA adlayers in higher ionic strength conditions supports a greater protein spreading as indicated by the recorded energy dissipation shifts and, therefore, a more stable attachment of the protein, most likely involving surface-induced protein denaturation that is typically identified with partial dehydration [9]. While such variations have been scrutinized in depth for adsorbed vesicles in the context of shape deformation, the general convention for QCM-D protein adsorption measurements has been associating greater/stronger adsorption with larger frequency shifts [23, 86].

Figure 4.7 Hypothetical schematic of the height difference in BSA adlayer formed on silica with increasing ionic strength.

Based on empirical data, the total uptake of adsorbed protein molecules (defined as adsorption) does increase with greater frequency shifts. However, with insufficient footprint per molecule, the protein-surface interaction is limited to the initial attachment and the relative strength of the interaction becomes irrelevant to the shift in frequency. In hindsight, the stability of the protein-surface interaction appears to be inversely related to the increase in frequency, when in fact, it is only secondary to the attribution of the surface area per protein molecule. In summary, the total uptake of BSA proteins adsorbed onto silica decreases with increasing ionic strength on account of greater protein spreading.

4.3.3 Desorption of BSA Protein.

To extend the newly gained insights, the effect of buffer rinsing and desorption rates have been investigated to further advance on the post-adsorption stability of protein adlayers. After reaching a plateau in the frequency shifts while running the QCM-D measurements, the protein solution in the measurement chamber was exchanged out with protein-free buffer solutions of equivalent ionic strengths. The washing continued until recordings were once again stabilized and the total displacement in frequency was normalized by the maximum frequency peak for desorption rate analysis. For all cases, partial desorption of protein was observed. At 50 mM or greater NaCl salt concentration, approximately 30% or more of the adsorbed proteins were removed by the buffer wash.

Figure 4.8 Desorption trends of the adsorbed BSA layer upon buffer wash each normalized to the corresponding maximum frequency shift via protein uptake.

By contrast, much less were removed for the 0 mM NaCl case. While the observations may appear to be in direct contradiction to earlier discussions in regards to the stability of protein-surface interactions, it suggests that there is less variability in the binding orientation of individual BSA molecules and less disruption on the surface-bound proteins. Furthermore, a higher packing density is expected with limited protein spreading on the surface, and the steric packing could contribute to some level of intermolecular stability that would be sufficient enough to withstand the buffer wash [87-89]. Indeed, the fraction of desorbed BSA protein at 50 mM or greater NaCl salt concentrations was equivalent across this ionic strength range, which supports that a distinct subpopulation of weakly bound protein was more or less forcefully removed in order to promote more favorable interactions among the remaining bound proteins (*e.g.*, greater spreading on the surface) [90] (Figure 4.8). Taken together, the findings indicate that the largest uptake of bound protein was recorded in buffer solution without NaCl salt. It also reinforces the notion that BSA protein adsorption on silica follows different pathways depending on the presence of NaCl salt, with the specific ionic strength of the solution further controlling the extent of protein spreading.

4.3.4 Utility for Surface Passivation.

BSA is widely used as a blocking agent to prevent nonspecific adsorption and fouling in many biological assays and industrial applications [91]. However, the use of BSA for surface passivation is vaguely defined lacking a clear protocol for optimal results, especially without regards to the solvent in use. Based on the findings, it can be hypothesized that surface passivation can be tuned by control over the ionic strength. To test the hypothesis, an extended QCM-D protocol was conducted to measure the blocking efficiency of adsorbed BSA films against fetal bovine serum (FBS), which is a cocktail of diverse proteins and other biological components [92] (Figure 4.9). Using the same protocols for the adsorption of 50 μ M of BSA in either 0 or 150 mM NaCl, the rinsing step was slightly modified by using Tris buffer containing 150 mM NaCl to effectively remove weakly bound proteins and to roughly mimic the ionic strength conditions of FBS. Upon washing, a reduction of approximately 90% in mass was observed for the case of the protein adlayer formed without NaCl, whereas an approximate 60% removal was

recorded for the 150 mM NaCl case. Next, 100% FBS was injected causing a large negative shift in the frequency signal due to a combination of fouling and differences in the bulk solution properties [93]. The bulk solution was then exchanged back to Tris buffer with 150 mM NaCl for the final wash, and the net displacement in frequency before and after FBS incubation was recorded to evaluate the blocking efficiency. In comparison to a 0% blocking reference value where the FBS was injected on a bare silica substrate, the BSA layers formed under 0 mM and 150 mM NaCl salt concentrations demonstrated 18 and 23% blocking efficiency, respectively, without statistical significance. From the results, the importance of the ionic strength conditions particularly during the post-adsorption phase became immediately apparent in defining the final structure of the protein adlayer, and evidently in determining the blocking efficacy.

Figure 4.9 Evaluation of BSA blocking performance against FBS fouling and schematic of blocking procedure. (a) Comparison of BSA adlayer performance formed with or without NaCl, (b) Blocking performance of BSA adlayer with glutaraldehyde cross-linker, (c) comparison of all surface passivation techniques, and (d) the schematic of the formed adlayer with or without glutaraldehyde crosslinking

Among the observed outcomes, the 0 mM NaCl case was especially interesting in terms of retaining a high bulk protein concentration on the surface, albeit with relatively weak attachments to the surface. To take advantage of the high protein uptake, a glutaraldehyde cross-linker was introduced, which would covalently link adsorbed protein molecules to other neighboring proteins [94-96], and establish a lateral stability that would help maintain the accumulated BSA on the silica surface. Initially, the 50 μ M BSA solution was adsorbed on the silica surface in the absence of NaCl to set up the protein layer, which was followed by incubation with 0.1% glutaraldehyde in an equivalent NaCl-free buffer solution. An increase in frequency shift from -56 Hz to -43 Hz was noted indicating the hardening of the protein film from the cross-linking activity [97, 98]. Once, the signals were stabilized, the solvent was exchanged to Tris buffer with 150 mM NaCl in which a negative frequency shift was observed, marking a sharp contrast from the previous run. The frequency shift stabilized at around -55 Hz, then the protein layer was challenged with 100% FBS followed by a subsequent buffer wash. In result, a 57% blocking efficiency was recorded which is nearly a 3-fold improvement from before.

As such, the findings indicate that while the adsorption behavior can be manipulated by controlling the ionic strength, it was insufficient by itself to improve passivation efficiency because of the conformational changes of the bound proteins and the corresponding effects on the protein-substrate interaction as well as the effect on packing density. As a result, the passivation efficiency is around 15-20% in all cases. However, by understanding how ionic strength specifically affects the adsorption behavior, one can take advantage of specific protocols to purposefully guide the protein adlayers to be structured with particular attributes that would make it favorable for additional treatments, such as for cross-linkage with glutaraldehyde as demonstrated in this study. Altogether, through a systematic approach to solving a fundamental problem, the investigation was able to shed light on the underpinning interactions within the adsorption process of BSA onto silica surfaces, which further lead to an extraction of a practical solution to a lingering problem in surface passivation strategies.

4.4 Conclusion

In summary, we have systematically investigated how ionic strength affects the solution properties and adsorption behavior of BSA protein and utilized this knowledge to establish an improved protein-based surface passivation strategy on silica surfaces. In particular, we discovered that ionic strength had only modest effects on the aggregation properties of protein molecules in solution, whereas it had a striking effect on the adsorption behavior of protein molecules onto silica surfaces and strongly influenced the degree of protein spreading, as reflected in the total protein uptake at saturation and corresponding viscoelastic modeling of the adlayer's effective thickness. In this regard, a key innovative aspect of our findings was that ionic strength modulation of protein adsorption was further aided by covalent cross-linking in order to stabilize protein molecules that were initially adsorbed under low ionic strength conditions. The covalent cross-linking provided a structural reinforcement that made the protein adlayer largely impervious to subsequent ionic strength modulation (*e.g.*, solvent-exchange to higher ionic strength conditions) and hence the bound protein molecules remained stable under physiologically relevant ionic strength conditions. As a result, the cross-linked BSA protein molecules that were deposited under low ionic strength conditions offered a marked improvement in surface passivation against serum fouling on the silica surface *via* pronounced steric blocking. Taken together, the findings in this work not only improve our understanding of how ionic strength influences BSA protein adsorption onto solid supports but provide guidance on how ionic strength conditions can be utilized for developing improved surface passivation strategies. Moreover, as BSA was found to be highly stable in aqueous solutions over long periods of time, its utility as a blocking agent is further reinforced and the role of ionic strength in improving surface passivation deserves attention across a wider range of hydrophobic and hydrophilic substrates in general.

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Chapter 5: Results Part II

Conformational Tuning of BSA Molecules for Improved Surface Passivation

Bovine serum albumin (BSA) is the most conventional protein used for surface passivation applications, but the inefficiency had been demonstrated especially when interacted with hydrophilic surfaces such as silica-based materials. Herein, the conformational properties of the protein had been tuned so as to devise a surface passivation protocol that would rely purely on the adsorption process without the need for additional reagents. The simple and versatile method increases the stickiness of BSA adsorption resulting in up to a 10-fold improvement in its antibiofouling properties. A critical point of irreversible BSA unfolding and protein oligomerization were identified by controlling the temperature and time and the adsorption behavior was analyzed using the quartz crystal microbalance-dissipation (QCM-D) system in conjunction with the localized surface plasmon resonance (LSPR) system. Results indicated that lower conformational stability allowed protein to maximize surface contact area with the surface while maintaining high surface coverage overall. From the findings, it was determined that surface passivation with predenatured BSA was indeed superior to the previous method, both in terms of blocking efficiency and coating time.

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5.1 Introduction

The design of biologically inert surfaces and interfaces is a key objective of materials science, and holds broad relevance for numerous applications. For example, preventing the nonspecific adsorption of fouling molecules onto measurement platforms is critical for sensor performance, while limiting protein adsorption onto and immune recognition of medical implant surfaces can improve biocompatibility [1-3]. In the chemical and materials science fields, hydrophilic polymer brushes are widely employed to create non-fouling interfaces and have been the subject of intense research and development [4-7]. However, in part due to simplicity and versatility as well as historical precedent, protein-based coatings remain the “gold standard” for many biological applications such as enzyme-linked immunosorbent assays (ELISA), and bovine serum albumin (BSA) reigns as the most commonly used surface antifouling agent [8-10]. Curiously, there are many different types of processed BSA available, and essentially negligible discussion in the scientific literature about which type of BSA is most suitable for surface passivation applications or how blocking performance can be optimized. From a materials science perspective, the methodology is incomprehensible and irrational because BSA has relatively weak, non-sticky interactions with commonly used surfaces such as silica-based materials [11]. Clearly, there is significant opportunity in the knowledge gap where blocking performance can be improved simply by exploiting the fundamental steps in the protein adsorption process to promote stronger protein-surface interactions.

To this end, a dense and stable passivation layer is idealized for maximum surface coverage and optimal antibiofouling properties [12], but would require the protein blocks to irreversibly adhere onto surfaces in bulk in order to quickly achieve saturation coverage in the coating protocols [13]. Central to the process of protein adsorption is the concept of surface-induced protein denaturation. In general, protein adsorption begins with the adhesion of contacting protein molecules to a material surface. This step is followed by surface-induced denaturation of adsorbed protein molecules, which is governed by protein-protein and protein-surface interactions, and plays a critical role in determining whether a bound molecule will be irreversibly (permanently) or reversibly

(transiently) attached [14, 15]. Surface-induced denaturation itself is a thermodynamically favorable process that involves protein unfolding and progressive conformational changes. As the protein breaks down and “spreads” on the surface, the number of contact sites increases between individual protein molecules and the material surface [3], and the non-electrostatic interactions take control to drive the post-adsorption restructuring process.

The extent of surface-induced protein denaturation dictates whether or not a bound protein molecule will remain adsorbed. Naturally, proteins with a greater fraction of amino acids in hierarchical secondary structures are associated with greater conformational stability, and hence greater resistance to denaturation. Such proteins often display weaker and reversible adsorption to the surface and are easily replaceable by proteins that are able to establish a greater footing along the surface. In the opposite case, the energetic barrier to undergo conformational changes are lower in proteins with a lower fraction of amino acids in the secondary structure, and therefore, readily denature to embrace the stronger and more stable protein-surface interactions. Experimentally, Karlsson *et al.* used engineered mutants of human carbonic anhydrase II with different levels of conformational stability to demonstrate a distinct trend in the adsorption behavior [16]. Mutants with disrupted secondary structure exhibited more extensive denaturation in the adsorbed state and hence greater adsorption irreversibility, as compared to the stable variants on all tested surface types (negatively charged, positively charged, hydrophilic and hydrophobic). Such insights suggest that pretreatment to alter protein conformation in bulk solution may enable control over irreversible adsorption onto solid surfaces, which could be linked to a possible design cue for better surface passivation.

To date, a few BSA adsorption studies have fundamentally investigated how solution-phase denaturation affects protein adsorption. Damodaran and Song reported a correlation of chemically denatured BSA to greater adsorption rate at the air-liquid interface compared to native BSA [17], suggesting that denatured BSA might have a lower desorption rate than native BSA and consequently a stronger protein-surface interaction. In another example, Shirahama and Suzawa showed that there was greater uptake of heat-denatured BSA to polymer lattices because of the accessible hydrophobic

regions of the protein to the material surface for greater binding interactions [18]. Building on these works, a temperature-induced reversible conformational changes in monomeric BSA molecules (decreased helicity) were found to be in correlation with the extent of surface-induced denaturation, providing direct experimental evidence of a relationship between lower conformational stability of single protein molecules in bulk solution and greater surface-induced denaturation in the adsorbed state [19]. Collectively, the existing fundamental works support that modulating the conformational stability of proteins in bulk solution influences protein adsorption and surface-induced denaturation, highlighting the potential benefits of intentionally decreasing the conformational stability of BSA molecules to promote greater irreversible adsorption and improved surface passivation.

Herein, the adsorption trends of heat-denatured BSA molecules were systematically investigated on silica surfaces, and the insights were optimally utilized to design a simple yet versatile surface passivation method that significantly improves the blocking performance of the resulting protein layer against serum biofouling. By combining various analytical techniques for solution-phase characterization and surface-sensitive techniques, the provided evidences validate how the conformational stability of protein in bulk solution can be intimately linked with the functional properties of its adsorption state. While the interaction of denatured BSA with material interfaces has been shown to improve luminescent properties [20, 21], charge transfer properties [22-24] as well as colloidal stability of nanomaterials [25, 26], this is the first report demonstrating how denatured BSA can offer a superior antifouling solution, both in terms of blocking efficiency and coating time.

5.2 Materials and Methods

5.2.1 Reagents

Bovine serum albumin (A2153), sodium dodecyl sulfate (SDS, L4390), and sodium chloride (NaCl, BioXtra) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Amresco (Solon, OH, USA). Ethanol (absolute grade, EMSURE) and hydrochloric acid (HCl, EMSURE) were

purchased from Merck Millipore (Billerica, MA, USA). All chemicals were used as-received without further purification.

5.2.2 Sample Preparation

A buffer solution consisting of 10 mM Tris [pH 7.5] with 150 mM NaCl was prepared using deionized water filtered through a Milli-Q water purification system (18.2 M Ω ·cm resistivity at 24 °C) followed by titration with 1 M HCl. A stock solution of BSA protein was prepared by dissolving the lyophilized protein in Tris-HCl buffer solution at a nominal protein concentration of 100 μ M. The protein concentration was verified using UV-vis spectroscopy (S-220, Boeco, Germany) at 280 nm wavelength. Where applicable, thermal denaturation was performed by heating 100 μ M BSA in a water bath at 70 °C for 30 min. Lower bulk concentrations of protein were prepared by diluting the 100 μ M protein solution with Tris-HCl buffer solution as appropriate.

5.2.3 Circular Dichroism (CD) Spectroscopy

The secondary structures of native and thermally denatured BSA were investigated by employing CD spectroscopy. CD spectra of the protein solutions (25 μ M concentration) were measured with an AVIV Model 420 spectrometer (AVIV Biomedical, Lakewood, NJ) using a quartz cuvette of 1 mm path length. The spectra were recorded in triplicate from 190 to 260 nm in 0.5 nm intervals with a 4 s averaging time and 1.0 nm bandwidth. The spectra were normalized by subtracting a background spectrum that was measured in equivalent buffer conditions. The resulting spectra were expressed in terms of molar residue ellipticity (MRE) by the following equation:

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

where n is the number of amino acid residues, c is the protein concentration, and l is the path length of the cuvette. The fractional helicity of individual BSA molecules was then calculated based on the MRE at 222 nm, $[\theta]_{222}$, by the following equation [27]:

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

5.2.4 Dynamic Light Scattering (DLS)

A NanoBrook 90Plus particle size analyzer (Brookhaven Instruments, Holtsville, NY, USA) was employed to measure the average hydrodynamic diameter and polydispersity of BSA protein molecules in solution. All measurements were performed with a 658.0 nm monochromatic laser and recorded at a scattering angle of 90°. Analysis of the intensity autocorrelation function was done by the BIC Particle Sizing software package to obtain the intensity-weighted Gaussian size distribution, and the average hydrodynamic diameter is reported from $n = 5$ technical replicates. All measurements were performed at 25°C in a temperature-controlled measurement chamber.

5.2.5 Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) of BSA protein molecules diffusing under Brownian motion in bulk solution was performed using a NanoSight LM10 instrument (Malvern Instruments, Malvern, UK), as previously described [28]. Briefly, protein samples were first diluted and manually introduced into the sample chamber by using a sterile disposable syringe. A 405 nm laser was then used to illuminate the sample. The light undergoes Rayleigh scattering by the protein molecules and this scattered light was observed by optical microscope (20× magnification) and recorded by a built-in sCMOS camera at a rate of 25 frames per second for a 3-min duration. The recorded data was then analyzed using the NTA 3.1 Build 3.1.46 software package. The Brownian motion of individual particles was tracked in real-time, and the Stokes-Einstein equation was used to calculate the hydrodynamic diameter of protein molecules. All measurements were conducted at room temperature.

5.2.6 Quartz Crystal Microbalance-Dissipation (QCM-D) Measurements

Protein adsorption onto silica surfaces was investigated by employing a Q-Sense E4 instrument (Biolin Scientific AB, Stockholm, Sweden), as previously described [29]. The treated sensor chips were enclosed within the measurement chambers, and liquid samples were introduced *via* a peristaltic pump (ISM833C, Ismatec SA, Switzerland) at a flow rate of 100 $\mu\text{L} \cdot \text{min}^{-1}$. Changes in resonance frequency (Δf) and energy dissipation (ΔD) of the sensor chips were recorded at multiple odd overtones and all measurements were

conducted at 25 °C. The normalized data at the fifth overtone (25 MHz) are reported. The QCM-D measurement data were analyzed by the Voigt-Voinova model [30] in order to extract the effective film thickness. For modeling analysis, the adsorbed protein layer was assumed to form a single, homogenous layer with a uniform effective density of 1300 kg·m⁻³, and the density and viscosity of the bulk aqueous solution were fixed at 1000 kg·m⁻³ and 0.001 Pa·s⁻¹, respectively.

5.2.7 Localized Surface Plasmon Resonance (LSPR)

An Insplorion XNano instrument (Insplorion AB, Gothenburg, Sweden) was employed to perform ensemble-averaged LSPR measurements in optical transmission mode, as previously described [31]. The sensor chips (Insplorion AB) consisted of well-separated (~8% surface coverage) gold nanodisk arrays on a fused silica substrate, as fabricated by hole-mask colloidal lithography [32]. The sensor surface was coated with a thin, conformal layer of silicon nitride (~10 nm thick). Immediately before experiment, the sensor chip was sequentially rinsed with Milli-Q water and ethanol and dried under a gentle stream of nitrogen gas followed by treatment with oxygen plasma at maximum radiofrequency power for 2 min (PDC-002, Harrick Plasma). The latter process results in the formation of a silica layer on the sensor surface. After surface treatment, the sensor chips were enclosed within a microfluidic flow-through chamber and liquid samples were introduced *via* a peristaltic pump at a flow rate of 50 µL·min⁻¹. Extinction spectra were recorded and analyzed in real-time (1 Hz resolution) by using the Insplorion software (Insplorion AB) and the centroid position [33] in the extinction spectrum was determined by high-order polynomial fitting.

5.2.8 Atomic Force Microscopy (AFM)

Atomic Force Microscopy (AFM) was employed to identify the morphological properties of the BSA protein adsorbed on a silica surface. A commercial AFM system (NX-Bio, Park Systems, Suwon, South Korea) equipped with an Eclipse Ti optical microscope (Nikon, Tokyo, Japan) was used to obtain the images of samples that were prepared by adsorbing either native or denature proteins on an oxygen plasma pretreated Silicon wafer (Latech, Singapore). Aluminum reflex coated silicon cantilever, PPP-NCHR

(Nanosensors, Switzerland) with a spring constant of ~ 42 N/m and a resonance frequency of 100~150 kHz in liquid condition, was used in the non-contact mode while the sample was submerged in aqueous buffer. The images were acquired with a 0.1-0.5 Hz scan rate in order to minimize damage in the samples and to mitigate the signal noise. To estimate the thickness of the protein layer, a square test was conducted to obtain the height contrast between the indentation and protein-covered surface.

5.2.9 SDS Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Silica nanoparticles were incubated in 1% normal human serum for 30 mins at 37 °C. After incubation, the samples were centrifuged at 16,000 x g for 5 mins and the collected supernatants were mixed with 4x Laemmli sample buffer. The samples were boiled at 95 °C for 5 mins before injected into the wells of 8% polyacrylamide gels along with a blank buffer control. SDS-PAGE was performed at 100 V for 2 h then transblotted onto nitrocellulose membranes at 300 mA for an additional 2 h. The membranes were blocked by TBST (Tris-buffered saline with 0.1% Tween 20) buffer solution containing either 3% native BSA monomers or 3% denatured BSA oligomers for 1 h at room temperature. Thereafter, the membranes were incubated with primary iC3b antibodies (diluted at 1:500 in blocking buffer) overnight, then washed three times with TBST and incubated with horseradish peroxidase conjugated anti-mouse goat antibodies (diluted at 1:2000 in blocking buffer) for 1 h. The membranes were washed four times then dipped in enhanced chemiluminescent solution for 5 minutes. Protein bands were detected and analyzed with an Amersham Imager 600 (GE Healthcare, Chicago, IL).

5.3 Principle Outcomes

5.3.1 Heat Denaturation of BSA Protein

Heat denaturation of BSA protein molecules in bulk solution is a well-studied phenomenon and was utilized here to prepare denatured BSA with lower conformational stability. With increasing temperature, BSA molecules undergo a series of reversible and/or irreversible conformational changes, whereby individual molecules transition from α -helical to β -sheet motifs before partially unfolding to yield random coil structures that

can oligomerize [34-38]. We first characterized how heat treatment processing affects the secondary structure and size of protein molecules in bulk solution. Circular dichroism (CD) spectroscopic measurements showed that BSA molecules at 25 °C had 66% fractional helicity, while the helicity of BSA molecules at 70 °C decreased to 53%. The decrease in helicity is consistent with BSA molecules undergoing partial unfolding and the corresponding magnitude is comparable to results obtained in previous reports [35-37]. The heat-treated protein solution was then cooled to room temperature, and subsequent measurements at 25 °C indicated that the fractional helicity remained at 53%. This finding verified that thermal denaturation induces irreversible conformational changes in the BSA protein structure and confirmed the presence of denatured BSA at room temperature.

Figure 5.1 Effect of the heat treatment cycle on BSA molecules and the corresponding circular dichroism spectra of BSA at each phase of the heat cycle.

The bulk solution containing denatured BSA molecules was further characterized with DLS and NTA measurements to consider the broad-spectrum effects of the volatile particles within the system. It is known that heat-induced irreversible conformational changes in BSA molecules are often accompanied by oligomerization as a means to stabilize newly exposed hydrophobic regions [15, 38-41]. Characterizing the size distribution of protein oligomers is important because the bulk diffusion properties of adsorbing protein molecules influences the diffusion-limited rate of protein adsorption [3].

Figure 5.2 BSA size distribution before and after heat treatment. (a) DLS measurement of native and denatured BSA, and (b) NTA analysis of denatured BSA.

Room-temperature DLS measurements indicated that the hydrodynamic diameter of native and heat-treated BSA protein molecules in solution were 8.2 ± 0.1 and 43.8 ± 4.7 nm, respectively, providing evidence to support that denatured BSA exists in an oligomeric state (Figure 5.2a). To confirm the presence of BSA oligomers, the size distribution of heat-denatured BSA was measured by NTA and it was determined that the size of the oligomers range from 50 to 150 nm in diameter (Figure 5.2b). Taken together, these findings are consistent with the CD spectroscopic measurements and confirm the occurrence of thermal denaturation upon heating at 70 °C, leading to permanent changes in BSA secondary structure (decreased helicity) which can be tested for surface passivation applications.

5.3.2 QCM-D Measurements on Native and Denatured BSA Adsorption

QCM-D experiments were next conducted to compare the room-temperature adsorption kinetics of native and denatured BSA on silica surfaces. Silica is a hydrophilic material and adsorption of the proteins onto silica surfaces is understood to be driven by a combination of long-range electrostatic forces as well as shorter-range hydrogen bonding and van der Waals interactions [42]. The QCM-D measure the shifts in resonance frequency and energy dissipation at real-time capable of analyzing the change in mass and viscoelastic properties of the adsorbed protein layer, respectively, capturing the full extent of the protein-surface interaction. To begin, a baseline signal was established with

aqueous buffer solution, and then the measurements were initialized by injecting 100 μM of native (nBSA) or denatured BSA (dBSA). The rather high protein concentration was selected to mimic the typical conditions of BSA blocking protocols (100 μM $\sim$ 6.6 $\text{mg}\cdot\text{mL}^{-1}$) [43]. For both cases, a monotonic decrease in the frequency signal was observed. At saturation, a frequency shift of around -37 Hz was observed for nBSA, while -44 Hz was recorded for dBSA (Figure 5.3a). The corresponding energy dissipation shifts were around 4×10^{-6} and 5×10^{-6} for nBSA and dBSA, respectively (Figure 5.3b). The stabilized adlayer was rinsed with buffer to remove loosely bound proteins from the formed adlayer resulting in final frequency values of -26 Hz and -38 Hz, and energy dissipation shifts of around 2×10^{-6} and 2.5×10^{-6} for nBSA and dBSA, respectively. The combined results demonstrated a greater mass uptake with dBSA, as expected for its oligomeric state, which would contribute to thicker adlayer as compared to nBSA monomers. Of note, the time scale to reach saturation was significantly shortened for dBSA as compared to nBSA, implying that the adsorption process of dBSA and the ensuing dBSA oligomers may be mechanistically different, evidently offering cues of a more efficient protocol to achieve surface passivation. This trend was a striking discovery since the overall protein adsorption process had been understood to be diffusion-limited [44], and therefore, larger-sized dBSA oligomers would be expected to exhibit a slower adsorption rate having a smaller diffusion coefficient in bulk solution than nBSA monomers. To contemplate with the experimental observations, it should be noted that BSA molecules inherently have weak attachments to silica surfaces [11], and since the QCM-D tracks the ensemble-average response of the net rate of adsorption and desorption of BSA protein molecules [45], the diffusion-limited process may not be fully reflected from the QCM-D signals alone. Previously, Kwok *et al.* reported that 99.3% of attaching BSA molecules desorb from the silica surface [11] and the effective rate of change in the QCM-D signal can be much lower than the rate predicted by the rate of diffusion-limited adsorption alone. Hence, a more plausible suggestion of the observed readings from the QCM-D measurements would be a significantly lower desorption rate of dBSA in comparison to that of nBSA.

This finding is supported by several related factors. First, dBSA has lower conformational stability in solution, so there is a greater degree of freedom for the bound

oligomers to unfold and conform to the silica surface, which is consistent with the intended goal of the passivation strategy. Second, regardless of the extent of spreading, larger-size oligomers of dBSA would have greater contacting surface area per adsorbing species, resulting in a stronger net interaction. Third, the two-dimensional Brownian motion exerted on larger-sized BSA oligomers is also lower, and hence the likelihood of desorption promoted by lateral forces is lower as well. The latter two points are supported by the work of Schwartz and colleagues [46], in which the adsorption of monomeric and oligomeric fibrinogen was studied. The authors proposed that favorable orientations for protein adsorption were more likely to occur for oligomers on account of their larger size, and less likely to desorb due to the slower diffusion rate at the surface. Taken together, the findings support that dBSA adsorbs and adheres tighter to silica surfaces than nBSA due to the lower conformational stability and greater surface contact area per adsorbing species. This combination of features results in greater and more efficient adsorption uptake on silica surfaces.

Figure 5.3 QCM-D measurement data comparison of native BSA monomers (red) and denatured BSA oligomers (blue) adsorbing onto silica surfaces as a function of time. The time point at which wash buffer is injected into the measurement chamber is indicated by the arrow.

5.3.3 Effect of BSA Concentration on QCM-D Adsorption Kinetics

Next, the adsorption pattern of nBSA and dBSA was observed across a range of bulk protein concentrations. In the case of nBSA, the trends of the adsorption kinetics and corresponding uptake rate were keenly dependent on the protein concentration. With reduction in the bulk protein concentration from 100 to 25 μ M, gradual impedance in the

protein uptake rate was observed causing the time requirement for saturation to double from 25 minutes to nearly 1 h, and correspondingly, reductions in the peak frequency and energy dissipation shifts were seen at saturation. The evident concentration dependent protein adsorption kinetics was consistent with past reports that suggested a general need for longer incubation times (or greater concentrations) for the coating of silica surfaces using BSA [15, 47-49]. Nonetheless, nBSA coating on the surface was verifiable as demonstrated by the consistent frequency and energy dissipation output after the washing step across all tested concentrations.

Figure 5.4 QCM-D results indicating the (a) frequency and (b) energy dissipation shifts of native BSA, and the (c) frequency and (d) energy dissipation shifts of denatured BSA on silica surfaces each measured at 25 μ M, 50 μ M, and 100 μ M bulk protein concentrations. Injection of wash buffer is indicated by black arrows in each plot.

In marked contrast, the adsorption kinetics of dBSA and the corresponding uptake on silica surfaces were unaffected by the change in bulk protein concentration (Figure 5.4c). Most noticeable was the rapid uptake of proteins to the surface regardless of the

concentration, achieving surface saturation almost instantaneously and with marginal difference in the resulting frequency shifts at saturation. Meanwhile, the energy dissipation shifts showed some variations with the different concentrations. This was likely a representation of the excess proteins that were loosely associated with the protein layer, especially considering its irrelevancy after the buffer wash.

The thickness of the BSA protein adlayers was also estimated by applying the Voigt-Voinova viscoelastic model to the QCM-D measurement data (Table 5.1) [50]. The modeling results clearly indicate that adlayers formed from dBSA were significantly thicker with an average of 6.13 nm compared to the adlayer of nBSA at an estimated average thickness of 3.97 nm. In general, the modeling results support that dBSA oligomers formed thicker adlayers than nBSA across the tested protein concentrations. Of note, for each type of BSA protein, the estimated adlayer thickness at saturation was greatest for the highest bulk protein concentration (100 μ M). This concentration-dependent effect is attributed to the rapid uptake of individual protein molecules outcompeting the post-adsorption rearrangement of surface-bound proteins [51]. Indeed, upon buffer washing, the adsorbed molecules reorganize such that the adlayer thicknesses would be similar across the tested protein concentrations with stable protein coatings. Overall, the observed trends indicate that dBSA oligomers form denser and thicker adlayers than nBSA monomers.

Table 5.1 Effective thickness of adsorbed native and denatured BSA layers at saturation

BSA Concentration	Thickness at saturation (nm)	Thickness after rinsing (nm)
25 μ M Native	5.1	3.8
50 μ M Native	5.8	3.9
100 μ M Native	6.9	4.2
25 μ M Denatured	8.1	6.4
50 μ M Denatured	8.3	5.8
100 μ M Denatured	11.5	6.2

5.3.4 LSPR Measurements on Native and Denatured BSA Adsorption

As a complimentary technique to QCM-D measurements, LSPR measurements were performed to gain more insights into the concentration-related adsorption behavior

of nBSA and dBSA. While both techniques are highly sophisticated surface-based techniques, QCM-D is an acoustic measurement that detects the mass of the adsorbing molecules as well as the hydrodynamic-coupled solvent, whereas the LSPR is an optical device that provides information on the “dry” mass of the adsorbing proteins [52-54]. Furthermore, LSPR is a highly surface sensitive device with a small probing volume making it suitable for analyzing minor conformational changes in the bound proteins [55, 56]. In a nutshell, the BSA samples were adsorbed onto silica-coated gold nanodisk arrays, and the resulting decay of evanescent electromagnetic field intensity emanating from the sensors were monitored as indications to protein-surface interactions [52-54].

Figure 5.5 LSPR measurements of concentration-dependent native and denatured BSA protein adsorption onto silica-coated gold nanodisk arrays.

The LSPR measurement trends for both nBSA and dBSA adsorption showed excellent agreement with the QCM-D measurements (Figure 5.5). For nBSA, the concentration-dependent variation in the uptake rate and the gradual decrease in peak intensity were clearly revealed, while in the case of dBSA, the rapid adsorption kinetics and the near-instantaneous saturation were reconfirmed. Peak shifts for nBSA recorded 0.76, 0.61, and 0.49 nm for 100, 50 and 25 μM protein concentrations, respectively, but were unrivaled to the results of dBSA peaks lingering at around 0.9 to 1.0 nm matching earlier reports from the QCM-D experiments. The desorption trends of the LSPR signals were also similar to QCM-D results but were with more clarity. In particular, the desorption rate of peak shifts was revealed to be concentration-dependent being fastest at a concentration of 100 μM for both cases, which is within reason considering the relative

amount of excess protein that needs to be removed from the adlayers. Another noteworthy trait was a spike in the peak shifts commonly appearing for both cases. The magnitude of the transient spike correlated with the bulk protein concentration that was used for coating, and is likely due to a structural rearrangement among bound BSA molecules in the adlayer whereby some molecules become closer to the silica surface while other protein molecules desorb [57, 58]. In the end, the post-desorption peak shifts for nBSA were distinguishable for each concentration conditions, whereas all post-desorption peak shift values tended to converge for dBSA. This distinction indirectly proves the importance of incubation time as a key factor to achieve optimal results for the conventional surface passivation protocol, which becomes nonessential by using dBSA as an alternative solution. Altogether, the LSPR measurements provided confirmatory evidences of dBSA coating on silica to be a robust, efficient and versatile option for surface passivation.

5.3.5 Topographical Analysis of Protein Coated Surface

Data driven analyses of the protein adsorption process and the characterization of coating properties have been consistent in pointing out a thicker and/or denser protein coating with dBSA in comparison to nBSA. Using an atomic force microscope (AFM), the topographical features of the two different adlayers were examined. To best mimic the protein adsorption conditions of the QCM-D and LSPR experiments, a clean silicon wafer was initially submerged in aqueous buffer, then transferred into a glass petri dish filled with 100 μM of either dBSA or nBSA solution. After 30 mins of incubation at room temperature with gentle agitation, the solution was carefully exchanged out with fresh buffer solution to minimize any unnatural disruption in the surface bound proteins. AFM imaging was performed while the samples were submerged in the buffer using a non-contact mode to eliminate the potential damage that can be done by the AFM tip.

The topographical features of the protein-coated surfaces presented in the images were strikingly different between the two samples (Figure 5.6). When incubated with nBSA, most of the bare surface was exposed while proteins were found sparsely adhering to the substrate. The lower than expected surface coverage is likely due to the unfavorable sample preparation conditions whereby many environmental outliers were

beyond control. Yet, the results are reflective of the weak interactive forces between BSA and silica, proving that nBSA coating on silica is an inconvenient and inefficient process. On the other hand, some apparent topographical roughness was featured in the images of the dBSA-coated substrate, but with an overall near-uniform surface as identified by the height profile characterization. Additional square tests were conducted on different regions to identify the content of the uniform layer, where the tip would be used to create indentation on the surface to distinguish soft matters (proteins) from hard materials (Figure 5.6c). In result, the uniform surface was identified to be a layer of proteins that had a height range of 4 nm to 15 nm depending on the region, which was relatively close to the estimated height derived from the Voigt-Voinova viscoelastic model. From the results, it is worthwhile to once again emphasize the effectiveness of dBSA coating being capable of carrying out surface passivation even in conditions that would be otherwise unfavorable.

Figure 5.6 2D and 3D AFM images of proteins adsorbed on silica with line height profiling. 2 μm x 2 μm images of (a) native BSA, (b) denatured BSA, and (c) square test of denatured BSA adlayer from a 10 μm x 10 μm image

5.3.6 Evaluation of Blocking against Serum Biofouling

Based on the different adsorption and coating properties, we next tested the functional efficacy of nBSA and dBSA coating on silica against fetal bovine serum (FBS). The antibiofouling capability was assessed by QCM-D experiments whereby the extent to which BSA coatings inhibited fouling by serum components was determined relative to

control experiments conducted on bare silica surfaces (Figure 5.7b) [28]. While fundamentally comparing the blocking performances of nBSA and dBSA, the incubation time was also taken into consideration in the experimental designs to evaluate the efficiency of the coating method. In brief, a measurement baseline was established with aqueous buffer, and then 100 μ M of either nBSA or dBSA was injected for 3 min, 30 min or 3 h before the buffer wash to complete the coating process. After reestablishing a stable signal on the protein coating, the surface was challenged with 100% FBS then was followed by a buffer washing step. The blocking efficiency was determined by comparing the final frequency shifts after FBS incubation to that of the results obtained by adsorbing FBS on bare silica.

Figure 5.7 Schematic of surface passivation methods using native or denatured BSA against FBS. (a) Frequency shifts of QCM-D measurements and (b) blocking efficiency of each proposed method.

The most blatant observation was the improvement in blocking performance with prolonged coating time. For each time increment, the blocking efficiency of nBSA coating improved significantly starting at 5.5%, then 14.9%, and finally at 37.8%, which can be safely assumed to be its maximum output. The coating quality of dBSA also improved with time but the performance was already at 58.9% with 3 min incubation,

which was gradually perfected with a blocking efficiency of 72.9% after 3 hours of incubation. In essence, the superiority of dBSA coating was clear registering up to a 10-fold difference in its blocking efficiency when comparing the 3 min coating results alone, and although with room for maturity, it was still able to outperform the perfected 3 hour coating of nBSA. This finding is consistent with the QCM-D and LSPR measurements indicating that denatured BSA forms a denser and thicker adlayer on the silica surface, hence reinforcing the connection between purposefully modulating the conformational properties of BSA protein in solution and achieving improved surface passivation in the adsorbed state.

Figure 5.8 SDS-PAGE/Western Blot analysis for performance comparison of nBSA monomers and dBSA oligomers as blocking agents. (a) Schematic illustration of step-by-step protocol using either nBSA monomers or dBSA oligomers as a blocking agent. Each gel was loaded with nanoparticle-treated serum (lanes 1-4), untreated serum (lane 5), and a blank buffer (lane 6) for the actual experiments and results are compared after blocking with (b) native BSA monomers or (c) dBSA oligomers. Target bands and nonspecific bands are indicated by red and blue arrows, respectively, while the red-dotted boxes point out the different blocking performances.

5.3.7 Evaluation of Blocking Performance in Western Blot Assay

The hypothesized superiority of dBSA oligomers as a blocking agent compared to nBSA monomers was further tested by Western Blot. The widely used immunochemical

assay requires blocking of the nitrocellulose membrane to enhance specificity of antibody-antigen interactions in which the conventional protocol recommends the use of nonfat dried milk or a 3-to-5% BSA solution. As presented in Figure 5.8a, the Western Blot assay consists of an electrophoresis step for the separation of individual proteins by molecular size then a blot transfer to the nitrocellulose membrane which then is incubated in primary and secondary antibodies for the detection of the target protein(s). Prior to the antibodies, a blocking step is normally required which would passivate the surface to prevent the nonspecific binding of antibodies, hence minimizing the background noise or false positive signals. Based on this approach, we investigated the immune reaction of human blood serum to bare silica nanoparticles by measuring the levels of complement C3b protein as the key biomarker of interest. Briefly, 1% normal human serum samples were incubated with or without silica nanoparticles for 30 mins at 37 °C and the supernatants were loaded in two separate 8% polyacrylamide gels in the following order: nanoparticle treated serum (lanes 1-4), untreated serum (lane 5), buffer blank (lane 6). The blotted membranes were then blocked with either 3% nBSA monomer blocking solution (Figure 5.8b) or 3% dBSA oligomer blocking solution (figure 5.8c) to prep the blots for immunostaining. After immunostaining, bands corresponding to C3b (~175 and ~104 kDa) and its inactive form iC3b (~63 and ~42 kDa) were detected (see red arrows) [59]. Alongside the expected bands, several background signals were also detected for both blots but a significant reduction in the nonspecific bands was observed when blocked with dBSA oligomers (see blue arrows). More importantly, new band patterns were observed only after blocking with dBSA oligomers enabling clear distinction for the nontreated serum from the silica treated serum samples, which was less apparent from the blot results after nBSA monomer blocking. Altogether, the Western blot results demonstrated promising application potential for dBSA oligomers especially as an alternative to nBSA monomers when dealing with samples showing insufficient signal-to-noise ratios. Further corroboration is needed to identify the mechanistical details of its performance and validate the superiority of dBSA oligomers, but the results presented herewith is undoubtedly astonishing and exciting.

5.4 Conclusion

In this study, a simple and versatile method is presented that increases the stickiness of BSA adsorbing onto silica surfaces that significantly improve surface passivation performances. The underlying concept of the protocol presented, herewith, is based on utilizing thermal denaturation to purposefully modulate the conformational stability of BSA molecules in bulk solution in order to promote greater adsorption uptake and more irreversible adsorption. These findings build on growing fundamental knowledge about the relationship between conformational stability and surface-induced protein denaturation, while presenting the first example of how this fundamental knowledge can be translated into a functional application with high performance. Given the wide usage of BSA as a surface-passivating agent and simple method to convert nBSA into dBSA, there are broad possibilities for utilizing dBSA in various application settings and for preparing improved protein-based coatings in general.

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Chapter 6: Results Part III

Understanding Protein Adsorption Behaviors by Probing the Relative Stabilities of Serum Albumin Homologues

Proteins exhibit unique behaviors in solution and upon depending on characteristics such as size, charge, hydrophobicity, and functional groups. In this chapter, the solution and adsorption behavior of homologous bovine serum albumin (BSA) and human serum albumin (HSA) were compared to probe the subtle differences in their behavior in the context of the outcomes discussed in the previous two chapters. For this study, the structural stabilities of the proteins in relation to ionic strength, temperature variation, and protein-surface interactions were defined as conformational, thermal, and adsorption stabilities, respectively. The study reveals that native HSA has a higher conformational stability than native BSA, but anionic strength dependency in their thermal stabilities, which is reversed in trend when heat-denatured. The adsorption stabilities were sensitive to protein structure but without correlation to conformational stability as native HSA demonstrated greater conformity to the surface despite the higher conformational stability. Furthermore, heat-denatured HSA showed greater adsorption stability than native HSA, which was opposite for BSA. Altogether, this study highlights the importance of separately looking into the different facets of structural stability to determine how subtle variations in protein sequence and structure result in different solution and adsorption behaviors of homologous proteins, before treating them as interchangeable model proteins.

6.1 Introduction

Understanding the solution and adsorption behaviors of proteins represents the key towards obtaining fundamental insights into a wide range of physiological processes [1-3] and is significantly relevant for biomedical applications such as pharmaceuticals [4-7] and biosensors that require antifouling capabilities [8-12]. In the previous chapter, the adsorption behavior of bovine serum albumin (BSA) had been examined on silica substrates against an ionic strength gradient to demonstrate its rather discerning performance as a blocking agent but provided fundamental insights and clues for possible improvements. The possibilities have been verified through the use of crosslinking agents but more prominently by altering the conformational state at which the protein molecule would appeal to the adsorbent. Because proteins are inherently soft biomaterials capable of changing conformations with external stimuli, the investigation on the inherent protein properties regarding conformation stability is very promising.

One of the most widely studied classes of protein is the serum albumins, owing to their abundance in the circulatory system and their popular application as a generic protein and blocking agent in solid-phase assays [10-15]. Physiologically, they perform several functions such as transporting and regulating the distribution of exogenous and endogenous compounds, as well as maintaining the oncotic pressure [14, 15]. While serum albumins from different species generally share many similarities in terms of sequence, structure and function, subtle differences exist as a consequence of evolution [15-17]. For example, all serum albumins are predominantly helical consisting of three similar domains arranged in an overall heart-like shape [15, 18]. Due to the close resemblance, the crystallographic model of BSA molecule have been reconstructed from human serum albumins (HSA) models. The amino acid sequences of bovine serum albumin (BSA) and human serum albumin (HSA), however, revealed only a 75.8% match [16] even though the basic physicochemical properties are in excellent agreement (*e.g.*, molecular weight of approximately 66.5 kDa and isoelectric point in the range of 4.8 to 5.6) [14, 15, 19]. This is believed to be the main cause for the significant behavioral differences in solution and upon adsorption [13, 20-22], and motivates the

need to apprehend the intermolecular and intramolecular occurrences to appropriately interpret and analyze the experimental observations.

The structural stability (*i.e.*, resistance of protein molecules to changes in secondary and tertiary structures) and colloidal stability (*i.e.*, resistance for protein molecules to aggregate) of serum albumins have been conventionally studied using temperature or ionic strength gradients [20, 23-27]. With increasing temperature, the breakdown of secondary structures (α -helices and β -sheets) are generally reported in the serum albumins, and the unsheathed domains are linked to the causes for protein aggregation. Borzova *et al.*, recently suggested that BSA takes on two forms of conformations in response to heat, each form containing different ratios of native to unfolded domains and with commensurate propensities to aggregate [28]. Eventually, this will result in aggregates of random sizes. As such, different arrangements and distributions of secondary structures within the protein can be decisive factors for the aggregation propensities even when comparing identical protein molecules. However, the influence of subtle structural deviations is difficult to gauge because thermal denaturation and aggregation are reported without separation for most cases. In comparison, ionic strength studies are insightful because the aggregation behavior of proteins can be modulated to a limited degree. First of all, an increase in ionic strength is able to stabilize the protein conformation as evinced by the increase in the temperature requirement for protein denaturation, which in turn also delays the heat-induced aggregation [24]. Additionally, the use of counterions in the mix mitigate electrostatic forces through charge shielding [29, 30], making it possible to investigate the effect of structural domains in a more electrostatically neutral setting. However, comparative studies between the homologous serum albumins are surprisingly rare even though the effect of temperature and ionic strength on the structural and colloidal stabilities of each serum albumin has been extensively evaluated, making it challenging to resolve the structure-stability relationships. Furthermore, a comprehensive study comparing the relative stabilities of denatured serum albumins is unprecedented, despite it being established to have the same degree of complexity to their native counterparts and likewise holds significant relevance in studies aimed at understanding protein stability [31-33].

The protein stability in solution influences the adsorption behavior and, in some cases, represents one of the driving forces for protein adsorption [34-37]. Previous works by Karlsson *et al.* employing engineered protein variants described how protein stability influences the orientation in which proteins approach the surface as well as the rate and extent in which protein undergoes conformational changes post-adsorption [38, 39]. Subtle variations at the submolecular level have dramatic influence on the adsorption behavior [40] and the final irreversible state of adsorbed proteins eventually determines the surface properties and degradation characteristics of the adsorbing material [41]. Having established that protein stability is influenced by the solution condition, several reports have discussed the adsorption behavior of serum albumins within the context of solution parameters such as temperature and ionic strength [42-53]. In particular, gradually raising the temperature generally led to an increased rate of serum albumin adsorption but resulted in different trends in saturation uptakes between HSA and BSA, depending on the solution and surface properties [42-44]. It was also noted that adsorbed BSA presents higher thermostability and lower α -helicity than its native form [23]. In terms of ionic strength, the addition of salt mediates adsorption through electrostatic protein-protein interactions [45-49], as well as surface potential, which influences protein-surface interactions [47, 51, 53]. While adsorption would be enhanced under favorable electrostatic interactions (*i.e.*, minimal protein-protein and protein-surface electrostatic repulsions), it was possible to adsorb BSA onto silica particles even under non-favorable electrostatic interactions [54]. This emphasizes that in the case of serum albumins, electrostatic interactions may not represent the primary driving force for adsorption [46, 47] and that the process can be entropically driven, as recently demonstrated for HSA adsorption onto titania [55]. However, despite extensive investigations into protein adsorption behavior using serum albumins as a model, with the exception of a few works [13, 21, 22], direct experimental comparisons between BSA and HSA are rare, especially from the perspective of their relative structural and colloidal stabilities. Comparative studies investigating the adsorption of denatured serum albumins, which have vastly different structural and colloidal stabilities than their native forms, are also lacking.

In this work, the solution and adsorption behaviors of BSA and HSA, and their denatured forms, are compared to reveal that subtle variations in protein sequence and structure can lead to significant differences in structural and colloidal stabilities. With increasing temperature and ionic strength, the solution behavior is characterized by tracking changes in secondary and tertiary structures using circular dichroism (CD) [56-61] and observing the extent of aggregation using dynamic light scattering (DLS) [62, 63]. Taken together, CD and DLS data provide information on the respective differences in structural and colloidal stabilities between the two proteins, and how this relates to the different solution behaviors. In a similar fashion, the adsorption behaviors of BSA and HSA on a planar silica surface as a function of denaturation temperature and ionic strength were characterized using the quartz crystal microbalance with dissipation (QCM-D) technique, which provides real-time information on the amount of adsorbed wet mass and the corresponding changes in adlayer viscoelasticity [64-67]. Accordingly, differences in structural and colloidal stabilities can be inferred from the different degrees of protein spreading during adsorption. Our work represents the first systematic study comparing both the solution and adsorption behaviors of native and denatured serum albumins in a sequential manner, enabling us to resolve variations in different types of structural stabilities as well colloidal stability between HSA and BSA. Our findings hold significant relevance in evaluating the respective serum albumins' suitability as a model protein in specific protein-surface interaction systems and are beneficial for optimizing conditions for applications involving protein formulations and surface coatings. From an experimental design perspective, the comparison of naturally occurring homologous serum albumins represents a simple investigational approach to reveal structure-stability relationships of proteins in general without the need for extensive protein engineering [38, 56, 68].

6.2 Materials and Method

6.2.1 Sample Preparation

Lyophilized bovine serum albumin (A2153) and human serum albumin (A3782) were purchased from Sigma-Aldrich (Singapore) and stored at 2-4 °C prior to use. Buffer

solutions were prepared using 10 mM tris(hydroxymethyl)aminomethane (Tris) and varying concentrations of sodium chloride (Sigma-Aldrich). All buffer solutions were adjusted to pH 7.5 with 1 M hydrochloric acid (Sigma-Aldrich), and filtered through 0.22 μm membrane filter before use. All protein samples were freshly prepared by dissolving a lyophilized amount of protein in buffer solution. The stock concentrations of the protein samples were determined by 280 nm absorbance measurements using molar extinction coefficient of 43,824 and 35,700 $\text{M}^{-1} \text{cm}^{-1}$ for BSA and HSA, respectively, and adjusted to the desired experimental concentration. For thermal investigations, stock aliquots of BSA and HSA were incubated in a water bath set at temperatures incrementing by 10 $^{\circ}\text{C}$ with a heat cycle consisting of 30 minutes of heating and 2 hours of cooling. Fresh aliquots were used for each temperature increment.

6.2.2 Circular Dichroism (CD)

CD spectrometry was performed using an AVIV Circular Dichroism Spectrometer Model 420 (Aviv Biomedical, USA). Experiments were conducted using a glass/quartz microcuvette with a 0.1 mm path length (Hellma Analytics, Germany) and the far-UV spectral range from 190 to 260 nm was analyzed in 1 nm segments. All measurements were recorded at 25 $^{\circ}\text{C}$ with a 4 s averaging time and a 1.0 nm bandwidth. 10 mM Tris buffer solution [pH 7.5] with 0 or 150 mM NaCl was used to record the background spectra, while all protein samples were prepared at a concentration of 50 μM in the same buffer solution for sample analysis. Further details on Molar Residue Ellipticity (MRE) can be found in Section 3.3.3.

6.2.3 Dynamic Light Scattering (DLS)

Size measurements for 50 μM BSA and HSA protein molecules in solution were analyzed with NanoBrook ZetaPALS Particle Analyzer (Brookhaven Instruments, USA) under varying ionic strength conditions and heat treatments. Initial measurements were performed at room temperature and subsequent measurements of 1 mL aliquots were conducted after a 30 min heat treatment (50, 60, 70, 80, or 90 $^{\circ}\text{C}$) followed by 2 hours of cooling at room temperature. All measurements were performed with a 658.0 nm monochromatic laser at a scattering angle of 90 $^{\circ}$ and data were collected using the BIC

Particle Size Software (Brookhaven Instruments). Ten measurements for each sample were recorded in the Cumulant mode and the average was reported with standard deviation to estimate the effective hydrodynamic diameter.

6.2.4 Quartz Crystal Microbalance with Dissipation (QCM-D)

QCM-D was employed for the investigation of protein adsorption. Q-Sense E4 (Biolin Scientific, Sweden) was utilized to monitor BSA and HSA adsorption on silicon oxide-coated sensor chip (QX303, Biolin Scientific). The surface of the sensor was rinsed with 1% SDS solution, Milli-Q water, and ethanol then dried under a gentle stream of nitrogen gas. The cleansed sensor chip was then pre-treated with oxygen plasma (PDC-002, Harrick Plasma, USA) for one minute immediately prior to the adsorption study. All solutions were introduced into the measurement chamber through a 0.76 mm-inner-diameter tube (Pharmed®, Ismatec SA, Switzerland) installed on a peristaltic pump (ISM833C, Ismatec SA) operating at a nominal flow rate of 50 $\mu\text{L}/\text{min}$. Real-time measurements of frequency and dissipation factors were recorded simultaneously at various overtones ($n=3, 5, 7, 9$, and 11). Protein solution injection was initiated upon stabilizing the frequency and dissipation baseline using protein-free buffer. The real-time shifts in frequency and dissipation were recorded up to the point of stabilization and the dataset from the fifth overtone (25 MHz) were presented in the main text.

6.3 Principle Outcomes

6.3.1 Thermal Stability of HSA vs BSA

The solution behavior of BSA and HSA was compared after a series of heating and cooling cycles with a 10 $^{\circ}\text{C}$ increment in between, in terms of conformational stability as well as colloidal stability (Figure 6.1a). DLS was employed to observe changes in particle size and the aggregative tendencies relative to the temperature, while the CD spectra of the proteins were obtained to compare the structural stability against thermal stress. Starting from room temperature, the contribution of the heat treatment became apparent only after reaching 60 $^{\circ}\text{C}$ as indicated by the DLS measurements whereby both HSA and BSA showed increase in size from 8~9 nm to ~12 nm and ~20

nm, respectively. At higher temperatures, the changes became more dramatic especially for HSA, which exceeded the DLS limit of detection at 80 °C, indicating a lower colloidal stability at higher temperatures in comparison to BSA.

Figure 6.1 Protein characterization with incrementing temperature for BSA and HSA. (a) DLS average measurement at various temperature settings and (b) secondary structure analysis from CD measurements.

Table 6.1 Secondary and tertiary structural analysis of BSA and HSA resolved from CD spectra MRE peak analysis

Protein	Temp (°C)	α -helicity (%) MRE ₂₂₂ (MRE ₂₀₈)	MRE ₂₂₂ /MRE ₂₀₈	Structure
BSA (150 mM NaCl)	20	55(55)	0.93	Single-stranded
	70	40(37)	0.85	Single-stranded
	80	38(33)	0.87	Single-stranded
HSA (150 mM NaCl)	20	62(63)	0.94	Single-stranded
	70	44(41)	0.89	Single-stranded
	80	32(11)	1.3	Coiled-coil

The CD spectra of the two proteins were examined for the heat treatment of 70 °C and 80 °C in comparison to their native form measured at room temperature (Figure 6.1b). The spectra from both serum albumins without heat treatment exhibited typical α -helical traits with peak minima Molar Residue Ellipticity (MRE) values at 208 nm and 222 nm [61], each corresponding to 55% and 62% α -helicity for BSA and HSA, respectively.

With heat treatment, progressive loss in the α -helicity was observed suggesting a transition in the secondary structure, and likely a consequent change in the tertiary structure [69, 70], which can be evaluated by comparing the ratios of the MRE at 222 nm and 208 nm [56, 71] as displayed in Table 6.1. Of note, the profiles of HSA and BSA were significantly different upon exposure to 80 °C heat treatment. While BSA showed consistency after the structural rearrangements at 70 °C, an extensive transformation was recorded for HSA with a change in α -helicity from 44% at 70 °C to 32% at 80 °C. The MRE ratio for HSA oligomers suggested a transition from a predominantly single-stranded α -helical structure at 70 °C into a predominantly coiled-coil structure at 80 °C (Table 6.1). Collectively, the aggregation trends and the conformational information suggest that HSA is more thermally sensitive than BSA at higher temperatures, which is separate from the fact that the initial denaturation temperature is lower for BSA. The CD spectra further hints that the greater aggregation propensity in HSA could be due to the extensive denaturation in HSA, which could also explain the intermolecular or intramolecular coiled-coiling of α -helices .

6.3.2 Ionic Strength Dependent Conformational Stability and Thermal Stability

Since the colloidal stabilities of proteins are often mediated by salt [25, 27], the effect of ionic strength was corroborated by repeating the same experiments with solutions conditioned at 0 mM, 50 mM, 150 mM, and 250 mM NaCl (Figure 6.2). Identical to earlier observations, change in size distribution was first witnessed at 60 °C for all tested conditions, which exacerbated with incrementing temperature with HSA aggregates being consistently larger than BSA, and unequivocally showing signs of uncontrolled HSA aggregation at 80 °C (Figure 6.2a and b). Notably, the extensive aggregation behavior of the proteins was observed only in the presence of NaCl whereas the aggregation trends were diminished in the absence of NaCl even at higher temperatures (Figure 6.2c and d), negating the fundamental assumption of aggregation to be an inevitable response to protein denaturation. Furthermore, the earlier hypothesis of thermal denaturation driven HSA aggregation is also scrutinized for further investigation on the structure-stability relationship of protein behaviors.

Figure 6.2 DLS measurement of BSA and HSA aggregation trends with incrementing temperature at different ionic strengths. (a) BSA size distribution, (b) HSA size distribution, and direct comparison of BSA and HSA at (c) 70°C and (d) 80°C relative to the different ionic strength conditions.

Taking note of the unique DLS measurements in NaCl-free solutions, the CD spectra of heat-treated proteins were reassessed in NaCl-free buffer as seen in figure 6.3. In salt-free conditions, the two proteins exhibited similar conformational stabilities even when heated to 80 °C, which is in contrast to the significant differentiation in secondary and tertiary structures previously noted. The superimposed CD spectra of the serum albumins in 0 mM and 150 mM NaCl buffer conditions further revealed native BSA to be the more instable protein as indicated by the increasing MRE with different ionic strengths. On the other hand, HSA conformation at room temperature were uninfluenced by the change in ionic strength but sensitized at higher temperature. The chronological order of events then can be predicted to begin with (1) thermal denaturation which then is

followed by (2) salt-mediated aggregation, and further down the road for HSA, (3) a salt-enabled entanglement of proteins to further advance the unfolding process, which is most likely a combinatory effect of thermal and protein-protein interaction induced denaturation. The data also suggests that there would be limitation to the extent of thermal denaturation of which an individual protein would undergo if without any other contributive factors such as protein-protein or protein-surface interactions (Table 6.2).

Figure 6.3 Structural analysis of BSA and HSA at 70°C or 80°C heat treatment under NaCl-free conditions. Inset figure shows direct comparison of CD spectra with or without NaCl for BSA (top) and HSA (bottom), separately.

Table 6.2 Secondary and tertiary structural analysis of BSA and HSA in the absence of NaCl resolved from CD spectra MRE peak analysis

Protein	Temp (°C)	α -helicity (%) MRE ₂₂₂ (MRE ₂₀₈)	MRE ₂₂₂ /MRE ₂₀₈	Structure
BSA (0 mM NaCl)	20	58(58)	0.92	Single-stranded
	70	44(43)	0.86	Single-stranded
	80	40(39)	0.83	Single-stranded
HSA (0 mM NaCl)	20	61(61)	0.95	Single-stranded
	70	45(44)	0.87	Single-stranded
	80	42(42)	0.83	Single-stranded

Often, most works describe the conformational stability of proteins broadly in terms of the resistance towards structural changes against the changing temperature, solution pH, ionic strength, chemicals or detergents [72-75]. However, as demonstrated by the salt-conditioned and salt-free protein behavior above, it was necessary to isolate conformational stability into different facets to better understand the proteins' response when exposed to different stimulants. Therefore, thermal stability was strictly defined to be the intrinsic resistance towards structural changes as a result of heat treatment, while conformation stability would be referred to as the extrinsic resistance towards structural changes as a result of ionic strength variations (*i.e.*, at room temperature), in all cases under physiological pH. With this distinction, the results can be summarized that HSA has a higher conformational stability than BSA, but a relative thermal stability depending on ionic strength, whereby HSA is less thermally stable than BSA in the presence of NaCl, but similar when absent of NaCl. In this respect, our observations differ from the findings by Michnik *et al.*, which revealed that HSA is thermally more stable than BSA in the absence of NaCl at pH 6 [20], and also deviates from the reports of Giancola *et al.*, which claims that the native conformation of proteins are reinforced with increasing ionic strength [76]. This highlights that the relative thermal stabilities vary greatly depending on the solution condition (*e.g.*, pH and ionic strength). Consequently, the colloidal stabilities also depend on ionic strength and generally decreases with increasing temperature. Interestingly, HSA has a lower conformational stability than BSA in its denatured form, which becomes more obvious with increasing thermal input. While the evaluation of thermal, conformational and colloidal stabilities of serum albumins have been mostly discussed using its native form as a starting point [24, 77-80], the relative stabilities of denatured serum albumins has yet to be discussed. This holds significant relevance especially for applications involving protein adsorption, whereby the tendency to denature can act as the driving force for greater protein-surface interactions (*i.e.*, entropically-driven protein adsorption) [52, 55].

6.3.3 Adsorption Stability of HSA and BSA

The respective adsorption behaviors of HSA and BSA on a planar silica surface were examined with the measured frequency and dissipation shifts from the QCM-D,

which relate to the adlayer mass and viscoelasticity changes, respectively. A comparison of frequency and dissipation shifts from the adsorption of the serum albumins at concentrations of 10 and 50 μM in 150 mM NaCl suggest that the intrinsic protein-surface interaction between native serum albumin and silicon oxide is stronger for HSA with faster adsorption rate, higher protein uptake and greater adlayer rigidity (Figure 6.4). Subsequent investigations were performed using a protein concentration of 50 μM in order to clearly observe the effects of conformational stability, especially in cases involving post-adsorption reorientation.

Figure 6.4 BSA and HSA adsorption trends relative to bulk protein concentration in solution (150 mM NaCl) measured by QCM-D in (a) frequency shifts and (b) energy dissipation shifts.

Since protein adsorption is ionic strength dependent [50, 51, 81, 82], the correlation between conformational stability and adsorption was investigated for HSA and BSA under varying ionic strengths. The QCM-D frequency shifts resulting from BSA adsorption showed a monotonic increase in mass uptake when ionic strength was gradually reduced, with the highest saturation uptake observed in the absence of salt (Figure 6.5a and b). The simultaneously measured dissipation shifts showed corresponding increase in adlayer viscoelasticity with mass uptake. In a similar fashion, the frequency shifts resulting from HSA adsorption also showed a monotonic increase in mass uptake but only when ionic strength was reduced from 250 mM NaCl to 50 mM NaCl (Figure 6.5c and d). In the absence of salt, the saturation uptake was lower than that of the 150 mM NaCl condition. Nonetheless, HSA adsorption occurred at a more rapid rate than BSA at all tested ionic strength conditions, and considering the similar

diffusivity of the serum albumins, this would suggest that there would be a significant difference in the desorption rate. The energy dissipation shifts for HSA adsorption were also consistently lower than BSA, and with overshooting features which is indicative of post-adsorption reorientation. Altogether, HSA appeared to have greater affinity to silica than BSA.

Figure 6.5 QCM-D results from investigating ionic strength dependent protein adsorption trends measured by the shifts in (a) BSA frequency and (b) BSA energy dissipation, and (c) HSA frequency and (d) HSA energy dissipation.

Taking note of the overshooting data, post-adsorption protein adlayer evolution was examined by analyzing the time-independent viscoelastic properties as a function of accumulating mass. Generally, the curves displayed two phases comprising of an uprising longer segment and a shorter segment that resumes after an abrupt change in direction, which correspond to the initial uptake of proteins until saturation and the post-adsorption reorientation phases, respectively. The earlier phase can reveal the relative viscoelastic

properties of each adsorbing protein molecule, and also hints on the binding strength for the protein-surface interaction as explicated by the hydration level. The proceeding shorter slope phase provides information on the post-adsorption denaturation and/or reorientation of proteins. Depending on the trajectory of the curve, the kinetic mode of the proteins can be predicted as seen in figure 6.6.

Figure 6.6 Simplified model of near-surface protein-surface interaction derived from the instantaneous trajectory of the f-D curve and schematics corresponding to each quadrant.

Figure 6.7 f-D curve trend comparisons for varying ionic strength conditions extracted from the QCM-D frequency and energy dissipation measurements for (a) BSA and (b) HSA.

The f - D curves of BSA adsorption showed distinguishable features with the change in solution's ionic strength conditions (Figure 6.7a). The trends are reflective of the aggregation events, whereby the most dynamic behavior was observed in the 150 mM NaCl conditioned experiment. Initially with greater viscoelasticity in the adlayer, which could be due to salt ion or water molecule couplings that associates with the individual protein molecules, the 150 mM NaCl conditioned sample exhibits the greatest degree in post-adsorption transitions as indicated by the change in direction of the slope and the progressive transitions in the plot. In contrast, the salt-free solution accumulated the greatest mass but without much variation in the viscoelasticity, which further supports the key role of salt in overseeing the protein behavior.

Meanwhile, the early phase adsorption trends of the HSA runs had less diversity amongst the different ionic strengths (Figure 6.7b), which was to be expected in view of its insensitivity to the ionic strength gradient as discussed earlier in the chapter. Also, the viscoelastic properties of the adsorbing protein molecules were more rigid than BSA suggesting a tighter protein-surface interaction. Still, significant post-adsorption transitions were observed for all samples, which is peculiarly similar to the aggregation trends and correlative multi-phase transitioning in its secondary structure with heat treatment.

Taken together, the QCM-D frequency and dissipation shifts and the f - D curves showed that the adsorption of BSA is more ionic strength dependent. This agrees well with the solution behavior where BSA in its native form was identified with greater sensitivity to ionic strength and hence, lesser conformational stability than HSA. However, in the post-adsorption phase, transitioning in the adlayer was greater in HSA despite it being understood to be more conformationally stable. This exemplifies the need to separate the different types of structural stability of proteins of which, in this case, is the structural stability factor measuring its resistance towards adsorbent-induced denaturation, or otherwise to be defined as adsorption stability. Therefore, it can be determined that while the conformational stability of HSA is higher than BSA, the adsorption stability of HSA is lesser than BSA .

6.3.4 Adsorption Behavior of Denatured HSA and BSA

Motivated by the finding that the relative conformational stability of heat denatured HSA (dHSA) is lower than heat denatured BSA (dBSA), which is a direct contradiction to their native counterparts, adsorption behavior of heat denatured serum albumins were also compared under varying ionic strength via QCM-D (Figure 6.8). Samples were heated up to 70°C to achieve heat denaturation while preventing the formation of micron-sized aggregates. In result, the frequency shifts for dBSA adsorption showed a monotonic increase in saturation uptake with increasing ionic strength with corresponding energy dissipation shift. With exception to the salt-free condition, overshooting behavior trends were observed but for both frequency and dissipation shifts in coordination (figure 6.8a and b). In the other hand, the frequency shifts for dHSA adsorption showed monotonic increase in ionic strength but only up to 150 mM NaCl. The adsorption of dHSA at 250 mM NaCl resulted in slightly lower uptake. The energy dissipation shifts corresponded well with the saturation uptake, which differed from the trends observed for native has (Figure 6.8d and e).

It was further revealed from the f - D curves that the heat denatured serum albumins did not exhibit traits of post-adsorption reorientation (Figure 6.8 c and f). This is expected since denatured serum albumins are generally in a thermodynamically favored, less-ordered state [78, 83, 84] and will therefore be less entropy-driven to undergo further conformational changes on the surface [85, 86]. Additionally, the denatured proteins are less likely to adsorb as monomers, limiting the degree of freedom for structural rearrangements mostly impeded by steric hindrance [87-89]. As for the overshooting in the dBSA adsorption trends, the resolved f - D curves revealed the adsorption phase and post-adsorption phase to overlap in opposite directions, which could then be seen as a momentary overcrowding effect near the surface followed by a bulk desorption. Still, ionic strength dependent slope gradients was observed whereby the outcome showed straightforward correlation to the charge-shielding effect (Figure 6.8c). The f - D curve profiles of the dHSA adsorption were distinct from its native form but differentiable from the dBSA adsorption behavior (Figure 6.8f). With exception to the salt-free solution, the curves indicate a constant softening of the adlayer with accumulating mass even at saturation and with minimal desorption. The observed

phenomenon can be attributed to protein-protein or oligomer-oligomer interaction near the surface, and potentially the establishment of multilayered protein adlayers. With increased sensitivity to ionic strength, the uptake kinetics of each curve show correlation to the salt contents, and a clear electrostatic influence in the total accumulated mass.

Figure 6.8 Ionic strength dependence adsorption trends measured by Frequency and energy dissipation shifts for (a and b) denatured BSA (dBSA) and (d and e) denatured HSA (dHSA), and the f-D curve analysis for (c) dBSA and (f) dHSA.

Comparing the two sets of experimental data (native proteins vs denatured proteins), the reversal of ionic strength dependency is most interesting. This could be due to the transitioning from ‘soft’ to ‘hard’ protein when the serum albumins are aggregating wherein the adsorption of soft proteins is generally entropy-driven and less affected by electrostatic interactions while the adsorption of hard proteins is more electrostatically driven [34, 90]. Indeed, a recent demonstration of the adsorption behavior of HSA was reported to differ greatly on silica, which is electrostatically-driven, than on titania, which is entropy-driven [55].

6.3.5 Adsorption Behavior in NaCl-free Solution

Figure 6.9 QCM-D measurements of the adsorption behavior of proteins before and after heat treatment in the absence of NaCl for (a) BSA and (b) HSA.

The salt-free conditions for both native and denatured proteins were separately collected and compared (figure 6.9) wherein the proteins in bulk solution were effectively denatured but resistant to oligomerization (See section 6.3.2). Referring back to chapter 4, the adsorption of native BSA without any supplementary aid was found to be insufficient in forming a sustainable protein layer along the surface. In contrast to the high uptake and high viscoelasticity readings of native BSA adsorption, the adsorption trends of dBSA monomers (and/or dimers) were similar to that of a protein spreading along the surface after adsorption (see chapter 4), which is highly plausible considering the destabilized structure of the molecule. The adsorption kinetics of the native HSA were also in resemblance of the surface spreading attribute (6.9b). As evinced by its f - D plot in figure

6.7b, this overshooting observation is representative of an extensive post-adsorption rearrangement. Furthermore, with earlier CD data results of HSA at elevated temperatures confirming the extensivity in its denaturation process, it can be argued that there is an intrinsic difference in its denaturation potential that allows HSA to use the momentum of denaturation (entropy-driven) to push forth protein-protein or protein-surface interactions. Interestingly, with heat treatment, the overshooting phenomenon of HSA was diminished, which further supports the importance of the native conformation for the entropy-driven process.

6.4 Conclusion

In this work, the behaviors of BSA and HSA were compared in solubilized state and at the liquid-solid interface. The homologous proteins were selected in consideration of the reported differences in their behaviors despite their close resemblance and near identical physicochemical properties in hope to uncover insights of fundamental protein adsorption processes that depend on intrinsic protein properties. Using temperature and ionic strength as the key parameters, conformational stability was evaluated in deciphering the protein-protein interactions and protein-surface interactions. Immediately, it was revealed that conformational stability was too broad of a definition to pinpoint the minute differences in the homologous proteins that were decisive factors in determining the characteristic behaviors. Based on the investigations, the proteins' capabilities to withstand denaturation were categorized into thermal (heat), conformational (ionic strength or pH), colloidal (aggregation-induced), and adsorption (surface-induced) stabilities. From the in depth analyses, an intrinsic action potential in the form of entropy is argued to be an important parameter in determining the extensiveness of post-adsorption protein denaturation. Between the pair of proteins, HSA was determined to be the better adsorbate which was able to undergo multiple conformational transitions and hence establish tighter interactions with the surface.

In view of the widespread use of BSA for many industrial and research applications, the integration of HSA as an alternative to BSA is promising. Although the FBS serum fouling study was omitted from this chapter, but it would be interesting to see

how it would perform in place of BSA as a passivation agent. More importantly, however, the experiments and the analytical models suggested herewith were purposefully designed so that it could be easily utilized for other comparative studies.

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Chapter 7

Implications and Future Outlook

Herein, the three-part series of the experimental results is summarized and broken down for further discussion. By observing the relative influence of ionic strength on protein-surface interactions, the inherent weakness of the BSA-silica interaction was confirmed. From there, the project was able to progress to reveal the reason for the apparent weakness and more importantly to address the puzzling rationale for considering BSA as a golden standard for surface passivation. Using insights gained from the due diligence, applicable and simple solutions were proposed for the BSA surface passivation technique. The most important aspect, however, is the realization of the knowledge gap between research and applications to emphasize the necessity of well-formulated projects for practical and intuitive solutions. The potential of protein adsorption studies is vast especially in consideration of the general lack of consensus within the works and with the experimental platform presented, herewith, there is relative ease in expanding out the research efforts in to various fields. Finally, glimpse of the preliminary results of the continuing investigations are presented to further emphasize the optimism in this field of study.

7.1 General Discussion

Protein adsorption is a common phenomenon that occurs spontaneously and equally for all exposed material surfaces. It is also a complicated process that had been studied for several decades, but to this end, there is no finite solution to completely repel or avoid the natural response. Nevertheless, understanding the fundamental biophysical mechanisms of protein adsorption represents the key towards obtaining the necessary insights to approaching a practical solution. Throughout the dissertation, the protein adsorption event had been systematically investigated through the use of bovine serum albumin (BSA) as the model protein and silica as the model substrate.

The three-part series of the project was initiated by looking at the effect of solution ionic strength on the protein behavior. Experimentally, a digression in the amount of protein uptake was observed with ionic strength. Theoretically, the opposite trend was to be expected since the effective charge shielding would promote greater protein-surface interaction. The theoretical assumptions were sound, and in fact, it was revealed that the data was actually in agreement only after realizing that the method of interpretation was at fault. Conventionally, a *greater adsorption* was associated with greater mass accumulation. However, with the energy dissipation readout, it was revealed that the *greater adsorption* was expressed in the form of protein spreading (*i.e.*, surface-induced denaturation) as indicated by the signs of dehydration in the protein adlayer. Consequently, the formed adlayer from the greater protein-surface interactions would be characterized with less mass and greater rigidity. The protein adlayers were tested against fetal bovine serum to evaluate their antifouling capabilities. Indeed, the protein adlayers formed at higher ionic strength were slightly better but equally unimpressive with a mere ~20% blocking efficacy. The study clearly demonstrated that the BSA-silica interaction is intrinsically weak, regardless of ionic strength conditioning, and that BSA adsorption by itself would be insufficient in forming a stable protein coating along the surface. To support this claim, a modified protein coating protocol was proposed using cross-linking agents, which demonstrated significant improvement in the blocking efficacy.

Following up on the protein spreading as a relative measure for protein-surface interactions, the structural stability aspect was vigorously challenged whereby the

adsorption trends of heat-denatured proteins were compared to proteins in its native form. As it turns out, the adsorption kinetics and uptake rate were both significantly improved with the disruption of the secondary structure of BSA. The distinction was even more obvious with the reduction of bulk protein concentrations in which the time requirement to reach surface saturation was significantly extended for native BSA while the variation was irrelevant for denatured BSA. AFM imaging revealed that the denatured BSA was able to form a complete dense protein layer on the surface with a thickness variation of 4 to 15 nm whereby the native BSA molecules were sparsely adsorbed exposing most of the bare silica surface. The passivation studies were particularly exciting being able to achieve 60% blocking efficiency only after 3 min of denatured BSA coating while the turnout from 3 h of native BSA incubation achieved only ~38% blocking efficiency.

The investigation provided clues to conformational stability having major impact over protein adsorption trends but how it affects the adsorption mechanism was not fully clear. The comparison of bovine serum albumin (BSA) to human serum albumin (HSA) was uniquely appropriate to proceed with the investigation since it had near identical physicochemical properties but a subtle difference in its amino acid makeup that was sufficient enough to make a marginal difference in its thermal stability. From this investigation, it was found that there were no direct correlation between its conformational stability and the extent of surface-induced denaturation. Rather, it was demonstrated that the proteins propensity to spread along the surface upon contact was an intrinsic entropy-driven attribute, which was more relatable to protein aggregation trends when disregarding the difference in the rate-limiting steps. The results of the comparative study showed that HSA was the better adsorbate, and although not tested, it would be predicted that HSA would show better blocking capabilities. Additionally, the mechanism in which thermally denatured proteins developed a thicker and denser adlayer appeared to be independent of entropy-driven factors, but instead by protein-protein interactions at the near surface.

In summary, entropy-driven post-adsorption rearrangements appeared to be the determining factor for the intimacy between proteins and surfaces, and were ultimately dependent on the intrinsic properties of proteins. BSA was a weak adsorbate, at least for silica surfaces, which could still stabilize on the repellent surface but only when mediated

with supplementary factors (*i.e.*, pre-denaturation, cross-linking agents). The solution conditions and material properties are both important factors that influence the resulting protein structure formed on the surface, but the results of this dissertation implies that the more mechanistic roles of such factors are aligned with how they influence the conformational stability of adsorbing proteins. In the perspective of a material scientist, the designing of a biocompatible, protein-repellant surface becomes close to impossible having to consider thousands of various protein species within a complex biological milieu. Therefore, surface coating with proteins or biomolecules that are already biocompatible show promising potential in fulfilling a bioinert material surface.

7.2 Future Outlook

7.2.1 Fundamental Studies

The scope of the project discussed within this dissertation is merely a tip of the iceberg in configuring the fundamental studies of protein adsorption, and more systematic studies are in demand to fully appreciate the detailed mechanisms of this phenomenon, especially in view of the eventual needs to look at physiologically similar settings. Nonetheless, the discussions presented herewith exemplifies that a substantial advancement in knowledge can be acquired with the current analytical techniques and an appropriate study design. Subsequent fundamental investigations will expand on the protein's conformational properties and consequent adsorption stabilities mainly by expanding on the library of proteins to be investigated, and the types of adsorbents to be used, but particularly with greater interest in blood-originated proteins (*e.g.*, fibrinogen, IgG, lysozyme, etc.) and biomaterials commonly used in invasive biomedical applications (*e.g.*, gold, silver, metal oxide materials, and polymers). One such investigation is the BSA and HSA adsorption behaviors on titania as a contingent project to the previous study on silica. Preliminary studies show that there are clear deviations from the observations made on silica, of which in general, the effect of ionic strength is less pronounced emphasizing the extensive protein-surface interaction potential of titania (Figure 7.1). The ongoing study will include an in depth analysis of the adsorption kinetics and a pH related experiment will be conducted to see the relative change in the

Figure 7.1 Preliminary QCM-D results of ionic strength dependent adsorption behavior of BSA (lef) and HSA (right) on either silica (top) or titania (bottom) surfaces.

adsorption behavior. Furthermore, the FBS blocking efficacy will be revisited to examine the blocking efficiency results and to better define the influence of the substrate property factors.

Another initiative that is currently in its planning and designing phase is the study of protein adsorption with particular focus on the Vroman effect[1], whereby the Vroman effect is described as the phenomenon of surface adsorbing proteins competing and eventually replacing other proteins that are typically characterized with less protein-surface stability. This project advances the current experimental designs from a one-protein system to a two (or more)-protein system, which will be practically relevant for the eventual need to study more complex biological systems. The investigation will

compare the resulting protein layers from a sequential adsorption protocol (*i.e.*, two or more different one-protein solutions adsorbed on surface in sequence) to that from a mixed adsorption protocol (*i.e.*, multiple-protein solutions simultaneously incubated on surface). Using QCM-D and fluorescent microscopy, the study will look at (1) the time resolved protein coating efficacy against Vroman effect; and (2) the competitive nature of protein adsorption and/or positive cooperative effects of proteins. Simultaneously, the surface passivation method using denatured protein species will also be challenged with the sequential adsorption protocol to effectively sort out the advantages and disadvantages of using thermally denatured proteins, or to identify any other overlooked attributes.

7.2.2 Protein Coating of Nanoparticles

Another area of study that requires the extensive fundamental knowledge of protein adsorption is the protein-nanoparticle interactions. Nanotechnology and the application of nanoparticles in medicine have revolutionized the approach in treating human diseases [2, 3]. However, nanoparticles exposed to *in vivo* or *ex vivo* conditions revealed a spontaneous accumulation of proteins surrounding the nanoparticles triggering unexpected biological complications impeding the product development and commercialization of nanotechnology [4, 5]. The protein structure formed around the nanoparticle, referred to as the *protein corona*, creates a new bioidentity overruling the intended functional designs of the nanoparticle as seen in figure 7.2 [6, 7].

Figure 7.2 Schematic of the protein corona on nanoparticles upon entry to a biological system with the mechanisms described based on fundamental protein adsorption schemes.

It is hypothesized that by diffusion-limited adsorption and Vroman effect, the protein corona would form a double layer consisting of a primary protein-surface interaction based “hard” corona and a secondary protein-protein interaction based “soft” corona [8, 9]. Depending on the composition of the protein corona, the protein-nanoparticle complex would trigger various preconditioned host responses such as cellular uptake, renal clearance [10] or acute immune reactions [11]. Recognizing its similarity to fundamental protein adsorption studies, it is possible that the complex nano-bio interactions could be delineated by simplified 2D models such as the experimental designs that had been presented in this dissertation.

Figure 7.3 Experimental design proposal for protein corona studies relying on translation of nanoparticle-protein interactions to 2D model and *vice versa*

On a separate but related note, a solution focused on mitigating the severity of protein adsorption on nanoparticles through protein coating had been devised using the surface passivation techniques discussed in the dissertation. Essentially, the goal is to camouflage the nanoparticle surface by mimicking the biological surroundings using host-friendly blood protein-coatings such as serum albumins or fibrinogen, so as to prevent abnormal accumulation of immune triggering proteins, namely the complement proteins that are capable of activating the acute immune response [12]. Complement proteins are commonly found associating with nanoparticles embedded within the protein corona of which play a pivotal role in the host’s defense [13-15]. In general, the

complement activity is recognized by the deposition of the third complement protein (C3) or its fragmented constituents (*i.e.* C3b, iC3b, and C3dg, *etc.*) of which are proliferated by the work of C3 convertases. There are three different pathways that initiates the assembly of the proteolytic convertases: (1) the classical pathway (CP) instigated by an antigen-antibody interaction; (2) the lectin pathway (LP) triggered by the interaction of mannin-binding lectin (MBL) to specific carbohydrates; and (3) the alternative pathway (AP) in response to non-self surfaces [16, 17]. Once activated regardless of the triggering factor, the complement system converges to a singular mode of action quickly tagging and eliminating the detected danger through phagocytosis, and even causing direct lysis of pathogens through the formation of membrane attack complexes (MAC) [18].

Figure 7.4 Schematic of C3b induced complement activation which includes the activation of C3b (blue). C3b adsorption, accumulation and amplification steps (green), membrane attacking complex (MAC) assembly pathway (white), and the proposed methods to mitigate the complement activation (red).

Host cells are equipped with complement regulatory factors (*i.e.*, factor H [19]) that counterbalances the amount of C3b opsonization. The pristine nanoparticles, on the other hand, would be fully exposed to this effect ending up being engulfed by

macrophages or causing detrimental side effects such as the complement activation related pseudo-allergy (CARPA) response [20]. To this extent, protein coating is hypothesized to be an effective method to mitigate the host's oversensitivity to nanoparticles as presented in figure 7.4.

The objective of the project is to completely passivate the nanoparticle surface with serum albumins (or fibrinogen) to render the surface inaccessible to the complement proteins (as represented by a blue colored coating in schematic), or by functionalizing the surface with factor H to mimic the host cell's complement regulatory mechanism to deactivate incoming complement proteins. Silica nanospheres with a 100 nm diameter have been used as a model system and the protein coating have been established by fluxing the nanoparticle in protein solution for 2 h at 37 °C as seen in figure 7.5.

Figure 7.5 Schematic of the protein coating process outlined by the following general steps: (i) solvent exchange of SiNPs and dilution in Tris buffer; (ii) incubation in protein solution at 37°C for two hours; (iii) centrifuge and wash to remove remnant proteins in supernatant; then, (iv) resuspend in solution.

The complement responses to silica nanospheres had been evaluated in its pristine form, with blood protein (*e.g.*, BSA, HSA, Fibrinogen) coating on the surface, with IgG

coating as a positive control, or with factor H, a regulatory protein of complement activation, coated on the nanoparticle surface. The nanoparticle-protein interaction was characterized with DLS and NTA, and each coating efficacies were assessed with ELISA and Western Blot. Preliminary results confirmed that HSA performed slightly better than BSA as a surface masking agent, but both options were outperformed by the larger fibrinogen and also in comparison to the functional factor H (Figure 7.6). Although the effect of protein coating was imperfect, the preliminary results were sufficient as a proof-of-concept work suggesting its potentials and more importantly reinforcing the value addition of establishing a solid fundamental foundation in protein adsorption. The preliminary studies will be further developed with the uncovering of more key insights related to protein adsorption.

Figure 7.6 Evaluation of complement activation intensity in either normal human serum or factor B depleted human serum (transparent columns) induced by silica nanospheres with or without protein coatings measured by ELISA kit sensitized to MAC.

7.3 Concluding Remarks

Conclusively, there is still a vast amount of work that needs to be covered in order

to fully appreciate the concept of protein adsorption. It is, nonetheless, a fascinating field that becomes more complex as the pieces come together, and it is definitely a field that requires the utmost patience and consistency. It is extremely important to establish a firm understanding of the elemental concepts before engaging in the more complex experimental models; otherwise the enduring journey may end up entangled in a never-ending Möbius loop. For example, until experimenting with the interactions and the adsorption of BSA to silica, there was no awareness of the inherent weakness of BSA because of its extensive use as a surface-passivating agent. Another example would be the mechanistic difference in the unfolding process depending on the source of denaturation. All such minor details will make a world of difference when it comes to solving problems with greater impacts, and hence the continuous emphasis on fundamental studies and empirical study designs.

The final destination for the collective investigations and the ongoing works is to engage in the protein-nanoparticle interactions to better understand and address the issues related to protein corona. As briefly mentioned above, the process in which proteins accumulate at the nanoparticle surface is essentially identical to the basic process of protein adsorption. However, when reviewing the literature related to protein corona, it is surprising to realize that the underlying assumptions in regards to protein adsorption is largely dismissed or misguided, much like the misalignment between protein aggregation studies and protein adsorption studies. Establishing a firm foundation through protein adsorption studies and based on preliminary results, the prospective studies exploring protein-nano interactions are optimistic.

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