Understanding of 2D human hair keratin platforms for cell culture applications

Tan, Bee Yi

2020

Tan, B. Y. (2020). Understanding of 2D human hair keratin platforms for cell culture applications. Doctoral thesis, Nanyang Technological University, Singapore.
<https://hdl.handle.net/10356/143096>

<https://hdl.handle.net/10356/143096>

<https://doi.org/10.32657/10356/143096>

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

Downloaded on 26 Apr 2023 20:04:11 SGT

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**UNDERSTANDING OF 2D HUMAN HAIR
KERATIN PLATFORMS FOR CELL CULTURE
APPLICATIONS**

TAN BEE YI

**SCHOOL OF MATERIALS SCIENCE AND
ENGINEERING**

2020

**UNDERSTANDING OF 2D HUMAN HAIR KERATIN
PLATFORMS FOR CELL CULTURE APPLICATIONS**

TAN BEE YI

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2020

Statement of Originality

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

9 June 2020

Tan Bee Yi

.....
Date

.....
Name

Supervisor Declaration Statement

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

9 June 2020

.....
Date

Ng Kee Woei

.....
Supervisor Name

Authorship Attribution Statement

*(B) This thesis contains material from 1 paper published in the following peer-reviewed journal which I am listed as an author.

Part of the content in Chapter 4 is published as Tan, B. Y., Nguyen, L. T., Kim, H. S., Kim, J. H., & Ng, K. W. (2017). Cultivation of human dermal fibroblasts and epidermal keratinocytes on keratin-coated silica bead substrates. *Journal of Biomedical Materials Research Part A*, 105(10), 2789-2798.

The contributions of the co-authors are as follows:

- A/Prof Ng Kee Woei and Prof Jae-Ho Kim provided the initial project direction.
- I prepared the manuscript drafts. The manuscript was then revised by Dr Luong and A/Prof Ng Kee Woei.
- I co-designed the study with Dr Luong and A/Prof Ng Kee Woei.
- I performed all the laboratory work at the School of Materials Science and Engineering and analyzed the data.
- Hyo-Sop Kim performed silica beads synthesis and keratin coating process.
- Materials characterizations including atomic force microscope, Fourier-transform infrared spectroscopy, Surface plasmon resonance, contact angle measurement, and zeta potential data were conducted and analyzed by Hyo-Sop Kim.
- All scanning electron microscopy, including sample preparation, was conducted by me in the Facility for Analysis, Characterization, Testing and Simulation.

9 June 2020

.....
[Date]

Tan Bee Yi

.....
[Name & Signature]

Abstract

Human hair proteins have shown great potential in biomedical applications due to their biocompatibility, biodegradability, and more importantly their ability to promote cell adhesion and proliferation. Hair proteins consist of two main components: keratin (intermediate filaments proteins) and keratin-associated proteins (KAP, matrix proteins). The overarching aim of this project was to establish an understanding of the fundamental interaction potential within and between the two hair proteins *in vitro*. This understanding would then be applied to produce novel keratin-based two-dimensional (2D) substrates for biomedical applications. Specifically, hair protein-based coatings and films were targeted for development. Protein immobilization on a surface is a well-adopted method to create bioactive surfaces but the short-term stability of the coating is a limitation. Thus, total hair proteins (THP) were immobilized covalently through chemical crosslinking via free-radical assisted methods, on plasma-treated polystyrene (PS) substrates, to achieve coatings that offer long term stability. THP in the reduced form can remain as a coating for up to 21 days in media incubated at 37 °C. In addition, the reduced THP shows significantly better protein retention on the substrates after sodium dodecyl sulphate (SDS) washing, indicating stronger interactions with the PS substrates. These interactions are likely to be dominated by covalent linkages due to the presence of active thiol groups which react readily with free radicals on the activated substrate surfaces after plasma treatment. Moreover, THP is found to prefer hydrophobic adsorption onto the substrates, which might be due to the dominant hydrophobic residues in the protein sequences. Besides THP extracts, it was also intriguing to understand the interaction capacity of the keratin and KAP fractions separately. Keratin and KAP were separated successfully by modifying a previously reported protocol. A keratin film that was mechanically stable and easy-to-handle was obtained by solvent casting at room temperature. The absence of KAP was found to decrease the brittleness of the film. The films obtained were characterized for physical properties (permeability, thickness, morphology, surface wettability, etc.),

mechanical properties, and thermal properties. This film was mechanically stable and exhibited 49.82 ± 6.08 MPa ultimate tensile strength (UTS) in a dry state which was nearly 50 times higher than films made with total human hair proteins with a plasticizer (1 ± 1 MPa). In the wet state, although keratin film exhibited a lower UTS 0.56 ± 0.18 MPa, it could be stretched extensively, which could never be achieved in a dry state. The tensile test results showed that the keratin film behaved as a viscoelastic material in the wet state tensile test. The keratin film also allowed 20kDa-FITC dextran to permeate in the case II diffusion mechanism. Interestingly, the keratin film showed a distinct surface and cross-sectional morphology and a protein secondary structure transformation. It is hypothesized that the keratin film could be formed by a hierarchical self-assembly process. Subsequently, keratin film was tested for basic cytocompatibility and cell physiology influences using human epidermal keratinocytes (HEKs). The keratin film was found to offer greater biocompatibility and cell response by HEKs in terms of enhanced cell proliferation, viability, K14 marker, and IL-1 α cytokine, in comparison to a collagen I coating. In conclusion, the human hair protein-based coatings and keratin films could be potential platforms for biomedical applications.

Lay Summary

Human hair is made up of proteins which can be further classified into two components: keratin, and keratin-associated proteins (KAP). Keratin is intermediate filament protein which forms a fiber-like structure that contributes to the mechanical strength of hair. KAP acts as a matrix and binds with keratin through disulfide crosslinking that further enhances the durability of hair. The purpose of this project is to establish an understanding of the interaction potential of hair proteins and produce novel hair proteins-based 2D platforms for biomedical applications.

Protein coating is a common way to create bioactive surfaces, but the short-term stability of the coating is a limitation. In this project, total hair proteins (THP) were coated on plasma-treated polystyrene substrates to achieve a layer of coating that offered long term stability. The coating was stable up to 21 days in the presence of cell culture media at 37 °C. In addition, 76.79 % of the THP remained on the surface after aggressive washing, showing that THP was strongly bonded on the surface.

Keratin and KAP were separated successfully through a 2-step extraction method called the Shindai method. A keratin film that was mechanically stable and easy-to-handle was obtained simply by solvent casting a keratin solution at room temperature. It was proposed that the absence of KAP reduced the brittleness of the film. Characterization showed distinct surface and cross-sectioned features, as well as a protein secondary structure transformation. Based on these findings, it was hypothesized that the keratin film could be formed by a hierarchical self-assembly process. The mechanical properties of the keratin film were characterized by tensile testing in wet and dry states. The ultimate tensile strength (UTS) exhibited by the keratin film was 49.82 ± 6.08 MPa in a dry state, which was nearly 50 times higher than other studies. In the wet state, the keratin film exhibited a much lower UTS, 0.56 ± 0.18 MPa, but it behaved as an elastomer which was able to be extensively stretched. Subsequently, the keratin film offered great

biocompatibility and cell responses. In addition, the animal study demonstrated the potential of keratin film as an alternative cell carrier for ocular applications. In conclusion, this project showed that human hair protein-based coatings and keratin films could be potential platforms for biomedical applications.

Acknowledgments

I would like to take this opportunity to express my profound gratitude to my supervisor, Associate Professor Ng Kee Woei, who gave me confidence and willing to make a risky decision, as well as extra effort to have me to work in his research group. The guidance given by him time to time shall carry me a long way in the journey of life on which I am about to embark. Besides my supervisor, I would like to thank my thesis advisory members: Associate Prof. Tan Lay Poh and Associate Prof Ali Miserez, for their insightful comments, and encouragement.

I would also like to express a deep sense of gratitude to Dr. Luong, for her exemplary guidance, mentoring and concerns. Without her cordial support, I believed that I would not be able to get through the difficulties from various stages and complete this task on time. I am obliged to all the researchers and students from Prof Ng's group for sharing useful advice and their valuable research experiences in their respective fields without any hesitation. Their help has given me a clearer direction which allowed me to aware of my weaknesses and improved it in a more effective way.

In addition, I would like to pay my special regards to the staff of FACTS for their assistance in FESEM and XPS. Furthermore, I would also like to acknowledge the technician staff from the Organic service lab especially Mr. Zi Li for his invaluable assistance for the mechanical test.

Lastly, I would like to thank my parents, sisters, and friends for their constant encouragement without which this assignment would not be possible.

Table of Contents

Abstract	i
Lay Summary	iii
Acknowledgments	v
Table of Contents	vii
Table Captions	xi
Figure Captions	xiii
Abbreviations	xvii
Chapter 1 Introduction	1
1.1 Background	2
1.2 Motivation	3
1.3 Hypotheses	6
1.4 Objectives	6
1.5 Scope	7
Chapter 2 Literature Review	9
2.1 Human Hair Structure and Proteins	10
2.3 Human Hair Proteins	11
2.3.1 Keratin	12
2.3.2 Keratin-associated Proteins (KAP)	16
2.4 Extraction Methods	18
2.5 Applications	20
2.5.1 Coating	24
2.5.2 Thin Films	27
2.5 Cell response of hair protein-based 2D substrates	33
2.6 Summary of literature review	33
Chapter 3 Experimental Methodology	35
3.1 Human hair proteins extractions	36

3.1.1	Total hair proteins (THP) extraction using Na ₂ S	36
3.1.2	Keratin and KAP extraction (Ethanol-assisted Shindai Method).....	36
3.2	Synthesis and characterization of physical coating on silica beads substrate	38
3.2.1	Silica bead synthesis	38
3.2.2	Keratin coating	38
3.2.3	Surface Plasmon Resonance (SPR)	39
3.2.4	Zeta potential	39
3.2.4	Characterization of Keratin Coated Silica Bead Surface	39
3.2.5	Cytokine Array	40
3.3	Immobilization of THP on Polystyrene (PS) Substrate.....	40
3.3.1	Free radical-assisted	40
3.3.2	Polymer-assisted	41
3.4	Quantification of Protein Concentration	42
3.4.1	BCA Protein Assay	42
3.4.2	Micro BCA Protein Assay	43
3.5	Ellman Assay	43
3.6	Keratin film fabrication by solvent casting	44
3.7	Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	44
3.8	Field Emission Scanning Electron Microscopy (FESEM)	45
3.9	Atomic Force Microscopy (AFM).....	45
3.10	X-ray photoelectron spectroscopy (XPS).....	45
3.11	Water contact angle measurement	46
3.12	Swelling test	46
3.13	Circular Dichroism (CD).....	47

3.14	Fourier-transformed infrared (FTIR) Spectroscopy	47
3.15	Differential Scanning Calorimetry (DSC)	48
3.16	Thermogravimetric Analysis (TGA)	48
3.17	Tensile Test	48
3.18	Nano-isothermal titration calorimetry (NanoITC)	48
3.19	Permeability test	49
3.20	Sterilization of keratin film	50
3.21	Cell Culture	50
3.22	Proliferation assay (PicoGreen Assay)	51
3.23	Metabolic assay (Alamar Blue Assay)	51
3.24	Immunoperoxidase Staining	51
3.25	ELISA Kit	52
3.26	Statistical analysis	53
	Chapter 4 Physical and covalent protein immobilization on substrate for cell culture applications	55
4.1	The Physical Coating on Silica Beads Substrate	56
	4.1.1 Characterizations of THP-coated silica beads substrate	56
	4.1.2 In-vitro work	61
4.2	Covalent coating on polystyrene substrate through plasma treatment	71
	4.2.1 Polymer-assisted protein immobilization	71
	4.2.2 Free-radical Assisted Protein Immobilization	74
	4.2.3 Interaction Study Between Purified Hair Proteins: Keratin and KAP	82
	Chapter 5 Keratin Films	93
5.1	Surface characterizations	94
5.2	Swelling Test	96
5.3	Protein Secondary Structure	97

5.3	Thermal Properties	100
5.4	Tensile test in dry and wet states	102
	Chapter 6 Biocompatibility of Keratin Films.....	107
6.1	Keratin film permeability.....	108
6.2	Cell morphology	109
6.3	Proliferation Assay	112
6.4	Cell metabolic activity	113
6.5	K14 staining.....	114
6.6	IL-1α quantification by ELISA Kit.....	115
	Chapter 7 Conclusions and Recommendations.....	117
7.1	Conclusions	118
7.2	Recommendations	120
	7.2.1 Exploit combination of keratin and KAP for protein immobilization and quantify the bonding present at interfaces	120
7.3	<i>In vitro</i> test	121
7.4	Exploring the self-assembly ability of keratin and the effects on properties	122
	7.4.1 Effect of drying process on keratin film structure	122
7.5	Interaction study between keratin and KAP	123
	References	125

Table Captions

Table 1 Summary of the subtypes from type I and type II keratins in amino acid content, isoelectric point, and molecular weight.	13
Table 2 Prominent amino acid contents of the three families in KAP: high sulfur (HS), Ultra-high sulfur (UHS), and High glycine-tyrosine (UHS) (Adapted from [38] with permission).	16
Table 3 Overview of the sequence characteristic of KAP in human hair (Adapted from [39] with permission).	17
Table 4 Summary of hair protein-based biomedical applications.....	21
Table 5 Summary of the key findings of hair protein-related thin film studies.	28
Table 6 Summary of the n value of the power law for different geometries [83].	32
Table 7 Thermal properties of non-treated PS, P-DI, and P-Na ₂ S obtained from TGA.....	78
Table 8 Summary of the sample collected for the dialysis optimization including sample collection time and sample name.....	85
Table 9 Summary of binding models exhibited between keratin and KAP titration n NanoITC at different concentration of urea.	88
Table 10 Summary of thermodynamic parameters obtained from sequential two sites in 2 M, 3 M, and 4 M of urea.....	90
Table 11 Mechanical properties of keratin thin film obtained from tensile testing.	102
Table 12 Summary of correlation coefficient (r^2 of keratin films corresponding to various mathematical models).....	108

Figure Captions

Figure 1 Schematic diagram of the human hair structure. (Reprinted from [20] with permission).....	11
Figure 2 Illustration of intermediate filament assembly from monomer to filament [26].....	15
Figure 3 Schematic of the reaction mechanism of reductive and oxidative extractions for hair proteins.	19
Figure 4 Possible functional groups of PS after plasma treatment followed by nitrogen and oxygen exposure.	25
Figure 5 Oxidized S-2p component of different samples: MP is pure microperoxidase-11; PTFE and PE referring to substrates without plasma treatment; DLC, PP, and NPP are plasma-treated samples with different gases during plasma deposition such as nitrogen/hydrogen, without nitrogen gas and nitrogen gas respectively. (Reprinted from [79]).....	27
Figure 6 Flow chart of ethanol-assisted Shindai Method for keratin and KAP separation. (Adapted from [50])	38
Figure 7 Schematic of THP immobilization through the free radical-assisted plasma.	41
Figure 8 Schematic of the binding mechanism of polymer-assisted keratin coating. (Adapted from [73] with permission)	42
Figure 9 Illustration of keratin film fabrication.	44
Figure 10 The representative microscopy image (left) and digital image (right) demonstrating the thickness measurement and top surface area measurement for swelling tests, respectively. Both measurements were conducted through Image J.	47
Figure 11 Illustrations of NanoITC set-up for interactions study between keratin and KAP.....	49
Figure 12 The pictures of sample preparation of permeability test and the schematic of the experiment set-up.....	50
Figure 13 Characterization of THP extracted by Na ₂ S: (a) SDS-PAGE; FESEM images of hair strand (b) before and (c) after extraction.....	57

Figure 14 AFM images of (a) bare silica substrate and (b) THP-coated substrate (average surface roughness shown in parentheses).	58
Figure 15 FTIR spectra of a bare silica substrate, a THP-coated substrate, and THP lyophilized powder. Two new IR absorbance peaks appeared at 1670 cm^{-1} and 3400 cm^{-1} regions in the THP-coated substrate compared to the bare silica substrate.	59
Figure 16 SPR measurement of surface density changes in coated THP during surface immobilization.....	60
Figure 17 Contact angle measurements of (a) bare silica substrate and (b) THP-coated substrate.	60
Figure 18 Cell morphology observation using optical microscope on day 4 for HEKs and HDFs cultured on different substrates.	62
Figure 19 Cell proliferation based on PicoGreen assay at day 2 and day 4 of HEKs and HDFs cultured on different substrates. * $p < 0.05$, $n = 6$	63
Figure 20 Cell metabolic activity based on Alamar Blue assay on day 2 and day 4 of HEKs and HDF cultured on different substrates. * $p < 0.05$, $n = 6$	64
Figure 21 Characterization of fibronectin and collagen I production by immunoperoxidase staining on day 2 and day 4 for different substrates.....	66
Figure 22 Cytokine profiles of HDFs cultured on different substrates on day 4. (a) Chemiluminescent images of cytokine arrays. Cytokines secreted at significantly different levels are labeled. (b-d) The mean pixel intensities of selected cytokines were normalized with proliferation assay results and plotted. THP-coated substrates enhanced the secretion of several cytokines related to wound healing.	70
Figure 23 Water contact angle image for polymer-assisted protein immobilization samples at various conditions.	71
Figure 24 Immunoperoxidase staining of polymer-assisted protein immobilizations on day 0, day 2 and day 5.	73
Figure 25 Immunoperoxidase staining of non-treated PS, P-DI, and P- Na_2S from day 0 to day 28.	75
Figure 26 Cross-sectional FESEM images of (a) non-treated PS, (b) P-DI, and (c) P- Na_2S	76

Figure 27 AFM images in amplitude and phase scanning modes for P-DI and P-Na ₂ S.....	77
Figure 28 TGA spectra of non-treated PS, P-DI, and P-Na ₂ S from 25 °C to 900 °C.	79
Figure 29 Immunoperoxidase staining of P-DI and P-Na ₂ S before and after SDS washing.....	80
Figure 30 Percentage of protein remained after SDS washing for P-DI and P-Na ₂ S via Micro BCA assay.....	80
Figure 31 XPS spectra of sulfur peaks deconvolution of samples: (a) P-Na ₂ S; (b) P-DI. *XPS spectra have been calibrated against carbon (284.8 eV). ...	82
Figure 32 SDS-PAGE of extracted KAP and keratin by ethanol-assisted extraction.....	83
Figure 33 FESEM images of delipidized hair (top), ‘KAP-free’ hair residue (middle) and hair residue after keratin extraction (bottom).....	84
Figure 34 The free-thiol concentration of dialysate collected from day 1 to day 3.....	86
Figure 35 The free-thiol concentration of dialyzed keratin solution on day 2 and day 3.....	87
Figure 36 Fluorescence intensity of keratin in various concentrations of urea.	88
Figure 37 NanoITC raw data heat rate graph of 1mg/ml KAP titrated into 0.02 mg/ml keratin in different concentrations of urea.....	90
Figure 38 FESEM images of keratin film. (a) surface image from top view; (b-d) cross-sectional images.	94
Figure 39 AFM height images of keratin film in 2D (left) and 3D (right) model.....	95
Figure 40 The water contact angle images show the wettability keratin film and THP film.....	96
Figure 41 The swelling test results of keratin film: volume change in percentage (left) and area change in percentage in the comparison of top surface area and cross-section area (right).....	97
Figure 42 CD spectra of keratin solution.....	98

Figure 43 Ratio percentage of secondary structures after FTIR deconvolution for (a) THP film (b) keratin film and (c) composition percentage of protein secondary structure for both samples.....	99
Figure 44 The DSC thermograms of keratin film and THP film.....	100
Figure 45 TGA and DTG spectra of keratin film (left) and THP film (right) with decomposition temperature stated.	101
Figure 46 Stress-strain curve of keratin film in both dry and wet states. ..	104
Figure 47 Stress-strain curves of keratin film obtained from cyclic test in the wet state at various fixed strain.....	105
Figure 48 The cumulative amount of FITC-20KDa permeated through keratin film as a function of time (a). The release profile was fitted into (b) Higuchi model, and (c) power law model.....	109
Figure 49 The microscopy images of HEKs at Day 3 and Day 5 cultured on different surfaces: control plate, 2 mg/ml collagen I, 20 mg/ml keratin, and 20 mg/ml THP.....	111
Figure 50 (a) The FESEM images of HEKs cultured on keratin film and (b) the area as indicated by red box in (a) at higher magnification.	112
Figure 51 The proliferation assay results of HEKs at Day 3 and Day 5 cultured on different surfaces: control plate, 2 mg/ml Collagen I, 20 mg/ml Keratin, and 20 mg/ml THP. ** $p < 0.05$, $n = 3$	113
Figure 52 The metabolic activity results (Alamar Blue assay) of HEKs cultured on different surfaces: control plate, 2 mg/ml Collagen I, 20 mg/ml keratin, and 20 mg/ml THP.....	114
Figure 53 The microscopy images showing the immunoperoxidase staining of K14 in HEKs cultured on keratin film.....	115
Figure 54 The concentration of IL-1 α secreted by HEK cultured on different coatings on Day 3 and Day 5. All data were normalized to corresponded dsDNA amount obtained from PicoGreen assay. * $p < 0.05$, ** $p < 0.01$, $n = 6$	116

Abbreviations

KAP	Keratin associated protein
2D	Two-dimensional
THP	Total hair protein
PS	Polystyrene
SDS	Sodium dodecyl sulphate
UTS	Ultimate tensile strength
ECM	Extracellular matrix
LDV	Leucine-aspartic acid-valine
EDS	Glutamic acid–aspartic acid-serine
3D	Three dimensional
HMSCs	Mesenchymal stem cells
ROS	Reactive oxidative species
HS	High sulfur
UHS	Ultra-High sulfur
HGT	High glycine-tyrosine
DTT	Dithiothreitol
TCEP	Tris (2-carboxyethyl) phosphine
sNHS	N-hydroxysulfo-succinimide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
PAA	Polyacrylic acid
PET	Polyethylene terephthalate
MP	Microperoxidase 11
XPS	X-ray photoelectron spectroscopy
HCA-T	Human corneal epithelial cell-transformed
APTES	Aminopropyltriethoxysilane
PBS	Phosphate buffer solution
DI water	Deionized water
SPR	Surface plasmon resonance system
FTIR-ATR	Fourier-transform infrared spectroscopy- attenuated total reflection

DMEM	Dulbecco modified Eagle medium
FBS	Fetal bovine serum
BSA	Bovine serum albumin
EDTA	Ethylenediaminetetraacetic acid
PDMS	Polydimethylsiloxane
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
FESEM	Field emission secondary electron microscopy
AFM	Atomic force microscopy
XPS	X-ray photoelectron spectroscopy
BE	Binding Energy
CD	Circular dichroism
DSC	Differential scanning calorimetry
TGA	Thermogravimetric analyzer
XRD	X-ray diffraction
NanoITC	Nano-isothermal titration calorimeters
HDF	Human dermal fibroblast
HEK	Human epidermal keratinocytes

Chapter 1 Introduction

This chapter briefly introduces the background of this research project including an overview of the current research achievements, as well as the research gaps in the field. Next, this chapter discusses the inspirations and motivations of the project, leading to the hypotheses and objectives. Lastly, the approaches to achieve the objectives and to prove or disprove the hypotheses are summarized in the project scope section.

1.1 Background

Polymer substrates are one of the most widely employed biomaterial platforms for biomedical applications. However, these are inherently not functionalized for optimal cell adhesion. Bioactive coatings are commonly used to improve cytocompatibility and facilitate cell adhesion on these polymer substrates by providing ligands for specific cell adhesion molecules expressed on cell membranes. Proteinaceous materials are often preferred as bioactive coatings because they can mimic the extracellular matrix (ECM) to promote cell-matrix and cell-cell interactions [1].

Keratins have emerged recently as alternative materials to be used as bioactive coatings to improve the cytocompatibility of surfaces [2]. Consequently, keratins from human hair and wool are leading the pack as sustainable keratin sources after decades of optimization in terms of extraction, purification, and characterization. In particular, human hair keratins have received significant interest since they are of human origin and are therefore more easily accepted for clinical translation. Essentially, keratins are one of the two main proteinaceous constituents of human hair, along with keratin associated proteins (KAP). Keratins, the more abundant of the two, belong to the intermediate filament protein family, and is rich in cysteines. KAP, on the other hand, is a matrix protein that is even more abundant in cysteines. The average cysteine content in keratins is 7.6% (residues/100 residues) while that for stratum corneum keratin is only 2.9% [3-5]. The abundance of cysteine facilitates disulfide bonding, providing a tough and durable structure to withstand external mechanical forces. In addition, hair proteins have been found to support cell attachment due to the presence of specific peptides such as leucine-aspartic acid-valine (LDV), and glutamic acid-aspartic acid-serine (EDS), which have been recognized as binding motifs in the ECM [6]. The highly repetitive protein sequences promote self-assembly within hair proteins, which results in a relatively homogenous protein secondary structure. Self-assembly enables superior processability of hair proteins into various types of platforms such as hydrogel [7-9], three-dimensional (3D) matrices [10], and thin films [11-15].

These studies show that hair proteins exhibit excellent prospects for advanced biomedical applications, where biocompatibility is highly essential.

The mechanisms of interactions between cells and treated substrates are complex because of multi-factorial interplays between the physical, chemical, and biological properties of the materials involved. Therefore, an appropriate and defined surface modification is desired to modulate surfaces for specific and consistent outcomes. Proteinaceous coatings can be coated onto substrates physically or chemically. Generally, physical coating is preferred as it can be simply obtained by incubating a protein solution on the substrate for a certain period of time. On the other hand, chemical (covalent) coating enables better stability and good preservation of the protein function over time due to the strong binding strength. Covalent coating can be achieved by wet chemical or plasma treatment, but there is no well-established technique to specifically achieve covalent coating of human hair proteins. Furthermore, some common raw materials used as bioactive coatings, such as fibronectin and collagen, are typically derived from animal sources, which give rise to disadvantages such as risks of pathogen transfer and potential for excessive immunological response. Thus, an autologous bioactive coating, which can be achieved using hair proteins, could become an optimal option for clinically relevant tissue regeneration purposes. Another type of two-dimensional (2D) substrate is a thin film. This alternative structure, which is an extension of coatings, has been widely used in various applications such as transdermal patches, ocular surface reconstruction, and cell delivery substrates. In these applications, mechanical resilience of the film becomes vital, in order to allow ease of handling during operation. Keratin films have the potential to provide the necessary mechanical properties, while supporting the bioactivity required as well.

1.2 Motivation

The degree and variety of biomaterials sophistication have increased as a result of fruitful investigations of the scientific community over recent

decades. Nature-derived biomaterials have been extensively studied as they are generally perceived as being more relevant for clinically viable therapies when compared to synthetic biomaterials. However, it is challenging to design a biomaterial which can achieve both commercial and clinical translation success. The ideal biomaterial has to be not only safe and efficacious but also cost-effective and practical. The goal is to design an advanced biomaterial that can modulate complex cell-cell interactions with native tissues and stimulate the patient's own regenerative ability for self-repairing. Tissues and biomaterials of human origin are often the gold standard in current clinical practice. Although naturally occurring biomaterials such as collagen and fibrin have been widely used in clinical applications, some issues related to foreign-body immunogenic reactions, and religion may arise as these are extracted from non-human origins. Therefore, human-derived biomaterials have been receiving more attention as the next generation of regenerative biomaterials. In the current market, there are a few FDA-approved products which are based on human materials. For example, fibrin-based scaffolds that are sourced from human pooled plasma (Tisseel, Artiss (Baxter)) and Evicel (Johnson & Johnson), and individual units of plasma (Cryoseal (Thermogenesis)). However, the fibrin-based scaffolds are relatively expensive because the amount of fibrin that can be extracted from plasma is limited. Furthermore, special handling is required during the blood collection from donors, the storage of products, and product transportation. In comparison, human hair is abundant, and its harvest does not require complex invasive procedures. This can be considered clinically advantageous as it encourages greater patient acceptance and compliance while reducing the chances of interspecies pathogen transfer and infections through blood-borne pathogen contact. Human hair proteins can also potentially minimize the risk of immunological rejection compared to animal-derived biomaterials, especially when autologous sources are used.

Physical proteinaceous coatings are widely used to improve the biocompatibility of commercialized bioimplants made of metal, synthetic polymers, and ceramics. The biggest drawback of physical-bonded proteinaceous coatings is their stability because proteins are easily degraded

over time. This would affect the biocompatibility of the coated substrate and might lead to premature biomedical implant failure, making them less desirable [1]. Therefore, a stable covalent-bonded protein immobilization is required for biomedical applications as it extends the service lifetime of the coating, as well as reduce the risk of an allergic response from the host. Plasma treatment is one of the widely used techniques to achieve covalent immobilization of a protein on an activated surface which is full of reactive free-radical molecules. In the case of hair proteins, the cysteines present undergo redox reactions as nucleophiles that exhibit antioxidant behaviour. The active thiol groups in cysteines are able to react with reactive oxidative species (ROS) to reduce oxidative stress [16]. Therefore, it is encouraging to use the reaction between the thiol groups in hair proteins and the free-radical molecules on plasma-treated substrates to achieve covalent protein immobilization.

Protein films prepared by using the proteins extracted from human hair and wool have been used to explore the biological as well as structural properties of self-assembled keratin. The films, however, showed poor strength and flexibility which limited their practical use. This is mostly due to the extensive disulfide crosslinking contributed by KAP. Hence, researchers shifted their focus on optimizing the mechanical properties of the films through heat treatments and plasticizer additions [14, 17-19]. However, it remains questionable whether these treatments affect the biological properties of the proteins. Therefore, instead of adding plasticizers, this study proposes that the removal of KAP could be an approach to reduce the brittleness of the keratin-based film. This “KAP-free” keratin film could be used to explore the extent of mechanical properties possible of keratin-based films. Human hair keratin was reported to exhibit a hierarchical structure as a result of the self-assembly process which started from heterodimers as the building blocks [20]. However, this well-established self-assembly mechanism has been mainly focussed at the molecular level. In this study, keratin film is expected to be used as a platform to observe if keratin self-assembly affects material structures on the macro-scale. This helps in designing, or customizing, the material to suit specific applications and to

enrich the fundamental understanding of the processability of human hair keratins.

1.3 Hypotheses

1. Total human hair proteins can be covalently coated on cell culture substrates via free radical cross-linking chemistry due to significant amounts of thiol groups presented in these proteins.
2. Keratin and KAP can improve the biocompatibility and functionality of cell culture substrates.
3. Keratin can interact with themselves, or KAP, through electrostatic interactions and/or thiol chemistry to self-assemble into organized structures with tunable physical, mechanical, and biochemical properties.

1.4 Objectives

1. To characterize cell culture surfaces coated with hair proteins, using XPS and stability studies.
2. To study the interaction between the extracted keratin and KAP through NanoITC.
3. To optimize and characterize the solution cast keratin films without plasticizer addition.
4. To validate the cytocompatibility of fabricated hair protein-based platforms using *in-vitro* models, in terms of cell adhesion, viability, and function.

1.5 Scope

The first phase of the project will focus on optimizing the extraction and characterization of the extracted hair proteins. Next, the extracted human hair protein will be immobilized, physically, on a silica beads substrate and covalently, via plasma treatment on conventional cell culture plates. Various characterizations and *in vitro* works will be conducted to determine the stability and biocompatibility of the coatings. Interaction studies between keratin and KAP will also be evaluated through NanoITC to exploit the potential of cooperating purified keratin for customized applications. In the latter phase of the project, the purified keratin will be fabricated to form a stand-alone film by solvent casting, and its physical properties will be examined. Next, *in vitro* works will be carried out to determine the biocompatibility of these hair protein-based platforms in terms of cell adhesion, viability, and function. In future work, further investigations will focus on developing a human hair protein-based platform that allows long-term stability and explore the possibility of using KAP as a crosslinker to improve the keratin film properties.

Chapter 2 Literature Review

This chapter starts with the introduction of human hair proteins, including the physical structure of human hair and the two main components of human hair proteins: keratin and KAP. The characteristics and functions of keratin and KAP will be discussed in detail. The next section demonstrates the theory of oxidative and reductive hair protein extraction. The last section discusses the hair protein-based 2D platforms for biomedical applications, especially focusing on coating and film applications. This includes the theory of plasma coating and a review of the various ways established to achieve covalent protein immobilization. It also summarizes all the significant studies about hair protein-based biomaterials for coating and film applications. The last section of this chapter reviews the cell response and biocompatibility of hair protein-based 2D platforms in the literature.

2.1 Human Hair Structure and Proteins

Human hair is often considered as waste, generated at approximately 300,000 tons per year worldwide [20]. Although hair is biodegradable, it consumes a large volume of space and may produce toxic gases during decomposition, which might cause environmental pollution and induce health problems [21]. Therefore, the bio-waste conversion is considered as one of the best solutions to address these problems. Since hair proteins are biocompatible and economically friendly, the study of hair protein-based biomaterials has become a popular research direction.

2.2 Physical Structure

One single strand of the human hair is approximately 50-100 μm in diameter. The principle compartments of hair fiber are the cuticle, cortex, and medulla. They are located at different regions of the fiber and consist of different proteins that serve different functions. The cuticle is the outermost layer, composed of a thin layer of over-lapping flattened cells, forming a scaly surface. The proteins in the cuticle provide a protective function to prevent hair from external damage. By comparison, they have a relatively high range of molecular weight (60-80 kDa) and are more difficult to be extracted than keratin and KAP located in the cortex. Underneath the cuticle, the cortex is considered as the main body of the hair fiber. Keratin and KAP are the two proteins present in the cortex. Moving forward into the center of the hair fiber, a region called the medulla is located. A schematic diagram of the human hair structure is shown below in Figure 1:

Figure 1 Schematic diagram of the human hair structure. (Reprinted from [20] with permission).

2.3 Human Hair Proteins

The hair proteins used in this study were extracted from the cortex. They are theoretically easier to be extracted and have been proved to be promising potential biomaterials in various fields such as biomedical, pharmaceutical, and wound healing. Within the cortex, there are two main groups of proteins: (1) keratin (MW 40-60 kDa) and (2) matrix proteins (MW 10-25 kDa). Collectively, the hair fiber consists of 50-60% keratin and 20-30% matrix proteins [11]. Keratin intermediate filament proteins, referred to simply as keratins, impart toughness to the hair fiber. The matrix proteins, also termed as keratin associated proteins (KAP), function primarily as a disulfide crosslinker or a glue that holds the cortical superstructure together [10]. In total, there are 17 keratin genes (11 type I; 6 type II) [10], and more than 85 KAP genes [12], which potentially contribute to the hair structure in humans.

2.3.1 Keratin

Keratin comprises 50-60% protein in the cortex and contains a relatively low sulfur content when compared to KAP. Keratin can be further divided into two families: type I (acidic) and type II (basic) keratin. Type I, acidic keratin contains 11 acidic subtypes with a distribution of molecular weight between 40-55 kDa, while type II, also called basic keratin, consists of 6 basic and neutral subtype proteins with a range of molecular weights of 55-65 kDa. Table 1 shows the summary of the details of all subtypes from type I and type II including amino acid percentage, isoelectric points, and molecular weights. Type I and type II will pair up to form heterodimers which are the smallest building blocks of the keratin [22]. Keratin is therefore heteropolymer and is only stable when equimolar of specific type I and type II keratins pair up. Furthermore, specific subtypes are also used as biomarkers as any mutations or defects of the paired-up keratin will lead to diseases.

It was found that type I and type II keratins were able to form stable heterodimers across different concentrations of urea starting from 8 M to 5 M [23]. The obligatory heterodimerization was reported as an ancient trait among keratin regardless of evolution and sequence drift. The high affinity of self-assembly between type I and type II has gained a lot of attention as they were regulated in a tissue-specific, differentiation-specific, and pairwise manners. The self-assembly features of hair keratin have been functionalized into hydrogels [7-9], 3D matrices [10], and thin films [11-15], mostly for biomedical applications.

Table 1 Summary of the subtypes from type I and type II keratins in amino acid content, isoelectric point, and molecular weight. ¹

Designation		Percentage of amino acid residues in each KIF protein sequence (%)																				Presence of LDV sequence	Isoelectric point (PI)	Mw (kDa)
		R	K	D	E	Q	N	H	S	T	Y	C	M	W	A	I	L	F	V	P	G			
Type I, acidic	K31 (Ha1)	7.2	2.9	3.6	10.8	7.2	7.2	0.7	9.6	5.8	2.4	6.0	0.5	0.5	5.3	3.4	12.5	1.9	5.5	5.3	1.7	1	4.5342	47.24
	K32 (Ha2)	6.9	2.7	4.2	10.0	6.9	5.4	0.9	8.5	5.6	2.7	5.8	3.3	0.4	7.4	3.1	10.9	1.3	6.0	4.7	3.1	1	4.4894	50.32
	K33a (Ha3-I)	7.4	3.0	4.0	11.4	7.7	6.9	0.7	9.7	5.4	2.7	5.4	0.5	0.5	5.4	3.5	12.6	1.7	5.4	3.2	2.7	1	4.4782	45.94
	K33b (Ha3-II)	7.2	3.0	3.5	11.4	8.2	7.4	0.7	9.4	5.2	3.2	5.0	0.7	0.5	5.4	3.5	12.4	2.0	5.2	4.0	2.2	1	4.4995	46.21
	K34 (Ha4)	6.2	4.1	3.4	10.8	7.8	6.7	1.4	9.9	5.5	2.5	5.7	0.7	0.5	5.0	4.6	12.4	1.6	4.8	3.7	2.8	1	4.7215	49.42
	K35 (Ha5)	7.0	3.7	4.4	10.3	5.7	4.2	0.4	10.8	4.0	2.9	5.1	1.8	0.7	8.1	2.9	11.9	1.5	5.1	4.4	5.3	1	4.5515	50.36
	K36 (Ha6)	6.6	3.6	3.9	10.7	6.9	3.9	1.1	8.8	5.4	2.6	4.3	1.3	0.6	6.4	5.1	10.9	2.1	6.4	4.3	5.1	1	4.6012	52.25
	K37 (Ha7)	5.1	4.2	4.0	10.7	6.9	4.9	2.2	8.0	5.3	2.0	5.3	2.0	0.2	8.0	4.0	11.8	1.6	4.5	4.2	4.9	1	4.6188	49.75
	K38 (Ha8)	5.5	3.7	4.2	10.7	7.0	4.8	1.8	7.9	5.9	1.8	6.1	1.8	0.2	7.7	4.4	11.8	1.3	3.9	4.8	4.6	1	4.4933	50.48
	K39 (Ka35)	5.5	5.5	3.7	10.2	7.5	6.1	1.2	8.1	6.7	2.6	5.5	1.4	0.8	5.3	6.3	10.6	1.0	4.5	4.3	3.1	1	4.8649	55.62
	K40 (Ka36)	4.6	3.0	4.9	12.1	7.0	4.6	0.9	10.2	6.7	2.3	7.7	1.2	0.7	6.3	3.2	13.9	1.2	3.9	2.6	3.0	1	4.5977	27.48
Type II, basic	K8 (Hb1)	6.9	4.8	3.4	10.1	4.4	4.2	0.8	8.9	4.0	2.6	6.7	1.2	0.2	7.7	5.0	8.9	2.4	6.5	2.4	9.1	0	5.2382	54.97
	K82 (Hb2)	6.4	6.0	3.3	9.6	5.1	4.1	1.2	8.4	3.5	2.9	4.7	1.9	0.4	7.2	4.7	10.7	2.5	5.7	3.5	8.2	1	6.7169	56.65
	K83 (Hb3)	7.3	4.9	3.4	10.3	4.7	4.7	1.0	7.9	4.1	2.6	6.3	1.2	0.2	7.9	4.5	8.9	2.6	6.7	2.6	8.1	0	5.1429	54.17
	K84 (Hb4)	6.8	4.7	3.7	7.5	4.7	4.2	1.0	9.8	4.7	2.2	3.3	1.7	0.5	8.3	4.0	9.2	3.7	7.0	3.3	9.8	0	7.6124	64.84
	K85 (Hb5)	8.1	4.9	3.2	10.3	3.9	3.9	0.8	10.7	3.7	3.0	5.3	1.4	0.4	8.7	4.5	8.3	2.6	6.5	2.8	7.1	0	6.4906	55.80
	K86 (Hb6)	7.6	4.9	3.5	10.5	4.3	4.3	0.8	8.6	4.3	2.9	6.0	1.2	0.2	8.2	4.5	8.4	2.3	7.6	2.5	7.2	0	5.3537	53.50

Note: Amino acid with the highest percentage for each subtype is highlighted in red.

Abbreviations: Arginine (R); Lysine (K); Aspartate (D); Glutamate (E); Glutamine (Q); Asparagine (N); Histidine (H); Serine (S); Threonine (T); (Tyrosine (Y); Cysteine (C); Methionine (M); Tryptophan (W); Alanine (A); Isoleucine (I); Leucine (L); Phenylalanine (F); Valine (V); Proline (P); Glycine (G)

¹ Compiled from Human Intermediate Filament Database: <http://www.interfil.org/>

Self-assembly of Keratin

All intermediate filaments share a similar monomer structure which consists of an α -helix in the center and a non-helical structure at both ends. Two monomers can form a so-called “coiled-coil” dimer through heterodimerization [24]. After that, these dimers assemble into unpolarized tetramers via antiparallel association. Subsequently, eight tetramers bundle into a cylindrical unit-filament [25]. Next, the unit-filament aggregates further with other unit-filaments to form intermediate filaments through an end-to-end aggregation mechanism (refer to Figure 2) [26]. After aggregation, the filaments undergo a compaction step during which the filament diameter shrinks to its final size of approximately 10 nm [27-29].

To study the mode of assembly of keratin, a motif with a designated sequence was synthesized [30, 31]. It was reported that this motif enabled good stability of the α -helical fold and coiled-coil conformation through intrachain and interchain ion pairings. It could nucleate the formation of long-range coiled-coil dimers. Wu *et al.* screened a series of motifs and found that the functional trigger site for coiled-coil formation was highly conserved among keratins [32]. These experiments indicated that the attractive forces mediating coiled-coil formation in keratin were strong, and co-factors were not essential [33].

Figure 2 Illustration of intermediate filament assembly from monomer to filament [26].

The self-assembly features of keratin, from heterodimerization through to bundling, allow keratin to acquire elasticity. Thus, keratin exhibits enhanced mechanical resilience which represents an intrinsic property of keratin. Yamada *et al.* reported that extensive bundling was promoted by changing the assembly conditions without the exogenous cross-linking activities [34]. On the other hand, Wang *et al.* successfully polymerized a pair of keratin by carefully maintaining a strongly reducing environment during assembly [35]. Furthermore, significant molecular realignments that were comparable to the mature wool fibers were observed when the keratin was shifted to an oxidizing environment. Therefore, this group speculated that the disulfide

bond formation arising in the late stage of self-assembly served to further stabilize the keratin. These studies indicated that the self-interactions exhibited by keratin changed with the environment, which significantly affected the resulting structural and mechanical properties.

2.3.2 Keratin-associated Proteins (KAP)

In the hair cortex, there are approximately 20-30 % proteins that belong to the KAP family [36]. KAP consists of relatively high amounts of sulfur due to the presence of cysteine residues. This allows abundant disulfide cross-links formation within KAP, as well as between KAP and keratin. Generally, KAP is further classified into three families based on their amino acid content, including high sulfur (HS), ultra-high sulfur (UHS), and high glycine-tyrosine (HGT) as summarized in Table 2 as well as Table 3, along with their sources and sequence characteristics.

The disulfide bonds contribute to the protection of keratin. Among the linkages present in the human hair proteins, disulfide bonds have the highest bond energy. Istrate *et.al.* reported that the thermal stability and mechanical properties of human hair were attributed to disulfide bonding [37]. In other words, KAP not only acts as a matrix protein that brings the keratin together by forming excessive disulfide bonds, but also maintains the thermal and mechanical stability of human hair.

Table 2 Prominent amino acid contents of the three families in KAP: high sulfur (HS), Ultra-high sulfur (UHS), and High glycine-tyrosine (UHS) (Adapted from [38] with permission).

Protein type	Amino acid content	Molecular weight range (kDa)
High sulfur (HS)	20-30 mol% cysteine	11-26
Ultra high sulfur (UHS)	>30% mol% cysteine	>20
High glycine-tyrosine (HGT)	20-40 mol% glycine 12-20% mol% tyrosine	6-9

Table 3 Overview of the sequence characteristic of KAP in human hair (Adapted from [39] with permission).

Family	Source	Designation	Members	No. Residues	Repeating length ¹	Sequence features ²
HS	Cortex	KAP1 (B2)	10	85-178	20	AB,A ₁ B, and A ₂ B with glycine-rich linker
	Cortex	KAP2 (BIIIA)	5	129	5	A, A ₁ , and A ₂ with glycine-serine-rich segment at N-terminus
	Cortex	KAP3 (BIIIB)	3	99	5	A
	Cortex	KAP15	1	138	-	-
	Cortex	KAP16	1	518	20	BA ₁ AA
	Cortex/cuticle	KAP23	1	66	-	-
	Cortex/cuticle/matrix	KAP13	4	161-176	10	BA
	Matrix	KAP11	1	164	5 and 10	(A ₁ -GGI/VS-S/T) ₄
UHS	Cortex	KAP4	15	107-211	10	A ₂ B
	Cortex	KAP9	9	155-251	10	A ₁ B
	Cuticle	KAP12	4	97-147	5	A
	Cuticle	KAP5	12	62-288	19	BABB' or BA'BB
	Cuticle	KAP10	12	222-402	42	(BA ₁ BXAAAB)-SS
	Cuticle	KAP17	1	106	5	B
HGT	Cortex	KAP6 (Type II)	3	63-111	2	(G-X) _{2,3,4,6}
	Cortex	KAP7 (Type I-C2)	1	88	-	13 residues segment devoid of glycine and tyrosine
	Cortex	KAP20	2	57-66	-	-
	Cortex	KAP21	2	80-84	-	-
	Cortex	KAP22	1	49	-	-
	Matrix	KAP8 (Type I-F)	1	64	2	(Glycine-X) _{2,3,4}
	Cuticle	KAP19	7	53-91	-	-

¹ The number of residues of the repeating unit presents in the KAP sequence.² X is referring to unspecific protein sequence; C is cysteine; P is proline; Q is glutamine; R is arginine; S is serine; T is threonine; A: CC-X-P-X; A₁: CCQP-X; A₂: CCRP-X; B: CC-X-S/T-S/T

2.4 Extraction Methods

Different methods for extraction of hair protein have been developed over the last 100 years, with the most widely used methods classified into reductive [2, 15, 40, 41] and oxidative methods [42-44]. Figure 3 shows the schematic for disulfide bond disruption during oxidative and reductive extractions. For reductive extraction, a reducing agent is used to reduce the covalent disulfide bonds (-S-S-) found in inter and intro cysteines into free thiol groups (-SH) and increase the solubilization of hair protein at the same time. Thus, the extracted hair protein is in reduced form and called kerateine, which can be re-oxidized to form disulfide bonds. 2-mercaptoethanol and reducing agents such as dithiothreitol (DTT), tris (2-carboxyethyl) phosphine (TCEP), and sodium sulfide (Na_2S) are commonly used in the reductive hair protein extractions. Alternatively, hair protein can be extracted through the oxidative method and the extracted hair protein is called keratose. Hair residues are treated with acid to obtain cysteic acid which consists of negatively charged sulfonic acid ($-\text{SO}_3^-$) groups. As compared to kerateine, keratose offers fewer free thiol groups. This makes keratose highly dependent on non-covalent interactions which leads to faster bulk degradation compared to kerateine [45]. On the other hand, Xie *et al.* proposed using an ionic liquid to extract wool protein which resulted in high solubility wool protein without any chemical changes [46]. In addition to these chemical methods, hair protein can also be extracted through enzymatic and microwave-assisted methods.

(a) Reductive method

(b) Oxidative method

Figure 3 Schematic of the reaction mechanism of reductive and oxidative extractions for hair proteins.

There are certain pros and cons for each of these reported hair protein extraction methods, which either promote or limit the end-product and corresponding applications. For instance, keratose is suitable for studying the biological and physiochemical properties of each different sub-fraction of the hair proteins, but the process is time-consuming and involves a large number of oxidative agents. Similarly, the low yield and high cost of production are the two major disadvantages for ionic liquid and enzymatic extractions. Although 2-mercaptoethanol can exhibit a good yield during reductive extraction, it is generally considered as an expensive chemical. Therefore, Na_2S is reported as a good alternative reducing agent due to the lower cost and comparable high yield production ability [47]. Furthermore, it is also challenging to completely remove chemicals used during extraction, such as sodium dodecyl sulphate (SDS). SDS is reported to be toxic to cells through

non-selective cell membrane destabilization and destruction [48], hence, the biocompatibility of the extracted hair proteins will be reduced if there is any remaining SDS presented. In order to solve this problem, Nakamura *et.al* proposed a novel human hair protein extraction called the “Shindai Method” which can produce a high hair protein recovery, approximately 60-70%, in the absence of SDS [49].

Since hair proteins composed of different fractions have specific functions, selective extraction of keratin has emerged. Until now, KAP and keratin have been separated successfully through keratin-suppression [50] and isoelectric precipitation [51, 52]. Fujii *et.al* reported a two-step human hair extraction through the Shindai Method [50]. The first step is to extract the KAP by suppressing the keratin dissolution through ethanol addition, followed by normal Shindai extraction. It is reported that ethanol can suppress the dissolution of keratin, but the mechanism is not fully understood yet.

2.5 Applications

The possible hair protein-based applications are summarized in Table 4. This report mainly focusses on the coating and thin-film applications.

Table 4 Summary of hair protein-based biomedical applications.

Source	Experimental features	Extraction method	Applications	References
Hoof	Characterize keratin extracted from bovine hooves and biocompatibility	Reductive	Potential biomaterials for tissue engineering and biomedical applications	[53]
Hoof, horn, and wool	Comparison of the composition of proteins from different sources	Reductive	A better understanding of the keratin distribution and compositions	[54]
Hoof, horn, and wool	Structural and chemical analysis	Reductive	Explore the potentials of keratin as biomass by conversion from textile and butchery wastes	[55]
Wool	Addition of Chitosan for making chitosan-keratin films	Reductive	Antibacterial and good substrate for cell culture	[56]
Wool	Self-assembled keratin	Reductive	Solvent-cast keratin films	[12]
Wool	Cultivation of mouse fibroblast on keratin coated substrate	Reductive	The coating which promotes cell proliferation	[57]
Wool/silk fibroin blends	Films and electrospun nanofibers	Oxidative	Biomedical devices, water filtration, textile fibers	[58]
Human hair	Optimization of keratin thin film formation through precipitation methods	Reductive (Shindai method)	Hair keratin film with similar properties to the native enzyme	[11]

Source	Experimental features	Extraction method	Applications	References
Human hair	Develop coating to modulate stem cell expansion in vitro	Reductive (Shindai method)	Potential coating to promote functioning stem cell growth	[59]
Human hair	Electrospun hair keratin matrix	Reductive (Shindai method)	Tissue regeneration	[10]
Human hair	Study the cellular influences and nano-topology changes of hair keratin coated substrate	Reductive (Shindai method)	Bioactive cell culture substrate coating	[2]
Human hair	Self-assembly 3D hair keratin hydrogel	Reductive	3D cell culture system	[7-9]
Human hair	Keratin based hydrogels	Oxidative	Regenerative medicine	[60, 61]
Human hair/silk fibroin	Silk fibroin and keratin blend 3D scaffold through electrospinning	Reductive (Shindai method)	3D scaffold for skin tissue engineering	[62]
Human hair/silk fibroin	The transition from random coil to β -sheets in the secondary structure of the composite	Reductive	Biomaterial with intrinsic biocompatibility and biodegradability	[63]
Human hair/PEO	Improved structural properties	Reductive	Scaffolds wound dressing & drug delivery membranes	[64]
Chicken feather	Evaluation ability as sorbent	Hydrolysed by NaOH	Removal of metal ions	[65, 66]

Source	Experimental features	Extraction method	Applications	References
Chicken feather	Effective extraction chicken feather keratin and thin film for drug release system	Reductive	Potential in controlled-drug release system	[67]
Chicken feather	Evaluate the effect of glycerol addition to the thin film properties	Reductive	Better understanding of the structure properties	[68]

2.5.1 Coating

Coatings are the most straightforward way to introduce biological cues on a surface and to modulate hydrophilicity and surface roughness, which can promote cell attachment and proliferation [69]. Particularly, coatings could bring breakthroughs as bioactive interfaces on various substrates such as metal oxides, quartz, polymers, and glass. These bioinert materials are able to exhibit cellular interaction after surface modification with short peptides or enzyme immobilization.

Plasma is generally considered as the fourth state of matter and it is composed of electrons and ionized species. The energetic molecules can be bombarded with high energy on the surfaces to induce surface modification. Plasma treatment is widely used in both industry and laboratory for many applications such as lithography, surface modification/activation, and sterilization due to its high efficiency and low cost. Amongst the different kinds of plasmas, the two most common plasmas are atmospheric and low-pressure plasma as they produce a high density of high energy species. Atmospheric plasma uses compressed air and usually, the plasma is injected through a mechanically controlled jet nozzle in an in-line manner, offering a short residence time and localized treatment. Low-pressure plasma has to be operated in an enclosed chamber to achieve a vacuum environment, hence, it offers a controlled volume and a wide choice of gas species. This allows more variety for surface modification. In addition, the homogenous plasma makes it suitable for mass operation, even with the samples having complex 3D geometry.

Plasma treatment is highly versatile and especially useful for surface modification by simply exposing the treated surface to different gas species during or after treatment. Argon and oxygen plasma are the two most commonly used gas species for plasma treatment. The most significant difference between them is that oxygen plasma enables surface modification while argon can only clean the surface without any further reaction, due to its inertness. Therefore, argon gas is usually mixed with oxygen during plasma,

especially for those materials which are susceptible to oxidation such as copper and silver. Other than argon, nitrogen is another popular complimentary gas for oxygen plasma. Generally, nitrogen and oxygen are mixed in the composition that replicates the atmospheric environment.

Oxygen plasma is very popular and widely used in biomedical applications, not only for sterilization but also for surface modification [70]. Polystyrene (PS), the most common material for tissue culture plates, is widely used for plasma treatment in biomedical related studies [71]. Oxygen and nitrogen can react with the free radicals generated by plasma and form into various stable functional groups, as shown in Figure 4:

Figure 4 Possible functional groups of PS after plasma treatment followed by nitrogen and oxygen exposure.

These resultant molecules can improve the biocompatibility of PS by increasing wettability which is a critical factor that significantly affects protein adsorption and cell adhesion [72]. Furthermore, these molecules can be used for functionalization such as covalent protein immobilization. The significant advantages of covalent protein immobilization are to provide stable binding that maintains protein functionality over a longer period. Covalent protein immobilization can be achieved by a wet chemical method with a crosslinker involvement [73], or by radical-assisted in absence of a crosslinker [74]. For the wet chemical method, the primary amine group (-NH₂) is the predominant target to chemically react with certain functional groups such as carboxylic acid (-COOH) in the presence of a crosslinker. 1-

ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is a common crosslinker for protein immobilization as it can link the amine group in proteins to carboxylic acid groups. The carboxylic acid can be obtained from either the plasma treated-polymer surface or the intrinsic functional group of certain polymers, such as polyacrylic acid (PAA).

However, it is worth noting that there is no direct experimental measurement to conclude whether the protein immobilization is covalently bonded on the substrate. The most well-established way to determine the covalency of the attachment is SDS-detergent washing. As an ionic surfactant, SDS can disrupt physisorption forces by unfolding the protein. This detergent washing process was first reported by Bohnert *et al.* in 1990 [75]. They found the binding affinity of albumin in blood plasma to polyethylene terephthalate (PET) surface was reduced after argon plasma treatment. Interestingly, the absorbed albumin showed better resistance to elution after the SDS washing step, thus revealing that the attachment could be attributed to a stronger binding mechanism rather than physical bonding. The SDS washing step was used in other studies to show that the protein attached to the substrate after cleaning was considered as a covalently bonded protein [76, 77].

Finally, in 2010, Yin *et al.* claimed that they have found direct evidence to prove the covalent immobilization by quantifying the oxidized sulfur component of cysteine from the immobilized protein [78]. In their study, they immobilized microperoxidase 11 (MP) through plasma treatment and quantified the covalently immobilized MP through X-ray photoelectron spectroscopy (XPS). SDS was used to remove physically bonded MP while only the covalently bonded MP was able to remain on the surface after the SDS cleaning. The covalent capacity was measured by quantifying the atomic ratio of the oxidized sulfur peak (S 2p) before and after the detergent cleaning. As shown in Figure 5, they reported that the plasma treatment significantly increased the amount of oxidized S 2p when compared to non-plasma treated surfaces. The result tallied with a previous study [79] which provided another approach for covalency measurement of protein immobilization, especially beneficial for the protein that contained cysteine residues.

Figure 5 Oxidized S-2p component of different samples: MP is pure microperoxidase-11; PTFE and PE referring to substrates without plasma treatment; DLC, PP, and NPP are plasma-treated samples with different gases during plasma deposition such as nitrogen/hydrogen, without nitrogen gas and nitrogen gas respectively. (Reprinted from [79])

2.5.2 Thin Films

Yamauchi *et al.* proved that keratin substrates allow better cell adhesion and proliferation of mouse fibroblast as compared to collagen and glass substrates [57]. Furthermore, Reichl *et al.* showed that the keratin films were able to improve cell growth for twelve cell lines originated from different tissues, including colon adeno-carcinoma, skin epidermis, corneal epithelium, corneal stroma, lung adeno-carcinoma, and nasal septum squamous cell carcinoma [14]. The common drawback of pure hair protein-based film is the high brittleness. Therefore, researchers shifted their focus to optimizing the mechanical properties of hair protein-based film while maintaining the biological properties. Significant efforts have been made to enhance the mechanical properties and accessibility of hair protein-based film such as heat treatment and plasticizer addition [14, 17-19]. These studies have strengthened the concept that human hair protein plays an important role in

cellular biology and it is worth exploiting. Table 5 is a summary of the key findings of recent hair protein-related thin film studies.

Table 5 Summary of the key findings of hair protein-related thin film studies.

Sources	Details	Key findings
Human Hair [14]	<ul style="list-style-type: none"> • Transparent films: solvent casting • Opaque films: trichloroacetic acid precipitation (TCA) • 2-3 μm thickness 	<ul style="list-style-type: none"> • Clear keratin coating promoted cell proliferation and attachment for epithelial cells especially Caco-2, which are required to express a tight cell layer • Proposed to be a high potential replacement for ocular reconstruction.
Human Hair [15]	<ul style="list-style-type: none"> • Aqueous and alkaline dialysate at different ratios (100, 90/10, 80/20, 70/30, and 50/50 respectively). • Solvent cast on PET as an intermediate substrate with glycerol (1-3 %) addition followed by curing at 110 $^{\circ}\text{C}$ for 2 hrs 	<ul style="list-style-type: none"> • Physically and chemically stable for ≤ 6 months • Suggested as a promising alternative for ocular surface reconstruction (<i>in vivo</i> included) • Inconclusive regarding the relationship between film composition, assembly of keratin, and ultrastructural morphology changes • Suggested steroids as an important factor to modulate matrix regeneration and tissue integration [41].

Sources	Details	Key findings
Human hair [13]	<ul style="list-style-type: none"> • Transferable thin film • Method: Hydrophobic coated PET [15] 	<ul style="list-style-type: none"> • A promising alternative for ocular cell scaffold due to its comparable cell migration, attachment, and proliferation • Human corneal epithelial cell-transformed (HCE-T) responses were preferred on non-treated PS • Conflicted results to Reichl [14] with a lower proliferation of HaCaT.
Wool [80]	<ul style="list-style-type: none"> • Transferable thin film <p>Method:</p> <ul style="list-style-type: none"> • Solvent casting • Two keratin solutions: dialyzed and dialyzed + diafiltered (desalted by multiple diafiltration wash cycles) were spread on a petri dish with the desired dimension and air dried at room temperature 	<ul style="list-style-type: none"> • Diafiltered thin film: highly transparent, no porosity • A potential substitute for biodegradable packaging materials due to its promising mechanical properties (13 MPa and 40 % extensibility) and biodegradability in less than 10 hrs. • The removal of polar amino acids through diafiltration hindered the cell attachment and proliferation as well as low water swelling (50%) • No additives added.

Sources	Details	Key findings
Silk fibroin/keratin [81]	<ul style="list-style-type: none"> • Silk fibroin/keratin blend film at different ratios (1:1, 1:3, 3:1) • Mixed with 0.25 g of starch and spread on PS plates, subsequently dried at 40 °C for 3 days 	<ul style="list-style-type: none"> • Starch increased the strength of the film and changed the part of the secondary structure from random coils to β-sheet • The presence of a β-sheet is suspected as the reason for the improved strength and thermal stability of the blend films.

Diffusion kinetics models

To understand the potential diffusion mechanism, many mathematical models have been derived to predict the release kinetics of drug delivery systems. Generally, the mathematical equations describe the relationship of the release dependency as a function of time. Basically, there are four mathematical models that are commonly adapted for biomaterials, which are Zero order, First order, Higuchi Model, and Korsmeyer-Peppas (power law). Macdonald *et al.* demonstrated that a film-based platform offered a controlled drug release system for transdermal drug delivery applications [82]. The release profile was fitted to the power law to reveal that the controlled release system was diffusion dependent. In this report, applying these empirical kinetics equations helped to understand the permeation mechanism of keratin films. This is important for film-based platforms for cell carrier application as adequate cellular infiltration and nutrient delivery are required in order to maintain the cell growth on the platforms.

The Zero-order proposes that the active agent is released at a constant rate as a function of time. This release model is driven by the concentration gradient and is independent of the concentration of the active agent. The graph is plotted as the cumulative released amount of active agent against time in hours.

The equation of the Zero-order release model is:

$$Q_t = Q_0 K_0 t \quad (1)$$

where Q_0 is the initial amount of drug, Q_t is the cumulative amount of drug at release time t , K_0 represents the zero-order release constant, and t is time in hours.

Opposite to the Zero-order, the First-order kinetics states that the active agent is released in an amount that is proportional to the amount of the active agent in the matrix. In other words, the model is concentration-dependent, and the release rate tends to decrease with respect to time.

The equation of First-order release model is:

$$Q_t = Q_0 e^{-Kt} \quad (2)$$

where Q_0 is the initial amount of drug, Q_t is the cumulative amount of drug at release time t , K_0 represents the first-order release constant, and t is time in hours.

The Higuchi model was established in 1961 and it has been the most widely used kinetic model to represent the release profile of drugs from matrix systems.

The equation of the Higuchi release model is:

$$Q = K_H t^{1/2} \quad (3)$$

where Q is the cumulative amount of drug released at time t , K_H is the Higuchi constant, and t is time in hours.

The Higuchi model is transposable and can be adapted to planar or spherical systems regardless of the homogeneity. Thus, the Higuchi model could be further expanded into a variety of mathematical approaches in order to classify the possible release profile in different forms. As such, the power law

was derived as a relatively more comprehensive kinetic model specifically for drugs released from polymeric systems.

The power law equation is:

$$(M_t / M) = K_m t^n \quad (4)$$

where M_t is the cumulative amount of drug released at time t , M is the total amount of drug, K_m is the kinetic constant, n is the release exponent, and t is the time in hours.

Table 6 Summary of the n value of the power law for different geometries [83].

Release mechanism	Geometry	Release exponent (n)
Fickian diffusion	Planar (thin films)	0.5
	Cylinders	0.45
	Spheres	0.43
Anomalous transport	Planar (thin films)	$0.50 < n < 1$
	Cylinders	$0.45 < n < 0.89$
	Spheres	$0.43 < n < 0.85$
Case I transport	Planar (thin films)	1
	Cylinders	0.89
	Spheres	0.85
Case II transport	Planar (thin films)	$n > 1$
	Cylinders	$n > 0.89$
	Spheres	$n > 0.85$

The power law is especially useful for systems with unknown release mechanisms. Generally, the power law graph representation will be $\log (M_t/M)$ versus $\log t$, while the release exponent (n) is the linear regression of the graph. If the graph shows good linearity, the n value can be used as an indicator to further classify the possible release mechanism. Table 6 shows the release mechanisms corresponding to different geometries based on the

power law equation. It shows that more than one transport mechanism is allowed in the same system.

2.5 Cell response of hair protein-based 2D substrates

Numerous studies have reported the use of human hair protein to produce 2D substrates for various biomedical applications. For instance, human hair protein showed the ability to promote cell adhesion and proliferation as a coating material for conventional tissue culture plates. Furthermore, Reichl investigated the cell growth behaviour of twelve cell lines on keratin coated substrates [14]. These cell lines belonged to epithelial and mesenchymal cells, which originated from a broad range of tissues, showing the potential of keratin as a coating material for a wide range of cell selection. The supportive cell attachment was mainly attributed to the intrinsic binding motif of hair keratin, LDV. In addition, cells cultured on a hair protein-based film have shown a high pronounced β_1 expression [84, 85]. It was suggested that LDV could be recognized by the integrin family, $\alpha_4\beta_1$, and consequently contributed to the supportive cell attachment behaviour of hair protein-based 2D substrates. Interestingly, another study showed cell adhesion on the hair protein substrate was not affected after blocking the integrin from the $\alpha_4\beta_1$ family [86]. Based on these scientific findings, the favorable cell attachment of hair protein-based 2D substrates was associated with both integrin- and non-integrin-receptor recognition. Although plenty of studies have shown the excellent biocompatibility of hair protein in terms of cell viability and proliferation, as well as cell adhesion, the actual mechanism and biological interactions between hair protein and cells still requires further study.

2.6 Summary of literature review

Biomaterials that originate from human sources are typically the preferred option to maximize the immunological compatibility and minimize cross-species contamination. However, limited resource is the biggest drawback. Therefore, human hair protein, an alternative human-derived biomaterial that

is found in abundance, could be an excellent option. The exceptional biological properties of hair proteins have gained significant interest in the development of protein-based biomaterials for a variety of applications, including composites, cosmetics, coating for drugs, biomedical implants, and tissue culture plates. The intrinsic binding motif, LDV, is the key attribute of human hair protein for improved cell attachment behaviour, which has been well reported.

Over the years, human hair protein-related studies were overwhelmed by biomaterials which were made up of total hair proteins. However, thin films made up of total hair proteins were too brittle to be handled. Plasticizers or blending with other elastic materials were used to reduce the brittleness. The high transparency and advanced cell attachment behaviour have led hair protein-based films to be extensively studied as an alternative for ocular reconstruction. Recently, selective extraction has received more attention. With the advances in the purification and extraction of hair proteins, keratin and KAP were successfully separated from total hair proteins. It is reported that various hair protein-based materials, produced by mixing keratin and KAP in different ratios, have shown different properties. This indicates that the properties of human hair protein-based biomaterials could be potentially optimized further to match the specific application.

Chapter 3 Experimental Methodology

This chapter presents the experimental methodology of this research project. The first part explains two different purification procedures to extract proteins from human hair. The second part shows the working principle of the approaches for protein immobilizations on polystyrene tissue culture plates and silica beads substrate. In the latter part of this chapter, the characterization techniques of this project is elaborated in detail.

3.1 Human hair proteins extractions

3.1.1 Total hair proteins (THP) extraction using Na₂S

Human hair was collected from a local hair salon. The hair was washed twice with detergents to remove impurities and then rinsed with absolute ethanol (Merck) to facilitate its drying process at room temperature. The dried hair was then soaked in a mixture of chloroform (Sigma) and methanol (Fisher Scientific) at a ratio 2:1 overnight in a fume hood for delipidization. After the solvents were completely evaporated, the delipidized hair was cut into lengths of 1 to 2 mm with scissors. THP, the mixture of keratin and KAP, was extracted by incubating 40 g of the cut hair in 1L of 0.125 M Na₂S•9H₂O (98+%, ACS reagent, ACROS Organics) at 40 °C for 1 hour. Next, the hair residues were removed by filtration and the resultant THP solution was dialyzed against deionized water (DI water) for the next 4 days in a cellulose tubing with 10 kDa molecular weight cut-off (Thermo Scientific SnakeSkin Dialysis Tubing) to remove the remaining Na₂S. The dialyzed THP solution was then freeze-dried to obtain THP powder and stored at -20 °C until further use.

3.1.2 Keratin and KAP extraction (Ethanol-assisted Shindai Method)

Keratin and KAP were extracted from human hair through a separation protocol described previously [50] and the procedure is demonstrated in

Figure 6. Briefly, the delipidized hair was incubated in a KAP extraction solution pH 9 Tris-HCl buffer (Sigma), which consisted of 8 M urea (Chem-Impex), 200 mM DTT (GoldBiotechnology), and 25 % ethanol at 50 °C for 72 hours. The extracted KAP solution was dialyzed against DI water for 24 hours in a cellulose tubing with 3.5 kDa molecular weight cut-off with water changing interval of every 2 hours. The remaining 'KAP-free' hair residues were rinsed with DI water and left to air-dry prior to the subsequent keratin extraction. The extraction of keratin was conducted in a pH 8.5 Tris-HCl buffer which consisted of 5 M urea, 2.6 M thiourea (Sigma), and 200 mM DTT at 50 °C for 24 hours. The extracted keratin solution was dialyzed against DI water for 9 water-changes (4 changes for the first 2 hours with 30 mins interval, followed by another 5 changes with 2 hours interval) in a cellulose tubing with 10 kDa molecular weight cut-off. To standardize the dialysis progress across different batches of samples, the free-thiol group presented in the dialyzed keratin solution was quantified by Ellman assay.

Figure 6 Flow chart of ethanol-assisted Shindai Method for keratin and KAP separation. (Adapted from [50])

3.2 Synthesis and characterization of physical coating on silica beads substrate

3.2.1 Silica bead synthesis

Monodispersed silica beads were synthesized using the Stöber method as described in the previous report [87]. The synthesized silica beads with an average diameter of 750 nm were purified by centrifugation and redispersed in ethanol.

3.2.2 Keratin coating

For THP immobilization on the surface of the silica beads, three surface modification steps were performed. All materials (reagent grade, Sigma Aldrich, USA) employed for the surface modification were used without further purification. In the first step, amine groups were generated on the bare silica surface by dispersing the beads in 0.1% 3-

aminopropyltriethoxysilane (APTES)/ethanol solution for 12 hours followed by centrifugation and dispersion in ethanol for three times to remove unreacted APTES. Secondly, amine-modified beads were dispersed in 1% glutaraldehyde /pH 8.0 phosphate buffer solution (PBS) with 20 mM sodium cyanoborohydride for 1 hour to implement aldehyde groups on the surface and the same washing procedures were repeated as above with DI water. Finally, the prepared silica beads were immersed in 0.1 mg/ml THP in pH 7.4 PBS solution for 6 hours and washed thoroughly with PBS buffer and DI water. Silica bead monolayer and THP coated bead monolayer formed at the air-water interface on a Langmuir-Blodgett trough were transferred into a 6-well plate. In order to provide a stable adhesion of the bead monolayer onto the polystyrene surface of the plate, transparent and non-toxic silicon polymer layers were coated prior to the transfer of the bead monolayer.

3.2.3 Surface Plasmon Resonance (SPR)

The surface density of the THP on beads was analyzed with a surface plasmon resonance system (SPR, Biacore2000, GE Healthcare, USA). The THP solution was injected into the Biacore instrument and the data was recorded until the signal reached equilibrium.

3.2.4 Zeta potential

Zeta-potential measurement unit (ELS-Z analyzer, Otsuka Electronics, Japan) was used to check surface property variation due to THP coating onto the beads in PBS solution at pH 7.4.

3.2.4 Characterization of Keratin Coated Silica Bead Surface

THP coated film of beads on the well plate was characterized using Fourier-transform infrared spectroscopy-attenuated total reflection (FTIR-ATR) (Nicolet 6700, Thermo Scientific, USA) equipped with Si window, atomic force microscopy (AFM, XE-100, Park systems, Korea), and contact angle measurement unit (Phoenix150, SEO, Korea). All AFM images were

recorded in non-contact mode with an acquisition frequency of 312 kHz. The contact angles were measured using DI water droplets.

3.2.5 Cytokine Array

Human XL Cytokine Array Kit (Proteome Profiler™ Array (ARY022B), R&D System, US) was used to investigate the cytokines secreted by HDFs when cultured on different substrates. All reagents were prepared according to the instructions provided by the supplier. On the first day, membranes were placed into a 4-well multi-dish containing 2 ml of the supplied blocking buffer in each well and then incubated for 1 hour. Next, the blocking buffer was replaced by 1.5 ml of sample diluted in the blocking buffer and incubated overnight at 4 °C. Subsequently, the membranes were washed three times for 10 min each with the wash buffer. They were then incubated in the detection antibody cocktail for 1 hour at room temperature followed by three washes as mentioned above. Each membrane was subsequently treated with 2 ml of 1X Streptavidin-HR for 30 mins and washed for another three times. Membranes were then removed, and the excess washing buffer was drained by touching one edge of the membrane onto absorbent paper wipes. 1ml of Chemi Reagent Mix was pipetted onto each membrane evenly which was then covered with a plastic sheet protector and incubated for 1 min. The excess reagent mix was drained off before capturing the image. All membranes were exposed for 10 min in a gel documentation system (ImageQuant LAS 4000 mini, GE Healthcare Life Sciences, US) and analyzed with ImageQuant TL software.

3.3 Immobilization of THP on Polystyrene (PS) Substrate

3.3.1 Free radical-assisted

THP powder was dissolved in DI water/0.125 M Na₂S₂O₈ aqueous solution at 5% w/v. The protein solutions were then centrifuged at 5000 rpm for 5 min to remove insoluble substances prior to immobilization. The plasma treatment

on PS substrates was carried out using Openair® Plasmatreater AS 400. The plasma was produced by applying 288 V in amplitude with 21 kHz in frequency, and the velocity of the plasma jet was fixed at 20 m/min. The protein solution was injected through a spray gun attached next to the plasma nozzle (refer to Figure 7 for the experimental set-up). A degradation test was performed by incubating the samples in Dulbecco modified Eagle medium (DMEM, PAN Biotech, Germany) supplemented with 10 % fetal bovine serum (FBS), 4 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37 °C for 28 days.

Figure 7 Schematic of THP immobilization through the free radical-assisted plasma.

3.3.2 Polymer-assisted

Polyacrylic acid (PAA, 45kDa, Sigma) powder was dissolved in 50 mM phosphate buffer solution (PBS, Gibco) and the pH was adjusted to 5.4 using 0.1 M hydrochloride (HCl). Next, N-hydroxysulfo-succinimide (sNHS, Thermofisher) and 1-Ethyl-3-(3-dimethylaminopropyl) (EDC, Thermofisher) were added to the PAA solution and stirred for 1 hour. The final concentrations of PAA, EDC, and sNHS in the solution was 0.5 % (w/v), 0.2

M, and 0.1 M, respectively. The purpose of the stirring is to activate the carboxyl groups of PAA to form sNHS ester. Concurrently, the PS surface was plasma irradiated at 100 W for 1 min with 22 ml/min oxygen flow volume at pressure 26.6 Pa. All plasma irradiated PS surfaces were exposed to air for 10 mins to maximize the carboxyl group formation. After that, the samples were incubated in PAA solution consisting of sNHS and EDC at room temperature for 2 hours. After the incubation, the samples were rinsed with DI water to remove the excess unreacted solution and incubated with 5% THP in PBS (pH7.8) for 1 hour. For the control experiment, a non-treated PS substrate was incubated with a 5% THP solution for 1 hour and rinsed with DI water to remove loosely bonded protein. The hypothesized immobilization mechanism of THP on plasma-treated PS with crosslinker is shown in Figure 8.

Figure 8 Schematic of the binding mechanism of polymer-assisted keratin coating. (Adapted from [73] with permission)

3.4 Quantification of Protein Concentration

3.4.1 BCA Protein Assay

The concentration of dialyzed keratin solution was quantified by PierceTM BCA Protein Assay Kit (ThermoFisher, Cat.23227). Briefly, the dialyzed keratin solution was diluted in DI water to meet the detection range of the BCA assay (20-2000 $\mu\text{g/ml}$). A series of dilutions of a known concentration of bovine serum albumin (BSA) was used as the standard curve. The working reagent was prepared according to the instructions provided by the supplier. The samples and the standards were placed in a 96-well plate, mixed with the

working reagent, and then incubated at 37 °C for 30 mins. After the incubation, the absorbance of the samples at 562 nm wavelength was measured using a plate reader (Infinite® M200).

3.4.2 Micro BCA Protein Assay

The detection range of Micro BCA assay (ThermoFisher, Cat.23235) is lower than BCA assay, which is between 0.5 - 40 µg/ml. It was used to quantify the concentration of THP in the SDS washing solution because the concentration of the THP was lower than the detection limit of BCA assay. To avoid the interference of SDS to the Micro BCA assay, the SDS washing solution was dialyzed against DI water before the assay. A series of dilutions of a known concentration of BSA was used as the standard curve. The working reagent was prepared according to the instructions provided by the supplier. The samples and the standards were placed in a 96-well plate, mixed with working reagent, and then incubated at 37 °C for 2 hours. After the incubation, the absorbance of the samples at 562 nm wavelength was measured using a plate reader (Infinite® M200, Tecan, Switzerland).

3.5 Ellman Assay

Ellman assay was used to quantify the free-thiol group concentration in the dialyzed keratin solution. The reaction buffer was prepared by dissolving 1 mM Ethylenediaminetetraacetic acid (EDTA) in 0.1 M sodium phosphate buffer at pH 8. A series of dilutions of a known concentration of L-cysteine was used as the standard curve (at a range of 0-1.5 mM). The Ellman's reagent stock solution was prepared at a concentration of 10 mM by dissolving Ellman's reagent powder (ThermoFisher, Cat.22582) in the reagent buffer. After that, 25 µL of each sample and the standards were placed in a 96-well plate. Next, each of the samples and the standards was mixed well with 5 µL of Ellman's reagent stock solution and 250 µL of reagent buffer and then incubated at room temperature for 15 mins. After the incubation, the absorbance of the samples at 412 nm wavelength was measured using a plate reader (Infinite® M200, Tecan, Switzerland).

3.6 Keratin film fabrication by solvent casting

Dialyzed keratin solution was adjusted to 20 mg/ml and poured into the customized polydimethylsiloxane (PDMS, Slygard 184) mold and well plate at $138.5 \mu\text{l}/\text{cm}^2$. It was left at room temperature until fully dried. Figure 9 shows the illustration of keratin film fabrication procedure.

Figure 9 Illustration of keratin film fabrication.

3.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

All chemicals and equipment for gel electrophoresis were purchased from Invitrogen unless otherwise stated. The final volume of protein sample was $20 \mu\text{l}$ which consisted of $10 \mu\text{l}$ of $1 \text{ mg}/\text{ml}$ protein sample, $3 \mu\text{l}$ of DI water, $5 \mu\text{l}$ LDS sample buffer (4x), and $2 \mu\text{l}$ reducing agent (10x). For the protein ladder, $5 \mu\text{l}$ of SeeBlue™ Plus2 Pre-stained protein standard was mixed with $15 \mu\text{l}$ of DI water. Next, the samples and the protein ladder were incubated at 95°C for 10 mins and then centrifuged at 13k rpm for 1 min before sample loading. NuPAGE gel (4-12%) was used at 150V for 45 mins with MES as a running buffer to resolve the molecular weights between 3 kDa to 198 kDa. After the run was completed, the gel was rinsed with DI water for 3 times and fixed on the rocking stage with a fixing solution (40 % ethanol and 10 % acetic acid) for 10 mins. The gel was then stained with colloidal Coomassie G-250 (Bio-rad) on the rocking stage overnight at room temperature. On the next day, the gel was destained with DI water until the background became clear.

3.8 Field Emission Scanning Electron Microscopy (FESEM)

Morphology of keratin film was analyzed under field-emission scanning electron microscope (FESEM; JSM-6340F, JEOL Co., Tokyo, Japan). The cross-section of the sample was obtained by breaking the samples into half after liquid nitrogen freezing. For the keratin film with cells cultured on the surface, it was fixed overnight in fixing solution containing 4% paraformaldehyde and 1.5 % glutaraldehyde. On the next day, the sample was dehydrated through a serial concentration of ethanol starting from 70%, 80%, 90%, and 100% for 2 hours interval in each. Finally, the sample was stored in a dry box until completely dried. Next, the sample was sputtered with platinum at 20 mA for 40 s prior to FESEM imaging. The accelerating voltage and current were set at 5 kV and 12 μ A, respectively. Thickness measurements were analyzed by using Image J. A total of six measurements from different spots of the cross-sectional image were used to get the average and standard deviation values.

3.9 Atomic Force Microscopy (AFM)

All AFM (Asylum Research, Cypher S) images were scanned in non-contact tapping mode. The parameters were used as follows: soft tip with 8 ± 2 nm diameter, 160 Hz resonance frequency, and 7.4 N/m force constant. Roughness and particle size were measured in height mode, while the phase distribution was obtained by phase mode through the software.

3.10 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) analysis was performed using an AXIS Supra spectrometer (Kratos Analytical) equipped with a hemispherical analyzer and a monochromatic Al K-alpha source (1487 eV) operated at 15 mA and 15 kV. A 3.1-volt bias was applied to the sample to neutralize charge build up on the sample surface. The high-resolution scans were recorded at 20 eV pass energy. Data analysis was done with ESCApe software (Kratos

Analytical). The binding energies (BEs) were charge-corrected based on the carbon 1s at 284.8 eV.

3.11 Water contact angle measurement

The water contact angle was measured at room temperature by using FTA32 Contact Angle and Surface Tension Analyzer (Analytical Technologies, Singapore). 6 µl of DI water was dispensed on the sample surface at 5 µl/s. Three measurements were taken from each sample to obtain the mean value.

3.12 Swelling test

The keratin film was cut into a rectangular piece while the two ends were sealed with scotch tape for gripping as shown in Figure 10. The film was fully immersed in PBS at room temperature for 5 mins and 72 hours. Percentage of volume change, as well as top surface and cross-section surface area changes, were quantified according to the following equations:

Total volume changes in percentage,

$$\frac{(V_t - V_o)}{V_o} \times 100\% \quad (5)$$

$$= \frac{(Length_{wet} \times Width_{wet} \times Thickness_{wet}) - (Length_{dry} \times Width_{dry} \times Thickness_{dry})}{(Length_{dry} \times Width_{dry} \times Thickness_{dry})} \times 100\%$$

where V_t is the swollen volume measured at wet state at time t , V_o is the original volume at dry state.

Top surface area changes in percentage,

$$\frac{(A_t - A_o)}{A_o} \times 100\% \quad (6)$$

$$= \frac{(Length_{wet} \times Width_{wet}) - (Length_{dry} \times Width_{dry})}{(Length_{dry} \times Width_{dry})} \times 100\%$$

Cross-section area changes in percentage,

$$= \frac{(Length_{wet} \times Thickness_{wet}) - (Length_{dry} \times Thickness_{dry})}{(Length_{dry} \times Thickness_{dry})} \times 100\%$$

Length and width were measured using ImageJ and thickness was measured using microscopy software. All samples were triplicated and at least 10 measurements were taken from each sample.

Figure 10 The representative microscopy image (left) and digital image (right) demonstrating the thickness measurement and top surface area measurement for swelling tests, respectively. Both measurements were conducted through Image J.

3.13 Circular Dichroism (CD)

Dialyzed keratin solution was prepared at 2 mg/ml and a quartz cuvette with an optical path length of 0.1 mm was used. Data were obtained on an AVIV 420 Circular Dichroism spectrometer in wavelength steps of 0.5 nm ranging from 180 to 260 nm, with an averaging time of 0.1 s over 3 scans. The CD spectrum was performed at 25 °C and plotted using OriginPro9.1 software.

3.14 Fourier-transformed infrared (FTIR) Spectroscopy

For keratin film samples, FTIR spectroscopy was carried out in attenuated total reflection (ATR) mode. The sample was pressed against the sample stage with a single reflection diamond element. The spectrum resolution was fixed at 4 cm⁻¹ and the sample was scanned in the wavenumber range between 400 and 4000cm⁻¹. FTIR deconvolution was done using Origin software to determine the protein secondary structure. Peaks within amide I were resolved through the second derivative.

3.15 Differential Scanning Calorimetry (DSC)

Differential scanning calorimeter (DSC, TA Instruments, U.S.) was used to evaluate the thermal properties of protein immobilization samples and film samples. Prior to DSC measurements, the samples were stored in a dry box overnight to remove moisture. The samples were cut into small pieces in order to fit into the standard aluminum DSC pan. The experiment temperature was increased from room temperature to 400 °C at 10 °C/min.

3.16 Thermogravimetric Analysis (TGA)

The thermogravimetric analysis (TA Instruments) was used to determine the decomposition temperature of the protein immobilization samples. Al₂O₃ crucible was used as a sample holder and heated from room temperature to 900 °C with a heating rate of 10 °C min⁻¹ in the nitrogen atmosphere.

3.17 Tensile Test

Mechanical Tester MTS C42 was used to study the mechanical properties of keratin film in the dry and wet states. Keratin film was cut into a standardized dumbbell shape with 63.5 mm in total length consisting of a gage section with 9.53 mm, 3.18 mm, and 4 mm in length, width, and thickness, respectively. The keratin film was pulled upwards by a 50 N load cell with a constant speed at 5 mm/s until broken. For the tensile test conducted in wet state, Bionix EnviroBath was filled with PBS (pH 7.2) and the keratin film was equilibrated in the PBS-filled chamber for 5 mins before the test.

3.18 Nano-isothermal titration calorimetry (NanoITC)

Figure 11 shows the illustration of the experimental set-up of NanoITC to study the interaction between keratin and KAP. In this report, the keratin represents ‘macromolecule’ due to the higher molecular weights while KAP is the ‘ligand’ due to the smaller molecular weight. Samples were centrifuged

at 13k rpm for 3 mins and the supernatants were used for the experiment. The supernatants were degassed for at least 10 min at 576 mmHg before injected into the cell and syringe. The stirrer speed was set at 300 rpm at 25 °C. All experiments were analyzed through NanoAnalyse software.

Figure 11 Illustrations of NanoITC set-up for interactions study between keratin and KAP.

3.19 Permeability test

Figure 12 shows the pictures of sample preparation for permeability test and the schematic of the experiment set-up. The membrane of the cell culture insert (Millicell Hanging Cell Culture Insert, PET 0.4 μ m, 24-well) was removed, replaced with keratin film, and then sealed with parafilm to prevent leakage (Figure 12 a). Prior to the permeability test, the keratin film was pre-soaked in PBS. After that, 50ul of 20 kDa FITC-dextran (Sigma, FD20S) was dropped on the center of keratin film (Figure 12 b). In the 24-well plate, 500 μ l of PBS was filled in the well at which the level just touched the bottom of the insert without overflowed. At each time point, the cell insert with keratin film was transferred to the next well, which was already filled with fresh PBS. The fluorescence measurement of the samples was measured using a plate reader (Infinite® M200, Tecan, Switzerland) at excitation/emission wavelengths of 490/580 nm.

Figure 12 The pictures of sample preparation of permeability test and the schematic of the experiment set-up.

3.20 Sterilization of keratin film

Keratin film was sterilized under UV for 10 mins on each side followed by immersion in 70% ethanol for 30 seconds. After that, the sterilized keratin film was soaked in cell culture media for 1 day (at least two changes of cell culture media) before seeding.

3.21 Cell Culture

Human dermal fibroblast (HDFs, ATCC® PCS-201-010™, US) was cultured in DMEM (PAN Biotech, Germany) supplemented with 10% FBS, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Human epidermis keratinocytes (HEKs, ATCC PCS-200-010™, US) was cultured in EpiGRO™ Human Epidermal Keratinocyte Complete Medium (Cat. #SCMK001, Millipore, US). The media were refreshed every 2-3 days and the cells were passaged when they reached 80% confluency.

For physical coating on silica beads experiment, both HEKs and HDFs cells were seeded onto 6-well plates at the same seeding density of 2×10^4

cells/well. For covalent coating on the PS substrate experiment, HEKs were seeded onto 24-well plates at the same seeding density of 4500 cells/well.

3.22 Proliferation assay (PicoGreen Assay)

Cell culture medium was removed from the well-plate prior to three-times wash with cold PBS. Cell lysis was then carried out by two freeze-thaw cycles of 20 mins each in cell lysis buffer (Cat. 9803S, Cell Signaling Technology, US). Subsequently, the cell lysates were mixed with PicoGreen (Molecular Probes®, US) working solution at a 1:1 ratio. All samples were incubated for 5 min at room temperature before fluorescence measurement with a plate reader (Infinite® M200, Tecan, Switzerland) at excitation/emission wavelengths of 480/520 nm.

3.23 Metabolic assay (Alamar Blue Assay)

The cell medium was replaced with Alamar blue reagent (Thermo Scientific, US) diluted in culture medium at 10:1 ratio and incubated for 2 hours at 37 °C, 95% humidity, and 5% CO₂. The samples were then measured with a plate reader (Infinite® M200, Tecan, Switzerland) for absorbance at the wavelengths of 570 nm and 600 nm upon color change observed.

3.24 Immunoperoxidase Staining

The cells were washed with cold PBS twice followed by fixation in 4% paraformaldehyde (Sigma, US) for 30 mins at room temperature. Subsequently, three-time washing with 0.05% Tween 20 in PBS (PBST) for 5 mins each and endogenous peroxidase quenching with Dako Real™ Peroxidase-Blocking solution (Agilent Technologies, US) for 30 mins at room temperature were performed. 10% goat serum in PBS was then applied as a blocking solution for 1 hour at room temperature. Two primary antibodies against fibronectin (ab23750, Rabbit polyclonal to fibronectin; Abcam, UK) and collagen I (ab88147, mouse monoclonal [3G3] to Collagen I; Abcam, UK) were used. Diluted primary antibodies (1:400 fibronectin and

1:100 Collagen I) were incubated with the samples in the blocking solution overnight at 4 °C. Subsequently, secondary antibodies (EnVision™+/HRP, anti-mouse, and anti-rabbit, Agilent Technologies, US) were applied onto the samples for 30 mins at room temperature, followed by DAB⁺ (Agilent Technologies, US) substrate development for 30 s – 2 mins. The reaction with DAB⁺ was stopped by pipetting tap water into the well once a brown color was observed. Counterstaining with haematoxylin (Thermofisher, US) was then performed. The samples were observed under an Olympus IX53 optical microscope and images at various magnifications were captured using the Q-capture Pro7 software.

For non-cell seeded samples, immunoperoxidase staining started from blocking step as fixation was not required. 10 % goat serum in PBS was spread onto the samples as a blocking solution for 1 hour at room temperature. The Primary antibody, anti-hair cortex cytokeratin (ab197395, Rabbit polyclonal to hair cortex cytokeratin) was purchased from Abcam, UK. Diluted primary antibody (1/200) was incubated with the samples in the blocking solution overnight at 4 °C. On the next day, secondary antibody (EnVision™+/HRP, anti-rabbit, Agilent Technologies, US) was applied to the samples for 30 mins at room temperature. The subsequent process was followed as stated in the previous paragraph. No counter stain was done for a non-cell seeded sample.

3.25 ELISA Kit

Human IL-1 α ELISA Kit (FGF2, Abcam ab46028) was used to investigate the IL-1 α secreted by HEK cultured on keratin coating. On day 3 and day 5, the cell culture medium was removed, followed by 3 times washing with cold PBS. The cell lysate was obtained by incubating the cells in cell lysis buffer (Cat. 9803 S, Cell Signaling Technology, US) for two freeze-thaw cycles of 20 mins each. The cell lysate was used for the ELISA kit and stored in -80 °C freezer for further use. All the ELISA protocol and reagents were prepared

according to the instructions provided by the supplier. The absorbance of the samples at 450 nm wavelength was measured using a plate reader (Infinite® M200, Tecan, Switzerland). The data was normalized by proliferation assay and plotted using OriginPro9.1 software.

3.26 Statistical analysis

All statistical analyses were done using Origin software by one-way analysis of variance (ANOVA) followed by Tukey's post hoc testing. $p < 0.05$ was considered statistically significant.

Chapter 4 Physical and covalent protein immobilization on substrate for cell culture applications

Chapter 4 discusses the findings of hair proteins immobilization on substrates for cell culture applications. Total hair proteins (THP) were first physically immobilized on silica beads substrate which could enrich HDFs behavior in culture, especially for wound healing therapies. In the second part of this chapter, THP was covalently coated on polystyrene substrates through plasma treatment by two approaches: polymer-assisted and free-radical assisted method. The former method demonstrates that THP could form homogenous and stable coating mainly through electrostatic interaction. The latter method offers a relatively promising covalent immobilization whereby the majority of the immobilized THP was retained on the surface after harsh washing.

4.1 The Physical Coating on Silica Beads Substrate

Human hair proteins have been extensively studied due to their biocompatibility, abundance, and potential for exploitation [1]. Hair protein coatings, in particular, can bring about new breakthroughs as bioactive interfaces on cell culture substrates, which has been shown to encourage ECM production [2] and moderate donor-to-donor variability while maintaining stem cell proliferative capacities [59]. In the current study, the THP was successfully coated on silica beads, which are advantageous as a cell culture substrate themselves.

4.1.1 Characterizations of THP-coated silica beads substrate

Total hair protein extraction using Na₂S

The extraction of THP using Na₂S as a reducing agent to cleave the disulfide bonds is a well-established method due to its high-yield production [2, 8, 9, 62]. According to the SDS-PAGE result in Figure 13 (a), two bands above 33 kDa and three bands below 28 kDa are observed, which corresponded to keratin and KAP fractions, respectively. The hair residue after the extraction was shown to be collapsed (Figure 13 c) while it was intact and appeared to be circular shape before extraction (Figure 13 b). Therefore, these results show that Na₂S is able to extract the THP from human hair successfully.

Figure 13 Characterization of THP extracted by Na_2S : (a) SDS-PAGE; FESEM images of hair strand (b) before and (c) after extraction.

As shown in Figure 14, AFM images of both bare silica and THP-coated substrates indicate that continuously packed monolayer particle films were successfully fabricated. The image of the THP-coated substrate in Figure 14 (b) shows that the THP coating did not cause any significant alteration of the surface morphology. A slight increase of less than 3 nm in average surface roughness for THP-coated substrate was observed due to the existence of proteins on the surface of the bead.

Figure 14 AFM images of (a) bare silica substrate and (b) THP-coated substrate (average surface roughness shown in parentheses).

Qualitative and quantitative analyses of THP coating were done using four different methods including FTIR, SPR, water contact angle, and zeta potential measurements. FTIR spectra in Figure 15 show the differences in the IR spectra of bare silica and THP-coated substrates. In the IR spectrum of the THP-coated substrate, two new IR bands appeared at around 1670 cm^{-1} and 3400 cm^{-1} regions, which originated from amide I and -OH groups, respectively. They are identical to the initial THP lyophilized powder.

Figure 15 FTIR spectra of a bare silica substrate, a THP-coated substrate, and THP lyophilized powder. Two new IR absorbance peaks appeared at 1670 cm^{-1} and 3400 cm^{-1} regions in the THP-coated substrate compared to the bare silica substrate.

The surface density of the keratin-coated substrate was $73.7 \pm 3.1\text{ ng/cm}^2$, which was estimated by SPR measurement, is shown in Figure 16. The surface coverage of THP on the bead surface was estimated at ca. 23% based on the molecular weight and size of keratin, which is a reasonable surface coverage compared to previous studies [2, 88].

Figure 16 SPR measurement of surface density changes in coated THP during surface immobilization.

The contact angle images of bare silica and THP-coated substrates are shown in Figure 17. The THP-coated substrate showed a contact angle ($74.1 \pm 3.7^\circ$) which is significantly higher than bare silica substrate ($83.3 \pm 4.2^\circ$). It is apparent that the THP-coated substrate has higher hydrophilicity due to the presence of THP which was extracted in an aqueous environment.

Figure 17 Contact angle measurements of (a) bare silica substrate and (b) THP-coated substrate.

In the case of the THP-coated substrate, the surface potential value was -30.6 ± 1.4 mV, which was slightly less negative than the value of bare silica substrate (-39.6 ± 2.8 mV). The higher surface energy and relatively lower net negative charge of the THP-coated surface may contribute to effective cell attachment in an initial cell adhesion period [89].

Silica beads are chemically inert and extremely stable; they provide a greater surface area for biomolecule and cell adhesion. Surface modification of the silica beads with various biomaterials including proteins is easy and well established [87]. Furthermore, silica bead films are transparent to visible light and allow convenient observation of cell behaviour during the cell culture process using phase contrast and fluorescent microscopy. However, silica is mostly associated with lower surface energy and is negatively charged, thereby generating repulsive forces that prevent cells from attaching to the tissue culture substrate. The presence of THP overcomes the limitations of silica as evidenced by the effective enhancement of hydrophilicity and surface roughness.

4.1.2 In-vitro work

Cell morphology

The morphologies of HDFs and HEKs cultured on different substrates are shown in Figure 18. The results show that the HDFs spreading was restrained on the bare silica substrate with cell aggregate formation by cells piling up onto one another on day 4, comparing to both control and the THP-coated substrates. For HEKs, colony formation was clearly observed and no significant difference in cell size and shape was visible across the three substrates.

Figure 18 Cell morphology observation using optical microscope on day 4 for HEKs and HDFs cultured on different substrates.

Cell Proliferation

The quantification of HDFs and HEKs proliferation is shown in Figure 19. The results reveal that the proliferation of HDFs on the THP-coated substrate was not significantly different from the bare silica substrate on day 2, while the cell proliferation on the coated substrate reduced significantly on day 4 as compared to the uncoated one. As shown in Figure 18, cell aggregation was observed as a distinct feature of HDFs cultured on the bare silica substrate, suggesting poorer affinity of cells to the bare silica substrate as compared to the THP-coated substrate. However, the reason for the greater proliferation of the bare silica substrate is not apparent. In contrast, HEKs proliferation on the THP-coated substrate was higher than the bare silica substrate on day 2, but there was no statistically significant difference between these two substrates on day 4.

Figure 19 Cell proliferation based on PicoGreen assay at day 2 and day 4 of HEKs and HDFs cultured on different substrates. * $p < 0.05$, $n = 6$.

Cell Metabolic Activity

The Alamar blue result was normalized with dsDNA quantities obtained from the proliferation assay in order to understand the effects of various substrates on the metabolic activity of HDFs and HEKs at different time points, as shown in Figure 20. Although the bare silica substrate had a higher number of HDFs on day 4, the cell metabolic activity on this substrate was remarkably lower than the THP-coated substrate. This suggests that the aggregated cells observed on the bare silica substrate in Figure 18 were less active. On day 2, the metabolic activity of HDFs on the THP-coated substrate was about 5 times higher than the bare silica substrate. As such, coating with THP on the bare silica substrate significantly enhanced the metabolic activity of HDFs. However, for HEKs, the THP coating did not increase cell metabolism.

Figure 20 Cell metabolic activity based on Alamar Blue assay on day 2 and day 4 of HEKs and HDF cultured on different substrates. * $p < 0.05$, $n = 6$.

Immunoperoxidase Staining

ECM production by cells in culture is an indicator of the compatibility of the substrate used. Specifically, a major function of dermal fibroblasts is the collagen synthesis. Collagen I and fibronectin are essential ECM components in the dermis that impart the mechanical strength required for the tissue and facilitate cell migration by supporting cell adhesion through cell adhesion molecules such as integrins. Therefore, immunoperoxidase staining was performed to investigate the expression and distribution pattern of collagen I by HDFs and fibronectin by both HDFs and HEKs on the different cultivation substrates.

The images in Figure 21 show that there was collagen I expression by HDFs and fibronectin expression by both HDFs and HEKs on day 2 and day 4 for both the THP-coated and bare silica substrates. This suggests the biocompatibility of these substrates to both cell types. In the HDFs cultures, fibronectin and collagen I expression were more extensive on both bare silica and THP-coated substrates compared to the control. Collagen 1 expression

by HDFs cultured on the THP-coated substrate was significantly more intense compared to the bare silica substrate. There was no significant difference in fibronectin expression observed in HEKs cultured on all three substrates, indicating that HEKs were less sensitive to the presence of the bare silica substrate and THP coating. The ability of the THP-coated substrate to induce collagen 1 and fibronectin production in HDFs was therefore promising and could have been facilitated by the presence of the LDV and EDS peptides, which were recognized as cell binding motifs to ECM [6]. Nonetheless, the detected ECM proteins remained largely intracellular in a relatively short culture time.

Figure 21 Characterization of fibronectin and collagen I production by immunoperoxidase staining on day 2 and day 4 for different substrates.

Cytokine Array

HDFs secrete a large number of cytokines and growth factors over the course of the wound healing cascade [90]. Wound healing is a well-studied subject and is recognized as an extremely complex process because there are numerous signaling pathways and responses involved. The overall wound healing process can be categorized into four stages: hemostasis, inflammation, proliferation, and remodeling. Impaired wound healing occurs if any of these stages is compromised. In this study, cytokines related to inflammation, proliferation, and remodeling phases of wound healing were evaluated as model readouts to understand the influence of the coatings. The results are presented in Figure 22.

According to Figure 22, it was observed that several cytokines were found to be moderated by the substrates used. For example, Angiopoietin-2 is highly expressed during vascular formation in wound healing [91]. Similarly, Endoglin is a major glycoprotein of vascular endothelium but it is also recognized as a highly expressed TGF- β receptor which regulates cell proliferation, migration, and adhesion towards injury site [92]. SDF-1 is found upregulated in the early phase of wound healing to promote vascular formation and fibroblast proliferation but it is nearly absent in the latter phase due to fibroblast differentiation in tissue remodeling [93]. Vitamin D regulates the expression of cathelicidin which modulates skin inflammation, induces angiogenesis, and improves re-epithelialization [94]. In addition, osteopontin is a cytokine existing in the ECM, promoting cell adhesion and migration apart from regulating inflammatory responses [95]. In contrast, the up-regulation of IFN- γ causes impaired wound healing by inhibiting collagen deposition and the TGF- β 1-mediated signaling pathway [96]. The results indicate the propensity of the THP-coated substrate to elevate cytokine secretion by HDFs.

Inflammatory cytokines are secreted in the inflammation phase to recruit immune cells such as macrophages, T cells, and lymphocytes towards the injured site to trigger the immune system [97, 98]. The results show that pro-

inflammatory cytokines such as IFN- γ , IL-1 α , IL-8, IL-17A, MCP-1, MIF, osteopontin, TARC, and Pentraxin-3 were secreted on all substrates (Figure 22 c). In fact, the THP-coated substrate consistently induced higher expression levels of the above-mentioned inflammatory cytokines compared with the bare silica substrate and control, indicating its potential to promote inflammation during wound healing. Among these pro-inflammatory cytokines, osteopontin recorded the highest relative expression level.

In the proliferation phase, the FGF family of cytokines including FGF basic (FGF-2), FGF-7, and FGF-19, which are growth factors responsible for fibroblast proliferation, were expressed on all substrates (Figure 22 d). FGF basic, FGF-7, and FGF-19 are beneficial to wound healing as they induce epidermal proliferation to promote re-epithelialization [99, 100]. Furthermore, angiogenesis-related cytokines such as Angiopoietin-2, Endoglin, SDF-1 and re-epithelialization regulator (Vitamin D), were also presented in all three sample groups. Thrombospondin-1, an adhesive glycoprotein, had the highest expression among the cytokines relevant to the proliferation phase. Notably, all the proliferation-related cytokines secreted by HDFs had the most significant expression on the THP-coated substrate compared to the other substrates, indicating the potential of the THP-coated substrate in supporting cell proliferation and angiogenesis during wound healing.

As shown in Figure 22 b, Serpin E1, a protease inhibitor, registered the highest signal among cytokines relevant to the remodeling phase. Other common proteases such as MMP-9, uPAR, and the inducer EMMPRIN which participates in fibroblasts differentiation into myofibroblasts [101] were limited in the expression. Similar to the inflammation and proliferation phases, HDFs cultured on the THP-coated substrate had the highest expression levels of cytokine relevant to the remodeling phase compared to the bare silica substrate and control.

Among the cytokines evaluated, Thrombospondin-1 is reported to inhibit the proliferation and migration of fibroblasts [102], as well as binding to various proteins (such as fibrinogen, fibronectin, and integrins), and involves in both cell-to-cell and cell-to-matrix interactions [103]. Retrospectively, the significant expression of this cytokine possibly contributed to the reduction of HDFs proliferation observed on day 4 of culture (Figure 19). Interestingly, Serpin E1 was the most highly expressed cytokine in this study. It is reported that Serpin E1 could protect ECM from degradation while assisting in more efficient cell migration by regulating cell detachment from vitronectin, fibronectin and collagen, and allowing subsequent re-adhesion of integrins to alternative matrix structural elements [104]. This was reflected in this study by the limited expression of MMP-9 and uPAR, which are responsible for ECM degradation. The expression of uPAR is known to enhance fibronectin binding by $\alpha 5\beta 1$ integrin [105]. Thus, it is hypothesized that uPAR expression may be encouraged by the significant expression of fibronectin as shown in the immunoperoxidase staining results (Figure 21). In addition, the significant expression of EMMPRIN on the THP-coated substrate is suggested to directly correlate to the elevated expression level of TGF- β [101]. Consequently, TGF- β signaling was likely responsible for the increased levels of fibronectin and collagen 1 expression recorded on both the THP-coated and bare silica substrates, as compared to control [106].

Collectively, the THP-coated substrate significantly enhanced the expression of several cytokines that are closely implicated in the wound healing cascade. The sustained level of ECM protein production and increased in cytokine secretion provided the reasons behind the elevation in metabolic activity recorded despite a reduction in cell proliferation. Essentially, HDFs transitioned from a proliferative state to a differentiated state when cultured on the THP-coated substrate. Further investigations are recommended to fully understand the biological significance of modulating HDFs cytokine production in culture, in relation to both fundamental research and expanding cells for clinical transplantation purposes.

Figure 22 Cytokine profiles of HDFs cultured on different substrates on day 4. (a) Chemiluminescent images of cytokine arrays. Cytokines secreted at significantly different levels are labeled. (b-d) The mean pixel intensities of selected cytokines were normalized with proliferation assay results and plotted. THP-coated substrates enhanced the secretion of several cytokines related to wound healing.

4.2 Covalent coating on polystyrene substrate through plasma treatment

4.2.1 Polymer-assisted protein immobilization

Surface wettability

Based on the contact angle results as shown in Figure 23, samples with plasma treatment showed relatively low water contact angle, which was in the range between 41° to 51° . It is worth noting that the water contact angle had a reduction of at least 10° for samples ‘plasma+THP’, ‘plasma+PAA+THP’, and ‘Plasma+PAA+EDC+THP’ after the THP coating. This result indicates that THP coating could improve the hydrophilicity of the coated surfaces effectively, which is also tallied with the previous study [107]. Hydrophilicity is treated as an important factor for biomedical related surfaces because hydrophilic surface is preferred for cell attachment. Therefore, the THP-coated surface can potentially have better cell attachment when compared to the non-treated polystyrene surface.

Figure 23 Water contact angle image for polymer-assisted protein immobilization samples at various conditions.

Degradation Test

All samples were incubated with DMEM for 5 days at 37 °C to determine the stability and ability of the coating retention on the surface with medium incubation. Immunoperoxidase staining was conducted at different time points: day 0, day 2, and day 5. All staining images are presented in Figure 24. THP-coated samples showed strong and homogenous staining up to day 2 but started to disintegrate after day 5 except for the sample without plasma ‘w/o plasma+THP’. This result suggests that hydrophobic adsorption between THP and the hydrophobic substrate surface (without any plasma treatment) could be the dominant binding mechanism. In fact, numerous studies have shown that hydrophilic surface offered more specific protein adsorption whereas hydrophobic surface tended to have unspecific adsorption [108]. In this study, the major amino acid residues presented in the THP are leucine and glycine. Both leucine and glycine are hydrophobic amino acids. Thus, they may provide more binding sites for hydrophobic interactions. In addition, proteins are sensitive to microenvironment and likely to have conformation changes in certain circumstance. Therefore, it is proposed that the hydrophobic surface may trigger protein structural changes and further characterization are required to identify the mechanism.

Figure 24 Immunoperoxidase staining of polymer-assisted protein immobilizations on day 0, day 2 and day 5.

4.2.2 Free-radical Assisted Protein Immobilization

In this free-radical assisted protein immobilization, 5 % THP lyophilized powder was dissolved in two solvents: DI water and 0.125 M Na₂S. The purpose is to determine how the THP interacts with the plasma treated polystyrene surface in neutral (DI water) and reduced (Na₂S) states. Plasma-treated sample with THP in DI water and 0.125 M Na₂S are named as P-DI and P-Na₂S respectively in the following discussions.

After the treatment, samples were incubated in DMEM at 37 °C. Immunoperoxidase staining was performed at different time points from day 0 to day 28 to observe the THP coating stability and distribution pattern. The morphology and topology were analyzed through FESEM and AFM. In addition, SDS detergent wash was used to determine the covalency of the coating. The washing SDS solution for each sample was collected and dialyzed against DI water for one day prior to the protein quantification by Micro BCA assay.

Degradation Test

The purpose of incubating samples P-DI and P-Na₂S in DMEM at 37 °C is to mimic the physiological condition. Another reason is to determine the stability of the coatings in the presence of media over 4 weeks. Immunoperoxidase staining microscopy images of all time points are summarized in Figure 25. Based on the staining results, the ‘droplet’ feature was observed for both P-DI and P-Na₂S samples. It is worth emphasizing that the THP staining was mainly presented in the surroundings of the ‘droplet’ feature of P-DI, whereas the THP staining was found predominantly trapped within the features and faded in the surroundings for P-Na₂S. It is suggested that those ‘droplet’ feature was generated by the spray gun during the THP injection and have formed covalent bonding towards the substrate. For P-Na₂S, abundant of cysteine was formed when the THP was in a reduced state. Cysteine is most likely to react simultaneously with active free radical

molecules due to its antioxidant behaviour. Therefore, the strong staining observed as ‘droplet’ feature in P-Na₂S are proposed to be covalent-bonded THP as the cysteine residues tended to react with free radical molecules generated by the plasma treatment. However, cysteine is susceptible to oxidation. Hence, those cysteines which are unable to react with free radical may have been oxidized to form disulfide bonds and physically bounded to the substrate.

Figure 25 Immunoperoxidase staining of non-treated PS, P-DI, and P-Na₂S from day 0 to day 28.

Thickness Measurement

According to the cross-sectional FESEM images shown in Figure 26, a layer of coating with a distinct structure was observed to be located on the top for both P-DI and P- Na_2S samples. It is suggested that this layer belonged to the immobilized THP through plasma treatment because it presented only on THP-coated samples and exhibited different structure when compared to the underneath PS substrate. The thickness of the P-DI was almost twice the value of P- Na_2S , which was approximately 509 ± 10 nm and 848 ± 18 nm, respectively. These cross-sectional images further supported that the protein immobilization was successful, but the equipment enabled chemical element mapping such as EDX or XPS are recommended as complementary characterization to quantify the elemental composition distribution.

Figure 26 Cross-sectional FESEM images of (a) non-treated PS, (b) P-DI, and (c) P- Na_2S .

Coating Homogeneity and Phase Distribution

Compared to the phase image of P- Na_2S shown in Figure 27, P-DI showed a relatively homogenous distribution of coating which is consistent with the immunoperoxidase staining result. On the other hand, the phase image of P- Na_2S showed a distinct ‘droplet’ feature which resembles the feature observed from the immunoperoxidase staining. In addition, the ‘droplet’ feature showed different contrast which was brighter than the surroundings.

During the phase image scanning, the amplitude motion of AFM tip changes corresponds to regions with different composition due to the interactions between the AFM tip and the surface chemistry. The amplitude changes of AFM tip are then converted into phase changes signals which resulted in bright and dark regions which represent hard/stiff and soft/more adhesion materials, respectively [109]. Thus, it is propounded that both bright and dark regions of P- Na_2S phase image belonged to the immobilized THP. It is because the ‘softer’ region (darker region) was unlikely to be the PS substrate which had a theoretically higher stiffness than the THP. Therefore, the contrast difference obtained from the phase image also indicates that the stiffness is potentially varied in different regions.

Figure 27 AFM images in amplitude and phase scanning modes for P-DI and P- Na_2S .

Thermal Stability

Table 7 Thermal properties of non-treated PS, P-DI, and P-Na₂S obtained from TGA.

Samples	Decomposition temperature (°C)	Deriv. weight (%/°C)
Non-treated PS	404	3.2
P-DI	405	3.1
P-Na ₂ S	403	3.2

Thermogravimetric analysis (TGA) thermogram provided evidence on the thermal stability of the protein in terms of weight loss and the rate of weight loss as a function of temperature. The corresponded decomposition temperatures and weight loss are summarized in Table 7. Figure 28 shows the TGA curves of non-treated PS, P-DI, and P-Na₂S. The weight loss of P-DI and P-Na₂S were 3.1 %/ °C and 3.2 %/ °C, respectively. The weight loss values are not much difference among the three samples. The decomposition temperature of P-DI was 405 °C, which was higher than the non-treated PS at 404 °C. These decomposition temperatures were likely contributed by the polystyrene. The thickness of PS substrate was $866.6 \pm 3.9 \mu\text{m}$, which was at least 1000 times larger than the thickness of THP coating of P-DI and P-Na₂S. This large difference in weight ratio might mask the signal from the THP. Although the current results are not conclusive, it shows the potential of using TGA as a complementary technique to determine the bonding of protein immobilization indirectly. It is because the chemically bonded THP was assumed to have better thermal stability and a peak shifted to higher decomposition temperature was expected due to the strong bonding presented between the interfaces. Finally, other characterization techniques such as XPS and FTIR are still required to reveal the chemical bonding presented, whether the protein immobilization is physically or chemically coated.

Figure 28 TGA spectra of non-treated PS, P-DI, and P-Na₂S from 25 °C to 900 °C.

Quantification of Covalent Bonded Protein

As mentioned, SDS washing was established to determine the covalency of the protein coating. Since SDS is an ionic detergent, it can rinse off proteins that are physically adsorbed, and the proteins retained on the coated surface are likely to be covalently bonded proteins. Immunoperoxidase staining was used to monitor the coating patterns before and after the SDS washing. Based on the optical microscopy images shown in Figure 29, the intensity of staining was reduced for both P-DI and P-Na₂S after the SDS washing. In addition, the ‘droplet’ feature was found to remain on P-Na₂S but disappeared on P-DI. The protein quantification was obtained using Micro BCA assay and the results are shown in Figure 30. The percentage of THP remained on the surface after SDS washing is the ratio of the amount of the THP retained on the surface to the total amount of the coated THP. P-Na₂S showed a significant higher substrate-bonded THP than P-DI, indicating that THP has great potential to be covalent-bonded protein through plasma treatment either in neutral or reduced conditions.

Figure 29 Immunoperoxidase staining of P-DI and P-Na₂S before and after SDS washing.

Figure 30 Percentage of protein remained after SDS washing for P-DI and P-Na₂S via Micro BCA assay.

In this study, XPS was used to identify and quantify the composition of the oxidized sulfur and nitrogen presented in the immobilized THP. The composition of nitrogen was normalized against carbon peak at 285.5 eV. The selected carbon peak was assumed to be the carbon contributed by the immobilized THP because it is not categorized as the fingerprints binding energy (BE) of the carbon contamination from the atmosphere. Similarly, the

nitrogen composition was presumed to belong to the amine group from immobilized THP as nitrogen was not supposed to be present on the substrate. The purpose of quantifying the normalized nitrogen composition from the XPS spectrum is to compare the amount of immobilized THP remained on the sample after SDS washing. The nitrogen/carbon ratio of P-Na₂S and P-DI were 0.6021 and 0.2215, respectively. As a comparison, P-Na₂S had a higher nitrogen/carbon ratio which showed the same trend obtained from protein quantification by Micro BCA assay. This result further indicates that P-Na₂S can retain more covalently immobilized proteins than P-DI.

Figure 31 shows the XPS spectra of sulfur peak deconvolution for P-Na₂S and P-DI. The sulfur peaks were deconvoluted to quantify the composition of the oxidized sulfur and free-thiol group. The peaks at higher BE are associated with more electropositive species. The deconvoluted sulfur peak with BE at 168.7 eV is the oxidized sulfur such as sulfates and sulfone. Another peak at 163.9 eV is very close to the thiol group reported in the literature. In this study, the peak ratio of the oxidized sulfur and free-thiol group was used as a guideline to determine the sample which offered relatively more free-thiol group. The ratios of P-Na₂S and P-DI were 1.22 and 2.37, respectively. The smaller ratio value indicates that the P-Na₂S possessed more free-thiol group as compared to P-DI. This observation supports the hypothesis that more available free-thiol group represent a higher amount of covalent bond as proposed.

Figure 31 XPS spectra of sulfur peaks deconvolution of samples: (a) P- Na_2S ; (b) P-DI. *XPS spectra have been calibrated against carbon (284.8 eV).

4.2.3 Interaction Study Between Purified Hair Proteins: Keratin and KAP

Except for hair keratin, skin keratin is the most widely studied keratin in literature. One of the functions of skin keratin is to protect epithelial cells from cell death by forming a plaque to provide additional mechanical strength to skin. This plaque is formed based on the connections between intermediate filaments and desmosome proteins. In fact, it was found that a similar plaque existed in hair [110]. However, hair is much more robust and having relatively higher mechanical strength when comparing to stratum corneum. Therefore, there are more complex mechanisms contributing to the robustness of hair yet to be explored. It is well-known that the disulfide crosslinking between KAP and keratin is responsible for the mechanical strength of hair, but it is limited to the cytoplasm. Matsunaga *et al.* found that KAP not only regulate the characteristic helical alignment of intermediate filaments, but also bind to desmosome proteins, forming an intercellular bridging [111]. These findings show the multi-functional character of hair proteins and it motivate our further investigation on the interactions between the purified keratin and KAP extracted by the Shindai method. The understanding of protein interactions can help to identify the factors that

affect interactions within hair proteins and optimize the properties of hair protein-based coating for specific applications.

Keratin and KAP separation using ethanol-assisted Shindai method

FESEM and SDS-PAGE were done to characterize the separation of keratin and KAP through ethanol-assisted Shindai method. Based on the SDS-PAGE result in Figure 32, the KAP fractions showed three bands between 28 kDa and 6 kDa, which tallied with the reported molecular weight distribution of KAP ranging from 10 kDa to 25 kDa [112]. On the other hand, the keratin fractions showed three visible bands at around 98 kDa, 55 kDa, and 45 kDa which represented dimer, basic, and acidic keratin, respectively. Basic and acidic keratin are the two main constituents of keratin which are essential to form heteropolymeric intermediate filaments. As such, the ethanol-assisted Shindai method was able to extract KAP and keratin separately from human hair.

Figure 32 SDS-PAGE of extracted KAP and keratin by ethanol-assisted extraction.

FESEM was done to observe the morphology of human hair before extraction (delipidized hair) and hair residues after each of KAP and keratin extraction. The FESEM images are shown in Figure 33. Interestingly, some fiber-like features were observed on the ‘KAP-free’ hair residues after the KAP extraction. It is postulated that these fiber features were belonged to keratin, which became visible after the KAs was completely extracted. FESEM images of the hair residue after keratin extraction were also obtained, which showed no intact hair residues but only a muddy paste left. As one of the main components of cortex, keratin contributed to the mechanical strength of hair due to the fiber-like features. Thus, the hair structure could have collapsed after the keratin was extracted. Therefore, the hair residue feature observed in these FESEM images could be the cuticle which might be broken during extractions and spread as a sheet.

Figure 33 FESEM images of delipidized hair (top), ‘KAP-free’ hair residue (middle) and hair residue after keratin extraction (bottom).

Table 8 Summary of the sample collected for the dialysis optimization including sample collection time and sample name.

Day	Time	Sample name
Day 1	4-6pm	1-1
		1-2
		1-3
		1-4
Day 2	10am-12pm	2-1
	12-2pm	2-2
	2-4pm	2-3
	4-6pm	2-4
Day 3	10am-12pm	3-1

Dialysis is a widely used technique in protein purification and buffer exchange. In this study, dialysis was performed and optimized to purify keratin effectively by removing the residual in the extraction solution including reducing agent and urea. It is because the urea presented in the extraction solution might cause urea crystallization, which was undesired for the subsequent film fabrication and material characterizations. Furthermore, the reducing agent in the extraction solution might affect the self-assembly of keratin and cause confusion to self-assembly study in the future work. Table 8 shows the sample collections time for the dialysis optimization experiment. To optimize the dialysis procedure, the free-thiol concentration of both dialysate and keratin solution were quantified by Ellman assay and the results are shown in Figure 34.

The free-thiol concentration of dialysate decreased gradually for the first four water-change and increased again for sample 2-1. Reducing agent and urea can interfere with the Ellman assay. Therefore, it is assumed that the increase of free-thiol concentration for sample 2-1 was due to the residues accumulated after the dialysis overnight. From sample 2-2 onwards, almost no free-thiol group detected in the dialysate. Therefore, it is proposed that the dialysis was completed after 5 water-change. To further confirm that the free-group concentration has reached the equilibrium, the free-thiol group

concentration of keratin solution was then determined and discussed in the next section.

Figure 34 The free-thiol concentration of dialysate collected from day 1 to day 3.

The free-thiol concentration detected in the keratin solution was normalized against the protein concentration to obtain free-thiol concentration per gram of protein and the results are shown in Figure 35. The purpose of the normalization is to standardize the result so that it is comparable across different batch of samples. According to Figure 35, the normalized free-thiol concentration of keratin solution was similar between samples collected at day 2 and day 3 dialysis process. Since there was no significant difference observed, it is concluded that the dialysis procedure was completed after 5 water-change. These results show that the dialysis process was optimized and provided a benchmark for the subsequent keratin extractions.

Figure 35 The free-thiol concentration of dialyzed keratin solution on day 2 and day 3.

Interaction studies on folding and unfolding conditions between keratin and KAP

Tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) are the three fluorescence amino acid residues that contribute to the intrinsic fluorescence of proteins [113]. These fluorescence residues are very sensitive to the environment and exhibit a red shift to the fluorescence emission, especially when the polarity of the environment increases, for instance, in the presence of noncovalent bonds or hydrogen bonds. Similarly, a blue-shift is expected when a protein is in a non-polar environment or native state [114]. Since the intrinsic fluorescence residues are highly influenced by the environment, they have been used to monitor the protein conformation change, ligand interaction, and protein denaturation. In this project, the intrinsic fluorescence behavior was used to monitor the unfolding state of keratin in various concentrations of urea.

As shown in Figure 36, the fluorescence intensity of keratin increased with an increasing in urea concentration. Usually, the hydrophobic tryptophan is buried and surrounded by hydrophilic residues in the native state, thus the fluorescence is hard to be detected. This result indicates that the fluorescence residues were highly exposed in a high urea concentration. The increased

fluorescence intensity therefore suggest that the keratin was fully or partially unfolded when dissolved in a higher concentration of urea.

Figure 36 Fluorescence intensity of keratin in various concentrations of urea.

Table 9 Summary of binding models exhibited between keratin and KAP titration n NanoITC at different concentration of urea.

Binding Models	Urea concentration (M)			
	2	3	4	8
Multiple Sites	✓	✓		
Sequential Two Sites	✓	✓	✓	
Sequential Three Sites		✓	✓	

The NanoITC raw data of the interaction study between keratin and KAP in 2 M, 3 M, 4 M, and 8 M urea are presented in Figure 37. Three fitting models: multiple sites, sequential two-sites, and sequential three-sites were well-fitted to different samples. The fitting results are summarized in Table 9. There was no model that could fit the 8 M urea result in the software, indicating that the higher degree of unfolding state keratin may exhibit a binding mechanism

which is not well-recognized by the system. Another possibility is the complete denaturation may occur and modify the potential binding sites to prevent the subsequent binding. Based on the summary in Table 9, 2 M and 3 M urea samples could be fitted into multiple sites and sequential two sites binding models while 4 M urea could be fitted into sequential two sites and sequential three sites binding models.

Multiple sites binding model refers to the availability of at least two non-identical and independent binding sites. On the other hand, sequential binding models are typically cooperative, and the binding sites can be either non-identical or identical. In other words, multiple sites binding model is relatively more restrictive and specific. According to the results, multiple site binding model was only applicable to 2 M and 3 M urea. This is because the structures of keratin and KAP were partially unfolded and the overall structures were still considered compact. Therefore, the available binding sites were limited and independent as they might be further away from each other. When urea concentration increased, the binding systems tended to become more unspecific. Hence, sequential binding models were preferred rather than multiple sites binding model. Higher concentration of urea allowed higher degree of unfolding, hence it is assumed that more potential binding sites were exposed under such circumstance. This result suggests that the binding mechanism between the highly unfolded keratin and KAP can facilitate proximal binding sites cooperatively and the degree of unfolding may influence the binding behavior between keratin and KAP.

Figure 37 NanoITC raw data heat rate graph of 1mg/ml KAP titrated into 0.02 mg/ml keratin in different concentrations of urea.

Table 10 Summary of thermodynamic parameters obtained from sequential two sites in 2 M, 3 M, and 4 M of urea.

Urea conc. (M)	Thermodynamic parameters					
	Association constant, K_a (M^{-1})		Enthalpy, ΔH (kJ/mol)		Entropy, ΔS (J/mol•K)	
	K_{a1}	K_{a2}	ΔH_1	ΔH_2	ΔS_1	ΔS_2
2	1.722×10^6	7.968×10^3	-992.3	5000	-3.209×10^3	1.684×10^4
3	1.194×10^5	1.352×10^6	520.6	-1415	1.843×10^3	-4.603×10^3
4	9.069×10^4	8.188×10^4	-10000	-5000	-3.345×10^4	-1.668×10^4

Another approach to predict the potential binding mechanism is analyzing the quantitative thermodynamic parameters. The thermodynamics parameters were obtained from the sequential two-sites binding model because it was the common model exhibited by 2 M, 3 M, and 4 M samples. The thermodynamic parameters were referring to the association constant (K_a), change in enthalpy (ΔH), and change in entropy (ΔS). All data is summarized in Table 10. Generally, the two most common binding sites of the sequential two-sites binding model are either ' $A+B \rightarrow AB$ ' or ' $AB+B \rightarrow ABB$ ' or other more complicated combinations. In this study, the ' AB ' binding was labelled as site 1 while ' ABB ' was labelled as site 2. Each site consisted of a set of three thermodynamics parameters as mentioned, for example, K_{a1} was association constant for site 1. Association constant is the affinity of binding, a higher value represents the likelihood of binding to occur. Other than association constant, change in enthalpy and entropy are another two important thermodynamic parameters. Typically, there will be an exothermic reaction when two species combine into one. This leads to a negative value of enthalpy (ΔH) and loss in entropy (ΔS).

K_{a1} was higher for 2 M urea while K_{a2} was slightly higher in 3 M urea. It is assumed that the preferred binding sites may change in different concentration of urea. Interestingly, no dramatic difference was observed between K_{a1} and K_{a2} for 4 M urea. This finding implies that the bindings became less specific when urea concentration was increased. It is proposed that the binding sites became less competitive as excessive binding sites were revealed under a higher degree of protein unfolding state. This assumption is further supported as the ΔH values obtained from 4 M urea were both negative and accompanied with low ΔS values, indicating a combination of two species (in this case could refer to the interactions between keratin and KAP) was preferred as the system released heat.

Ultimately, the association constants of 3 M urea were the highest among the four samples. This indicates that the 3 M urea could provide an environment with a higher affinity for the binding between keratin and KAP. Furthermore, the overall enthalpy values became more negative as compared to 2 M urea.

This shows that the system was closely resembled a transition from endothermic to exothermic binding in nature and most likely exhibited different binding sites due to conformation changes [115]. Therefore, this result suggests that 3 M urea may be a suitable condition for subsequent studies, for examples, to determine the specific binding sites and the interaction mechanism between keratin and KAP in an unfolded state.

Chapter 5 Keratin Films

This chapter demonstrates another type of human hair proteins-based platform, a stand-alone film made from the purified keratin. Unlike other highly brittle keratin-based films reported in the literature, this keratin film was mechanically stable and easy to handle. It was obtained by solvent casting the purified keratin at room temperature without additional crosslinkers or plasticizers. To explore the properties of this keratin film, various characterization techniques were used to study its surface features, internal structure, mechanical properties, and thermal properties.

5.1 Surface characterizations

Surface and cross-sectional morphology by FESEM

The cross-section and surface morphologies of the keratin film were characterized by FESEM, as shown in Figure 38. The cross-section of the keratin film showed porous and sheet-like structures with dispersed granular particles, while the surface was smooth, and no visible pore was observed. Some spherical grooves were found on the film surface which might be the granular particles observed in the cross-sectional images. Those granular particles could be the melanosomes from hair. Interestingly, distinct and significant structures were observed across the surface and cross-sectional images. This finding implies that the keratin film could have exhibited large-scale molecular re-arrangement during the drying process.

Figure 38 FESEM images of keratin film. (a) surface image from top view; (b-d) cross-sectional images.

Topology by AFM

In Figure 39, the AFM height image shows the topology of the keratin film consisting of granules features. The average surface roughness was 33 ± 5 nm. Similar keratin granules were observed by Hartrianti *et al.* but the average roughness was much lower [59]. It was reported that cell culture surface coated with nanosized keratin globules enhanced fibronectin production effectively in HDFs.

Figure 39 AFM height images of keratin film in 2D (left) and 3D (right) model.

Surface wettability by water contact angle

The water contact angle was used to characterize the surface hydrophilicity of the keratin film. Another film made of THP was characterized as a control, which was referred to as THP film in the following discussions. According to Figure 40, the water contact angle of keratin film was $88^\circ \pm 4^\circ$ while THP film was $68^\circ \pm 1^\circ$. The water contact angle of keratin film was significantly higher than THP film, indicating that the keratin film surface was more hydrophobic. The hydrophobicity of the keratin film surface could be due to the smooth surface structure which generally exhibited low water retention ability. In addition, the unfolded hydrophobic residues could be exposed on the film surface during the air-drying process, which resulted in a relatively hydrophobic surface.

Figure 40 The water contact angle images show the wettability keratin film and THP film.

5.2 Swelling Test

According to Figure 41, the percentage change in the total volume of keratin film has no significant difference after immersing in PBS for 5 mins and 72 hours. It indicates that the keratin film has reached an equilibrium state at 5 mins. Except for the percentage change in total volume, the percentage change in the top surface and cross-sectional areas were quantified and compared. Interestingly, the percentage change in the cross-section area was significantly higher than the percentage change in the top surface area. This suggests that the swelling of the keratin film was directional, which was mainly affected by the cross-sectional area. This is probably due to the internal structure of the keratin film, as shown in Figure 38. It proposes that the porous and hierarchical internal structures allowed more water retention and resulted in a higher swollen ratio. As a comparison, the top surface area was relatively restrained from swelling. This could be due to the smooth surface with no visible pores observed under FESEM images, AFM topology scanning, and low surface hydrophilicity, which are unlikely to allow high water absorption.

Figure 41 The swelling test results of keratin film: volume change in percentage (left) and area change in percentage in the comparison of top surface area and cross-section area (right).

5.3 Protein Secondary Structure

Circular Dichroism spectroscopy

Circular dichroism (CD) is an excellent technique to determine the secondary structure of protein rapidly. Briefly, the optical transitions of the chromophores of the amides presented in the backbone of proteins would be captured and resulted in characteristic CD spectra corresponding to specific protein secondary structures [116]. In this study, CD was used to determine the protein secondary structures of keratin in a liquid state, which represented the state before being processed into the keratin film. According to the CD spectrum in Figure 42, there was one positive band at around 190 nm and two negative bands between 210 nm and 230 nm, which represented the characteristic bands of α -helical proteins [116].

In addition, the ratio of the peak intensities at 222 nm to 208 nm ($\theta_{222}/\theta_{208}$) is an indication to identify the likelihood of the helical structure being isolated or formed into the coiled-coil structure [117-119]. According to this theoretical proposition, a $\theta_{222}/\theta_{208}$ ratio of less than 0.9 represents the presence of isolated α -helix while a ratio greater than 1 indicates coiled-coil formation. In this study, the $\theta_{222}/\theta_{208}$ ratio was approximately 1. It indicates that the coiled-coil structure was preserved after extraction. Since the coiled-

coil structure is an important building block for keratin filaments, maintaining this structure after extraction is desired and essential for the subsequent self-assembly process.

Figure 42 CD spectra of keratin solution.

Deconvolution of Amine I peak by FTIR-ATR mode

Deconvolution of amine I peak from the FTIR spectrum is a semi-quantitative method for protein secondary structure analysis. The ratio percentage of each secondary structure was obtained from the area under the corresponding deconvoluted peak (Figure 43). For keratin film, β -sheet was the dominant protein secondary structure, followed by the random coil and β -turn. Interestingly, the keratin solution consisted of α -helix as the dominant composition based on CD results whereas the keratin film exhibited a relatively lower amount of α -helix. In other words, it is highly possible that most of the helical proteins could have transformed into β -sheet, β -turn, and random coil during the film formation process. This is supported by the presence of 3_{10} helices which was proposed to be the intermediate unit during the transitions between helical and non-helical states. [120].

The transition from α to β phase has been suggested as a universal mechanism of filamentous α -helical proteins to exhibit advanced mechanical properties [121]. In this study, THP film consisted of a higher β -sheet amount compared to keratin film. The presence of the high amount of β -sheet could lead to high brittleness because β -sheet has been reported to correlate with the mechanical properties of proteinaceous biomaterials [122]. The changes in protein conformations are affected by a few factors, such as mechanical stress, solvent, heat treatment, freezing temperature, and freezing duration. In this study, the slower drying process that provided sufficient time for the molecule re-arrangement may have affected the mechanical properties. Tamada *et al.* reported the effect of drying methods on protein secondary structures transformation by manipulating freezing and drying temperatures. Self-aggregated fragments were partially transformed into β -sheet structure from random coil structure during silk-based sponge formation by the freeze-drying method [123]. In this study, the difference of α -helical composition between keratin solution and film suggests that the transformation of protein conformation occurred during the drying process.

Figure 43 Ratio percentage of secondary structures after FTIR deconvolution for (a) THP film (b) keratin film and (c) composition percentage of protein secondary structure for both samples.

5.3 Thermal Properties

Thermal stability by DSC

DSC was used to study the thermal properties of keratin film. In Figure 44, the water evaporation heat transfer peak was observed for both keratin film and THP film within the range between 80 °C and 120 °C. Similarly, the second endothermic heat transfer of both samples occurred at around 230 °C. This peak represented the denaturation of helical protein while the enthalpy acted as the measurement of fiber crystallinity [124]. Lastly, the broad endothermic peak observed in the THP film at around 325 °C was due to the beta-protein denaturation. As a comparison, the endothermic peak exhibited by keratin film was relatively small and less pronounced, suggesting that the amount of beta-protein was lesser. This observation was tallied with the protein secondary structure quantification by FTIR deconvolution. Since there was no significant peak shift observed, the DSC results indicate that the thermal stability of keratin film and THP film are comparable.

Figure 44 The DSC thermograms of keratin film and THP film.

Decomposition temperature by TGA

TGA thermogram was used as a complementary characterization technique to investigate the thermal stability of keratin film in terms of weight loss as a function of temperature. Thermogravimetric (TG) curve determines the mass decrement during the heating process whereas the derivative thermogravimetry (DTG) curve determines the maximum temperature at the maximum rate of mass loss. Figure 45 shows the TGA thermograms of keratin film and THP film. The region between 25 °C and 200 °C was the first stage of mass loss which attributed to the evaporation of water. The second stage of the mass reduction occurred between 200 °C and 400 °C, which is mostly associated with the degradation of protein arising from the breaking of the polypeptide backbone. The decomposition temperatures of the maximum rate of weight loss of THP film and keratin film were comparable, which were obtained at 321 °C and 319 °C, respectively. The decomposition temperatures corresponded to the findings in DSC where the β -protein denaturation occurred at around 325 °C. In addition, some minor steps were observed in the DTG curves in both samples, which may correspond to the multiplicity of protein conformations of the films.

Figure 45 TGA and DTG spectra of keratin film (left) and THP film (right) with decomposition temperature stated.

5.4 Tensile test in dry and wet states

Table 11 Mechanical properties of keratin thin film obtained from tensile testing.

Dry/ wet state	Ultimate Tensile Strength, UTS [MPa]	Modulus [MPa]	Strain at break [%]
Dry	49.8 ± 6.1	1050 ± 90	6.1 ± 0.3
Wet	0.6 ± 0.2	1.0 ± 0.5	179 ± 17

The ultimate tensile strength (UTS), modulus, and strain at break-point of keratin film in wet and dry states are summarized in Table 11. The stress-strain curves are shown in Figure 46. The THP film was not included because it was too fragile for the tensile tests. The largest drawback of the THP film was the high brittleness which led to poor handling and limited accessibility. Due to the brittle behaviour, hair protein-based biomaterials were unavailable for the tensile test unless plasticizers were added. It is mostly due to the high cysteine content of KAP which resulted in a large amount of disulfide bonds crosslinking. The disulfide crosslinking is reported not only to maintain the structural stability but also to limit the degree of freedom of the internal structure.

The tensile testing results show that the keratin film exhibited UTS at 49.8 ± 6.1 and 0.6 ± 0.2 MPa in dry and wet state, respectively. In the dry state, this is encouraging as the tensile strength of keratin film was higher than the keratin film with the addition of glycerol (1 MPa) and keratin/chitosan blend film (27-34 MPa) reported elsewhere [56]. Therefore, it is suggested that keratin can form a film with better mechanical properties yet provide better handling without plasticizer addition. Keratin film exhibited much higher modulus in the dry state but lower extensibility when compared to the wet state. In the dry state, it obtained 1050 ± 90 MPa modulus which is almost 1000 times higher than it was in the wet state (1.0 ± 0.5 MPa), but the strain at break-point was only $6.1 \pm 0.3\%$. This suggests that the structure was

relatively compact which limited the flexibility. Hence, higher strength was required during plastic deformation.

Plasticizer has been reported to reduce the brittleness of hair protein film effectively, but the effect was compromised when exposed to wet conditions. The hair protein film plasticized by glycerol was found to lose the flexibility and regain the brittleness as a result of the dissolution of glycerol in water [125]. As a comparison, the keratin film in this study allowed better flexibility in wet conditions by showing $179 \pm 17\%$ of strain at the fracture point. This result suggests that the keratin film showed optimum extensibility and flexibility without the presence of plasticizers and matrix proteins. This phenomenon might be associated with the penetration of water molecules into the keratin film. It is suggested that the penetrated water molecules incorporated with the system to cause swelling and create space within the structure. This could enable the rearrangements between keratin intermediate filaments which contributed to the flexibility [126].

In addition, the stress-strain curve of the keratin film in wet state presented in a J-shape, which was reported as a characteristic mechanical behaviour of viscoelastic soft tissues [127]. The tensile test in the wet state met the criterion, which provided an overview of how the keratin film behaved mechanically when exposed to the liquid condition. Fudge *et al.* used hagfish as an intermediate filaments (IF) model to study the mechanics of IFs in a hydrated state [128]. The results suggested that the α - β transition occurred during the stretching. The model was strained under the seawater environment and exhibited a stress-strain profile similar to this study. There were two similarities between these two studies: firstly, the samples were IFs based models; secondly, both samples showed similar stress-strain profile. These induced the speculation that the keratin film of this study would likely exhibit the similar mechanics.

Mechanical test is always a challenge for pure hair protein-based materials due to their high brittleness unless additive/plasticizer is added. It is considered as an advantage for this study when a pure keratin-based film

(without plasticizer) was allowed for the tensile test in both dry and wet states. The tensile test in a dry state provided an opportunity to compare the mechanical properties of the keratin film of this study to other studies. In short, this tensile test result shows that the keratin film behaved differently according to the environment: ductile in a dry state and elastomer behaviour in a wet state. These observations encourage further optimization to achieve more desired properties for specific applications.

Figure 46 Stress-strain curve of keratin film in both dry and wet states.

To further explore the mechanical stability of keratin film in the wet state, a cyclic test was conducted with various fixed strains ranging between 5 % and 25 %. At each fixed strain, two rounds of cyclic test were conducted while each round consisted of three continuous cycles. The keratin film didn't fracture and showed a hysteresis loop for all the cycles (refer to Figure 47). The stress-strain curves shifted down with an increasing number of cycles for a fixed strain at 10% onwards. This stress-relaxation phenomenon is known as preconditioning which is commonly observed for viscoelastic materials subjected to cyclic stretching. In other words, the viscoelastic behaviour of the keratin film was manifested when it showed hysteresis loops and stretched to 10% strain or more by the stress-relaxation as a function of the number of

cycles [128]. The downshifted hysteresis loops became narrow with the increasing number of cycles. This circumstance is related to one or several of the following effects, the changes of internal structure, damage of the native internal structure [129], or hydration [130] of the sample.

Figure 47 Stress-strain curves of keratin film obtained from cyclic test in the wet state at various fixed strain.

Chapter 6 Biocompatibility of Keratin Films

This chapter briefly discusses the biocompatibility evaluation of keratin film using human epidermal keratinocytes (HEKs). In the first section of this chapter, HEK's response to different coatings is discussed. HEKs were cultured on a 24-wells plate with different coatings including collagen I, total hair proteins, and keratin. To evaluate the HEKs response to these coatings, various assays and cell-specific marker stainings such as cell proliferation assay, cell viability assay, ELISA, and K14 staining were conducted.

6.1 Keratin film permeability

Table 12 Summary of correlation coefficient (r^2 of keratin films corresponding to various mathematical models).

Mathematical models	correlation coefficient (r^2)
Zero-order	0.52
First-order	0.27
Higuchi	0.87
Power Law	0.95

To explicate the permeation characteristic of the keratin films, the permeation of 20 kDa FITC-dextran through keratin film was fitted into various models and summarized in Table 12. Compared to zero and first-order models, the permeation data was able to fit both Higuchi and power law models better, by showing relative higher correlation coefficients (r^2). Figure 48 shows the cumulative release profile of 20 kDa FITC-dextran permeated through keratin film and the profiles fitted into Higuchi model and power Law. The results show that the permeation mechanism was not only dependent on diffusion, which is driven by the concentration gradient but also associated with matrix erosion or dissolution.

According to the kinetic data listed in Table 12, the r^2 of the power law (0.95) was slightly higher than Higuchi model (0.87). Since the power law is derived from the Higuchi equation, it is reasonable that one release data can fit into both models. The purpose of deriving power law is to determine whether the release system is specific to diffusion-controlled or matrix erosion-controlled. The release mechanisms are classified by the release exponent (n) according to different geometries (refer to Table 6). In this results, the n value was 0.67 which fall between the range of $0.5 < n < 1$. This indicates that the release mechanism of 20 kDa FITC-dextran is specific to anomalous transport (Fickian diffusion together with matrix erosion).

Figure 48 The cumulative amount of FITC-20KDa permeated through keratin film as a function of time (a). The release profile was fitted into (b) Higuchi model, and (c) power law model.

6.2 Cell morphology

HEKs were cultured on 24-well plates with three different coatings: 20 mg/ml keratin, 20 mg/ml THP, and 2 mg/ml collagen I (as a positive control). The morphologies of HEKs were monitored up to day 5 through optical microscopy and FESEM as shown in Figure 49 and Figure 50, respectively. According to the images, HEKs were able to attach and form colonies on both collagen I and keratin coating. The confluency was comparable among control, collagen I, and keratin coating by visualizing through optical microscope images. This is mostly due to the presence of a well-known cell motif, LDV in keratin. Bush *et al.* have revealed that the positively charged and hydrophobic surfaces allowed more cell-binding sites to keratinocytes, which enhanced cell spreading and cell adhesion [131]. Thus, the relatively

low wettability of keratin film might have also enhanced the affinity of HEKs to keratin film in terms of cell adhesion and cell spreading. However, HEKs were barely attached to 20 mg/ml THP coating. They were not spread and were unable to form colonies on THP coating. Our previous study has reported that THP was able to improve the biocompatibility of a bioinert surface but it was done at a lower concentration compared to this study [107]. Therefore, it is suspected that the poor attachment of HEKs on THP coating observed in this study is mostly due to the concentration issue.

Figure 49 The microscopy images of HEKs at Day 3 and Day 5 cultured on different surfaces: control plate, 2 mg/ml collagen I, 20 mg/ml keratin, and 20 mg/ml THP.

According to the optical microscopy images in Figure 49, the distribution of HEKs at day 5 was similar among control, collagen I, and keratin coating. This observation indicates that the proliferation rate of HEKs cultured on the keratin coating was comparable to the two controls. In addition, the colony observed on the keratin coating further supported that HEKs proliferated in a healthy state when cultured on the keratin coating. Similar colony features

were observed through FESEM images in Figure 50. Several micropodia and ultramicroextensions were observed in Figure 50 (b). Furthermore, the two cells (highlighted with yellow arrows) in Figure 50 (a) were rounded and detached from the substrate. This pair of rounded cells is considered as a feature of HEKs in proliferation state, suggesting that they were proliferating before fixation. This observation was treated as an additional evidence to support such conclusion qualitatively. Since the FESEM images in Figure 50 was used as a complementary result to analyze the morphology of HEKs qualitatively, only one representative image has been shown.

Figure 50 (a) The FESEM images of HEKs cultured on keratin film and (b) the area as indicated by red box in (a) at higher magnification.

6.3 Proliferation Assay

The proliferation of HEKs was quantified by PicoGreen assay and the results are shown in Figure 51. On day 3, the proliferation of HEKs was not significantly different between control, collagen I, and keratin coating. However, it was significantly lower in THP coating when compared to other

samples. This is mostly due to the poor cell attachment of HEKs as discussed in the previous section. Nearly no live cells were detected on day 5 for THP coating. Although the HEKs proliferation on keratin coating was significantly lower than collagen I coating, it was still comparable to the control. This is encouraging and indicates that the keratin coating is a potential alternative cell culture substrate for HEKs.

Figure 51 The proliferation assay results of HEKs at Day 3 and Day 5 cultured on different surfaces: control plate, 2 mg/ml Collagen I, 20 mg/ml Keratin, and 20 mg/ml THP. ** $p < 0.05$, $n = 3$

6.4 Cell metabolic activity

To understand the metabolic activity of HEKs on various coatings, the Alamar Blue result was normalized with dsDNA quantities obtained from the proliferation assay. The Alamar Blue results are summarized in Figure 52. On day 3, except for collagen I and keratin coating, other samples were significantly different from each other. At day 5, the metabolic activity of HEKs was comparable among control, collagen I, and keratin coating. However, THP coating showed significantly lower metabolic activity compared to all samples at day 3. No signal was detected from the THP coating as most of the cells were dead on day 5. This result shows that the cell growth response of HEKs cultured on keratin coating is satisfactory as it is comparable to the collagen I and control.

Figure 52 The metabolic activity results (Alamar Blue assay) of HEKs cultured on different surfaces: control plate, 2 mg/ml Collagen I, 20 mg/ml keratin, and 20 mg/ml THP.

6.5 K14 staining

Keratin 14 (K14) is a prototypic specific marker of mitotically active basal keratinocytes, pairing with Keratin 5 (K5). They help to maintain cell shape and provide resistance to mechanical stress. More importantly, this pair of keratin modulates cell proliferation and negatively regulates cell differentiation through P13K/Akt and Notch 1 signaling pathways [132]. Mutation of K14 or K5 would cause defects in nasal cell compartment and lead to skin blistering disease such as epidermolysis bullosa simplex (EBS). Therefore, K14 is required for the normal development of keratinocytes.

In this study, immunoperoxidase staining was used as a complementary characterization to determine the capability of keratin coating in maintaining the functionality of HEK. According to the optical microscopy images in Figure 53, the HEKs cultured on keratin coating showed extensive expression of K14. This implies that the cells were functional and proliferative. This

observation further suggests that the keratin coating is a substrate that can allow HEKs to stay functional and proliferative.

Figure 53 The microscopy images showing the immunoperoxidase staining of K14 in HEKs cultured on keratin film.

6.6 IL-1 α quantification by ELISA Kit

IL-1 family is the mitogen of inflammatory cytokines while IL-1 β and IL-1 α are the two dominant cytokines. Although these two IL-1 members bind to the same receptor (IL-1Ra), they have distinct functions. IL-1 β is expressed only in tumor sites while IL-1 α is released at a relatively higher level in the healthy epidermis. For normal epidermis, the ratio between IL-1 α to IL-1 β was reported to be 40:1[133]. According to the ELISA assay result in Figure 54, the IL-1 α expression in keratin coating was higher than the control and collagen I coating on day 3 and day 5. The result suggests that keratin might cause more inflammation in HEKs when compared to collagen I and control. It is worth noting that IL-1 α has been reported as a cytokine which can

promote wound healing by stimulating proliferation as well as migration of HEKs [134]. In other words, IL-1 α expression is positive and required for effective wound healing. Despite of drawing conclusion from this face value, the implication of this observation will need to be further investigated.

Figure 54 The concentration of IL-1 α secreted by HEK cultured on different coatings on Day 3 and Day 5. All data were normalized to corresponded dsDNA amount obtained from PicoGreen assay. * $p < 0.05$, ** $p < 0.01$, $n = 6$

Chapter 7 Conclusions and Recommendations

This chapter summarizes and concludes the significant findings of this project. The project is separated into two parts: coating and film. THP was extracted and coated physically onto silica bead substrates. The coating successfully improved the cell response of the bioinert silica bead substrates in terms of cell spreading, cell proliferation, and cytokine interactions related to wound healing. Furthermore, covalent immobilization was conducted via plasma treatment. Free-radical crosslinking is proposed to be a promising covalent crosslinking mechanism for cysteine-rich hair proteins. In the second section of this project, the main components of hair proteins, keratin and KAP, were extracted separately. Keratin was able to form a stand-alone keratin film simply by solvent evaporation without additives. The keratin film exhibited high modulus in a dry state and high strain in a wet state. Hierarchical internal structure was found and prompted to be the evidence of self-assembly. Primary cell line, HEKs, was successfully seeded and showed comparable cell proliferation and cell viability to the conventional tissue culture plate and the collagen I coating.

7.1 Conclusions

In this report, hair proteins were assembled into 2D substrates via coating and film formation. Hair proteins were extracted using two methods: Na_2S extraction and ethanol-assisted Shindai method. The former extracted THP whereas the latter extraction method extracted keratin and KAP separately. The aim is to provide a better understanding on the interactions within hair proteins to produce viable 2D substrates for specific applications. The THP extracted by Na_2S was successful. A combination of bands belonged to keratin and KAP were observed in SDS-PAGE, which tallied with previous studies. According to the SDS-PAGE results of ethanol-assisted Shindai method, keratin showed three visible bands at around 98 kDa, 55 kDa, and 45 kDa that represented dimers, type II and type I keratin, respectively. In addition, the extracted KAP also showed three bands between 10-25 kDa, showing the separation of keratin and KAP by ethanol-assisted Shindai method was successful. This is further supported by the FESEM images which show fiber-like features and the collapse of hair cuticle before and after extraction.

THP was successfully coated on monodispersed silica beads which was spread as a monolayer on tissue culture plate. Smooth and uniform surface coatings were achieved, resulting in a silica beads substrate with higher wettability and lower negative charge. The coating complemented a monolayer silica bead by facilitating more desirable HDFs spreading, heightened metabolic activity, sustained ECM protein production, and increased cytokine secretion when compared to the bare silica bead substrate. To produce covalent coating, THP was immobilized on a PS substrate through polymer-assisted and free-radical assisted methods. For the polymer-assisted method, the formation of ester covalent bonding between the plasma-treated substrate and $-\text{COOH}$ functional group was inadequate. Thus, the keratin coating did not last beyond 5 days in the cell culture medium. Meanwhile, the untreated PS dish (without plasma treatment and crosslinking reaction) revealed the presence of keratin coating up to day 5. This could be due to the hydrophobic behaviour of keratin that preferred hydrophobic

interactions. Further characterizations are required to determine chemical bonds present at the interface and the efficiency of keratin crosslinking with the polymer.

For free-radical assisted method, the PS dish coated with the THP dissolved in Na₂S was able to retain more proteins after the SDS washing. Based on the XPS results, P-Na₂S showed higher nitrogen/carbon ratio, which indicates that more proteins were retained on the surface. In addition, the deconvoluted sulfur band showed that P-Na₂S provided more free-thiol groups, suggesting that more covalent binding points are available. These results indicate that the free thiol groups in the THP can potentially react with the free-radical molecules on the activated substrate to form covalent bonds, thereby assisting THP immobilization on the PS substrate. Furthermore, keratin and KAP in various urea concentrations were used to study the interactions between them via NanoITC. It is suggested that the bindings between keratin and KAP became more unspecific when the urea concentration was increased. This is because more binding sites were revealed and become less competitive to each other at high degrees of unfolding condition.

Keratin fraction can form a keratin film with easier handling than the THP film without plasticizer. It is proposed that the absence of KAP reduced the disulfide crosslinking which resulted in a less rigid and more flexible structure. Furthermore, FESEM images showed distinct features between a smooth surface and a porous cross-sectional structure. It is encouraging to further explore the extent of keratin self-assembly in different conditions by varying keratin concentrations and drying parameters. The *in vitro* study also shows that the keratin films were able to offer satisfied biocompatibility and cell response by HEKs. In conclusion, this project shows that human hair protein-based coatings and keratin films can be the potential platforms for biomedical applications.

7.2 Recommendations

7.2.1 Exploit combination of keratin and KAP for protein immobilization and quantify the bonding present at interfaces

Combination of keratin and KAP at different ratio for protein immobilization

The reduced keratin solution shows strong coating retention after the harsh washing step. This is a positive result, indicating that the thiol-chemistry can be exploited to produce viable 2D substrates, due to the presence of abundant cysteine residue in this unique class of material. The two main components of hair proteins, keratin and KAP, exhibit different properties based on their protein sequence and cysteine contents. KAP consists of abundant cysteine residues while keratin contain a binding motif, LDV. Thus, the next step is to mix keratin and KAP in a different ratio for 2D substrate coating, to exploit their distinct behaviors and to facilitate different use in biomedical applications.

X-ray photoelectron spectroscopy (XPS)

There are two characterization modes in XPS, which are surface profiling and depth profiling. Surface profiling can detect all the elements presented on the surface while depth profiling allows nanoscale-etching followed by a surface profiling. It is worth to emphasize that depth profiling not only provide element analysis data, but also become a way to determine the thickness of the coating. The etching rate can be optimized and repeated until the interface between the coating material and substrate material is reached. However, it is challenging to obtain accurate bonding interaction data analysis through depth profiling. It is because a precise thickness estimation is required in order to achieve the correct etching cycle that falls in between the interfaces. Morphology imaging through FESEM, element mapping through confocal Raman microscopy with 785 nm laser, and AFM phase scanning mode are

the alternate approaches to achieve thickness estimation for protein immobilization. Another challenge is raised when the coating thickness is more than 1 μm . It is because the etching process is done by ions while the recommended maximum etching thickness for XPS is approximately less than 500 nm. Hence, it is recommended to minimize the exposure time to ions for biological samples as well as for an economic consideration.

Quantitative analysis for bond strength, type of bonds, and composition of different bonds are feasible in XPS. These can be done by the peak deconvolution for each interested element spectrum and then quantified by the XPS software. Therefore, XPS will be the primary characterization technique for bonding interaction study of protein immobilization in this project.

7.3 *In vitro* test

Our group has previously reported the biocompatibility of several cell lines for hair protein-coated substrates including HDFs, HEKs, and MSCs. MSCs is a well-studied adult stem cell in literatures as they showed promising capacity and long term multipotentiality when induced in *ex vivo*. Most importantly, they offer immunosuppression as privileged over other cell types. In future, we will use stem cells as an advance *in vitro* work for keratin film and protein immobilized substrates. The cell morphology will be monitored during the cultivation through optical microscope. Alamar blue and PicoGreen assays will be conducted to determine the cell viability and toxicity. Furthermore, cell attachment assay will be performed to check the ability of cell attachment on different platforms at different time points. Those unattached cells will be rinsed off by PBS, while the attached cells will then be trypsinized and the number of cells will be counted.

Mono cell culture provides important information as to our knowledge in the specific cell response to the keratin film. Establishing a co-culture system on a keratin film can be a strategy to evaluate cell-cell interaction by both

paracrine and direct cell-cell contacts. HDFs and HEKs have been widely used in the co-culture system, thus it is recommended to co-culture these two cell lines on a keratin film as the first step of establishing co-culture system in this study. In recent years, a paradigm shift from 2D cell culture to 3D cell culture was developed rapidly. 3D cell culture can mimic the microenvironment and affect the cell functions in a similar fashion to the *in vivo* response. For instance, HDFs and HEKs have been used in the 3D skin model to reproduce the dermis and epidermis in the presence of collagen I which acts as a matrix. To study cell-cell interaction more precisely, refinements are required to convert the 2D keratin film cell culture platform into 3D cell culture system to maximize the simulation to *in vivo* environment. This is considered as the pioneer step of exploring the feasibility of the keratin film in the co-culture and 3D cell culture system.

7.4 Exploring the self-assembly ability of keratin and the effects on properties

7.4.1 Effect of drying process on keratin film structure

It was reported that prolong drying process provided sufficient time for molecules to rearrange among themselves and resulted in a more organized structure. Therefore, varying the drying process is the next step to characterize the self-assembly ability of keratin and how it affects the properties of the keratin film.

Keratin film will be fabricated in the following conditions: open-air-dried, vacuum dried, oven-dried, air-dried with high humidity, and air-dried with low humidity. While open-air-dried is the existing fabrication for keratin film, vacuum-dried is used to produce a keratin film in a shorter time to minimize the possibility of oxidation. The purpose of the oven-dried is to check the viability of keratin assemble in an elevated temperature as well as its thermostability. Humidity is also applied to prolong the drying process.

FESEM will be used to observe the morphology as well as measure the thickness and granular particles sizes dispersed in the structure. In addition, crystallinity and protein secondary analysis will be done by XRD and FTIR amide I peak deconvolution, respectively. Furthermore, keratin film will be immersed in PBS to determine the duration to remain intact before disintegration in the presence of liquid.

7.5 Interaction study between keratin and KAP

The interaction study between keratin and KAP will be continued by using NanoITC as the primary characterization technique. The interaction study will be performed by varying different factors such as pH, temperature, and solvents. As mentioned in the literature review, keratin consists of type I and II keratin with 11 and 6 subtypes, respectively. These subtypes possess different but similar isoelectric points. It is worth to study how these keratin subtypes behave differently and the possible interactions in different pH or dissolving in different solvents. Furthermore, varying temperature is also an option in the protein interaction study to monitor the changes of the behavior of proteins before and after the denaturing temperature. In addition, it is interesting to know if keratin can reassemble under these circumstances. These attempts are essentials to provide an overview for the interactions study between keratin and KAP. It can be an opportunity to obtain more insight into the specific interaction within human hair proteins and fully utilize them in potential application with a specific function.

References

- [1] J. G. Rouse and M. E. Van Dyke, "A review of keratin-based biomaterials for biomedical applications," *Materials*, vol. 3, no. 2, pp. 999-1014, 2010.
- [2] F. Taraballi S. Wang, J. Li, F. Y. Y. Lee, S. S. Venkatraman, W. R. Birch and K. W. Ng, "Understanding the nano-topography changes and cellular influences resulting from the surface adsorption of human hair keratins," *Advanced healthcare materials*, vol. 1, no. 4, pp. 513-519, 2012.
- [3] W. Crewther, J. Gillespie, B. Harrap, and A. Inglis, "Low-sulfur proteins from α -keratins. Interrelationships between their amino acid compositions, α -helix contents, and the supercontraction of the parent keratin," *Biopolymers: Original Research on Biomolecules*, vol. 4, no. 8, pp. 905-916, 1966.
- [4] H. P. Baden and L. Bonar, "The alpha-fibrous proteins of epidermis," *The Journal of investigative dermatology*, vol. 51, no. 6, p. 478, 1968.
- [5] R. Fraser, T. MacRae, and G. E. Rogers, "Keratins: their composition, structure and biosynthesis," 1972.
- [6] V. Verma, P. Verma, P. Ray, and A. R. Ray, "Preparation of scaffolds from human hair proteins for tissue-engineering applications," *Biomedical materials*, vol. 3, no. 2, p. 025007, 2008.
- [7] L. R. Burnett, M. B. Rahmany, J. R. Richter, T.A Aboushwared, D. Eberli, C. L. Ward and M. E. Van Dyke, "Hemostatic properties and the role of cell receptor recognition in human hair keratin protein hydrogels," *Biomaterials*, vol. 34, no. 11, pp. 2632-2640, 2013.
- [8] S. Wang, F. Taraballi, L. P. Tan, and K. W. Ng, "Human keratin hydrogels support fibroblast attachment and proliferation in vitro," *Cell and tissue research*, vol. 347, no. 3, pp. 795-802, 2012.
- [9] S. Wang, Z. Wang, S.E.M. Foo, N.S. Tan, Y. Yuan, W. Lin, Z. Zhang, and K. W. Ng, "Culturing fibroblasts in 3D human hair keratin hydrogels," *ACS applied materials & interfaces*, vol. 7, no. 9, pp. 5187-5198, 2015.

-
- [10] W. T. Sow, Y. S. Lui, and K. W. Ng, "Electrospun human keratin matrices as templates for tissue regeneration," *Nanomedicine*, vol. 8, no. 4, pp. 531-541, 2013.
- [11] T. Fujii, D. Ogiwara, and M. Arimoto, "Convenient procedures for human hair protein films and properties of alkaline phosphatase incorporated in the film," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 1, pp. 89-93, 2004.
- [12] K. Yamauchi, A. Yamauchi, T. Kusunoki, A. Kohda, and Y. Konishi, "Preparation of stable aqueous solution of keratins, and physiochemical and biodegradational properties of films," *Journal of Biomedical Materials Research Part A*, vol. 31, no. 4, pp. 439-444, 1996.
- [13] Y. Feng, M. Borrelli, T. Meyer-ter-Vehn, S. Reichl, S. Schrader, and G. Geerling, "Epithelial wound healing on keratin film, amniotic membrane and polystyrene in vitro," *Current eye research*, vol. 39, no. 6, pp. 561-570, 2014.
- [14] S. Reichl, "Films based on human hair keratin as substrates for cell culture and tissue engineering," *Biomaterials*, vol. 30, no. 36, pp. 6854-6866, 2009.
- [15] S. Reichl, M. Borrelli, and G. Geerling, "Keratin films for ocular surface reconstruction," *Biomaterials*, vol. 32, no. 13, pp. 3375-3386, 2011.
- [16] M. Valko, C. Rhodes, J. Moncol, M. Izakovic, and M. Mazur, "Free radicals, metals and antioxidants in oxidative stress-induced cancer," *Chemico-biological interactions*, vol. 160, no. 1, pp. 1-40, 2006.
- [17] T. Fujii and Y. Ide, "Preparation of translucent and flexible human hair protein films and their properties," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 9, pp. 1433-1436, 2004.
- [18] T. Tanabe, N. Okitsu, and K. Yamauchi, "Fabrication and characterization of chemically crosslinked keratin films," *Materials Science and Engineering: C*, vol. 24, no. 3, pp. 441-446, 2004.
- [19] S. Reichl and C. C. Müller-Goymann, "Keratin film made of human hair as a nail plate model for studying drug permeation," *European*

- Journal of Pharmaceutics and Biopharmaceutics*, vol. 78, no. 3, pp. 432-440, 2011.
- [20] H. Lee, K. Noh, S.C. Lee, I.K. Kwon, D.W. Han, I.S. Lee and Y.S. Hwang, "Human hair keratin and its-based biomaterials for biomedical applications," *Tissue Engineering and Regenerative Medicine*, vol. 11, no. 4, pp. 255-265, 2014.
- [21] A. Gupta, "Human Hair "waste" and Its Utilization: Gaps and Possibilities," *Journal of Waste Management*, vol. 2014, p. 17, 2014, Art. no. 498018.
- [22] H. Zahn, "Progress report on hair keratin research," *International journal of cosmetic science*, vol. 24, no. 3, pp. 163-169, 2002.
- [23] M. Hatzfeld and W. W. Franke, "Pair formation and promiscuity of cytokeratins: formation in vitro of heterotypic complexes and intermediate-sized filaments by homologous and heterologous recombinations of purified polypeptides," *The Journal of Cell Biology*, vol. 101, no. 5, pp. 1826-1841, 1985.
- [24] T. A. Smith, S. V. Strelkov, P. Burkhard, U. Aebi, and D. A. Parry, "Sequence comparisons of intermediate filament chains: evidence of a unique functional/structural role for coiled-coil segment 1A and linker L1," *Journal of structural biology*, vol. 137, no. 1-2, pp. 128-145, 2002.
- [25] A. V. Sokolova, K. Laurent, W. Tatjana, M. Norbert, I. S. Dmitri, H. Harald, A. Ueli, and V. S. Sergei, "Monitoring intermediate filament assembly by small-angle x-ray scattering reveals the molecular architecture of assembly intermediates," *Proceedings of the National Academy of Sciences*, vol. 103, no. 44, pp. 16206-16211, 2006.
- [26] T. Hohmann and F. Dehghani, "The Cytoskeleton—A Complex Interacting Meshwork," *Cells*, vol. 8, no. 4, p. 362, 2019.
- [27] P. Steinert, L. Marekov, and D. Parry, "Diversity of intermediate filament structure. Evidence that the alignment of coiled-coil molecules in vimentin is different from that in keratin intermediate filaments," *Journal of Biological Chemistry*, vol. 268, no. 33, pp. 24916-24925, 1993.

-
- [28] S. Georgakopoulou, D. Möller, N. Sachs, H. Herrmann, and U. Aebi, "Near-UV circular dichroism reveals structural transitions of vimentin subunits during intermediate filament assembly," *Journal of molecular biology*, vol. 386, no. 2, pp. 544-553, 2009.
- [29] N. Mücke, "Wedig T, Burer A, Marekov LN, Steinert PM, Langowski J, Aebi U, Herrmann H," *Molecular and biophysical characterization of assembly-starter units of human vimentin. J Mol Biol*, vol. 340, pp. 97-114, 2004.
- [30] R. A. Kammerer, T. Schulthess, R. Landwehr, A. Lustig, J. Engel, U. Aebi and M.O. Steinmetz, "An autonomous folding unit mediates the assembly of two-stranded coiled coils," *Proceedings of the National Academy of Sciences*, vol. 95, no. 23, pp. 13419-13424, 1998.
- [31] M. O. Steinmetz, A. Stock, T. Schulthess, R. Landwehr, A. Lustig, I. Faix and R.A. Kammerer, "A distinct 14 residue site triggers coiled-coil formation in cortexillin I," *The EMBO Journal*, vol. 17, no. 7, pp. 1883-1891, 1998.
- [32] K. C. Wu, J. T. Bryan, M. I. Morasso, S. I. Jang, J. H. Lee, J. M. Yang and P. M. Steinert, "Coiled-coil trigger motifs in the 1B and 2B rod domain segments are required for the stability of keratin intermediate filaments," *Molecular biology of the cell*, vol. 11, no. 10, pp. 3539-3558, 2000.
- [33] H. Herrmann, L. Kreplak, and U. Aebi, "Isolation, characterization, and in vitro assembly of intermediate filaments," in *Methods in cell biology*, vol. 78: Elsevier, 2004, pp. 3-24.
- [34] S. Yamada, D. Wirtz, and P. A. Coulombe, "Pairwise assembly determines the intrinsic potential for self-organization and mechanical properties of keratin filaments," *Molecular biology of the cell*, vol. 13, no. 1, pp. 382-391, 2002.
- [35] H. Wang, D. A. Parry, L. N. Jones, W. W. Idler, L. N. Marekov, and P. M. Steinert, "In vitro assembly and structure of trichocyte keratin intermediate filaments: a novel role for stabilization by disulfide bonding," *The Journal of cell biology*, vol. 151, no. 7, pp. 1459-1468, 2000.

-
- [36] W. Crewther, R. Fraser, F. Lennox, and H. Lindley, "The chemistry of keratins," *Advances in protein chemistry*, vol. 20, pp. 191-346, 1965.
- [37] D. Istrate, C. Popescu, M. E. Rafik, and M. Möller, "The effect of pH on the thermal stability of fibrous hard alpha-keratins," *Polymer degradation and stability*, vol. 98, no. 2, pp. 542-549, 2013.
- [38] P. E. Bowden, S. Hainey, G. Parker, and M. B. Hodgins, "Sequence and expression of human hair keratin genes," *Journal of dermatological science*, vol. 7, pp. S152-S163, 1994.
- [39] D. A. Parry, T. A. Smith, M. A. Rogers, and J. Schweizer, "Human hair keratin-associated proteins: sequence regularities and structural implications," *Journal of structural biology*, vol. 155, no. 2, pp. 361-369, 2006.
- [40] L. R. Williams, F. M. Longo, H. C. Powell, G. Lundborg, and S. Varon, "Spatial-temporal progress of peripheral nerve regeneration within a silicone chamber: Parameters for a bioassay," *Journal of comparative neurology*, vol. 218, no. 4, pp. 460-470, 1983.
- [41] M. Borrelli, N. Joepen, S. Reichl, D. Finis, M. Schoppe, G. Geerling and S. Schrader, "Keratin films for ocular surface reconstruction: evaluation of biocompatibility in an in-vivo model," *Biomaterials*, vol. 42, pp. 112-120, 2015.
- [42] G. D. Rosson, E. H. Williams, and A. L. Dellon, "Motor nerve regeneration across a conduit," *Microsurgery*, vol. 29, no. 2, pp. 107-114, 2009.
- [43] A. Farole and B. T. Jamal, "A bioabsorbable collagen nerve cuff (NeuraGen) for repair of lingual and inferior alveolar nerve injuries: a case series," *Journal of Oral and Maxillofacial Surgery*, vol. 66, no. 10, pp. 2058-2062, 2008.
- [44] M. t. J. Bertleff, M. F. Meek, and J.-P. A. Nicolai, "A prospective clinical evaluation of biodegradable neurolac nerve guides for sensory nerve repair in the hand," *The Journal of hand surgery*, vol. 30, no. 3, pp. 513-518, 2005.
- [45] R. C. de Guzman, S. M. Tsuda, M.-T. N. Ton, X. Zhang, A. R. Esker, and M. E. Van Dyke, "Binding interactions of keratin-based hair fiber

- extract to gold, keratin, and BMP-2," *PloS one*, vol. 10, no. 8, p. e0137233, 2015.
- [46] H. Xie, S. Li, and S. Zhang, "Ionic liquids as novel solvents for the dissolution and blending of wool keratin fibers," *Green Chemistry*, vol. 7, no. 8, pp. 606-608, 2005.
- [47] S. Sharma, A. Gupta, S. M. S. T. Chik, K. C. Gek, P. K. Podde, J. Thraisingam and M. Subramaniam, "Extraction and characterization of keratin from chicken feather waste biomass: a study," in *Proceedings of the national conference for postgraduate research (NCON-PGR 2016)*, Universiti Malaysia Pahang (UMP), Pekan, 2016, pp. 693-699.
- [48] Â. S. Inácio, K. A. Mesquita, M. Baptista, J. Ramalho-Santos, W. L. Vaz, and O. I. V. Vieira, "In vitro surfactant structure-toxicity relationships: implications for surfactant use in sexually transmitted infection prophylaxis and contraception," *PLoS One*, vol. 6, no. 5, p. e19850, 2011.
- [49] A. Nakamura, M. Arimoto, K. Takeuchi, and T. Fujii, "A rapid extraction procedure of human hair proteins and identification of phosphorylated species," *Biological and Pharmaceutical Bulletin*, vol. 25, no. 5, pp. 569-572, 2002.
- [50] T. Fujii, S. Takayama, and Y. Ito, "A novel purification procedure for keratin-associated proteins and keratin from human hair," *Journal of biological macromolecules*, vol. 13, no. 3, 2013.
- [51] J. R. Richter, R. C. De Guzman, O. K. Greengauz-Roberts, and M. Van Dyke, "Structure-property relationships of meta-keratine biomaterials derived from human hair," *Acta biomaterialia*, vol. 8, no. 1, pp. 274-281, 2012.
- [52] H. Thomas, A. Conrads, K.-H. Phan, M. van de Löcht, and H. Zahn, "In vitro reconstitution of wool intermediate filaments," *International Journal of Biological Macromolecules*, vol. 8, no. 5, pp. 258-264, 1986.
- [53] P. Kakkar, B. Madhan, and G. Shanmugam, "Extraction and characterization of keratin from bovine hoof: A potential material for biomedical applications," *SpringerPlus*, vol. 3, no. 1, p. 596, 2014.

-
- [54] R. C. Marshall and J. Gillespie, "The keratin proteins of wool, horn and hoof from sheep," *Australian Journal of Biological Sciences*, vol. 30, no. 5, pp. 389-400, 1977.
- [55] M. Zoccola, A. Aluigi, and C. Tonin, "Characterisation of keratin biomass from butchery and wool industry wastes," *Journal of molecular structure*, vol. 938, no. 1, pp. 35-40, 2009.
- [56] T. Tanabe, N. Okitsu, A. Tachibana, and K. Yamauchi, "Preparation and characterization of keratin-chitosan composite film," *Biomaterials*, vol. 23, no. 3, pp. 817-825, 2002.
- [57] K. Yamauchi, M. Maniwa, and T. Mori, "Cultivation of fibroblast cells on keratin-coated substrata," *Journal of Biomaterials Science, Polymer Edition*, vol. 9, no. 3, pp. 259-270, 1998.
- [58] M. Zoccola, A. Aluigi, C. Vineis, C. Tonin, F. Ferrero, and M. G. Piacentino, "Study on cast membranes and electrospun nanofibers made from keratin/fibroin blends," *Biomacromolecules*, vol. 9, no. 10, pp. 2819-2825, 2008.
- [59] P. Hartianti, L. Ling, L. M. M. Goh, K. S. A. Ow, R. M. Samsonraj, W. T. Sow and K. W. Ng, "Modulating mesenchymal stem cell behavior using human hair keratin-coated surfaces," *Stem cells international*, vol. 2015, 2015.
- [60] P. Sierpinski, J. Garrett, J. Ma, P. Apel, D. Klorig, T. L. Smith and M. Van Dyke, "The use of keratin biomaterials derived from human hair for the promotion of rapid regeneration of peripheral nerves," *Biomaterials*, vol. 29, no. 1, pp. 118-128, 2008.
- [61] P. J. Apel, J. P. Garrett, P. Sierpinski, J. Ma, A. Atala, T. L. Smith and M. Van Dyke, "Peripheral nerve regeneration using a keratin-based scaffold: long-term functional and histological outcomes in a mouse model," *The Journal of hand surgery*, vol. 33, no. 9, pp. 1541-1547, 2008.
- [62] N. Bhardwaj, W. T. Sow, D. Devi, K. W. Ng, B. B. Mandal, and N.-J. Cho, "Silk fibroin-keratin based 3D scaffolds as a dermal substitute for skin tissue engineering," *Integrative Biology*, vol. 7, no. 1, pp. 53-63, 2014.

-
- [63] K. Lee and W. Ha, "DSC studies on bound water in silk fibroin/S-carboxymethyl keratine blend films," *Polymer*, vol. 40, no. 14, pp. 4131-4134, 1999.
- [64] C. Tonin, A. Aluigi, C. Vineis, A. Varesano, A. Montarsolo, and F. Ferrero, "Thermal and structural characterization of poly (ethylene-oxide)/keratin blend films," *Journal of Thermal Analysis and Calorimetry*, vol. 89, no. 2, pp. 601-608, 2007.
- [65] I. A. Aguayo-Villarreal, A. Bonilla-Petriciolet, V. Hernández-Montoya, M. A. Montes-Morán, and H. E. Reynel-Avila, "Batch and column studies of Zn ²⁺ removal from aqueous solution using chicken feathers as sorbents," *Chemical Engineering Journal*, vol. 167, no. 1, pp. 67-76, 2011.
- [66] P. Sun, Z.-T. Liu, and Z.-W. Liu, "Chemically modified chicken feather as sorbent for removing toxic chromium (VI) ions," *Industrial & Engineering Chemistry Research*, vol. 48, no. 14, pp. 6882-6889, 2009.
- [67] X. C. Yin, F. Y. Li, Y. F. He, Y. Wang, and R. M. Wang, "Study on effective extraction of chicken feather keratins and their films for controlling drug release," *Biomaterials Science*, vol. 1, no. 5, pp. 528-536, 2013.
- [68] G. R. P. Moore, S. M. Martelli, C. Gandolfo, P. J. do Amaral Sobral, and J. B. Laurindo, "Influence of the glycerol concentration on some physical properties of feather keratin films," *Food Hydrocolloids*, vol. 20, no. 7, pp. 975-982, 2006.
- [69] H. Mohammadifarda, A. Arpanaeia, M. Vosoughib, M. Kabirsalmania, and M. Shakibaeia, "Alginate-silica nanocomposite beads for the 3D cell culture: preparation and study of the mechanical stability and cell proliferation and viability," in *Proceedings of the 4th International Conference on Nanostructures*, 2012, vol. 1, pp. 843-845.
- [70] C. Oehr, "Plasma surface modification of polymers for biomedical use," *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, vol. 208, pp. 40-47, 2003.

-
- [71] J. M. Goddard and J. Hotchkiss, "Polymer surface modification for the attachment of bioactive compounds," *Progress in polymer science*, vol. 32, no. 7, pp. 698-725, 2007.
- [72] Y. Arima and H. Iwata, "Effect of wettability and surface functional groups on protein adsorption and cell adhesion using well-defined mixed self-assembled monolayers," *Biomaterials*, vol. 28, no. 20, pp. 3074-3082, 2007.
- [73] Y. Sasai, Y. Tanaka, S.-i. Kondo, Y. Yamauchi, and M. Kuzuya, "Plasma-based Surface Functionalization of Polystyrene Substrate for Cell Culture Application."
- [74] Y. Sasai, N. Matsuzaki, S.-i. Kondo, Y. Yamanashi, and M. Kuzuya, "Surface engineering of polystyrene dish for improvement of cell adhesion using plasma techniques," *Journal of Photopolymer Science and Technology*, vol. 21, no. 2, pp. 277-280, 2008.
- [75] J. L. Bohnert, B. C. Fowler, T. A. Horbett, and A. S. Hoffman, "Plasma gas discharge deposited fluorocarbon polymers exhibit reduced elutability of adsorbed albumin and fibrinogen," *Journal of Biomaterials Science, Polymer Edition*, vol. 1, no. 4, pp. 279-297, 1989.
- [76] A. Kondyurin and M. Bilek, *Ion beam treatment of polymers: application aspects from medicine to space*. Newnes, 2014.
- [77] M. M. Bilek and D. R. McKenzie, "Plasma modified surfaces for covalent immobilization of functional biomolecules in the absence of chemical linkers: towards better biosensors and a new generation of medical implants," *Biophysical reviews*, vol. 2, no. 2, pp. 55-65, 2010.
- [78] Y. Yin, M. M. Bilek, and D. R. McKenzie, "Direct Evidence of Covalent Immobilisation of Microperoxidase-11 on Plasma Polymer Surfaces," *Plasma Processes and Polymers*, vol. 7, no. 8, pp. 708-714, 2010.
- [79] Y. Yin, D. Bax, D. R. McKenzie, and M. M. Bilek, "Protein immobilization capacity and covalent binding coverage of pulsed plasma polymer surfaces," *Applied surface science*, vol. 256, no. 16, pp. 4984-4989, 2010.

-
- [80] H. Atri, E. Bidram, and D. E. Dunstan, "Reconstituted Keratin Biomaterial with Enhanced Ductility," *Materials*, vol. 8, no. 11, pp. 7472-7485, 2015.
- [81] Y. Srisuwan, A. Thonpho, and P. Srihanam, "Effect of starch on property of silk fibroin/keratin blend films," *International Journal*, vol. 11, no. 28, pp. 2870-2873, 2016.
- [82] M. Macdonald, N. M. Rodriguez, R. Smith, and P. T. Hammond, "Release of a model protein from biodegradable self assembled films for surface delivery applications," *Journal of Controlled Release*, vol. 131, no. 3, pp. 228-234, 2008.
- [83] M. L. Bruschi, *Strategies to modify the drug release from pharmaceutical systems*. Woodhead Publishing, 2015.
- [84] R. MAKAREM and M. J. HUMPHRIES, "LDV: a novel cell adhesion motif recognized by the integrin $\alpha 4\beta 1$," ed: Portland Press Limited, 1991.
- [85] R. Makarem, P. Newham, J. A. Askari, L. J. Green, J. Clements, M. Edwards and A. P. Mould, "Competitive binding of vascular cell adhesion molecule-1 and the HepII/IIICS domain of fibronectin to the integrin alpha 4 beta 1," *Journal of Biological Chemistry*, vol. 269, no. 6, pp. 4005-4011, 1994.
- [86] J. R. Richter, R. C. de Guzman, and M. E. Van Dyke, "Mechanisms of hepatocyte attachment to keratin biomaterials," *Biomaterials*, vol. 32, no. 30, pp. 7555-7561, 2011.
- [87] J. H. Kim, H. S. Kim, J. H. Lee, S. W. Choi, Y. J. Cho, and J. H. Kim, "Hexagonally close packed langmuir-blodgett films from monodispersed silica nanoparticles," *Journal of nanoscience and nanotechnology*, vol. 9, no. 12, pp. 7007-7011, 2009.
- [88] H. P. Erickson, "Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy," *Biological procedures online*, vol. 11, no. 1, p. 32, 2009.
- [89] K. Webb, V. Hlady, and P. A. Tresco, "Relative importance of surface wettability and charged functional groups on NIH 3T3 fibroblast attachment, spreading, and cytoskeletal organization," *Journal of biomedical materials research*, vol. 41, no. 3, p. 422, 1998.

-
- [90] S. Werner, T. Krieg, and H. Smola, "Keratinocyte–fibroblast interactions in wound healing," *Journal of Investigative Dermatology*, vol. 127, no. 5, pp. 998-1008, 2007.
- [91] P. C. Maisonpierre, C. Suri, P. F. Jones, S. Bartunkova, S. J. Wiegand, C. Radziejewski and T. J. Daly, "Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis," *Science*, vol. 277, no. 5322, pp. 55-60, 1997.
- [92] M. Valluru, C. A. Staton, M. W. R. Reed, and N. J. Brown, "Transforming growth factor- β and endoglin signaling orchestrate wound healing," *Frontiers in physiology*, vol. 2, p. 89, 2011.
- [93] A. Toksoy, V. Müller, R. Gillitzer, and M. Goebeler, "Biphasic expression of stromal cell-derived factor-1 during human wound healing," *British Journal of Dermatology*, vol. 157, no. 6, pp. 1148-1154, 2007.
- [94] W. Z. Mostafa and R. A. Hegazy, "Vitamin D and the skin: Focus on a complex relationship: A review," *Journal of advanced research*, vol. 6, no. 6, pp. 793-804, 2015.
- [95] C. Hunter, J. Bond, P. C. Kuo, M. A. Selim, and H. Levinson, "The role of osteopontin and osteopontin aptamer (OPN-R3) in fibroblast activity," *Journal of Surgical Research*, vol. 176, no. 1, pp. 348-358, 2012.
- [96] Y. Ishida, T. Kondo, T. Takayasu, Y. Iwakura, and N. Mukaida, "The essential involvement of cross-talk between IFN- γ and TGF- β in the skin wound-healing process," *The Journal of Immunology*, vol. 172, no. 3, pp. 1848-1855, 2004.
- [97] R. Gillitzer and M. Goebeler, "Chemokines in cutaneous wound healing," *Journal of leukocyte biology*, vol. 69, no. 4, pp. 513-521, 2001.
- [98] S. W. Spiekstra, M. Breetveld, T. Rustemeyer, R. J. Scheper, and S. Gibbs, "Wound-healing factors secreted by epidermal keratinocytes and dermal fibroblasts in skin substitutes," *Wound repair and regeneration*, vol. 15, no. 5, pp. 708-717, 2007.
- [99] S. Werner, K. G. Peters, M. T. Longaker, F. Fuller-Pace, M. J. Banda, and L. T. Williams, "Large induction of keratinocyte growth factor

- expression in the dermis during wound healing," *Proceedings of the National Academy of Sciences*, vol. 89, no. 15, pp. 6896-6900, 1992.
- [100] A. Komi-Kuramochi, M. Kawano, Y. Oda, M. Asada, M. Suzuki, J. Oki and T. Imamura, "Expression of fibroblast growth factors and their receptors during full-thickness skin wound healing in young and aged mice," *Journal of Endocrinology*, vol. 186, no. 2, pp. 273-289, 2005.
- [101] E. Huet, B. Vallée, D. Szul, F. Verrecchia, S. Mourah, J. V. Jester and E. E. Gabison, "Extracellular matrix metalloproteinase inducer/CD147 promotes myofibroblast differentiation by inducing α -smooth muscle actin expression and collagen gel contraction: implications in tissue remodeling," *The FASEB Journal*, vol. 22, no. 4, pp. 1144-1154, 2008.
- [102] M. Streit, P. Velasco, L. Riccardi, L. Spencer, L. F. Brown, L. Janes and M. Detmar, "Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice," *The EMBO journal*, vol. 19, no. 13, pp. 3272-3282, 2000.
- [103] A. Hirao, I. Hamaguchi, T. Suda, and N. Yamaguchi, "Translocation of the Csk homologous kinase (Chk/Hyl) controls activity of CD36-anchored Lyn tyrosine kinase in thrombin-stimulated platelets," *The EMBO Journal*, vol. 16, no. 9, pp. 2342-2351, 1997.
- [104] R.-P. Czekay, C. Wilkins-Port, S. P. Higgins, J. Freytag, J. M. Overstreet, R. M. Klein and P. J. Higgins, "PAI-1: an integrator of cell signaling and migration," *International journal of cell biology*, vol. 2011, 2011.
- [105] E. Monaghan, V. Gueorguiev, C. Wilkins-Port, and P. J. McKeown-Longo, "The receptor for urokinase-type plasminogen activator regulates fibronectin matrix assembly in human skin fibroblasts," *Journal of Biological Chemistry*, vol. 279, no. 2, pp. 1400-1407, 2004.
- [106] R. Samarakoon, J. M. Overstreet, and P. J. Higgins, "TGF- β signaling in tissue fibrosis: redox controls, target genes and therapeutic opportunities," *Cellular signalling*, vol. 25, no. 1, pp. 264-268, 2013.
- [107] B. Y. Tan, L. T. Nguyen, H. S. Kim, J. H. Kim, and K. W. Ng, "Cultivation of Human Dermal Fibroblasts and Epidermal

- Keratinocytes on Keratin-Coated Silica Bead Substrates," *Journal of Biomedical Materials Research Part A*, 2017.
- [108] F. Zen, M. D. Angione, J. A. Behan, R. J. Cullen, T. Duff, J. M. Vasconcelos and P. E. Colavita, "Modulation of protein fouling and interfacial properties at carbon surfaces via immobilization of glycans using aryldiazonium chemistry," *Scientific reports*, vol. 6, p. srep24840, 2016.
- [109] S. Magonov, V. Elings, and M.-H. Whangbo, "Phase imaging and stiffness in tapping-mode atomic force microscopy," *Surface science*, vol. 375, no. 2-3, pp. L385-L391, 1997.
- [110] H. W. Harding and G. E. Rogers, "I ϵ -(γ -Glutamyl) lysine cross-linkage in citrulline-containing protein fractions from hair," *Biochemistry*, vol. 10, no. 4, pp. 624-630, 1971.
- [111] R. Matsunaga, R. Abe, D. Ishii, S. I. Watanabe, M. Kiyoshi, B. Nöcker and K. Tsumoto, "Bidirectional binding property of high glycine-tyrosine keratin-associated protein contributes to the mechanical strength and shape of hair," *Journal of structural biology*, vol. 183, no. 3, pp. 484-494, 2013.
- [112] M. A. Rogers, L. Langbein, S. Praetzel-Wunder, H. Winter, and J. Schweizer, "Human hair keratin-associated proteins (KAPs)," *International review of cytology*, vol. 251, pp. 209-263, 2006.
- [113] C. A. Royer, "Probing protein folding and conformational transitions with fluorescence," *Chemical reviews*, vol. 106, no. 5, pp. 1769-1784, 2006.
- [114] A. B. Ghisaidoobe and S. J. Chung, "Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Förster resonance energy transfer techniques," *International journal of molecular sciences*, vol. 15, no. 12, pp. 22518-22538, 2014.
- [115] J. Leppiniemi, T. Grönroosm J. A. Määttä, M. S. Johnson, M. S. Kulomaa, V. P. Hytönen and T. T. Airenne, "Structure of Bradavidin-C-Terminal Residues Act as Intrinsic Ligands," *PloS one*, vol. 7, no. 5, p. e35962, 2012.
- [116] N. J. Greenfield, "Using circular dichroism spectra to estimate protein secondary structure," *Nature protocols*, vol. 1, no. 6, p. 2876, 2006.

- [117] S. C. Kwok and R. S. Hodges, "Stabilizing and destabilizing clusters in the hydrophobic core of long two-stranded α -helical coiled-coils," *Journal of Biological Chemistry*, vol. 279, no. 20, pp. 21576-21588, 2004.
- [118] S. Lau, A. Taneja, and R. Hodges, "Synthesis of a model protein of defined secondary and quaternary structure. Effect of chain length on the stabilization and formation of two-stranded alpha-helical coiled-coils," *Journal of Biological Chemistry*, vol. 259, no. 21, pp. 13253-13261, 1984.
- [119] M. C. Manning and R. W. Woody, "Theoretical CD studies of polypeptide helices: examination of important electronic and geometric factors," *Biopolymers: Original Research on Biomolecules*, vol. 31, no. 5, pp. 569-586, 1991.
- [120] G. L. Millhauser, "Views of helical peptides: a proposal for the position of 310-helix along the thermodynamic folding pathway," *Biochemistry*, vol. 34, no. 12, pp. 3873-3877, 1995.
- [121] A. Zhmurov, O. Kononova, R. I. Litvinov, R. I. Dima, V. Barsegov, and J. W. Weisel, "Mechanical Transition from α -Helical Coiled-Coils to β -Sheets in Fibrin (ogen)," *Journal of the American Chemical Society*, vol. 134, no. 50, p. 20396, 2012.
- [122] S. H. Hiew and A. Miserez, "Squid sucker ring teeth: multiscale structure-property relationships, sequencing, and protein engineering of a thermoplastic biopolymer," *ACS Biomaterials Science & Engineering*, vol. 3, no. 5, pp. 680-693, 2016.
- [123] Y. Tamada, "New process to form a silk fibroin porous 3-D structure," *Biomacromolecules*, vol. 6, no. 6, pp. 3100-3106, 2005.
- [124] M. Spei and R. Holzem, "Thermoanalytical determination of the relative helix content of keratins," *Colloid & Polymer Science*, vol. 267, no. 6, pp. 549-551, 1989.
- [125] T. Tanabe, N. Okitsu, A. Tachibana, and K. Yamauchi, "Preparation and characterization of keratin–chitosan composite film," *Biomaterials*, vol. 23, no. 3, pp. 817-825, 2002.
- [126] D. S. Fudge and J. M. Gosline, "Molecular design of the α -keratin composite: Insights from a matrix–free model, hagfish slime threads,"

- Proceedings of the Royal Society of London. Series B: Biological Sciences*, vol. 271, no. 1536, pp. 291-299, 2004.
- [127] F. Xu and T. Lu, "Skin Mechanical Behaviour," in *Introduction to Skin Biothermomechanics and Thermal Pain*: Springer, 2011, pp. 87-104.
- [128] D. S. Fudge, K. H. Gardner, V. T. Forsyth, C. Riekel, and J. M. Gosline, "The mechanical properties of hydrated intermediate filaments: insights from hagfish slime threads," *Biophysical journal*, vol. 85, no. 3, pp. 2015-2027, 2003.
- [129] G. Kang and X. Wu, "Ratchetting of porcine skin under uniaxial cyclic loading," *Journal of the mechanical behavior of biomedical materials*, vol. 4, no. 3, pp. 498-506, 2011.
- [130] G. Jemec and J. Serup, "Epidermal hydration and skin mechanics. The relationship between electrical capacitance and the mechanical properties of human skin in vivo," *Acta dermato-venereologica*, vol. 70, no. 3, pp. 245-247, 1990.
- [131] K. Bush, P. Driscoll, E. Soto, C. Lambert, W. McGimpsey, and G. Pins, "Designing tailored biomaterial surfaces to direct keratinocyte morphology, attachment, and differentiation," *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, vol. 90, no. 4, pp. 999-1009, 2009.
- [132] J. C. Chamcheu, T. Roy, M. B. Uddin, S. Banang-Mbeumi, R. C. N. Chamcheu, A. L. Walker and S. Huang, "Role and Therapeutic Targeting of the PI3K/Akt/mTOR Signaling Pathway in Skin Cancer: A Review of Current Status and Future Trends on Natural and Synthetic Agents Therapy," *Cells*, vol. 8, no. 8, p. 803, 2019.
- [133] M. J. Humphries, S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada, "Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion," *The Journal of cell biology*, vol. 103, no. 6, pp. 2637-2647, 1986.
- [134] J. D. Chen, J.-C. Lapiere, D. N. Sauder, C. Peavey, and D. T. Woodley, "Interleukin-1 α stimulates keratinocyte migration through an

epidermal growth factor/transforming growth factor- α -independent pathway," *Journal of investigative dermatology*, vol. 104, no. 5, pp. 729-733, 1995.