Understanding the biochemical properties of human hair keratins : self-assembly potential and cell response

Lai, Hui Ying

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**Understanding the Biochemical Properties of Human Hair Keratins:
Self-assembly Potential and Cell Response**

LAI HUI YING

**Interdisciplinary Graduate School
Nanyang Environment and Water Research Institute @ NTU**

2020

**Understanding the Biochemical Properties of Human Hair Keratins:
Self-assembly Potential and Cell Response**

LAI HUI YING

INTERDISCIPLINARY GRADUATE SCHOOL

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

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LAI HUI YING

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ASSOC PROF NG KEE WOEI

Authorship Attribution Statement

Chapter 4 is patented as Ng Kee Woei, Lai Hui Ying. (2020). Self-Assembly of Solubilized Human Hair Proteins into Intermediate Filament Networks (SG Patent Application No. 10202003536S). Singapore Patent.

The contributions of the co-inventors are as follows:

- A/Prof Ng Kee Woei provided the initial project direction and finalized the patent drafts.
- I conducted the experimental work and prepared the patent drafts.

Chapter 4 is published as Lai, H. Y., M. I. Setyawati, A. R. Ferhan, K. Divakarla, Chua, H.M., W. Chrzanowski, N. J. Cho, and Ng, K. W. (2020). Self-assembly of Solubilized Human Hair Proteins into Intermediate Filament Networks. *ACS Biomaterials Science & Engineering*. DOI: 10.1021/acsbiomaterials.0c01507.

The contributions of the co-inventors are as follows:

- I conducted the experimental design, experimental work, data analysis and manuscript draft writing.
- Dr. Magdiel Ingrid Setyawati provided constructive advices on the manuscript design and proofread the manuscript draft.
- Dr. Abdul Rahim Ferhan provided technical support and consultation on LSPR related experimental work.
- Ms. K. Divakarla conducted data collection on high-resolution AFM imaging.
- Ms. Chua Huei Min assisted on TEM imaging.
- A/Prof Wojtek Chrzanowski provided the support on high-resolution AFM imaging and data processing.
- Prof Cho Nam-Joon provided the access and supply of LSPR system, sensor chips and consumable to facilitate the LSPR experimental work.
- A/Prof Ng Kee Woei provided the initial project direction and finalized the manuscript drafts.

Chapter 5 is under preparation for manuscript submissions. Lai, H. Y., Nguyen, L. T., Adav S. S., Chua, H. M., Loke, J. J., Miserez, A., Schmidtchen, A., Ng, K. W. (2021). Top-down Approach: Purification of Enriched Human Hair Keratins for Behavior Study.

The contributions of the co-inventors are as follows:

- I conducted the experimental design, experimental work, data analysis and manuscript draft writing.
- Dr. Nguyen TH Luong provided constructive advices on the experimental design.
- Dr. Advac Sunil Shankar provided support on sample preparation for MALDI-ToF protein identification and data analysis.
- Ms. Chua Huei Min assisted on sample preparation for TEM imaging.
- Mr. Loke Jun Jie provided technical support and constructive advices on the semi-preparative FPLC experiment.
- A/Prof Miserez Ali provided access on the semi-preparative FPLC instrument.
- Prof Schmidtchen Artur provided access on the HPLC instrument.
- A/Prof Ng Kee Woei provided the initial project direction and finalized the manuscript drafts.

Chapter 6 is under preparation for manuscript submissions. Lai, H. Y., Setyawati, M. I., Vizetto-Duarte C., Chua, H. M., Low, C. T., Ng, K. W. (2021) Dissection of human hair extracts' antioxidant capacity: ROS scavengers for *in vitro* application.

The contributions of the co-inventors are as follows:

- I conducted the experimental design, experimental work, data analysis and manuscript draft writing.
- Dr. Magdiel Ingrid Setyawati provided constructive advices on qPCR experiment, manuscript design and proofread the manuscript draft.
- Dr. Caterina Vizetto-Duarte provided constructive advices on antioxidant experiment.
- Ms. Chua Huei Min assisted on sample preparation and thiol quantification assays.
- Ms. Low Choon Teck provided support on assay preparation.

- A/Prof Ng Kee Woei provided the initial project direction and finalized the manuscript drafts.

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LAI HUI YING

Abstract

Keratin is a class of cysteine rich intermediate filament (IF) proteins, existing in abundance and readily available in bio-wastes such as human hair. A total of 17 keratin types are present in human hair, which can be further classified as Type I and II keratin subtypes. They associate in 1:1 ratio to form strongly bonded heterodimers and further assemble into microfibrils (7 – 10 nm). A mature hair fiber is formed through a highly regulated morphogenesis process. In recent decades, keratin as a novel natural biomaterial has shown excellent bioactivity, biocompatibility, and angiogenic properties in a wide range of biomedical applications. Much work has been done on extracting and understanding the profile of keratins from hair since the 20th century. Much efforts were also made to elucidate their detailed structure and to understand their molecular assembly kinetics. Although the conditions for assembly of soluble IF proteins into characteristic 10 nm wide filaments vary, no co-factors are required, this thereby makes biochemical studies of IF practical. However, the chemistry and biology of hair keratin subtypes expression and their potential interaction mechanisms are still yet to be understood. In fact, among all the past studies, limited effort has been invested in understanding the molecular self-assembly of crude hair keratin and no study has been performed to fractionate hair keratins, which would be a critical step to unravel the current knowledge gaps of interaction and function of these proteins.

This Ph.D. project, therefore, aims to understand and evaluate the self-assembling potential of the hair keratin extracts and further perform separation of the different keratin subtypes within the extracts for behavior study. It is hypothesized that the enriched specific hair keratin subtypes would present unique cell-material behaviors and characteristics. In order to validate this hypothesis, a number of objectives were identified, and the scope of the experiments was formulated. Specifically, the self-assembly potential of hair keratins was evaluated and cellular response to the crude and purified keratins solution were explored. The antioxidant properties of total hair proteins, keratins proteins and keratin associated proteins were compared. Potential applications of such materials could range from biomedical to water remediation.

The protocol to reconstruct self-assembled intermediate filaments from crude keratin extracts was established via step-down dialysis in acidic buffer conditions. Continuous self-assembled fibers were achieved, which demonstrated average diameters ranging from 6 to 10 nm when assembled in buffer below pH 3.3. Furthermore, coating casted with the assembled protein fibers were able to remain stable up to five days in fully supplemented culture media and were cell compatible. Surface morphology and integrity of the assembled coating were characterized using Atomic Force Microscopy (AFM), optical microscope and immunohistochemistry.

As type I and II keratin subtypes are strongly bound as dimers and have similar molecular weights and isoelectric points (pI), it is extremely challenging to isolate the individual subtypes. Among the different chromatography approaches evaluated Gel Permeation Chromatography (GPC), Asymmetrical Field Flow Fractionation (AFFF) and High-Performance Liquid chromatography (HPLC), HPLC utilizing a weak anion-exchange (WAX) column revealed six distinct peaks detected by the UV detector, indicating the highest possibility in fractioning the keratin extracts based on pI differences. A two-step salt elution method was ascertained to achieve type II enriched fractions.

Lastly, antioxidant properties of the purified fractions were found to be retained and comparable to the crude keratin extracts. Interestingly, the crude keratin extracts showed similar DPPH scavenging ability (IC_{50}) compared to KAPs, although the latter possesses greater cysteine content. Additionally, within the same concentration range, KAPs induced cell toxicity in the *in vitro* study as it formed precipitates. Keratins exhibited antioxidant properties as a media supplement, which was comparable to the well-established antioxidant compound, Acetylcysteine (NAC) at 1 mM. Human dermal fibroblasts (HDFs) were protected from acute hydrogen peroxide-induced oxidative stress and showed increased proliferation rate in the presence of 40 μ M keratin.

In conclusion, the above findings provide new perspectives into the self-assembly and antioxidant ability of crude and purified human hair keratins, which can be further exploited as an advanced biomaterial across a wide discipline of applications.

Lay Summary

Conventionally, human hair is primarily perceived as an aesthetic feature, as compared to other parts of body, and have been heavily targeted by the cosmetics and fashion industries. Hair fiber diameter ranges between 17 to 181 μm , and it is common to shed up to 100 hair fibers daily. Some urban countries with high population encounter the dilemma of disposing huge amount of hair waste, which causes blockage of drainage systems, disuse of large space volume, and various downstream waste processing issues. However, with proper treatment and handling, these protein-rich hair fibers can be recycled and utilized in various applications including agriculture, composite materials, water purification, cosmetics, and even in pharmaceuticals and biomedical usage. Each human hair fiber contains 60% by weight of the protein keratin, which can be extracted and solubilized chemically by breaking the strong interlaced networks. These keratin proteins can be subsequently fabricated into different forms, such as sponges, thin films, hydrogel, fibers, etc. These have demonstrated competent efficiency in wound healing applications, among others. Although only few keratin-derived products have been successfully translated into clinical trials and subsequently commercialized, the potential of human hair keratins in the biomedical field is still in its infancy stage and therefore more understanding on keratin's properties and mechanisms of interactions should be developed and advanced. The formation of each hair fiber is highly conserved and organized at which the smallest protein fragments are bundled in defined configurations to form a stable and mature fiber. To further appreciate such highly intricate yet precise phenomena, this project aims to study and separate the crude keratin mixture into different fractions. It was revealed foremost that self-assembly of keratin proteins into nanofilament network can be induced in acidic conditions, and they can be cast into coatings that possess good stability in physiological environment for up to 5 days. In addition, partial isolation of the keratin mixture was achieved, and the fractions retained antioxidant properties comparable to the crude keratin mixture. These novel findings provide new insights into the mechanisms of keratin interaction and unlock more potential uses of this asset in human hair fibers.

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Table Captions

Table 2. 1 Summary of recent applications of keratin-derived materials, sources of the keratin material, the respective extraction method, and unique properties.

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Table 3. 1 Compilation of buffer composition and pH conditions used during the step-down dialysis in this study. The stability of keratins solution when added to 2.5 mM and 20 mM KCl were described either as Soluble, Precipitated or not available “-”.

Table 4. 1 Compilation of the acidic buffer composition, pH condition and pH changes during the step-down dialysis in this study. The stability of the dialyzing and assembled keratins solution was described either as Soluble (S), Precipitated (P) or Not Available “N/A”.

Table 5. 1 The yield percentage of the fractions obtained via an A) analytical and B) semi preparative WAX column were compiled and compared.

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Figure Captions

Schematic 1 Structure of a hair fiber.

Figure 2. 1 Schematic of a growing hair fibre showing the different zones in its follicle. Reproduced from permission from Robbins et al. with permission [5]. Copyright © 1988, Springer Science Business Media New York.

Figure 2. 2 Schematic of a wool fiber drawn by Bruce Fraser and Tom MacRae. Reproduced from R. D. B. FRASER et al. with permission [14]. Copyright (c) 2012-2020 CSIRO Australia.

Figure 2. 3 Separation methods in a typical isolation and purification. Reproduced from Takács et al with permission [69]. Copyright © 2000 John Wiley & Sons, Ltd.

Figure 2. 4 Cross section view of a flow FFF channel. Reproduced from J. Calvin Giddings et al. with permission [73]. Copyright © 1977 Published by Elsevier Inc.

Figure 2. 5 SDS-gel electrophoresis of A3B4 and A5B3 CNBr fragments from Glycinin subunits sample. Reproduced from Staswick et al. with permission [78]. Copyright © 1984, American Soc For Biochemistry & Molecular Biology.

Figure 2. 6 (b) Coomassie Blue-stained gel and immunoblot analysis of the fractions using (c) AE3 antibody and (d) AE 1 antibody. Reproduced from R Eichner et al. with permission [81]. Copyright, Rockefeller University Press.

Figure 2. 7 TEM images showing human recombinant K8/K18 assembled in a) 25 mM Tris-HCl, 50 mM NaCl (pH 7.5) and b) 10 mM Tris-HCl (pH 7.5). While K5/K14 were c) assembled and d) dialyzed in 10 mM Tris-HCl (pH 7.5). Scale bar: 100 nm. Reproduced from Harald Herrmann et al. with permission [103]. Copyright © 2002 Elsevier Science (USA).

Figure 2. 8 TEM images of human hair recombinant keratin proteins A) K31, B) K81, and C-D) K31/K81 dialyzed in 10 mM Na₂HPO₄, 75 mM NaCl, 5 mM DTT (pH 8). Reproduced from Tijana Z et al. with permission [102]. © 2017 Wiley Periodicals, Inc.

Figure 2. 9 Schematic diagram showing assembly of keratins from heterodimeric tetramers into heterogenous full-width filaments by lateral and nearly concomitant longitudinal assembly. Reproduced from Harald Herrmann et al. with permission [96]. Copyright © 2004 by Annual Reviews.

Figure 3. 1 Flow diagram of the human hair keratin proteins extractions framework and the involved downstream applications (boarded in red).

Figure 3. 2 Flow diagram of the self-assembled human hair keratin network on a 24 well plates format for cell viability study.

Figure 3. 3 Schematic diagram of the staining procedures for TEM imaging.

Figure 3. 4 Schematic figure of the immunohistochemical staining reaction. Reproduced from Kim, S. W. et al. with permission [11]. Copyright © 2016 The Korean Society of Pathologists/The Korean Society for Cytopathology.

Figure 3. 5 Schematic diagram of the working principle of localized surface plasmon resonance (LSPR) technique in stimulating the proteins deposition mechanism. Reproduced from Jackman, J. A. et al. with permission [24].

Figure 3. 6 CD spectra of polypeptides and proteins with respective intrinsic secondary structures. Poly-L-lysine in its (1, black) α -helical at pH 11.1, (2, red) antiparallel β -sheet conformations, and (3, green) extended conformations at pH 5.7 [28]. Placental collagen in its (4, blue) native triple-helical and (5, cyan) denatured forms [27, 29]. Reproduced from Greenfield, N. J. et al. with permission [27]. Copyright © 2007, Springer Nature.

Figure 3. 7 Separation principle in Asymmetrical Field Flow Fractionation. Reproduced from Müller, D. et al. with permission [34]. Open access, © 2015 Müller, Cattaneo, Meier, Welz and de Mello.

Figure 3. 8 Illustration of the separation principle underlying gel permeation chromatography (GPC). Reproduced from Dyamenahalli, K. et al. with permission [37]. Copyright © 2015 Elsevier Ltd.

Figure 3. 9 Diagram of Reverse-phase chromatography separation (Left) and Anion Ion exchange chromatography (Right). Reproduced from Salvato, F. et al. with permission [40]. Open access, IntechOpen.

Figure 3. 10 Schematic diagram of gel electrophoresis, transfer, incubation, and detection. Reproduced from Trevor Henderson et al. with permission [44]. Materials obtained from Servier Medical Art and Noun Project.

Figure 3. 11 Reaction mechanism of 5,5'-dithio-bis-(2-nitrobenzoic acid (DTNB) with sulfhydryl groups. Reproduced from Gromer, S. et al. with permission [45]. Copyright © 2002, Academic Press.

Figure 3. 12 Reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. Reproduced from Liang, N. et al. with permission [50]. Open access, MDPI.

Figure 3. 13 Proposed mechanism of DCFH-DA reagent adapted from Bass et al. [53]. DCFH-DA is deesterified to DCFH, which is oxidized to fluorescent DCF by reactive oxygen species, after entering the cells. The similar deesterification can also be achieved via chemical activation for acellular testing purpose [52]. Reproduced from Bass, D. A. et al. with permission [53]. Copyright © 1983, American Association of Immunologists.

Figure 4. 1 Representative TEM images of SA-keratin at neutral to basic buffer condition, A) Low Tris buffer (LT) consists of 2 mM Tris HCl, 1m M DTT, pH 9.0; B) Elongation Buffer (EB) consists of 10 mM Tris-HCl, pH 7.3 and C) 0.7 mM sodium phosphate buffer consist of 1 mM DTT.

Figure 4. 2: A) Schematic of the conformation changes of the human hair keratin proteins from irregular beads like mesh into regular and elongated self-assembled nano filaments when the pH is lowered. B) Representative TEM images of SA-keratin at different pH and concentration of KCl salt. All pH stated above were prepared with Citric acid buffer except pH 7.5, which was prepared in 0.7 mM phosphate buffer. All SA-keratin were left for 1 h before fixation with 0.1% glutaraldehyde (scale bar: 50 nm).

Figure 4. 3 Representative TEM images of SA-keratin reconstituted in 55.3 mM acetic acid buffer (pH 2.9) at different concentrations of KCl salt.

Figure 4. 4 Fiber diameters obtained using ImageJ software via scale bar calibration. 200 data points were taken randomly from 5 – 6 TEM images of different replicates to yield the average fiber diameters.

Figure 4. 5 Measured fiber diameter of SA-keratin (n=200) in different buffer condition. (A-D) keratins self-assembled in Citric acid buffer at different pH condition, and E) in acetic acid buffer. The minimum and maximum boundary lines of each colored box indicate the 25th and 75th percentile values, respectively. The line within the box marks the mean. Whiskers (above and below each box) indicate the range within 1.5IQR. F) The effect of pH and salt condition to fiber diameter is summarized. Data presented as mean $\pm$ standard deviation (SD).

Figure 4. 6 Circular Dichroism profile of 0.5 mg/ml SA-keratin in different buffer conditions and salt concentrations. (A-D) Keratin self-assembled in Citric acid buffer at different pH condition, and (E) in acetic acid buffer.

Figure 4. 7 A) Representative TEM and AFM images of SA-keratin prepared in 2.5 mM citric acid buffer (pH 2.9) after initiation of self-assembly for 1 h. AFM images were presented in enhanced color mode and normal mode using XEI Software. B) Z-height profile obtained from the AFM line scan were labelled in black, blue and red, corresponded to the different KCl concentration. C) Thickness of the SA-keratin coating at varied KCl concentration, obtained from Surface Profilometer.

Figure 4. 8 Representative AFM images of SA-keratin showing individual fibers in (A-C) normal view and (D-E) 3D view, prepared in 2.5 mM citric acid buffer (pH 2.9) after initiation of self-assembly for 1 h. High-resolution AFM analysis was carried out by Miss K. Divakarla and Prof. W. Chrzanowski from the University of Sydney.

Figure 4. 9 (A-B) Illustration of self-assembly procedure and duration leading to different fiber morphology. The modification was emphasized in red font. (C-D) Representative TEM images of keratin assembled via the two different methods. The SA-keratin were dialyzed in 2.5 mM citric acid buffer (pH 2.9) at the presence of 20 mM KCl.

Figure 4. 10 (A) Schematic diagram showing the formation of SA-keratin coating network. (B-E) Localized Surface Plasmon Resonance (LSPR) analysis to simulate the coating deposition using (B-C) pH 2.9 SA-keratin solution and (D-E) pH 5.5 keratin solution in the absence of salt. The red arrow indicates the injection of keratins solution into the LSPR sensor region, while the blue arrows indicate the multiple rinsing steps.

Figure 4. 11 A) Immunoperoxidase staining profile of SA-keratin coating in DMEM media over 15 days and B) the corresponding time course stability of coating based on absorbance mapping at 468 nm. Data presented are means $\pm$ SD at $n=3$. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, $*p<0.05$, compared to Day 0 of the corresponding sample group.

Figure 4. 12 Representative TEM images of the five main fractions (F1 – F5) eluted from a weak-anion exchange column, after undergoing step-down dialysis in 2.5 mM citric acid buffer (pH 2.9).

Figure 5. 1: A snapshot of the method and parameters window in the NovaFFF software showing the three main steps during a fractionation, a) focus step, b) constant crossflow step, c) crossflow reduction step and d) rinse step.

Figure 5. 2 Representative AFFF fractogram showing the effect of A) injection time, B) crossflow rate, C) crossflow decline slope and D) crossflow power slope on the peak resolution.

Figure 5. 3 BSA proteins were used as a comparator to estimate the mass distribution of the unresolved human hair keratin proteins. Peaks 1 and 2 on the BSA proteins profile (red) corresponds to its monomer and dimer, respectively.

Figure 5. 4 Fractions under the region of interest A) were collected during the separation and were desalted using protein concentrator (7000 rcf, 15 min) and subjected to B) SDS PAGE for Coomassie blue staining. RK represents the raw keratin proteins before purification, while the fractionated type I (40 kDa) and type II (55 kDa) keratin bands were demarked with the red boxes. STD represents the protein molecular weight standard.

Figure 5. 5 Human hair keratin proteins (10 mg/ml) were dissolved in three different solvent, including A) DI water, B) MES buffer and C) BA buffer (50 mM acetate acid, 6 M urea), respectively. The molar masses of the peak of interest detected using refractive index (RI) and UV detectors were computed using ASTRA software and shown on the right of each graph. All run was conducted at room temperature and the flow rate was maintained at 0.8 ml/min.

Figure 5. 6 A) BCA proteins and B) PEG polymers were used as calibration for molar mass distribution and validation for ASTRA computation arithmetic.

Figure 5. 7 Twelve fractions from the separation profile A) were collected from three runs, dried using Eppendorf Concentrator Plus (20 mbar, 1,400 rpm, room temperature, overnight) and subjected to B) Coomassie blue stained after SDS PAGE. The elution buffer used in this study was 30-70% Acetonitrile with 0.1% Trifluoroacetic acid (ACN/ 10% TFA) with HPLC grade water (0.1% TFA).

Figure 5. 8 Chromatogram of human hair keratin proteins eluted in a gradient of A) 0 – 0.2 M NaCl and B) 0 – 0.1 M NaCl over 60 min duration. The NaCl concentration was presented in red line while the red asterisk indicates the salt concentration in relation to the appearance of peaks.

Figure 5. 9 Chromatogram of human hair keratin proteins eluted in a A) two-step gradient method, which the salt concentration was indicated by the red line. Three representative batches of separation profile using an B) analytical column and C) semi preparative column via the two-step gradient method and the peaks were labelled from 0 – 5, respectively.

Figure 5. 10 Chromatograph of human hair keratin proteins separated using a semi preparative column via a two-step gradient method at pH 5. A) Six regions of interest were highlighted and labelled from 0 to 5, respectively. B) Silver stained profile of the corresponded six fractions and the type I and II bands were indicated by the red and black arrow, respectively.

Figure 5. 11 Immunoblotting analysis of the six fractions, which were collected in previous chromatogram (Figure 5.10A), using A) KRT34 and B) AE3 antibodies. The antibodies recognize the type I (yellow box) and II keratins (red box), respectively. The molecular mass of standard was indicated at the left of the panel.

Figure 5. 12 A) Chromatogram showing fraction of interested collected from three different run and B) 30 µg of each fractions were subjected to SDS PAGE and Coomassie blue staining. The red and black arrow on the right of the gel indicated the type I and II bands, respectively, while RK denoted to the unpurified raw keratins protein. C) Ten visible bands were incised and labelled accordingly before sending out for MALDI-TOF-MS analysis.

Figure 5. 13 Matrix diagram showing the proteins score distribution of the detected keratin subtypes (red – type II; black – type I) in the purified fractions. The lowest protein score threshold was set at 30 to ensure statistical confidence.

Figure 6. 1 SDS PAGE profile of keratin associated proteins (KAP), keratins and total hair proteins (THP) stained with Coomassie Blue Dye.

Figure 6. 2 DPPH radical scavenging activity of A) L-cysteine, B) total hair proteins, C) keratin, D) KAP and E) keratin-NEM. F) The IC₅₀ values of these samples were estimated with DoseResp model.

Figure 6. 3 The quenching effect of keratin (blue), keratin-NEM (green) and KAP (orange) proteins on DCF signal induced by the present of 0.2 μ M HRP and elevated H₂O₂ concentration. Three protein concentrations at 3.6 μ M, 9 μ M and 20 μ M were introduced in this study. Data presented are means $\pm$ SD at n=3. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, *p<0.05, compared between the different concentration groups.

Figure 6. 4 A) Chromatogram of human hair keratins showing the six fractions of interested, labelled from 0 to 5. B) Immunoblot of the collected fractions against KRT34 and AE3 proteins markers, which indicate the type I and II keratin bands, respectively. Panel A and B have been presented in the previous chapter as Figure 5.10 & 5.11 and are presented here to facilitate discussion in this chapter.

Figure 6. 5 Biocompatibility of SA-keratin coating (pH 2.9) was demonstrated for (A-B) HDF and (C-D) HEK cells. Metabolic activity of A) HDF and C) HEK over the course of 5 days obtained using PrestoBlue Assay. Bright field images showing the respective cell morphology of B) HDF and D) HEK grown on SA-keratin coating on day 5.

Figure 6. 6 Representative immunofluorescent images of A) HDF and B) HEK grown on SA-keratin coating (pH 2.9). ECM protein fibronectin (green) and focal adhesion protein vinculin (green) was visualized with immunofluorescence. The actin network and nuclei were stained red and blue, respectively. The staining intensities of C) fibronectin and D) vinculin expressed by HDF and HEK were quantified using ImageJ. Scale bar: 25 μ m.

Figure 6. 7 A) Metabolic activity and B) cell number of HDF treated with keratin and KAP at varied concentration over 1h, 4h and 24h. C) Representative phase contrast images showing morphology of HDF cells after 24 h treatment. Data presented are means $\pm$ SD at n=3. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, *p<0.05, compared to 0 μ M of the corresponding time point groups.

Figure 6. 8 A) Metabolic activity and B) cell numbers of HDF in response to 500 μ M H₂O₂ and designed treatment groups. Data presented are means $\pm$ SD at n=4. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, *, #p<0.05,

compared to Ctr corresponded to 0 μ M and 500 μ M H₂O₂ at each timing point, respectively.

Figure 6. 9 Representative bright field images of HDF cell morphology after introduction to various treatments for 4 h.

Figure 6. 10 A) Normalized ROS signal and B) cell numbers of HDF in response to 500 μ M H₂O₂ and designed treatment groups. Data presented are means $\pm$ SD at n=4. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, *,#p<0.05, compared to Ctr (no keratin treatment) corresponded to 0 μ M and 500 μ M H₂O₂, respectively.

Figure 6. 11 A) ROS intensity collected from 100 HDF cells using ImageJ software "Color Threshold" function. The intensity reading was gathered from 5 - 6 frames of images. B) Live HDF cells were stained with CellROX (green) and Hoechst 33342 (blue) fluorescent dyes and fixed for imaging purpose. Images from two channels were merged using ImageJ software (See Figure A.3 for complete set of images). Data presented are means $\pm$ SD at n=100. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, *,#p<0.05, compared to Ctr corresponded to 0 μ M and 500 μ M H₂O₂, respectively.

Figure 7. 1 TEM images of human hair keratin dialyzed in 8 M urea, 1 mM, and 2.5 mM citric acid at pH 2.9. (Image credit to Marin Yee)

Figure 7. 2 Cross-sectional SEM images of freeze-dried SA-keratin (pH 2.9) sponges, fabricated at A) 1 mg/ml and B) 4 mg/ml.

Figure A. 1 Initiate adsorption rate of keratins solution (pH 2.9 and pH 5.5) during Localized Surface Plasmon Resonance (LSPR) analysis.

Figure A. 2 Localized Surface Plasmon Resonance (LSPR) analysis in triplicated run to stimulate the coating deposition using SA-keratin solution at A) pH 2.9 and B) pH 5.5 in the absence of KCl salt.

Figure A. 3 Live HDF cells were fluorescent stained with CellROX (green) and Hoechst 33342 dyes (blue) and fixed with 4% PFA for imaging purpose. Images from two channels were merged using ImageJ software.

Abbreviations

ACN	Acetonitrile
AFFF	Asymmetrical Field Flow Fractionation
AFM	Atomic Force Microscopy
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CD	Circular Dichroism
DCF	Dichlorofluorescein
DCFH	Dichlorofluorescin
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DTT	Dithiothreitol
ECM	Extracellular Matrices
FBS	Fetal Bovine Serum
GPC	Gel Permeation Chromatography
HDF	Human Dermal Fibroblast
HEK	Human Epidermal Keratinocytes
HPLC	High-Performance Liquid Chromatography
IEX	Ion Exchange
IPC	Interfacial Polyelectrolyte Complexation
KAPs	Keratin Associated Proteins
KCl	Potassium Chloride
L-Cys	L-Cysteine
LDV	Leu-Asp-Val
LSPR	Localized Surface Plasmon Resonance
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MALS	Multi Angle Light Scattering
MS	Mass Spectrometry
NAC	Acetylcysteine
NC	Nitrocellulose
PBS	Phosphate-Buffered Saline

PFA	Paraformaldehyde
PVDF	Polyvinylidene fluoride
RI	Refractive Index
ROS	Reactive Oxygen Species
RP	Reverse Phase
SA	Self-assembled
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPR	Surface Plasmon Resonance
TCEP	Tris(2-carboxyethyl)phosphine
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
UA	Uranyl Acetate
UV	Ultraviolet

Chapter 1

Introduction

The beginning of this thesis demonstrates insights and a few problem statements on the current human hair keratin research, which justify the needs and interests of this contemplated proposal. Some technical limitations and anticipated challenges in rationalizing and progressing from the present knowledge are discussed. Three objectives were designed and strategized in order to validate the hypotheses. A concise overview of the full dissertation is provided, followed by the identified originality and novel findings.

Keywords: Human hair keratin; Intermediate filament proteins

1.0 Introduction

The term “keratin” was originally defined as structural fibrous proteins extracted from corneous tissues [1]. It is an intermediate filament protein that predominantly exists in vertebrate epithelia and epidermal appendages. Sources of keratin include animal horns, hooves, chicken feather, wools, nails, as well as human hair [2]. In human hair, keratins constitute approximate 60% of the total mass. Environmental issues such as drainage system blockage, eutrophication, release of toxic gas and hygiene problems will arise if such amino acid-rich biomass is not disposed appropriately. Although hair shedding rates could vary to aging and other external factors, it is generally assumed that each individual sheds or trims off 100 g of hair annually [3]. A world population of 7.5 billion would produce a tremendous amount of 750 million kg of hair waste every year [4]. With this in mind, effective bio-waste recycling could be achieved if we can fully utilize this abundant and economical resource.

There are 17 keratin types present in human hair, which can be further classified as Type I and II keratin subtypes. They form strongly bonded heterodimers in 1:1 ratio manner, and further assemble into microfibrils (7 – 10 nm). A schematic diagram below displays the structural hierarchy of a hair fiber which is formed through a highly regulated morphogenesis process. The microfibrils consisting keratin IF proteins are further embedded within the amorphous matrix proteins (keratin associated proteins, KAPs) to form macrofibrils. The intrinsically high content of cysteine residues in keratin allows covalent disulphide bridges to be formed at the inter- or intra- molecular level. These bonds contribute to the considerable mechanical properties and chemical inertness of keratin. As a natural biopolymer, keratin has been reported to possess high biocompatibility, biodegradability, neuroinductivity, and flexibility to be fabricated into different forms that are widely used in biomedical related research [5, 6]. Although numerous studies have been conducted on transforming keratin into functional biomaterial templates, such as hydrogels, sponges, coating, etc., the exact science and assembly mechanism behind this biopolymer remain unsolved.

Schematic 1 Structure of a hair fiber.

Within the last decade, several protocols on keratin extraction from human hair as well as the separation of keratin proteins have been well established and patented [7]. Nevertheless, existing studies have not reported on the self-assembly mechanism of the naturally extracted human hair proteins or attempted in purifying the different keratin subtypes. The classification of human hair keratin into type I and type II were established on their molecular mass and isoelectric point differences. Although there exist highly conserved amino acid sequences among the subtypes, only some subtypes contain the LDV peptide sequence which is a cell adhesion motif. As such, the potential to exploit human hair waste as a natural source of biomaterial and the existing knowledge gaps in human hair keratin research. Instigated the idea of this Ph.D. project, which aims to study the self-assembly mechanism of crude human hair proteins and to purify and understand the keratin subtypes from the extracted human hair keratin proteins.

Chemical extraction methods have been shown to achieve a high yield of human hair keratin protein, at which the highest keratin yield of 70% was reported by Fuji *et al.* using the Shindai Method [8]. Within our group, a more efficient extraction protocol using sodium sulphide as a reducing agent was established, requiring a shorter incubation period albeit having a lower dry yield percentage of around 20-40% per batch [9, 10]. In comparison to the recombinant approach, the use of human hair keratin extracts for the investigation of self-assembly mechanism and purification of keratin subtypes appears to be more promising and realistic due to its abundance, even though the complex protein mixtures would complicate the fractionation process. Undeniably, the former approach would be able to produce highly purified and specific subtypes, however, one concern is the

uncertainty of the post translational modification (PTM) process. Naturally, the formation of keratin fibers involve highly modulated and complex cascade of events, where it is essential to undergo thorough PTM, to ensure that the authentic structure and functionality of the keratin heteromers are unaltered [11].

In addition to the potential of using human hair-extracted keratin as functional materials, replicating the self-assembled fibrous nature of keratin proteins *in vitro* should be viable and translatable into a favorable template given a finely tuned environment. Likewise, separation of the two main classes of keratin subtypes, Types I and II, can be achieved by combining the knowledge of molecular mass and isoelectric point differences with proper sample preparation and suitable chromatography techniques. Considering the rich amount of highly reactive cysteine residues in the keratin extract, the separation of subtypes with high similarity or overlapping molecular mass/isoelectric point is the main obstacle faced in this project. Therefore, garnering a strong understanding of the material and developing effective protocols to extract and enrich the keratin subtypes are crucial.

1.1 Hypothesis

Following the literature review, it is hypothesized that,

- I. *Crude/purified hair keratin can self-assemble through electrostatic interactions and/or thiol chemistry to form fibrous networks.*
- II. *Specific hair keratin subtypes can be separated from total hair protein extracts using combinations of liquid chromatography techniques.*
- III. *Hair keratins have the potential to act as antioxidants due to the presence of thiol groups, to protect cells from oxidizing agents.*

1.2 Objectives

To prove the hypotheses, the following objectives need to be achieved.

Objective 1: *To develop and optimize a protocol for inducing self-assembly of hair keratins into fibrous networks*

Parameters such as pH condition, salt concentration, keratin concentration, and assembly duration will be optimized to induce and facilitate the fibrous networks formation. Characterization techniques including SEM, TEM, CD, AFM, and surface profilometer will be included to study morphology, assembly ability, and conformation changes of the keratin fractions.

Objective 2: *To develop and optimize a protocol for separation of Types I and II hair keratins.*

Different separation techniques will be explored to separate the keratin subtypes from the crude extracts. These include Asymmetrical Field Flow Fractionation (AFFF), Gel permeation chromatography (GPC) and High-Performance Liquid Chromatography (HPLC). After identification of the suitable techniques, optimization of the separation resolution including sample preparation, mobile phase, and column selection will be conducted. For downstream characterization and application, the obtained fractions will be subjected to concentration and buffer exchanges.

Objective 3: *To evaluate the potential of hair keratin extracts as antioxidizing supplements for in vitro cell culture applications.*

Free radical scavenging ability of hair proteins extracts will be investigated and compared using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay and de-esterified 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) reagent. Hair keratin extracts at varied concentration will be introduced to human dermal fibroblast (HDF) cells as antioxidizing

supplements in the presence of hydrogen peroxide (H_2O_2). The cell viability and reactive oxidative stress (ROS) condition will be evaluated using biological assays, including PicoGreen, PrestoBlue and CellROX assays, in combination with immunofluorescent staining.

1.3 Dissertation Overview

This thesis presents the following few chapters in accordance with the objective to study and understand the interaction and behavior of human hair keratin proteins.

Chapter 1 provides a rationale for the human hair keratin research and outlines the goals and scope of this project.

Chapter 2 reviews the literature concerning the overall timeline of human hair keratin research and the current knowledge and shortage about keratin-derived biomaterials

Chapter 3 elaborates the methodology and principles behind the techniques adopted in this thesis, including the development of a unique self-assembled protein network, fractionation of the keratin subtypes from crude human hair keratin proteins and finally cellular studies on the human hair proteins solution and platform.

Chapter 4 depicts the optimization procedure in achieving a novel self-assembled human hair keratin proteins nanofilaments network and characterization to understand the kinetic and mechanism of the self-assembled 2D platform.

Chapter 5 discusses the employed purification techniques and characterization method, including errors and aborted attempts.

Chapter 6 elaborates on the cell culture studies on human hair keratin proteins solution and the self-assembled human hair keratin proteins network. Antioxidant properties of the human hair proteins will also be compared and discussed.

Chapter 7 converges all findings and recapitulate the significant achievement accordance with the proposed hypotheses. Future opportunities and strategies to reinforce the disclosed findings will be described in this final chapter.

1.4 Findings and Outcomes/Originality

This research led to several novel outcomes:

1. Established the conditions needed to form self-assembled human hair keratin nanofilamentous networks (patented)
2. Developed an ion exchange HPLC approach to obtain partially purified human hair keratin subtypes
3. Provided quantitative comparison of the antioxidant properties of human hair proteins

References

- [1] H. H. Bragulla and D. G. Homberger, "Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia," *Journal of anatomy*, vol. 214, no. 4, pp. 516-559, 2009.
- [2] M. Feughelman, "A note on the water-impenetrable component of α -keratin fibers," *Textile Research Journal*, vol. 59, no. 12, pp. 739-742, 1989.
- [3] C. R. Robbins and C. R. Robbins, *Chemical and physical behavior of human hair*. Springer, 2002.
- [4] A. Gupta, "Human hair "waste" and its utilization: gaps and possibilities," *Journal of waste management*, vol. 2014, 2014.
- [5] A. Vasconcelos and A. Cavaco-Paulo, "The use of keratin in biomedical applications," *Current drug targets*, vol. 14, no. 5, pp. 612-619, 2013.

- [6] F. Wunner, S. Florczak, P. Mieszczanek, O. Bas, E. De-Juan-Pardo, and D. Hutmacher, "Electrospinning with polymer melts—state of the art and future perspectives," in *Comprehensive Biomaterials II*: Elsevier, 2017, pp. 217-235.
- [7] M. E. Furth, A. Atala, and M. E. Van Dyke, "Smart biomaterials design for tissue engineering and regenerative medicine," *Biomaterials*, vol. 28, no. 34, pp. 5068-5073, 2007.
- [8] A. Nakamura, M. Arimoto, K. Takeuchi, and T. Fujii, "A rapid extraction procedure of human hair proteins and identification of phosphorylated species," *Biol. Pharm. Bull.*, vol. 25, no. 5, pp. 569-572, 2002.
- [9] S. Wang et al., "Culturing fibroblasts in 3D human hair keratin hydrogels," *ACS applied materials & interfaces*, vol. 7, no. 9, pp. 5187-5198, 2015.
- [10] P. Hartrianti et al., "Fabrication and characterization of a novel crosslinked human keratin - alginate sponge," *Journal of tissue engineering and regenerative medicine*, 2016, doi: 10.1002/term.2159.
- [11] N. T. Snider and M. B. Omary, "Post-translational modifications of intermediate filament proteins: mechanisms and functions," *Nature reviews Molecular cell biology*, vol. 15, no. 3, p. 163, 2014.

Chapter 2

Literature Review

This chapter covers the fundamental knowledge of human hair fiber assembly and current understanding of hair keratin extraction, and their functionalities. In particular, the well-known properties of keratins and keratin-based biomaterial platforms developed since the 16th century are presented. A thorough review of the inter-disciplinary applications of keratin-derived biomaterials originating from different sources, including wool, feathers, and human hair are discussed. In addition, several case studies showing different purification approaches, including a newly emerged technique, field flow fractionation technique, are also elaborated as part of this project's attempt to purify hair keratin subtypes. The unique properties of self-assembled intermediate filament proteins are described in the final section in this chapter.

Keywords: Protein purification; Intermediate filament proteins; Self-assembly; Keratin-derived biomaterial

2.0 Background

The foremost documentation of hair in medical applications was in a 16th century literary work titled “Ben Cao Gang Mu” by renowned Chinese herbalist, Li Shi-Zhen [1]. The effect of pyrolyzed human hair on acceleration of wound healing process was reported. Moving into the 18th century, keratin research was initiated on material sources like hooves and horns and eventually shifted to wool and human hair [2]. Human hair keratin, a naturally derived material, has been gaining more attention in biomedical research as it reportedly possesses the cell adhesion motif – leucine-aspartic acid-valine (LDV), and demonstrates biocompatibility for a wide range of regenerative applications, notably in wound healing treatment [2-4]. In this chapter, an introduction of human hair fibers and keratins are first presented, followed by an in-depth illustration of keratin derived biomaterials and their applications. To execute the fractionation of the human hair keratin solution, purification scenario on various complex biological or proteins systems are reviewed and studied. Subsequently, self-assembly behavior of keratin proteins in various conditions are compiled and discussed.

2.1 Human Hair Keratins

Beneath the epidermis, the formation of a hair fibre begins at the hair follicle, where the bulb and dermal papilla are located. Hair cells, melanocytes, and important growth factors can be found in this active layer of growing hair. While the hair cells differentiate and move upward into the keratinized layers, the hair fibres now gain stability by the formation of cystine linkage. A permanent hair fibre is then formed and developed into three distinct layers including a fully cornified cuticle, cortex and sometimes medulla (Figure 1) [5]. The most abundant amino acids present in human hair keratins are Serine, Glutamic acid, and Cysteine, which constitute approximately 10.3, 12, and 16.7 mol% of the total amino acid content, respectively [6]. The strong intermolecular disulphide bonding between the cysteine residues within the entire structure makes keratin fibres robust and less soluble compared to other proteins. These structural proteins can be categorized based on their distinct structures, sulphur content and regulatory function [7]. Due to the importance and

growth of research interest in intermediate filaments proteins, studies on assembly mechanism and structure of intermediate filaments including keratin have been conducted extensively using crystallographic modelling and crosslinking combined technique [8-12]. Figure 2.2 shows the schematic of a wool fiber, which is similar as the human hair superstructure except for the alpha to gamma keratin ratio. It has been revealed that the alpha keratins form heterodimers in helical coiled-coil structure and two of such dimers will further assemble into a tetramer in an antiparallel fashion [13].

Figure 2. 1 Schematic of a growing hair fibre showing the different zones in its follicle. Reproduced from permission from Robbins *et al.* with permission [5]. Copyright © 1988, Springer Science Business Media New York.

Figure 2. 2 Schematic of a wool fiber drawn by Bruce Fraser and Tom MacRae. Reproduced from R. D. B. FRASER *et al.* with permission [14]. Copyright (c) 2012-2020 CSIRO Australia.

The outer most scaly and tubular cuticle layer consists of flattened cells followed by the densely packed keratinous protein layer called cortex. The medulla embedded in the core of a hair fibre is usually absent in fine fibres [15, 16]. Among the three main structures, cortex comprises the most fiber mass with cortical cells arranged in the parallel orientation to the fiber axes. The elongated cavities, referred as nuclear remnants, are derived at the center of the developing cortical cells which are constructed by numerous closely packed microfibrils. These microfibrils are assembled in a highly organized matter within the amorphous cysteine-rich matrix, which are also known as keratin associated proteins (KAPs) matrix. Bundles of microfibrils are collected in quasi-hexagonal arrays that form into macrofibrils [17]. Zooming out to the scale-like cuticle layer, each scale cell consists of two distinct layers; the exocuticle, which is a keratinous outer layer, and the endocuticle, which is a non-keratinous inner layer that derived from cytoplasmic debris. The exocuticle was reported to complex with a cysteine-rich outer layer called epicuticle.

2.2 Extraction Methods and Keratin Derived Material Applications

To further characterize and understand the chemistry of hair keratins, different methods have been introduced to break down the hair fibre structure and extract the keratins into soluble and stable forms. Oxidative, reductive, and enzymatic extraction approaches were initially implemented on wool, hooves, and feathers and eventually adopted for human hair.

A review paper, by David R. Goddard *et al.* summarized keratin extractions done by several groups using oxidative procedures which involve bromine, permanganate, and H_2O_2 . It was claimed that these reactions are slow and the oxidants do not specifically attack the disulphide bonds, thus affecting the other protein composition [18]. In contrast, reductive extraction methods using thioglycolic acid, potassium cyanide, sodium sulphide and sodium sulphite required a shorter duration and the reaction acted specifically on the

disulphide bond [18-21]. In recent years, peracetic acid, thioglycolic acid with sodium hydroxide, and sodium sulphide are commonly used by several groups in human hair keratin extraction [3, 4, 22-28]. The protocol developed by Goddard and Michaelis [18] was modified and adopted by J. R. Richter *et al.* [29] using thioglycolic acid to extract kerateines. The same group also conducted oxidative extraction method by implementing Alexander and Earland's [30] procedure, which involved reaction using 2% peracetic acid for 10 h.

In addition, Fuji and his co-worker developed a convenient and high yield extraction protocol called "Shindai method" to produce detergent free keratin proteins from rat hair, chicken feathers, wool, human nails and as well as human hair [31]. Addition of thiourea into the extraction buffer was able to increase the yield percentage of more than 75% as compared to the 10-15% yield obtained using conventional methods [32, 33]. Furthermore, our group reported a simple, inexpensive, rapid and consistent procedure which able to dissociate human hair proteins using sodium sulphide giving approximately 20% dry weight yield [34]. In short, keratins can be extracted from the hair cuticle using above approaches, by either converting keratins into keratoses or kerateines, using oxidative or reductive method, respectively. The oxidized form of cystine (-SS) is cysteic acid (-SO_{3H}) while the reduced form is cysteine (-SH) [3].

Both kerateines and keratoses behave chemically different due to the backbone polarity and functional group differences. For instance, keratoses contains the oxidized cysteic acid which possess electron withdrawing properties leading to its water soluble and hygroscopic properties [28]. It is non-disulphide crosslinkable and will degrade within days to weeks in vivo due to hydrolytic degradation. In contrast, kerateines are less polar and insoluble in water due to their ability to be re-crosslinked upon oxidation of cysteine groups. Biomaterials made of kerateines have a relatively longer degradation period from several weeks up to months [35]. Keratin derived materials fabricated into various platforms such as hydrogel, porous scaffolds, thin films, and coatings were reported to possess intrinsic cell adhesion, excellent biocompatibility and angiogenesis properties [4, 13, 18]. Due to the medical potential, applications for these keratin-based biomaterials further transcended

into peripheral nerve, skin, and bone regenerations [2, 24, 33]. Metal uptake and antioxidant studies were also explored using keratin proteins from various sources due to their high cysteine content [36-40]. Extractions, applications, and properties of keratin-derived materials from human hair, wools and feathers are compiled in Table 2.1 below.

Table 2. 1 Summary of recent applications of keratin-derived materials, sources of the keratin material, the respective extraction method, and unique properties.

Keratin Source	Extraction method	Fabrication form	Application	Unique properties	Ref.
Human Hair	Reducing agent, Na ₂ S	3D Hydrogel	Living cell encapsulation	Fibroblast infiltration	[34]
	Reducing agent, Na ₂ S	Electrospun nanofibrous scaffold	Mimic ECM for cellular activity	Improve human dermal fibroblast infiltration and facilitate tissue regeneration	[25]
	Reducing agent, Na ₂ S	Coating	Surface coating material for cell culturing	Improve mouse fibroblast adhesion and proliferation	[27]
	Shindai Method	Post-casted film	Investigation of histamine release from rat mast cells	Antiallergic properties	[41]
	Reducing agent, TGA	Coating	Study of hepatocyte adhesion to keratin substrates	Hepatic ASGPR as receptor for keratin biomaterial	[42]
	Oxidizing agent, CH ₃ CO ₃ H (Peracetic acid)	Scaffold	In vivo study in mouse subcutaneous tissue pockets	Non-toxic, biodegradable and remodel with natural collagen ECM	[28]
	Shindai Method	Coating	Study of 12 cell line on keratin CLEAR coating and TCA coating	Most cell types show best attachment and proliferation on CLEAR coating	[43]
	Pretreated with acid/alkali with Na ₂ S	Physical fibre	Metal absorption	Metal removal ability (Cu, Hg)	[44]
Wool	Reducing agent, 2-ME	Coating	Peptide modified keratin cultured with mouse fibroblast	Enhanced cell adhesion	[45]

	Reducing agent, $\text{Na}_2\text{S}_2\text{O}_5$	S-sulfo keratin sponges	Cell tissue engineering scaffold	Controllable porosity and pore size for	[46]
	Reducing agent, 2-ME	Sponge	Cell cultivation using mouse fibroblast	Scaffolds for long-term high-density cell cultivation	[47]
	Reducing agent, metabisulphite	Microparticulate	Drug Delivery System	Biocompatible, non-toxic.	[48]
	Reducing agent, 2-ME	Sponge	Osteoblast	Differentiation of osteoblast on hybrid sponge started at day 5.	[49]
	Reducing agent, 2-ME	Film	Keratin-Chitosan film for cell culture	Waterproof, antibacterial, support fibroblast	[50]
	Reducing agent, Tributylphosphine	Powder	Metal ion uptake test	Able to absorb 3.15mM of Hg(II)/g of wool	[37]
Feather	Enzymatic method, <i>Bacillus subtilis</i> AMR	Hydrolyzed keratin peptides	Hair care products: Mild Shampoo and rinse-conditioner	Better hydration to hair fibers	[51]
	Ball milling, ultrasonic exposed	Physical Fibers	Metal absorption	Metal removal ability (Pb, Cu, Hg)	[52]
	Reducing agent, 2-ME	Hybrid membrane (PU)	Metal removal	Maximum removal rate for Chromium is 32%	[53]
	Enzymatic method, <i>Bacillus subtilis</i> AMR	Hydrolysate	Free radical scavenging activity	Antioxidant potential	[39]

Keratin derivatives demonstrated a wide range of potential applications, albeit, biomaterials made of pure keratins, such as keratins thin films, are usually poor in mechanical strength as they are extremely brittle [54]. Therefore, incorporation of natural and synthetic polymers, including chitosan, silk fibroin, hydroxyapatite particles, PVA, PCL, PEO, and etc. were necessary to improve the physical properties while retaining the bioactivity of these keratin-based biomaterials [50, 55-58]. All in all, continual progress is

persisted to make keratin a mainstream biomaterial and be translated to more clinical trials in the future.

2.3 Classification of Human Hair Keratin

Keratin intermediate filament proteins extracted from human hair are classified into three types, alpha-, beta-, and gamma keratins [4]. Alpha-keratins are also more commonly named as keratin proteins, which can be further classified into 2 subgroups: type I and type II. Among the 28 type I and 26 type II human keratins which were recorded in the current database, 17 keratins are expressed in human hair, including K31 to K40 (type I) and K81 to K86 (type II) keratins, while the rest belongs to epithelial keratin. It is also interesting to note that K84 only present in the cytoskeletal extracts of the human tongue [59]. Keratins are mostly present within hair cortex in helical tertiary structure and low in Sulphur (~9 mol%), showing molecular weight ranges between 40-80 kDa. On the other hand, Beta-keratins are extremely difficult to be extracted and they serve an important function to protect the hair structures as the cuticle layer. Moreover, the globular gamma keratins are also described as keratin-associated proteins (KAPs) or matrix proteins, which hold the cortical microfibrils together acting as disulphide crosslinkers [24, 60, 61]. The KAPs are composed of high sulphur content (~20 – 30 mol%) and are relatively low in molecular weight, ranging between 6 and 30 kDa [62, 63]. To date, 89 KAPs have been identified in the human hair [63]. Existing studies have revealed that hair fibers possess 50-60% of keratin and 20-30% of KAPs [15].

Among all existing extraction protocols, a modified version of Shindai Method is most well-known for extracting the keratins and KAPs fractions separately. The study has proven with the addition of 25% ethanol in the extraction solution, the dissociation of keratin proteins was inhibited, hence the dissociation of KAPs fractions from keratins can be achieved [31]. However, the definite explanation of keratin proteins being suppressed in the presence of ethanol even with high reducing and denaturing condition is still unknown. The isoelectric points and molecular weight of all 11 type I and 6 type II keratins are shown in Table 2.2, whereby data was obtained from Human Intermediate Filament Database [64].

Furthermore, the molecular weight of type I and II keratins obtained via gel electrophoresis in different studies were further elucidated at 40 - 56.5 kDa and 54 – 57 kDa, respectively [9, 15, 35]. By undergoing highly proliferative processes, these type I and II keratin are able to self-assemble into heterodimers and further elongated into fibres inside the follicle and form immensely stable hair structure after extruded through the skin [3].

Table 2. 2 Isoelectric point and molecular weight of type I and type II keratin.

	Keratin	Isoelectric Point	Molecular Weight
Type I	K31/Ha1	4.53	47.24 kDa
	K32/Ha2	4.49	50.32 kDa
	K33a/Ha3-I	4.48	45.94 kDa
	K33b/Ha3-II	4.50	46.21 kDa
	K34/Ha4	4.72	49.42 kDa
	K35/Ha5	4.55	50.36 kDa
	K36/Ha6	4.60	52.25 kDa
	K37/Ha7	4.62	49.75 kDa
	K38/Ha8	4.49	50.48 kDa
	K39/Ka35	4.86	55.62 kDa
	K40/Ka36	4.60	48.15 KDa
Type II	K81/Hb1	5.24	54.97 kDa
	K82/Hb2	6.72	56.65 kDa
	K83/Hb3	5.14	54.17 kDa
	K84/Hb4	7.61	64.84 kDa
	K85/Hb5	6.49	55.80 kDa
	K86/Hb6	5.35	53.50 kDa

The excellent biocompatibility, cell attachment and infiltration properties of keratin-derived materials are assumed to be correlated to the presence of LDV cell adhesion motif. Table 2 shows the LDV motif sequence which is present in all the type I keratins and K82 for the type II keratin group. Thus, there is motivation to attempt fractionation of the keratin subtypes and further study the behavior of the purified keratin as this might bring new insights or beneficial improvements in the mechanistic and regenerative medicine applications. However, the isoelectric points and molecular weights of the two keratin groups are highly proximate, which makes separation of these subtypes challenging.

Table 2. 3 Partial amino acid sequences of human hair keratins (LDV cell adhesion motif sequences shown in highlighted font) [64].

Keratin	Partial Amino Acid Sequence	pI	MW
K31/Ha1	TESEARYSSQ LSQVQSLITN VESQLAEIRS DLERQNQEYQ VL LDV RARLE 301 350	4.53	47.24kDa
K32/Ha2	LAQMCMITN VEAQLAEIRA DLERQNQEYQ VL LDV RARLE GEINTYRSL 351 400	4.49	50.32kDa
K34/Ha4	SQLSQVQSLI TNVESQLAEI RCDLERQNQE YQVL LDV RAR LECEINTYRS 351 400	4.72	49.42kDa
K35/Ha5	QLAQMCMIT NVEAQLAEIR ADLERQNQEY QVL LDV RARL ECEINTYRGL 351 400	4.55	50.36kDa
K36/Ha6	MQCLISNVEA QLSEIRCDLE RQNQEYQVLL LDV KARLEGEI ATYRHLEGE 351 400	4.60	52.25kDa
K37/Ha7	AEDRYGTELA QMQLISNLE EQLSEIRADL ERQNQEYQVL LDV KARLENE 351 400	4.62	49.75kDa
K38/Ha8	AEDRFGTELA QMQLISNVE EQLSEIRADL ERQNQEYQVL LDV KTRLENE 351 400	4.49	50.48kDa
K39/Ka9	LTQIQSLIDN LEAQLAEIRC ALERQNQEYE IL LDV KSRLE CEITTYRSL 351 400	4.86	55.62kDa
K40/Ka10	IDNLENQLAE IRCDLERQNG EYQVL LDV KA RLEGEINTYW GLLSEDSRL 351 400	4.60	48.15kDa
K33a/Ha3-I	TESEARYSSQ LSQVQRLITN VESQLAEIRS DLERQNQEYQ VL LDV RARLE 301 350	4.48	45.94kDa
K33b/Ha3-II	TESEARYSSQ LSQVQSLITN VESQLAEIRS DLERQNQEYQ VL LDV RARLE 301 350	4.50	46.21kDa
K82/Hb2	DFLKSLYEEE ICLLSQISE TSVIVKMDNS RE LDV DGHA EIKAQYDDIA 251 300	6.72	56.65kDa

2.4 Protein Purification Methods

Understanding of protein's physical and chemical properties is crucial to conduct any separation process. The most common and well-developed chromatography techniques involve column packing materials, such as gel-filtration media, ion-exchangers, affinity adsorbents, reversed phase packings and etc. [65]. These columns were packed with various diameters media ranges from few microns to hundredth microns, to fulfill different applications and cost efficiency. Nevertheless, samples pretreatment and extractions steps

are also critical to achieve successful purification for downstream characterizations and applications [66].

Of all the various chromatographic techniques, affinity chromatography offers the best purification and isolation capabilities [67, 68]. The targeted polypeptide chains can be purified using immobilized immunoglobulin columns combined with genetic engineering approaches by attaching “tags” or “affinity tails” to the protein chains. After which, enterokinase is used to remove the affinity tags [69]. However, this method often incurs high cost due to the absorbent design and it is only limited to small columns. Moreover, analytes can be separated based on variation in charges, structures, sizes, hydrogen bonding or hydrophobicity. These parameters and the respective purification methods were included in Figure 2.3.

Method	Separation basis	Isolation, purification, capability
Centrifugation	Density/size	+
Filtration	Size	+
Liquid–liquid extraction	Solubility	+
Precipitation	Solubility	+
Chromatography:		
Anion-exchange	Charge	+++
Cation-exchange	Charge	+++
Hydrophobic interaction	Hydrophobicity	++
Affinity:	Structure	+++++
Immunoaffinity		
Heparin affinity		
Mimetic™ dye–ligand affinity		
Metal–chelate affinity		
Immobilized affinity “tails”		
Reversed-phase	Hydrophobicity	++++
Chromatofocusing	Charge	+++
Preparative isoelectric focusing	Charge	++++
Immobilized artificial membrane	Hydrophobicity	+++
Adsorption	Charge/shape	++
Gelfiltration	Size/Shape	++

The + symbol refers to the purification capability of each method, ranging from low (+) to high (+++++) level.

Figure 2. 3 Separation methods in a typical isolation and purification. Reproduced from Takács *et al* with permission [69]. Copyright © 2000 John Wiley & Sons, Ltd.

Field flow fractionation (FFF) was invented back in 1966 which acts as a mild method to

separate colloids, macromolecules, and particles. FFF technique does not rely on analytes partitioning or adsorption which reduces the extent of sample loss. The purification of a diversity of specimens including nanotubes, stem/cancer cells, inorganic/polymeric particles, surface adsorption study, cell apoptosis, proteins etc. have been explored using FFF techniques [70-72]. A schematic of an FFF separation event is shown in Figure 2.4, illustrating the narrow zone of solute molecules being confined in the FFF channel (slab shaped rectangular) [73]. When a flow is introduced into the channel (from the left), a parabolic stream is formed in which the greatest stream velocity locates in the center of the channel. Thus, the solute molecules carried down by the stream is affiliated to the distance from the channel wall, which is governed by the diffusion coefficient of the solutes. This explains that the sample peaks appearing in an FFF spectrum are strictly based on the diffusion coefficients differences. Hence, diffusivity (D), Stokes radii and friction coefficient of a fractionated mixture can be calculated based on the retention time [73]. FFF is a relatively mild and gentle separation method, which can retain the integrity of fragile particulates or native protein samples during separation [73]. With such characteristic and capabilities, FFF has yet to become a mainstream analytical technique. This might be due to the complexity of FFF technique, which requires sufficient knowledge on the various forms, including sedimentation, electrical, dielectrophoretic, magnetic and acoustic FFF instruments [74-77].

Figure 2. 4 Cross section view of a flow FFF channel. Reproduced from J.Calvin Giddings *et al.* with permission [73]. Copyright © 1977 Published by Elsevier Inc.

Demonstration of sample extraction and preparation followed by sequential purification methods was demonstrated in an interesting study conducted by Nielsen *et al.* performed the purification of soybean cultivar storage polypeptides and reported successful identification of the Glycinin's acidic and basic subunit complexes [78-80]. In this study, the Glycinin was first reduced using acetone powder with 0.4 M NaCl, 0.02% NaN₃, 0.01 M β -mercaptoethanol in 0.035 M potassium phosphate at pH 7.6 and further S-alkylated with 4-vinylpyridine in 6 M guanidine HCl. On the other hand, unreduced Glycinin was prepared using the same procedure in the absence of sulfhydryl-reducing agent. Prior to electrophoresis, unreduced Glycinin was broken down into subunits by mixing with β -mercaptoethanol. Subsequently, the Glycinin samples were cleaved using CNBr digestion and were first fractionated on a Sephadex G-75 column (2.5 x 100 cm) in 9% formic acid (eluent) and purified by reverse phase HPLC. In addition, the NH₂-terminal sequences of the purified peptides were determined using a Beckman 890C Sequencer. Increased mobility of the reduced Glycinin was observed from the SDS gels indicated S-S bond formation in the fragment (Figure 2.5). Intriguingly, it was concluded that only one intermolecular disulfide bond was responsible in connecting the Glycinin's acidic and basic polypeptides in this study [78].

Figure 2. 5 SDS-gel electrophoresis of A₃B₄ and A₅B₃ CNBr fragments from Glycinin subunits sample. Reproduced from Staswick *et al.* with permission [78]. Copyright © 1984, American Soc For Biochemistry & Molecular Biology.

Another study, which is more relevant to this dissertation research, was performed by Ueli Aebi *et al.* on the separation of keratins extracted from skin epidermis [81]. A weak anion exchange DEAE-column was used in the reported protocol, followed with a Sephacryl S-400 SEC column in the second gel permeation chromatography (GPC) separation. All solutions used in extraction, dialysis, and elution contained reducing agent, DTT and denaturant, urea. The eluent was titrated to pH 8.2 for ion exchange chromatography (IEX) and pH 7.5 for GPC method. This paper has shown successful separation of four keratins, using AE1 and AE3 monoclonal primary antibody in Western blotting. In addition, filament reassembly properties of these keratin complexes were also investigated. Notably, the presence of both type I and II keratins are compulsory to induce filaments formation [81].

Figure 2. 6 (b) Coomassie Blue-stained gel and immunoblot analysis of the fractions using (c) AE3 antibody and (d) AE 1 antibody. Reproduced from R Eichner *et al.* with permission [81]. Copyright, Rockefeller University Press.

2.5 Self-assembly of Keratin Derivatives and Keratin Intermediate Filament Proteins

Apart from the excellent bioactivities which support cellular proliferation and infiltration, keratin derivatives also possess intrinsic self-assembly properties at both nano and macroscale [82]. The self-assembly phenomenon of keratin is well documented in the highly conserved and reproducible superstructure of hair fibers. Numerous studies have

reported the effect of freeze-drying, crosslinkers, freeze-thaw cycles and the use of electrospinning to induce self-assembly and polymerization of keratin proteins into 3-dimensional scaffolds, containing homogeneous nano- to macro-sized pores or fibrous structures [83-87]. The synergistic effect of having cell binding motifs (LDV residues in human hair and RGD residues in wools) and the inherent ability to self-assemble broadens the potential of keratin-based biomaterials in different platforms and applications [64].

A study conducted by Paulina Sierpinski *et al.* reported spontaneous formation of self-assembled hydrogels by addition of water/PBS to the extracted and freeze-dried wool keratins [24]. The microstructure of the hydrogels was investigated under SEM, showing fibers diameter at around 2 to 20 μm and pore sizes ranged from 20 to 50 μm . The keratin hydrogels were further utilized in nerves regeneration study and demonstrated good neuroconductivity, possibly denoted to the growth factors or regulatory molecules, which were expressed in the hair follicles and retained in the hair fibers [24, 88]. Another interesting finding by Fumiyoshi Ikkai *et al.* reported the assembly of chemically modified wool keratins gel by heat-induced gelation was imperatively dependent on the hydrophobic interaction between α -helix keratin chains [89]. The gelation could be achieved even in the absence of disulfide [89]. The reported circular dichroism (CD) data further clarified the keratins conformation is rod like and the majority have α -helix structures in order to associate the hydrophobic forces for gel formation [89]. In the absence of crosslinking agent, improvement of human hair keratin gels in thermal, mechanical, and swelling resistance properties have been proven by sufficient self-crossing via multiple freeze-thaw cycles [86]. However, it was anticipated that the assembled network was enhanced through physical cross-linking and disulfide bonding during the freeze-thaw process [86].

Furthermore, electrically charge polymeric materials with nano- or micro-fibrous structure (nonwovens), were widely fabricated using electrospinning technique. Synergistic effect of wool keratins and silk fibroin at equal blend ratio was reported to forge finer nanofibers, which were assembled as random coils or α -form secondary structures [90]. Inhibition of the keratin protein chains' molecular arrangement and crystallization were also revealed by a higher rate of fiber formation, resulting in less thermally stable nanofibers [90].

Another study by Aluigi *et al.* investigated the physical and thermal properties of electrospun keratin/PEO mat, and described destabilization of the self-assembly nature of keratin leading to a less complex protein conformation [91]. Albeit, practical utilization of these keratin/PEO blend fibers mat was limited due to poor mechanical properties and water instability [91]. These studies demonstrated the effect of modification and processing methods which affect the macro scale architecture and the scaffolds' properties.

Moving on, intermediate filaments (IFs) are portrayed as important “stress buffering” elements present in the cytoskeleton and nuclear of animal cells [92]. IFs proteins are recognised for its insolubility in physiological pH / ionic strength and ability to reassemble *in vitro* conditions without any cofactors [93]. Among the five IFs categories, the dynamic and point mutation in keratin IFs were reported in relation to genetically related Epidermolytic diseases which show reddening, scaling, and severe blistering of skin [94-96]. More recently, human keratin mutation was reported to be associated with livers, pancreas and bowels related disorder [97-99]. Understanding of the keratin IFs molecular assemble mechanism is therefore crucial to acquire diagnosis, cure, and prevention for such diseases.

The heterodimerization or self-assembly of keratins at nano scales has been previously demonstrated in native/recombinant epithelial keratins and recombinant hair keratins [100-103]. Low ionic strength and neutral / high pH buffer were reported to initiate assembly of epithelial keratins IFs from its denatured form in concentrated urea *via* step down dialysis manner [104]. However, differences in the assembly condition of epithelial (K8/K18) and epidermal keratins (K5/K14) were also reported, as such when the keratins were dialyzed into 2 mM Tris-HCl (pH 9) buffer and added to the assembly solution of 10 mM Tris-HCl (pH 7.5), K8/K18 was still thoroughly soluble (Figure 2.7A & B) while K5/K14 demonstrated adequate filament formations (Figure 2.7C & D) [103]. Another study by Pierre A. Coulombe *et al.* adopted a neutral pH self-assembly condition, by using 50 mM Tris HCl, 10 mM 5-mercaptoethanol (pH 7.25) [100]. This study reported that the assembly of K5/K14 occurred even in 9.5 M urea condition and the concentration for filament formation was detected as low as 37.5 µg/ml, which was below the assigned critical

assembly concentration (50 $\mu\text{g/ml}$) for keratins purified from mammalian cells [100]. Although the filament formation of keratin through heterodimerization was constantly emphasized, a study conducted by Rachael N. Parker *et al.* observed one-dimensional nanostructures from homooligomerization of hair recombinant K31 and K81, attributed to their high cysteine content [102]. When dialyzing in 10 mM Na_2HPO_4 , 75 mM NaCl, 5 mM DTT (pH 8), K31 alone was able to assemble into fibers of varying morphologies (Figure 2.8A) while K81 appeared relatively thicker and shorter (Figure 2.8B) compared to the normal IF morphology (Figure 2.8C & D) [102].

Figure 2. 7 TEM images showing human recombinant K8/K18 assembled in a) 25 mM Tris–HCl, 50 mM NaCl (pH 7.5) and b) 10 mM Tris–HCl (pH 7.5). While K5/K14 were c) assembled and d) dialyzed in 10 mM Tris–HCl (pH 7.5). Scale bar: 100 nm. Reproduced from Harald Herrmann *et al.* with permission [103]. Copyright © 2002 Elsevier Science (USA).

Figure 2. 8 TEM images of human hair recombinant keratin proteins A) K31, B) K81, and C-D) K31/K81 dialyzed in 10 mM Na₂HPO₄, 75 mM NaCl, 5 mM DTT (pH 8). Reproduced from Tijana Z *et al.* with permission [102]. © 2017 Wiley Periodicals, Inc.

A three step schematic is shown in Figure 2.9 to explain the assembly of keratin proteins, which began with the lateral growth of heterodimeric tetramers (type I and II) into a unit length filament (ULF), and further annealed longitudinally into a full-width intermediate filament [103, 105]. The ULFs during the assembly event were observed only when the protein concentration is decreased below 0.1 mg/ml [103, 106]. However, due to the strong and specific interactions during the keratin heterodimers formation, the exact sequence motifs and molecular understanding of the reaction have not been revealed.

Figure 2. 9 Schematic diagram showing assembly of keratins from heterodimeric tetramers into heterogenous full-width filaments by lateral and nearly concomitant longitudinal assembly. Reproduced from Harald Herrmann *et al.* with permission [96]. Copyright © 2004 by Annual Reviews.

Few studies have been performed to investigate the interference of keratin mutation to the fiber assembly process. A study reported by Allison K. Wilson *et al.* highlighted the importance of tails, heads, and carboxyl sequences of K5/K14 domains in filament assembly [107]. Interestingly, the tail domain of both K5 and K14 attributed to filament stabilization, while only K5 head domain was required in filament elongation and lateral alignments. The carboxyl sequences were revealed to be crucial for lateral alignment but are not obligatory for filament elongation [107]. Mutation R125H in K14 and R89C in K18 are most closely associated to a form of Epidermolysis bullosa simplex (EBS), however, these two mutations did not show any disruption on *in vitro* keratin heterodimerization assembly process [103, 108, 109]. Furthermore, mutations in epithelial K8 G62C expressed by inflammatory bowel disease patients showed significant influences of the keratin cytoskeleton assembly dynamics by forming short and disorganized filaments aggregates in epithelia cells model [99]. Filaments formed during *in vitro* polymerization appeared to be less uniform and shorter whereby small particulate aggregates was observed in K18 and mutated K8 (G62C, I63V, and K464N) [99].

References

- [1] L. Zhen and B. C. G. Mu, "The Time Literature & Art Press: Changchun," Jilin, China, 2005.
- [2] 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.
- [3] P. Hill, H. Brantley, and M. Van Dyke, "Some properties of keratin biomaterials: kerateines," *Biomaterials*, vol. 31, no. 4, pp. 585-593, 2010.
- [4] H. Lee et al., "Human hair keratin and its-based biomaterials for biomedical applications," *Tissue Engineering and Regenerative Medicine*, vol. 11, no. 4, pp. 255-265, 2014.
- [5] C. R. Robbins and C. R. Robbins, Chemical and physical behavior of human hair. *Springer*, 2002.

- [6] A. Kuzuhara, "Analysis of structural changes in bleached keratin fibers (black and white human hair) using Raman spectroscopy," *Biopolymers*, vol. 81, no. 6, pp. 506-514, 2006.
- [7] J. Schweizer et al., "New consensus nomenclature for mammalian keratins," *Int. J. Biochem. Cell Biol.*, vol. 174, no. 2, pp. 169-174, 2006.
- [8] R. Fraser, T. P. MacRae, D. Parry, and E. Suzuki, "Intermediate filaments in alpha-keratins," *Proceedings of the National Academy of Sciences*, vol. 83, no. 5, pp. 1179-1183, 1986.
- [9] H. H. Bragulla and D. G. Homberger, "Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia," *Journal of anatomy*, vol. 214, no. 4, pp. 516-559, 2009.
- [10] P. M. Steinert, L. N. Marekov, R. B. Fraser, and D. A. Parry, "Keratin intermediate filament structure: crosslinking studies yield quantitative information on molecular dimensions and mechanism of assembly," *Journal of molecular biology*, vol. 230, no. 2, pp. 436-452, 1993.
- [11] R. B. Fraser and D. A. Parry, "Molecular packing in the feather keratin filament," *Journal of structural biology*, vol. 162, no. 1, pp. 1-13, 2008.
- [12] J.-H. He, Z.-F. Ren, J. Fan, and L. Xu, "Hierarchy of wool fibers and its interpretation using E-infinity theory," *Chaos, Solitons & Fractals*, vol. 41, no. 4, pp. 1839-1841, 2009.
- [13] C. Popescu and H. Höcker, "Hair—the most sophisticated biological composite material," *Chem. Soc. Rev.*, vol. 36, no. 8, pp. 1282-1291, 2007.
- [14] R. Fraser, T. MacRae, and G. E. Rogers, "Keratins: their composition, structure and biosynthesis," 1972.
- [15] W. Crewther, R. Fraser, F. Lennox, and H. Lindley, "The chemistry of keratins," *Advances in protein chemistry*, vol. 20, pp. 191-346, 1965.
- [16] J. E. Plowman, D. P. Harland, and S. Deb-Choudhury, *The hair fibre: Proteins, structure and development*. Springer, 2018.
- [17] R. Fraser and T. MacRae, "The structure of the α -keratin microfibril," *Biosci. Rep.*, vol. 3, no. 6, pp. 517-525, 1983.

- [18] D. R. Goddard and L. Michaelis, "A study on keratin," *Journal of Biological Chemistry*, vol. 106, no. 2, pp. 605-614, 1934.
- [19] D. R. Goddard and L. Michaelis, "Derivatives of keratin," *Journal of biological chemistry*, vol. 112, no. 1, pp. 361-371, 1935.
- [20] I. O'donnell and E. Thompson, "Studies on reduced wool," *Australian Journal of Biological Sciences*, vol. 17, no. 4, pp. 973-978, 1969.
- [21] J. Maclaren, "The extent of reduction of wool proteins by thiols," *Australian Journal of Chemistry*, vol. 15, no. 4, pp. 824-831, 1962.
- [22] M. E. Furth, A. Atala, and M. E. Van Dyke, "Smart biomaterials design for tissue engineering and regenerative medicine," *Biomaterials*, vol. 28, no. 34, pp. 5068-5073, 2007.
- [23] S. Wang et al., "Culturing fibroblasts in 3D human hair keratin hydrogels," *ACS applied materials & interfaces*, vol. 7, no. 9, pp. 5187-5198, 2015.
- [24] P. Sierpinski et al., "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.
- [25] 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.
- [26] L. R. Burnett et al., "Hemostatic properties and the role of cell receptor recognition in human hair keratin protein hydrogels," *Biomaterials*, vol. 34, no. 11, pp. 2632-2640, 2013.
- [27] F. Taraballi et al., "Understanding the nano-topography changes and cellular influences resulting from the surface adsorption of human hair keratins," *Adv. Healthcare Mater.*, vol. 1, no. 4, pp. 513-519, 2012.
- [28] R. C. de Guzman, M. R. Merrill, J. R. Richter, R. I. Hamzi, O. K. Greengauz-Roberts, and M. E. Van Dyke, "Mechanical and biological properties of keratose biomaterials," *Biomaterials*, vol. 32, no. 32, pp. 8205-8217, 2011.
- [29] J. R. Richter, R. C. De Guzman, O. K. Greengauz-Roberts, and M. Van Dyke, "Structure–property relationships of meta-kerateine biomaterials derived from human hair," *Acta Biomater.*, vol. 8, no. 1, pp. 274-281, 2012.

- [30] P. Alexander and C. Earland, "Structure of wool fibres: isolation of an α -and β -protein in wool," *Nature*, vol. 166, no. 4218, p. 396, 1950.
- [31] T. Fujii, S. Takayama, and Y. Ito, "A novel purification procedure for keratin-associated proteins and keratin from human hair," *J. Biol. Macromol.*, vol. 13, no. 3, 2013.
- [32] A. Nakamura, M. Arimoto, K. Takeuchi, and T. Fujii, "A rapid extraction procedure of human hair proteins and identification of phosphorylated species," *Biol. Pharm. Bull.*, vol. 25, no. 5, pp. 569-572, 2002.
- [33] M. E. Van Dyke, C. R. Blanchard, S. F. Timmons, A. J. Siller-Jackson, and R. A. Smith, "Implantable prosthetic or tissue expanding device," ed: Google Patents, 2005.
- [34] 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.
- [35] G. Dan Mogosanu, A. Mihai Grumezescu, and M. Carmen Chifiriuc, "Keratin-based biomaterials for biomedical applications," *Current drug targets*, vol. 15, no. 5, pp. 518-530, 2014.
- [36] M. Masri and M. Friedman, "Effect of chemical modification of wool on metal ion binding," *J. Appl. Polym. Sci.*, vol. 18, no. 8, pp. 2367-2377, 1974.
- [37] M. S. Masri and M. Friedman, "Interactions of keratins with metal ions: uptake profiles, mode of binding, and effects on properties of wool," in *Protein-Metal Interactions: Springer*, 1974, pp. 551-587.
- [38] H. Y. Lai, S. Wang, V. Singh, L. T. Nguyen, and K. W. Ng, "Evaluating the antioxidant effects of human hair protein extracts," *J. Biomater. Sci. Polym. Ed.*, vol. 29, no. 7-9, pp. 1081-1093, 2018.
- [39] D. M. Kumar, P. Priya, S. N. Balasundari, G. Devi, A. I. N. Rebecca, and P. Kalaichelvan, "Production and optimization of feather protein hydrolysate from *Bacillus* sp. MPTK6 and its antioxidant potential," *Middle-East journal of scientific research*, vol. 11, no. 7, pp. 900-907, 2012.

- [40] S. Alahyaribeik and A. Ullah, "Methods of keratin extraction from poultry feathers and their effects on antioxidant activity of extracted keratin," *Int. J. Biol. Macromol.*, 2020.
- [41] T. Fujii, S. Murai, K. Ohkawa, and T. Hirai, "Effects of human hair and nail proteins and their films on rat mast cells," *Journal of Materials Science: Materials in Medicine*, vol. 19, no. 6, pp. 2335-2342, 2008.
- [42] 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.
- [43] 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.
- [44] T. Tan, C. Chia, and C. Teo, "Uptake of metal ions by chemically treated human hair," *Water Research*, vol. 19, no. 2, pp. 157-162, 1985.
- [45] K. Yamauchi, H. Hojo, Y. Yamamoto, and T. Tanabe, "Enhanced cell adhesion on RGDS-carrying keratin film," *Materials Science and Engineering: C*, vol. 23, no. 4, pp. 467-472, 2003.
- [46] K. Katoh, T. Tanabe, and K. Yamauchi, "Novel approach to fabricate keratin sponge scaffolds with controlled pore size and porosity," *Biomaterials*, vol. 25, no. 18, pp. 4255-4262, 2004.
- [47] A. Tachibana, Y. Furuta, H. Takeshima, T. Tanabe, and K. Yamauchi, "Fabrication of wool keratin sponge scaffolds for long-term cell cultivation," *J. Biotechnol.*, vol. 93, no. 2, pp. 165-170, 2002.
- [48] F. Cilurzo, F. Selmin, A. Aluigi, and S. Bellosta, "Regenerated keratin proteins as potential biomaterial for drug delivery," *Polymers for Advanced Technologies*, vol. 24, no. 11, pp. 1025-1028, 2013.
- [49] A. Tachibana, S. Kaneko, T. Tanabe, and K. Yamauchi, "Rapid fabrication of keratin-hydroxyapatite hybrid sponges toward osteoblast cultivation and differentiation," *Biomaterials*, vol. 26, no. 3, pp. 297-302, 2005.
- [50] 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.

- [51] A. L. V. Villa et al., "Feather keratin hydrolysates obtained from microbial keratinases: effect on hair fiber," *BMC Biotechnol.*, vol. 13, no. 1, p. 1, 2013.
- [52] P. Kar and M. Misra, "Use of keratin fiber for separation of heavy metals from water," *Journal of Chemical Technology and Biotechnology*, vol. 79, no. 11, pp. 1313-1319, 2004.
- [53] V. Saucedo-Rivalcoba, A. Martínez-Hernández, G. Martínez-Barrera, C. Velasco-Santos, J. Rivera-Armenta, and V. Castaño, "Removal of hexavalent chromium from water by polyurethane–keratin hybrid membranes," *Water, Air, Soil Pollut.*, vol. 218, no. 1-4, pp. 557-571, 2011.
- [54] A. Shavandi, T. H. Silva, A. A. Bekhit, and A. E.-D. A. Bekhit, "Keratin: dissolution, extraction and biomedical application," *Biomaterials science*, vol. 5, no. 9, pp. 1699-1735, 2017.
- [55] A. Vasconcelos, G. Freddi, and A. Cavaco-Paulo, "Biodegradable materials based on silk fibroin and keratin," *Biomacromolecules*, vol. 9, no. 4, pp. 1299-1305, 2008.
- [56] X. Zhao et al., "Calcium phosphate coated Keratin–PCL scaffolds for potential bone tissue regeneration," *Materials Science and Engineering: C*, vol. 49, pp. 746-753, 2015.
- [57] A. Aluigi, C. Vineis, A. Varesano, G. Mazzuchetti, F. Ferrero, and C. Tonin, "Structure and properties of keratin/PEO blend nanofibres," *Eur. Polym. J.*, vol. 44, no. 8, pp. 2465-2475, 2008.
- [58] A. A. El-Kheir, C. Popescu, S. Mowafi, M. Salama, and H. El-Sayed, "Physico-chemical properties of keratin-polyvinyl alcohol composite," *Fibers and Polymers*, vol. 16, no. 3, pp. 537-542, 2015.
- [59] L. Langbein, M. A. Rogers, H. Winter, S. Praetzel, and J. Schweizer, "The catalog of human hair keratins II. Expression of the six type II members in the hair follicle and the combined catalog of human type I and II keratins," *Journal of Biological Chemistry*, vol. 276, no. 37, pp. 35123-35132, 2001.
- [60] 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.

- [61] Y. Shimomura and M. Ito, "Human hair keratin-associated proteins," in *Journal of Investigative Dermatology Symposium Proceedings*, 2005, vol. 10, no. 3: *Elsevier*, pp. 230-233.
- [62] 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.
- [63] H. Gong et al., "An updated nomenclature for keratin-associated proteins (KAPs)," *Int. J. Biol. Sci.*, vol. 8, no. 2, p. 258, 2012.
- [64] I. Szeverenyi et al., "The Human Intermediate Filament Database: comprehensive information on a gene family involved in many human diseases," *Human mutation*, vol. 29, no. 3, pp. 351-360, 2008.
- [65] B. Ersson, L. Rydén, and J. C. Janson, "Introduction to protein purification," *Protein Purification: Principles, High Resolution Methods, and Applications*, pp. 1-22, 2011.
- [66] R. R. Burgess, "Refolding solubilized inclusion body proteins," in *Methods in enzymology*, vol. 463: Elsevier, 2009, pp. 259-282.
- [67] N. Sonenberg, K. M. Rupprecht, S. M. Hecht, and A. J. Shatkin, "Eukaryotic mRNA cap binding protein: purification by affinity chromatography on sepharose-coupled m7GDP," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 76, no. 9, pp. 4345-4349, 1979.
- [68] P. Cuatrecasas, "Protein purification by affinity chromatography derivatizations of agarose and polyacrylamide beads," *J. Biol. Chem.*, vol. 245, no. 12, pp. 3059-3065, 1970.
- [69] B. J. Takács, *Protein purification: Theoretical and methodological considerations*. Wiley Online Library.
- [70] S. K. Ratanathanawongs Williams and D. Lee, "Field-flow fractionation of proteins, polysaccharides, synthetic polymers, and supramolecular assemblies," *J. Sep. Sci.*, vol. 29, no. 12, pp. 1720-1732, 2006.
- [71] E. Urbánková, A. Vacek, and J. Chmelfík, "Micropreparation of hemopoietic stem cells from the mouse bone marrow suspension by gravitational field-flow

- fractionation," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 687, no. 2, pp. 449-452, 1996.
- [72] K. D. Caldwell, Z.-Q. Cheng, P. Hradecky, and J. C. Giddings, "Separation of human and animal cells by steric field-flow fractionation," *Cell Biophys.*, vol. 6, no. 4, pp. 233-251, 1984.
- [73] J. C. Giddings, F. J. Yang, and M. N. Myers, "Flow field-flow fractionation as a methodology for protein separation and characterization," *Anal. Biochem.*, vol. 81, no. 2, pp. 395-407, 1977.
- [74] J. C. Giddings, F. J. Yang, and M. N. Myers, "Application of sedimentation field-flow fractionation to biological particles: molecular weights and separation," *Separation Science*, vol. 10, no. 2, pp. 133-149, 1975.
- [75] P. Reschiglian, A. Zattoni, B. Roda, E. Michelini, and A. Roda, "Field-flow fractionation and biotechnology," *Trends Biotechnol.*, vol. 23, no. 9, pp. 475-483, 2005.
- [76] R. Sanz, B. Torsello, P. Reschiglian, L. Puignou, and M. Galceran, "Improved performance of gravitational field-flow fractionation for screening wine-making yeast varieties," *J. Chromatogr.*, vol. 966, no. 1-2, pp. 135-143, 2002.
- [77] L. Sun, M. Zborowski, L. R. Moore, and J. J. Chalmers, "Continuous, flow-through immunomagnetic cell sorting in a quadrupole field," *Cytometry: The Journal of the International Society for Analytical Cytology*, vol. 33, no. 4, pp. 469-475, 1998.
- [78] P. Staswick, M. Hermodson, and N. Nielsen, "Identification of the cystines which link the acidic and basic components of the glycinin subunits," *J. Biol. Chem.*, vol. 259, no. 21, pp. 13431-13435, 1984.
- [79] P. E. Staswick, M. A. Hermodson, and N. C. Nielsen, "Identification of the acidic and basic subunit complexes of glycinin," *J. Biol. Chem.*, vol. 256, no. 16, pp. 8752-8755, 1981.
- [80] M. Moreira, M. Hermodson, B. Larkins, and N. Nielsen, "Partial characterization of the acidic and basic polypeptides of glycinin," *Journal of Biological Chemistry*, vol. 254, no. 19, pp. 9921-9926, 1979.

- [81] R. Eichner, T.-T. Sun, and U. Aebi, "The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments," *Int. J. Biochem. Cell Biol.*, vol. 102, no. 5, pp. 1767-1777, 1986.
- [82] J. McLellan, S. G. Thornhill, S. Shelton, and M. Kumar, "Keratin-based biofilms, hydrogels, and biofibers," in *Keratin as a Protein Biopolymer: Springer*, 2019, pp. 187-200.
- [83] A. Rahmani Del Bakhshayesh et al., "Recent advances on biomedical applications of scaffolds in wound healing and dermal tissue engineering," *Artificial cells, nanomedicine, and biotechnology*, vol. 46, no. 4, pp. 691-705, 2018.
- [84] A. Patrucco, L. Visai, L. Fassina, G. Magenes, and C. Tonin, "Keratin-based matrices from wool fibers and human hair," in *Materials for Biomedical Engineering: Elsevier*, 2019, pp. 375-403.
- [85] F. Costa, R. Silva, and A. Boccaccini, "Fibrous protein-based biomaterials (silk, keratin, elastin, and resilin proteins) for tissue regeneration and repair," in *Peptides and Proteins as Biomaterials for Tissue Regeneration and Repair: Elsevier*, 2018, pp. 175-204.
- [86] X. Cui et al., "Freeze–thaw cycles for biocompatible, mechanically robust scaffolds of human hair keratins," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 107, no. 5, pp. 1452-1461, 2019.
- [87] S. Hamasaki, A. Tachibana, D. Tada, K. Yamauchi, and T. Tanabe, "Fabrication of highly porous keratin sponges by freeze-drying in the presence of calcium alginate beads," *Materials Science and Engineering: C*, vol. 28, no. 8, pp. 1250-1254, 2008.
- [88] M. Blessing, L. Nanney, L. King, C. Jones, and B. Hogan, "Transgenic mice as a model to study the role of TGF-beta-related molecules in hair follicles," *Genes & Development*, vol. 7, no. 2, pp. 204-215, 1993.
- [89] F. Ikkai and S. Naito, "Dynamic light scattering and circular dichroism studies on heat-induced gelation of hard-keratin protein aqueous solutions," *Biomacromolecules*, vol. 3, no. 3, pp. 482-487, 2002.
- [90] 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.

- [91] A. Aluigi et al., "Electrospinning of keratin/poly (ethylene oxide) blend nanofibers," *J. Appl. Polym. Sci.*, vol. 104, no. 2, pp. 863-870, 2007.
- [92] H. Herrmann and U. Aebi, "Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics," *Curr. Opin. Cell Biol.*, vol. 12, no. 1, pp. 79-90, 2000.
- [93] J. M. Starger, W. E. Brown, A. E. Goldman, and R. D. Goldman, "Biochemical and immunological analysis of rapidly purified 10-nm filaments from baby hamster kidney (BHK-21) cells," *Int. J. Biochem. Cell Biol.*, vol. 78, no. 1, pp. 93-109, 1978.
- [94] H. Winter et al., "Mutations in the hair cortex keratin hHb6 cause the inherited hair disease monilethrix," *Nat. Genet.*, vol. 16, no. 4, pp. 372-374, 1997.
- [95] L. D. Corden and W. McLean, "Human keratin diseases: hereditary fragility of specific epithelial tissues," *Exp. Dermatol.*, vol. 5, no. 6, pp. 297-307, 1996.
- [96] H. Herrmann and U. Aebi, "Intermediate filaments: molecular structure, assembly mechanism, and integration into functionally distinct intracellular scaffolds," *Annu. Rev. Biochem.*, vol. 73, no. 1, pp. 749-789, 2004.
- [97] D. M. Toivola, N. O. Ku, E. Z. Resurreccion, D. R. Nelson, T. L. Wright, and M. B. Omary, "Keratin 8 and 18 hyperphosphorylation is a marker of progression of human liver disease," *Hepatology*, vol. 40, no. 2, pp. 459-466, 2004.
- [98] G. M. Cavestro et al., "Association of keratin 8 gene mutation with chronic pancreatitis," *Dig. Liver Dis.*, vol. 35, no. 6, pp. 416-420, 2003.
- [99] D. Owens et al., "Human keratin 8 mutations that disturb filament assembly observed in inflammatory bowel disease patients," *J. Cell Sci.*, vol. 117, no. 10, pp. 1989-1999, 2004.
- [100] P. A. Coulombe and E. Fuchs, "Elucidating the early stages of keratin filament assembly," *Int. J. Biochem. Cell Biol.*, vol. 111, no. 1, pp. 153-169, 1990.
- [101] C.-H. Lee, M.-S. Kim, B. M. Chung, D. J. Leahy, and P. A. Coulombe, "Structural basis for heteromeric assembly and perinuclear organization of keratin filaments," *Nat. Struct. Mol. Biol.*, vol. 19, no. 7, p. 707, 2012.
- [102] R. N. Parker, K. L. Roth, C. Kim, J. P. McCord, M. E. Van Dyke, and T. Z. Grove, "Homo-and heteropolymer self-assembly of recombinant trichocytic keratins," *Biopolymers*, 2017.

- [103] H. Herrmann, T. Wedig, R. M. Porter, E. B. Lane, and U. Aebi, "Characterization of early assembly intermediates of recombinant human keratins," *J. Struct. Biol.*, vol. 137, no. 1-2, pp. 82-96, 2002.
- [104] R. D. Paladini, K. Takahashi, N. S. Bravo, and P. A. Coulombe, "Onset of re-epithelialization after skin injury correlates with a reorganization of keratin filaments in wound edge keratinocytes: defining a potential role for keratin 16," *Int. J. Biochem. Cell Biol.*, vol. 132, no. 3, pp. 381-397, 1996.
- [105] H. Herrmann and U. Aebi, "Intermediate filament assembly: fibrillogenesis is driven by decisive dimer-dimer interactions," *Curr. Opin. Struct. Biol.*, vol. 8, no. 2, pp. 177-185, 1998.
- [106] H. Herrmann, M. Häner, M. Brettel, N.-O. Ku, and U. Aebi, "Characterization of distinct early assembly units of different intermediate filament proteins," *J. Mol. Biol.*, vol. 286, no. 5, pp. 1403-1420, 1999.
- [107] A. K. Wilson, P. A. Coulombe, and E. Fuchs, "The roles of K5 and K14 head, tail, and R/KLLEGE domains in keratin filament assembly in vitro," *Int. J. Biochem. Cell Biol.*, vol. 119, no. 2, pp. 401-414, 1992.
- [108] E. Fuchs and K. Weber, "Intermediate filaments: structure, dynamics, function and disease," *Annu. Rev. Biochem.*, vol. 63, no. 1, pp. 345-382, 1994.
- [109] N.-O. Ku et al., "Keratin 8 and 18 mutations are risk factors for developing liver disease of multiple etiologies," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 10, pp. 6063-6068, 2003.

Chapter 3

Experimental Methodology

This chapter presents the detailed methodologies into three part, to 1) achieve a novel self-assembled nanofilaments network from the crude human hair keratin extracts, 2) fractionate the keratin subtypes and 3) evaluate the antioxidant properties of hair keratins. Surface morphology, proteins conformation and construct thicknesses were obtained via SEM, TEM, CD, AFM and Profilometer. Justification of the selected liquid chromatography purification techniques, characterization methods and optimization rationale are discussed. Quantification of protein and thiol concentration were obtained by colorimetric assays such as Micro BCA Assay, Bradford Assay and Ellman Assay. Gel electrophoresis combined with Silver Staining, Western blot and MALDI-ToF mass spectrometry provided information of protein identity. Lastly, PrestoBlue, PicoGreen, DPPH Assay and CellROX reagent were used to study the biological activities and antioxidant activity of our keratin solutions in vitro.

Keywords: Self-assembly; Liquid chromatography; HPLC; Western Blot; Antioxidant

3.1 Rationale for Methods

In pursuance of the stated objectives and scopes, the experimental methodology is divided into four main segments, starting from the foremost human hair proteins extraction method (Section 3.2). The two main types of proteins that are present in human hair are keratins and keratin associated proteins (KAPs). As solubilized keratins exist in helical structures, it is targeted in the adjacent section (Section 3.3), involving a unique procedure in achieving self-assembled nanofilament network and related characterization techniques.

Total hair keratin mixtures containing 17 subtypes were used in separation approaches (Section 3.4) to obtain subtypes enriched fractions. Liquid chromatography techniques including asymmetrical field flow fractionation (AFFF), Gel Permeation Chromatography (GPC) and High-Performance Liquid Chromatography (HPLC) were exploited to fractionate the two main subtypes (type I, II keratins) based on their molecular mass and isoelectric points differences.

Finally, comparison and evaluation of the radical scavenging ability of the keratins, KAPs and total hair proteins (THP, consisting of both keratin and KAPs) was conducted (Section 3.8) using DPPH and H₂DCFDA antioxidant assays. The antioxidant potential of total human hair proteins and keratins obtained from feathers has been previously demonstrated [1-3]. Herein, further in-depth understanding and comparison of the antioxidant capacity of hair keratins and hair matrix proteins is sought. Methodologies for techniques comprising calorimetry assays, proteins characterization and cell culture procedures are grouped under Sections 3.9 and 3.10.

3.2 Extraction of Human Hair Proteins

Random human hair samples sourced from local hair salons were first cleaned with soap, dried with 70% ethanol and rinsed thoroughly with water. The clean hair samples were air-dried and soaked in a mixture of chloroform and methanol (2:1 v/v) for 24 h to remove the

lipid content. Delipidized hair samples were air-dried and cut into 1 cm long fragments for subsequent Na_2S and Shindai extraction.

An established protocol by our group were used in order to obtain total hair proteins (THP) [4]. Briefly, 2.5 g of cut hair fragment were immersed in 50 ml 0.125 M Na_2S solution and incubated at 40 °C for 1 h. The extracted mixture was filtered and exhaustively dialyzed for 4 days using 10 kDa MWCO (molecular weight cut off) snakeskin cellulose tubing (Thermo Scientific) with 4 L deionized (DI) water. The mixture was then freeze and freeze-dried until fully dried and was stored in -20 °C freezer for further characterization and study.

Figure 3. 1 Flow diagram of the human hair keratin proteins extractions framework and the involved downstream applications (boarded in red).

A two-step extraction procedure reported by Fuji *et al.* was adopted to obtain KAPs and keratins separately [5]. 2.5 g of delipidized hair was incubated at 50 °C for 72 h in a solution containing 25 mM Tris HCl (pH 9.5), 2.6 M thiourea, 8 M urea, 200 mM DTT and 25 v/v% ethanol. This step is to extract the KAPs from the hair structure by suppressing the solubility of the keratins in the extraction solution. After 72 h, the KAPs free hair was filtered, washed thoroughly and air dried. Keratins extraction solution consisted of 25 mM

Tris HCl (pH 8.5), 2.6 M thiourea, 5 M urea and 200 mM DTT were used to incubate the hair at 50 °C for 24 h. Following the extraction, the extracted keratin solution was centrifuged at 13,000 rpm for 15 min and dialyzed against the required buffer solutions in a step-down dialysis manner (Section 3.3.1), in a 10 kDa MWCO cellulose tubing.

3.3 Self-assembly of Solubilized Human Hair Keratin Proteins into Intermediate Filament Networks

Several reported self-assembly protocols were adopted and modified in our human hair keratin self-assembly study, including the use of Tris-HCl and sodium phosphate buffer at neutral-basic pH conditions [6, 7]. Such protocols were commonly used in studying cytokeratin, hair recombinant keratin and type III / type V intermediate filament proteins, such as vimentin, lamin, etc. [7-9]. In this dissertation, self-assembly of human hair keratin proteins in a range of acidic condition were performed in comparison to the conventional method (Table 3.1).

3.3.1 Step Down Dialysis

The acidic buffer solution used for keratin self-assembly was prepared using citric acid (Sigma). A range of citric acid concentration included 50 mM, 2.5 mM and 0.7mM were prepared at pH 2.5, pH 2.9, pH 3.3, pH 4.5 and pH 5.5. For pH 4.5 and pH 5.5, sodium hydroxide (NaOH) was used to adjust the pH of the 0.7 mM citric acid to achieve the desired pH values. 1.25 mM hydrochloric acid (HCl) and 55.3 mM acetic acid were used to adjust the dialysis buffer to pH 2.9 in lieu of 2.5 mM citric acid. The extracted keratin solution was first dialyzed against the corresponding buffer solution consisting of 8 M urea and 1 mM DTT overnight. Moving on, the keratin solution was dialyzed against the corresponding dialysis buffer by lowering the urea concentration in a stepwise manner, from 4 M to 2 M Urea for 3 - 4 h at each step. The keratin solution was left overnight in the final dialysis buffer (0 M Urea) for complete urea removal.

Table 3. 1 Compilation of buffer composition and pH conditions used during the step-down dialysis in this study. The stability of keratins solution when added to 2.5 mM and 20 mM KCl were described either as Soluble, Precipitated or not available “-”.

pH & Buffer ^{a)}	0 mM KCl ^{b)}	2.5 mM KCl	20 mM KCl
pH 2.5 (50 mM Citric acid)	Soluble	Soluble	Soluble
pH 2.9 (2.5 mM Citric acid)	Soluble	Soluble	Soluble
pH 3.3 (0.7 mM Citric acid)	Soluble	Soluble	Soluble
pH 4.5 (0.7 mM Citric acid + NaOH)	Precipitated	-	-
pH 5.5 (0.7 mM Citric acid + NaOH)	Soluble	Soluble	Precipitated
pH 2.9 (1.25 mM Hydrochloric acid)	Precipitated	-	-
pH 2.9 (55.3 mM Acetic Acid)	Soluble	Soluble	Soluble
pH 7.3 (10 mM Tris HCl) ^{c)}	Soluble	-	-
pH 9.0 (2 mM Tris HCl) ^{d)}	Soluble	-	-
pH 7.5 (0.7 mM Sodium Phosphate) [7]	Soluble	Soluble	Soluble

^{a)} All buffer stated in table above consists of 1 mM DTT; ^{b)} This column shows the condition of keratin solution at the final stage of step-down dialysis, before introducing to any salt; ^{c)} This buffer condition will also be referred as Low Tris buffer (LT) and ^{d)} Elongation Buffer (EB) in the discussion [6].

3.3.2 Incorporation of Self-assembly Buffer

The concentration of keratin proteins after step down dialysis was obtained using the Bradford assay (Biorad). Keratin proteins were diluted to approximate 0.2 – 0.5 mg/ml with their corresponding dialysis buffers and filtered through 0.22 μ m syringe filter before

initiation of self-assembly by adding an equal volume of 5 mM KCl or 40 mM KCl dissolved in the corresponding buffer solution (hereon referred to as SA solution), for a few mins up to a few h. After incubating in the SA solution for the desired duration, the keratin solution was added to the same volume of fixing solution containing 0.2% glutaraldehyde and 2.5 mM KCl or 20 mM KCl, for 3 – 5 min, and thereon ready to be applied to the intended surface to deposit the self-assembled networks. For characterization, this was done on glow discharged carbon-coated grids and negatively stained with 2% Uranyl Acetate (UA) solution for TEM imaging (Section 3.6).

Remark:

1. **Step down dialysis buffer solution** : 0.7 - 50 mM, pH 2.3 - 5.5 citric acid
2. **Self-assembly solution (SA solution)** : 5 - 40 mM KCl dissolved in the corresponding Citric acid buffer
3. **Fixing solution** : SA solution + 0.2% glutaraldehyde

3.3.3 Formation of Self-Assembled Human Hair Keratin Networks for Cell Culture and Stability Study

The self-assembled keratin (SA-keratin) network was casted in the 24 well-plates by adding 100 μ L of SA solution followed by same volume of keratin solution (final concentration: 0.5 mg/ml). The samples were kept static for 1 h for coating deposition. For cell culture and stability test purpose, the fixation step was eliminated. The coated wells were then washed with 500 μ L DI water for 3 times and air dried in the tissue culture hood upon seeded with human dermal fibroblast (HDF) and human epidermal keratinocytes (HEK) primary cells at 2,000 cells/ well and 4,000 cells/well, respectively. Fresh media was changed after PrestoBlue assay, which was conducted every 2 days to check the cells metabolic activity. At day 5, the cells were stained with Hoechst 33342 Dye (1 μ g/mL) for 30 min to quantify the cell number. For stability study, 500 μ L of supplemented DMEM media was added into the well plates and kept at 36 °C up to 15 days, fresh media was replenished to the wells as replicates of the *in vitro* study. Immunohistostaining were conducted using cytokeratin antibody (Abcam AE13, 1:250) antibodies (Detail protocol

could be found in Section 3.3.5).

Figure 3. 2 Flow diagram of the self-assembled human hair keratin network on a 24 well plates format for cell viability study.

3.3.4 Sample Preparation for TEM, AFM Imaging and Surface Profilometer

Type A-Carbon coated grids (Ted Pella) were glow discharged for 60 seconds before used. 5 μL of keratins and/or SA-keratin solutions were loaded on the grid for 30 s followed by 4 s blotting to remove excessive solution. The grids were washed with 5 μL of DI water for 30 s followed by 4 s blotting. Finally, 5 μL of 2 % UA was used to negatively stain the keratin for 30 s, followed by a 4 s blotting. The grids were air dried for at least 15 min before storing in a desiccator overnight prior to TEM imaging (TEM Carl Zeiss LIBRA® 120, Acceleration Voltage: 120 kV, Resolution: 0.34 nm). In contrary, 4 μL of SA buffer was first dropped on a clean Silicon wafer substrate followed by an equal volume of keratins solutions. The droplets were left still for 1 h for coating deposition. The droplets were removed gently by pipetting and fixing solutions were introduced to the drop casted area for 1 min. The coated area was washed with 10 μL DI water for 3 times and left for air dry before imaging under AFM (Park Systems NX10). Similarly, surface profilometer (Alpha-Step IQ Profilometer) was used to measure the thickness of the coating in comparison with the AFM data.

The higher magnification AFM images were measured and processed by our collaborators, Miss K. Divakarla and Prof. W. Chrzanowski, from the University of Sydney, using AFM operating in ScanAsyst mode (Bruker, USA) equipped with SCOUT 350R tip (NuNano, UK). Sample scan was undertaken at various scan sizes (10 μm , 5 μm , 2.5 μm , 1 μm and 500 nm), with the 1 μm scan being used for analysis using the MountainsSPIP 8 software

(Image Metrology, Denmark).

Figure 3. 3 Schematic diagram of the staining procedures for TEM imaging.

3.3.5 Immunohistostaining for SA-keratin Coating

Immunohistochemistry (IHC) utilizes the principle of antibodies affinity (Figure 3.4) to specific antigens in biological tissues, cells or thin construct and visualizes the binding via enzymes such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP) to catalyze and produce color adducts [10]. This sensitive technique is widely used to detect the distribution and localization of target antigens. 3,3'-Diaminobenzidine (DAB) as chromogen was used to enhance signal in conjunction with HRP-based immunostaining systems. The water/alcohol insolubility and stability of the dark brown adducts derived from DAB enable long-term storage of the stained product. Hence, IHC staining technique was adopted to detect the intactness of the human hair proteins network after incubation in cell culture media up to 15 days.

The casted SA-keratin coating in 24 well-plates were first blocked in 200 µL of 10% Bovine Serum Albumin (BSA, Sigma) in PBS for 1 h. Thereafter, 100 µL of anti-hair cortex cytokeratin antibody AE13 (1:250) in 10% BSA solution were introduced to the sample wells for overnight incubation (4 °C). The wells were subsequently washed in 500 µL of tap water for 2 x 5 min followed by 5 min PBST (PBS supplemented with 0.05% Tween 20) wash. A drop of anti-mouse secondary antibody (Envision™ +/HRP) were added to

each well at room temperature for 30 min, followed by tap water and PBST wash as described previously. Color development was done by adding 100 μ L of DAB⁺ solution (prepared as per supplier's instruction) to the samples for 2 min. The reaction was stopped by introducing tap water to the wells. The stained samples were air dried and the absorbance mapping was taken at 468 nm using microplate reader (Tecan M200 Infinite).

Figure 3. 4 Schematic figure of the immunohistochemical staining reaction. Reproduced from Kim, S. W. *et al.* with permission [11]. Copyright © 2016 The Korean Society of Pathologists/The Korean Society for Cytopathology.

3.3.6 Localized Surface Plasmon Resonance Analysis (LSPR)

Surface plasmon resonance technique (SPR) emerged as a versatile and label-free optical biosensor for real time bimolecular interaction detection [12, 13]. Bond types such as protein-protein, protein-DNA, receptor-drug, lipid membrane-proteins and etc. can be determined by using such sensitive and powerful technology [14-17]. Plasmonic materials that possess negative real and small positive imaginary dielectric constant such as gold, silver, copper and aluminum exhibit plasmon resonance effect by coherently oscillating of the surface conduction electrons [18]. The conventional SPR technique utilizes bulk metal film as the sensing platform; while LSPR enhanced nanoscale metallic structures ergo both retains the sensitivity to changes of the local dielectric environment [19, 20]. In short, excitation of the conduction band of electrons in metal nanoparticles by incident light can result in a localized surface plasmon resonance (LSPR). This gives rise to a maximum

incident light intensity at certain wavelength ($\lambda_{\max}$) [21, 22]. This enables the detection of binding events along with structural changes in biomacromolecular conformation via a spectrometer (Figure 3.5) [23, 24]. Hence, LSPR was adopted to stimulate the formation of self-assembled human hair keratin networks.

The LSPR experiment was conducted on an Xnano System (Insplorion) equipped with an Xnano Sensor Chip (9.5 x 9.5 x 1 mm, SiO₂ coated). Herein, SA-keratin solution prepared in 2.5 mM citric acid buffer (pH 2.9) and 0.7 mM citric acid buffer (pH 5.5) were pumped into the LSPR system at the lowest flow rate (50 μ L/min). Once the proteins solution entered the sensor chip, the flow was paused and left for 30 min to allow deposition and equilibrium. The unbound or loosely bound proteins were then flushed off with citric acid buffer followed by 8 M urea and the shift of centroid were collected and analyzed via the Insploration Software.

Figure 3. 5 Schematic diagram of the working principle of localized surface plasmon resonance (LSPR) technique in stimulating the proteins deposition mechanism. Reproduced from Jackman, J. A. *et al.* with permission [24].

3.3.7 Circular Dichroism Spectrometry

Secondary and tertiary structure of solution-based proteins, nucleic acid, and higher structures form by association of these molecules can be explored using circular dichroism (CD) techniques. CD as a type of spectroscopic technique is essentially used to understand in comparison to native conformation, structural changes due to association/dissociation of

ligands and during unfolding/refolding of biomolecules [25]. Plane polarized light (PPL) oscillates in a single plane can be achieved by polarizing unpolarized light using Polaroid, Nicol prism and etc. [26]. Equal or unequal absorption of the polarized light component further result in characteristic CD profiles for symmetric and asymmetric molecules, respectively. For instances, α -helical proteins are known to show negative bands at 208 nm and 222 nm and a positive band at 193 nm (Figure 3.4a) [27]. Few representative intrinsic CD profiles of β -sheet, random coil (extended) and triple helix conformation are shown in Figure 3.6.

Figure 3. 6 CD spectra of polypeptides and proteins with respective intrinsic secondary structures. Poly-L-lysine in its (1, black) α -helical at pH 11.1, (2, red) antiparallel β -sheet conformations, and (3, green) extended conformations at pH 5.7 [28]. Placental collagen in its (4, blue) native triple-helical and (5, cyan) denatured forms [27, 29]. Reproduced from Greenfield, N. J. *et al.* with permission [27]. Copyright © 2007, Springer Nature.

Herein, the secondary structures of the stepwise dialyzed keratin solutions were characterized by Circular Dichroism (CD) using a Chirascan spectropolarimeter (Model 420, AVIV Biomedical Inc.). The proteins were diluted to 1 mg/mL prior to each set of CD measurements. Measurements were obtained in duplicate across wavelengths ranging from 180 to 260 nm, using a 1 nm step size and 1 nm bandwidth. Spectra were smoothed using a 2 points FFT method in Origin software.

3.4 Purification of Keratin Proteins solutions

One of the aims of this Ph.D. project is to fractionate the different keratin subtypes and conduct functional testing on their properties. In fact, type I and type II keratins possess high similarity in their molecular weights and isoelectric points. A good understanding of the protein's chemistry is crucial to identify the suitable treatment and technique for effective separation. Herein, three liquid chromatography approaches were adopted to fractionate the crude keratin extracts.

3.4.1 Purification Using Asymmetrical Field Flow Fractionation (AFFF)

Asymmetrical Field Flow Fractionation (AFFF) is a separation technique applicable to nanoparticles, supramolecular polymers, native proteins and etc. over a wide size range [30, 31]. The working principle of AFFF system depends solely on the diffusion coefficient of the macro/sub-micrometer solutes, at which smaller/lighter solutes will diffuse further away from the membrane wall, hence carried down the channel with a higher velocity [32]. In contrary, bigger/denser solutes are highly compressed against the membrane wall due to the cross flow, hence demonstrating retained elution. This gentle, noninvasive and versatile separation technique enable analysis of complex biological samples, such as proteins, nucleic acids, viruses, enzymes, subcellular units, or even whole cells [33].

Figure 3. 7 Separation principle in Asymmetrical Field Flow Fractionation. Reproduced from Müller, D. *et al.* with permission [34]. Open access source.

Postnova Multi Flow FFF (AF2000) aqueous-metal-free analytical channel, coupled with

Ultraviolet (UV) detector was used for the keratin proteins separation purpose. Freeze dried human hair keratin sample was dissolved in DI water/ 8 M urea/ 0.125 M Na₂S to make up a concentration of 4 mg/ml. The eluent comprised of 0.1 M NaCl, 0.001% SDS and 0.2% NaN₃, and must be filtered through a Millipore 0.2 µm membrane filter before subjected to the AFFF system. The system was flushed and equilibrated with running buffer for at least 20 min or until system pressure was stabilized. A sample volume 3 times greater than the injection volume (20 µL) was manually injected into the system. 10 kDa MWCO PES membrane and 500 µm spacer were equipped throughout the experiment. Injection flow and detector flow rate was set at 0.2 ml/min and 0.5 ml/min, respectively. The signal was detected at 280 nm UV wavelength. Fractions were pooled from 5 identical runs were concentrated using Pierce Protein Concentrator (5 - 20 ml), at 7000 rcf for 15 min before subjected to SDS PAGE and Coomassie staining. min

3.4.2 Purification Using Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC), is also commonly referred as size exclusion chromatography (SEC), fractionates macromolecules based on their molecular size differences [35]. Cross-linked polydextran gels with diverse pore sizes were used as stationary phase at the earlier development [36]. These gel beads swell into a three-dimensional network after immersed and equilibrated with water, serving as a molecular sieve. During a separation event, molecules of small hydrodynamic radius (low molecular weight) penetrate and retain in the gel particle pores, thus elute at a later order compared to the bigger molecules. Illustration of the separation event in a GPC column is shown in Figure 3.8.

Agilent Bio SEC-3 (100 Å, size 7.8 x 300, particle size 3 µm) obtained under Buy and Try program was coupled to Shimadzu GPC system. This column is packed with spherical, narrowly dispersed 3 µm silica particles coated with hydrophilic layers. Eluent used during the run was 100 mM NaCl, 100 mM sodium phosphate buffer (pH 7.2). The flow rate was set at 1.0 ml/min and column temperature was either set at room temperature or 60 °C. 50 µL of sample was injected into the system, and the total run time is 15 - 20 min. UV,

Refractive Index (RI) and Multi Angle Light Scattering (MALS) detectors were coupled in line with the system. ASTRA software was used to evaluate the chromatogram in order to obtain molar masses information. Due to the absence of fraction collector in this GPC system, no sample fraction was obtained.

Figure 3. 8 Illustration of the separation principle underlying gel permeation chromatography (GPC). Reproduced from Dyamenahalli, K. *et al.* with permission [37]. Copyright © 2015 Elsevier Ltd.

3.4.3 Purification Using Ultra High-Performance Liquid Chromatography (RP and IEX HPLC)

Solutes with distinct polarity or hydrophobicity can be fractionated using reverse phase (RP) chromatography technique. Mobile phases (eluent) used in this technique are usually a mixture of aqua-organic solvent at varied polarity, while the stationary phases are constructed nonpolar and hydrophobically. As protein molecules appear in denatured form in a highly hydrophobic environment, RP chromatography are suitable in analyzing or purifying peptides or proteins in denatured state [38].

Contrarily, ion exchange chromatography (IEX) is established for separation of charged molecules. Proteins of interest are absorbed to an oppositely charged matrices (stationary phase) and desorbed when eluent were introduced to neutralize the electrostatic forces [39]. Hence, pH control and salts concentration in the eluent are the crucial factors in protein separation using IEX chromatography technique.

Figure 3. 9 Diagram of Reverse-phase chromatography separation (Left) and Anion Ion exchange chromatography (Right). Reproduced from Salvato, F. *et al.* with permission [40]. Open access, IntechOpen.

Reverse phase column: Poroshell 300SB-C18 (size 2.1x 75 mm, 5 μm silica particles with a solid core and 0.25 μm thick shell of 300 Å pores), under Buy and Try program, was attached to an Agilent-1290 system. Eluent A comprised acetonitrile (ACN), 0.1% Trifluoroacetic acid (TFA) while eluent B were made of HPLC grade water and 0.1% TFA. 10 μL of sample was injected to the column at each run, at 0.5 ml/min flow rate and 3 min run time. Absorbance signal was collected at 280 nm using a UV detector. 12 fractions were collected every 0.25 min within the 3 min run, fractions over three runs were collected and concentrated using Eppendorf Concentrator Plus (20 mbar, 1,400 rpm, RT, overnight) before conducting SDS PAGE and Coomassie staining.

Ion exchange column: Weak Anion Exchange column, Agilent Bio-WAX 5 μm 300 Å (stainless steel semi-preparative column, 10 x 250 mm) was used in fractionating 20 mg/ml keratin samples. The sample was dissolved in the running buffer A, constituted of 50 mM sodium acetate, 8 M urea, 5 mM TCEP.HCl (pH 5.04), filtered with 0.22 μm syringe filter before subjected to the column. The elution buffer was prepared with 50 mM sodium acetate, 8 M urea, 5 mM TCEP.HCl, 1 M NaCl (pH 5.05). 150 μL of sample was injected into the column, and gradually eluted under a two elution-step method by introducing 0 – 17.5 mM NaCl in the first 15 min, followed by 32.5 to 100 mM NaCl for the next 15 min

and lastly, 500 mM NaCl over 20 min. 0.5 ml/min flow rate was maintained throughout the run. Signal was collected using UV detector at 280 nm and 254 nm. Six fractions of interested were collected from multiple runs and dialyzed against DI water, concentrated using CentriVac before subjected to SDS PAGE and Coomassie staining.

3.5 Gel Electrophoresis and Western Blotting

Migration and separation of charged particles/ions within an inert gel matrix under the effect of electric field is called gel electrophoresis. Agarose and polyacrylamide with variable pore sizes are widely used to produce mechanically strong and transparent gel [41, 42]. When the electric field is exerted, the reduced protein samples with different masses and mobility will migrate and separate down the gel. Subsequently, various dyes such as Coomassie Blue and Amido Black dye were used to stain and analyze the separated proteins. In order to identify or localize a more specific target component, the separated zone in the gel were transferred on a membrane matrix, made of nitrocellulose (NC) or polyvinylidene difluoride (PVDF) and perform immunoblotting [43]. Herein, the membrane matrix will be probed firstly with an antibody specific to the target proteins (primary antibody) followed with fluorescence-labeled, radiolabeled or enzyme conjugated secondary antibody for development and detection.

Figure 3. 10 Schematic diagram of gel electrophoresis, transfer, incubation, and detection. Reproduced from Trevor Henderson *et al.* with permission [44]. Materials obtained from Servier Medical Art and Noun Project.

Keratin solution was mixed with 2 μ L sample reducing agent and 5 μ L sample buffers. The protein-loading amount for Coomassie Blue staining and MALDI/TOF mass spectrometry was 20 – 30 μ g while Western blotting and Silver staining only required 1 – 2 μ g of protein. The mixed samples were heated at 100 °C for 10 min before subjected to gel electrophoresis in NuPAGE Novex 4-12% Bis-TRIS gels (MES running buffer), at 150 V for 60 min. For Western blotting, samples were run in 8% Blot Tris-Bis Gel and Bolt MOPS running buffer at 120 V for 110 min to improve bands separation. Gels were subsequently rinsed in DI water for three times and were fixed in 40% ethanol/ 10% acetic acid for 15 min or overnight before proceeding to QC Colloidal Coomassie Stain (Bio Rad) staining or Silver Staining (Pierce). After Coomassie staining, destaining was done by gently washing with DI water for 3 h for at least three water changes.

To perform Western blotting, the gel was transferred onto a NC membrane using iBlot² Dry Blotting System (20 V, 7 min) after electrophoresis. The membrane was then kept in blocking buffer made up of 5% Bovine serum albumin (BSA) dissolved in PBS containing 0.05% Tween-20 (PBST), under constant shaking for 1 h. Primary antibodies (KRT40 and AE3) against specific human hair keratin (KRT40 - 1:2000; AE3 - 1:200) were diluted in blocking buffer and incubated with the membrane overnight at 4 °C. Anti-Keratin 34 Antibody (KRT34) and Anti-AE3 antibody (AE3) will specifically stain the human hair type I and II keratins, respectively. Unbound primary antibodies were rinsed off with PBST, followed by incubation of membranes with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 in blocking buffer) for 1 h. Chemiluminescent signals were developed using the SuperSignal West Pico Chemiluminescence substrate (Thermo Scientific) and visualized using a luminescent image analyzer (ChemiDoc MP System).

3.6 Protein Quantification Assays

Human hair proteins solution was prepared under different buffer condition at an outspread concentration range (few $\mu\text{g/ml}$ to few tenth thousand $\mu\text{g/ml}$). Therefore, three protein assays were adopted to perform the quantification more appropriately. Prediluted Bovine Serum Albumin (BSA) Standards (Pierce™) were used as calibrants for all protein quantification assays.

3.6.1 BCA Assay

Pierce Bicinchonic Acid (BCA) Protein Assay Kit (Thermo Scientific) was used to obtain the human hair proteins concentration (linear range: 20–2000 $\mu\text{g/mL}$) after dialyzed in DI water. The reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid (BCA). 25 μL of proteins solution were mixed with 200 μL (triplicates) of Reagent A and reagent B (50: 1) thoroughly in 96 well plates and were incubated at 37°C for 30 min. After the incubation, purple-colored product, formed by the chelation of two molecules of BCA with one cuprous ion, will be read at 562 nm absorbance using a microplate reader (Tecan, NanoQuant).

3.6.2 MicroBCA Assay

Pierce MicroBCA Assay Kit (Thermo Scientific) was an adaptation from Pierce BCA Protein Assay Kit and has been optimized for the use with diluted proteins samples (0.5-20 $\mu\text{g/mL}$). The concentration of the extremely diluted and iota keratin proteins fractions (suspended in DI water) after purification will be tested with this ultra-sensitive assay in contrary to the previous assay. 100 μL of the collected fractions (dilute up to 200 times) were mixed well with the 100 μL of working reagent composed of Reagent A, B and C (25: 24: 1) and were incubated at 37 °C for 2 h. The absorbance of the colored adducts were read at 562 nm using a microplate reader.

3.6.3 Bradford Assay

Protein determination of Bradford assay (Bio-Rad) involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. This reagent has low interference to detergent, denaturant, basic buffer, reducing agents etc. which makes it suitable for the quantifying keratin proteins solution prepared in varied buffer conditions required in the self-assembly study. The linear range of these assays for BSA is 125 – 1,000 $\mu\text{g/ml}$. The 1x dye reagent was removed from 4°C storage and warm to ambient temperature before used. 5 μL of keratin proteins samples were mixed thoroughly with 250 μL Bradford reagent and were incubated at least 5 min at room temperature. When the Bradford dye binds to the proteins, a stable unprotonated blue adduct was formed, which absorbance can be measured at 595 nm using a microplate reader.

3.7 Thiol Quantification Assay

Pierce Ellman's Reagent (DTNB, (5,5'-dithio-bis-(2-nitrobenzoic acid))) (Thermo Scientific) reacts with sulfhydryl groups (-SH) and releases a yellow-colored product (TNB, 2-nitro-5-thiobenzoic acid). It is widely used to measure reduced cysteines and other free sulfhydryls in neutral pH solution. L-cysteine (Sigma) solution was used as calibrant in this assay (linear range: 0.25 mM to 1.5 mM). Reaction buffer (0.1M sodium phosphate 1 mM EDTA, pH 8) were first mixed thoroughly with Ellman reagent (10 mM stock) at 1:50 ratio. 250 μL of the combined solution were then added to 5 μL of proteins solution (triplicates) and incubated at room temperature for 15 min. The absorbance of the colored adducts were read at 412 nm using a microplate reader.

Figure 3. 11 Reaction mechanism of 5,5'-dithio-bis-(2-nitrobenzoic acid (DTNB) with sulfhydryl groups. Reproduced from Gromer, S. *et al.* with permission [45]. Copyright © 2002, Academic Press.

3.8 Antioxidant Assays

Free radicals present in biological systems or exogenously could cause various degenerative disorders, such as carcinogenesis, ageing, etc. [46]. Antioxidants such as lipid soluble compounds (Vitamin E, β -carotene, and coenzyme) and water-soluble compounds (ascorbic acid, N-acetylcysteine, uric acid, and bilirubin) works synergistically and interdependently on one another in a biological system [47, 48]. Thiols contain compounds are well known as free radical scavengers due to its high reactivity and prone to deprotonation. In this dissertation, DPPH and acellular H_2DCFDA antioxidant assays were used to quantify the free radical scavenging ability of the extracted human hair proteins.

3.8.1 DPPH Assay

The 2, 2 diphenyl-1-picrylhydrazyl (DPPH) is an accurate, sensitive, and stable free radical reagent soluble in organic solvent. The additional of antioxidant will reduce the DPPH molecule by hydrogen atom transfer, causing discoloration and decrease in absorbance at 515 – 528 nm, which can be utilized to evaluate antioxidant activities [49]. The principle of the antioxidant activity measured by DPPH method is shown as Figure 3.12 below. DPPH assay was conducted by adding 22 μ L of sample to 200 μ L of DPPH reagent (0.0048% w/v in methanol). After incubating in the dark at room temperature for 30 min, the absorbance was measured at 517 nm using a microplate reader. The percentage of scavenging activity was calculated using the equation as below:

$$\text{Scavenging activity percentage (\%)} = \frac{1 - (A_{\text{sample}} - A_{\text{sample background}})}{A_{\text{control}} - A_{\text{control background}}} \times 100$$

Figure 3. 12 Reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. Reproduced from Liang, N. *et al.* with permission [50]. Open access, MDPI.

3.8.2 Acellular ROS assay

The de-esterified 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) is oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of hydroxyl radicals, HRP & H₂O₂, peroxyntirite, or nitric oxide [51]. This can also be accomplished by acid- or base-catalyzed esterolysis in the absence of cells [52]. In the presence of reactive oxygen species (ROS), DCFH is rapidly oxidized to highly DCF. The mechanism of DCFDA conversion is shown in Figure 3.13. H₂DCFDA was first dissolved in methanol (0.049% w/v or equivalent to 1 mM). To perform deesterification from H₂DCFDA to DCFDA, 500 µL of the H₂DCFDA solution was added to 2 mL of 0.01 N NaOH for 30 min. Afterward, 10 mL of a 25 mM phosphate buffer at pH 7.4 was then added to the DCFDA/methanol/ NaOH solution to neutralize the combined solution. Lastly, 50 µL of this solution was added to 150 µL of the sample solution/standard solution to achieve final concentration of 10 µM DCFDA . Upon exposure to H₂O₂ and HRP mixture, the formation of DCF was quantitated spectrofluorimetrically with excitation and emission wavelengths of 490 nm and 520 nm, respectively.

Figure 3. 13 Proposed mechanism of DCFH-DA reagent adapted from Bass *et al.* [53]. DCFH-DA is deesterified to DCFH, which is oxidized to fluorescent DCF by reactive oxygen species, after entering the cells. The similar deesterification can also be achieved via chemical activation for acellular testing purpose [52]. Reproduced from Bass, D. A. *et al.* with permission [53]. Copyright © 1983, American Association of Immunologists.

3.9 Cell Culture Studies

3.9.1 Cell Viability Test of Self-assembled Keratins Nanofilaments Network

Primary Human Dermal Fibroblasts (HDFs, ATCC® PCS-201-010TM, US) were cultured in high glucose DMEM (PAN Biotech, Germany) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). Primary Human Epidermal Keratinocytes (HEKs, ATCC PCS-200-010TM, US) were cultured in serum-free EpiGRO™ Human Epidermal Keratinocyte medium (Chemicon, SCMZ-BM) supplemented with growth factors (Chemicon, SCMK001-S). The cells were cultured in a humidified environment at 37 °C and 5% CO₂ until they reached 80% confluency. HDFs at passage 7 -10 and HEKs at passage 4 – 6 were used in the experiments. Growing phase HDFs and HEKs were harvested by trypsinization (0.125% w/v) and counted using a hemocytometer. Both HDFs and HEKs were seeded in

SA-keratin coated or untreated 24-well plates at 2,000 cells/cm² density. To evaluate the proliferation activity of the HDFs and HEKs, PrestoBlue assay was performed on day 1, 3 and 5. Briefly, 150 µL of PrestoBlue reagent (1:10) in serum free media was added into each well. After 1 h of incubation at 37 °C, 100 µL of supernatant were transferred from the well to a new 96-well plate and fluorescent readings were measured at excitation and emission wavelengths of 560 nm and 590 nm. Upon completion of the PrestoBlue assay on day 5, all well were rinsed with 200 µL PBS and incubated with Hoechst 33342 (1 µg/mL) Dye for 30 min to quantify the cell counts at excitation and emission wavelengths of 350 nm and 461 nm.

3.9.2 Evaluation of the Protective Effect of Human Hair Proteins

Human Dermal Fibroblasts (HDFs, ATCC® PCS-201-010TM, US) were cultured in high glucose DMEM (PAN Biotech, Germany) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/ml streptomycin, and 10% FBS. Prior to seeding, growing phase HDFs were harvested by trypsinization (0.125% w/v), and counted using a hemocytometer. To evaluate the protective role of the hair proteins against oxidative stress, cells were treated with H₂O₂, in serum free DMEM media, and analyzed with PicoGreen and/or PrestoBlue assay. After seeding 60,000 cells/cm² cell density in 96-well plates for one day, cells were exposed to freshly prepared cell culture media containing 500 µM H₂O₂ with either keratin, KAP solution or only DMEM (negative control). 1, 4 and 24 h after H₂O₂ treatment, cell numbers in each group were determined by quantifying their metabolic activity using PrestoBlue assay first and then quantify their double strand DNA (dsDNA) using the PicoGreen assay. Briefly, 150 µL of PrestoBlue reagent (1:10) in serum free media was added into each well. After 1 h of incubation at 37 °C, 100 µL of supernatant were transferred from the well to a new 96-well plate and take absorbance reading at 570 nm. Next, all well were rinsed with 200 µL of PBS and added with 150 µl cell lysis buffer (0.005% SDS) and incubated at room temperature for at least 2.5 h. After that, 100 µL of dsDNA standards or cell lysate samples were mixed with 100 µL PicoGreen working solution in a 96-well plate. Florescent readings were measured at excitation and emission wavelengths of 480 nm and 520 nm, using a microplate reader. Relative dsDNA amount

was calculated based on the dsDNA calibration and directly correlated to cell numbers with the usage of a standard curve.

3.9.3 Oxidative Stress Induction and Protection

For ROS assay, cells were seeded at a density of 10,000 cells/cm² in a 96-well plate overnight. Cells were treated with different media as mentioned above. After 2 h of incubation, the cells were washed with PBS and loaded with CellROX Green dye (5 μ M) and Hoechst 33342 (1 μ g/mL) for 30 min. Thereafter, samples were rinsed again in PBS to remove excessive dye, before fluorescence readings were measured at excitation/emission wavelengths of 485/520 nm (CellROX Green) and 350/461 nm (Hoechst 33342), respectively, using a microplate reader. The ROS readings were normalized against cell numbers obtained by Hoechst Dye.

Alternatively, the cells were seeded on Nunc Lab-Tek II Chamber Slide System (Thermofisher Scientific) at the same cell density and underwent the similar treatments. Afterward, cells were then fixed using 4% paraformaldehyde (PFA) after staining with CellROX Green, Hoechst dye followed with PBS washes. The quantification of positively stained cells was counted manually, and the intensity of ROS signal was quantified using “Threshold color” function in Origin Software. A total of 100 cells were counted for each treatments group, which were taken from 5 – 6 frames of images.

3.10 Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF)

Protein bands were excised from the Coomassie blue stained gel and were sent to NTU SBS Proteomics and Mass Spectrometry Services for MALDI-TOF protein identification (ID) analysis. SwissProt-Human data base was used for data search.

References

- [1] H. Y. Lai, S. Wang, V. Singh, L. T. Nguyen, and K. W. Ng, "Evaluating the antioxidant effects of human hair protein extracts," *J. Biomater. Sci. Polym. Ed.*, vol. 29, no. 7-9, pp. 1081-1093, 2018.
- [2] M.-Y. Wan, G. Dong, B.-Q. Yang, and H. Feng, "Identification and characterization of a novel antioxidant peptide from feather keratin hydrolysate," *Biotechnol. Lett.*, vol. 38, no. 4, pp. 643-649, 2016.
- [3] D. M. Kumar, P. Priya, S. N. Balasundari, G. Devi, A. I. N. Rebecca, and P. Kalaichelvan, "Production and optimization of feather protein hydrolysate from *Bacillus* sp. MPTK6 and its antioxidant potential," *Middle-East journal of scientific research*, vol. 11, no. 7, pp. 900-907, 2012.
- [4] F. Taraballi *et al.*, "Understanding the nano-topography changes and cellular influences resulting from the surface adsorption of human hair keratins," *Adv. Healthcare Mater.*, vol. 1, no. 4, pp. 513-519, 2012.
- [5] T. Fujii, S. Takayama, and Y. Ito, "A novel purification procedure for keratin-associated proteins and keratin from human hair," *J. Biol. Macromol.*, vol. 13, no. 3, 2013.
- [6] J. Deek, F. Hecht, L. Rossetti, K. Wißmiller, and A. R. Bausch, "Mechanics of soft epithelial keratin networks depend on modular filament assembly kinetics," *Acta Biomater.*, vol. 43, pp. 218-229, 2016.
- [7] H. Herrmann, T. Wedig, R. M. Porter, E. B. Lane, and U. Aebi, "Characterization of early assembly intermediates of recombinant human keratins," *J. Struct. Biol.*, vol. 137, no. 1-2, pp. 82-96, 2002.
- [8] I. Hofmann, H. Herrmann, and W. W. Franke, "Assembly and structure of calcium-induced thick vimentin filaments," *Eur. J. Cell Biol.*, vol. 56, no. 2, pp. 328-341, 1991.
- [9] R. N. Parker, K. L. Roth, C. Kim, J. P. McCord, M. E. Van Dyke, and T. Z. Grove, "Homo-and heteropolymer self-assembly of recombinant trichocytic keratins," *Biopolymers*, 2017.

- [10] P. K. Nakane and G. B. Pierce Jr, "Enzyme-labeled antibodies for the light and electron microscopic localization of tissue antigens," *Int. J. Biochem. Cell Biol.*, vol. 33, no. 2, pp. 307-318, 1967.
- [11] S.-W. Kim, J. Roh, and C.-S. Park, "Immunohistochemistry for pathologists: protocols, pitfalls, and tips," *Journal of pathology and translational medicine*, vol. 50, no. 6, p. 411, 2016.
- [12] H. H. Nguyen, J. Park, S. Kang, and M. Kim, "Surface plasmon resonance: a versatile technique for biosensor applications," *Sensors*, vol. 15, no. 5, pp. 10481-10510, 2015.
- [13] S. Löfås, M. Malmqvist, I. Rönnerberg, E. Stenberg, B. Liedberg, and I. Lundström, "Bioanalysis with surface plasmon resonance," *Sensors Actuators B: Chem.*, vol. 5, no. 1-4, pp. 79-84, 1991.
- [14] M. Kim, K. Park, E.-J. Jeong, Y.-B. Shin, and B. H. Chung, "Surface plasmon resonance imaging analysis of protein–protein interactions using on-chip-expressed capture protein," *Anal. Biochem.*, vol. 351, no. 2, pp. 298-304, 2006.
- [15] J. Majka and C. Speck, "Analysis of protein–DNA interactions using surface plasmon resonance," in *Analytics of protein–DNA interactions*: Springer, 2006, pp. 13-36.
- [16] R. L. Rich *et al.*, "Kinetic analysis of estrogen receptor/ligand interactions," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 13, pp. 8562-8567, 2002.
- [17] O. L. B. Acevedo and D. Pauron, "Protein-lipid interaction analysis by surface plasmon resonance (SPR)," 2014.
- [18] D. V. Nesterenko and Z. Sekkat, "Surface plasmon sensing with different metals in single and double layer configurations," *Appl. Opt.*, vol. 51, no. 27, pp. 6673-6682, 2012.
- [19] K. L. Kelly, E. Coronado, L. L. Zhao, and G. C. Schatz, "The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment," ed: ACS Publications, 2003.
- [20] M. M. Miller and A. A. Lazarides, "Sensitivity of metal nanoparticle surface plasmon resonance to the dielectric environment," *The Journal of Physical Chemistry B*, vol. 109, no. 46, pp. 21556-21565, 2005.

- [21] A. R. Ferhan, G. J. Ma, J. A. Jackman, T. N. Sut, J. H. Park, and N.-J. Cho, "Probing the interaction of dielectric nanoparticles with supported lipid membrane coatings on nanoplasmonic arrays," *Sensors*, vol. 17, no. 7, p. 1484, 2017.
- [22] K. A. Willets and R. P. Van Duyne, "Localized surface plasmon resonance spectroscopy and sensing," *Annu. Rev. Phys. Chem.*, vol. 58, pp. 267-297, 2007.
- [23] K. M. Mayer and J. H. Hafner, "Localized surface plasmon resonance sensors," *Chem. Rev.*, vol. 111, no. 6, pp. 3828-3857, 2011.
- [24] J. A. Jackman, A. R. Ferhan, and N.-J. Cho, "Nanoplasmonic sensors for biointerfacial science," *Chem. Soc. Rev.*, vol. 46, no. 12, pp. 3615-3660, 2017.
- [25] B. Banerjee, G. Misra, and M. T. Ashraf, "Circular dichroism," in *Data Processing Handbook for Complex Biological Data Sources*: Elsevier, 2019, pp. 21-30.
- [26] K. Wilson and J. Walker, *Principles and techniques of biochemistry and molecular biology*. Cambridge university press, 2010.
- [27] N. J. Greenfield, "Using circular dichroism spectra to estimate protein secondary structure," *Nat. Protoc.*, vol. 1, no. 6, p. 2876, 2006.
- [28] N. J. Greenfield and G. D. Fasman, "Computed circular dichroism spectra for the evaluation of protein conformation," *Biochemistry*, vol. 8, no. 10, pp. 4108-4116, 1969.
- [29] H. BENTZ, H. P. BÄCHINGER, R. GLANVILLE, and K. KÜHN, "Physical Evidence for the Assembly of A and B Chains of Human Placental Collagen in a Single Triple Helix," *Eur. J. Biochem.*, vol. 92, no. 2, pp. 563-567, 1978.
- [30] S. K. Ratanathanawongs Williams and D. Lee, "Field-flow fractionation of proteins, polysaccharides, synthetic polymers, and supramolecular assemblies," *J. Sep. Sci.*, vol. 29, no. 12, pp. 1720-1732, 2006.
- [31] J. C. Giddings, F. J. Yang, and M. N. Myers, "Flow field-flow fractionation as a methodology for protein separation and characterization," *Anal. Biochem.*, vol. 81, no. 2, pp. 395-407, 1977.
- [32] J. C. Giddings, F. J. Yang, and M. N. Myers, "Application of sedimentation field-flow fractionation to biological particles: molecular weights and separation," *Separation Science*, vol. 10, no. 2, pp. 133-149, 1975.

- [33] P. Reschiglian, A. Zattoni, B. Roda, E. Michelini, and A. Roda, "Field-flow fractionation and biotechnology," *Trends Biotechnol.*, vol. 23, no. 9, pp. 475-483, 2005.
- [34] D. Müller, S. Cattaneo, F. Meier, R. Welz, and A. J. deMello, "Nanoparticle separation with a miniaturized asymmetrical flow field-flow fractionation cartridge," *Frontiers in chemistry*, vol. 3, p. 45, 2015.
- [35] S. Mori and H. G. Barth, *Size exclusion chromatography*. Springer Science & Business Media, 2013.
- [36] J. Porath, "Cross-linked dextrans as molecular sieves," in *Advances in protein chemistry*, vol. 17: Elsevier, 1963, pp. 209-226.
- [37] K. Dyamenahalli, A. Famili, and R. Shandas, "Characterization of shape-memory polymers for biomedical applications," in *Shape memory polymers for biomedical applications*: Elsevier, 2015, pp. 35-63.
- [38] K. O. Eriksson and M. Belew, "Hydrophobic interaction chromatography," *Protein purification: principles, high resolution methods, and applications*, vol. 3, pp. 165-181, 2011.
- [39] E. Karlsson and I. Hirsh, "Ion exchange chromatography," *Protein Purification: Principles, High Resolution Methods, and Applications*, pp. 93-134, 2011.
- [40] F. Salvato and A. de Lima Leite, "Strategies for protein separation," in *Integrative Proteomics*: IntechOpen, 2012.
- [41] J. L. Brunelle and R. Green, "One-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE)," in *Methods in enzymology*, vol. 541: Elsevier, 2014, pp. 151-159.
- [42] B. Johansson, "Agarose gel electrophoresis," *Scand. J. Clin. Lab. Invest.*, vol. 29, no. sup124, pp. 7-19, 1972.
- [43] B. Caballero, L. C. Trugo, and P. M. Finglas, *Encyclopedia of food sciences and nutrition*. Academic, 2003.
- [44] P. MICHELLE DOTZERT. "Southern vs Northern vs Western Blotting Techniques." Lab Manager. <https://www.labmanager.com/insights/southern-vs-northern-vs-western-blotting-techniques-854> (accessed 17 April, 2020).

- [45] S. Gromer, H. Merkle, and R. H. Schirmer, "Preparation and Inhibitor Studies," *Methods Enzymol.*, vol. 347, no. 075.687902, p. 535.00, 2002.
- [46] S. Singh and R. Singh, "In vitro methods of assay of antioxidants: an overview," *Food Rev. Int.*, vol. 24, no. 4, pp. 392-415, 2008.
- [47] M. Murthy, "Coenzyme-Q and related isoprenoid compounds: biosynthesis, regulation, functions and biomedical implications," ed: Prominent Press Scottsdale, Arizona, 2001, pp. 231-346.
- [48] B. Frei, R. Stocker, and B. N. Ames, "Antioxidant defenses and lipid peroxidation in human blood plasma," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 85, no. 24, pp. 9748-9752, 1988.
- [49] M. S. Blois, "Antioxidant determinations by the use of a stable free radical," *Nature*, vol. 181, no. 4617, pp. 1199-1200, 1958.
- [50] N. Liang and D. D. Kitts, "Antioxidant property of coffee components: assessment of methods that define mechanisms of action," *Molecules*, vol. 19, no. 11, pp. 19180-19208, 2014.
- [51] C. P. LeBel, H. Ischiropoulos, and S. C. Bondy, "Evaluation of the probe 2', 7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress," *Chem. Res. Toxicol.*, vol. 5, no. 2, pp. 227-231, 1992.
- [52] R. Cathcart, E. Schwieters, and B. N. Ames, "Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay," *Anal. Biochem.*, vol. 134, no. 1, pp. 111-116, 1983.
- [53] D. Bass, J. W. Parce, L. R. Dechatelet, P. Szejda, M. Seeds, and M. Thomas, "Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation," *The Journal of Immunology*, vol. 130, no. 4, pp. 1910-1917, 1983.

Chapter 4

Self-assembly of Solubilized Human Hair Proteins into Intermediate Filament Networks

Reassembly of the crude human hair keratin extracts was conducted in a range of pH conditions, from the isoelectric point of the keratin proteins (pH 5.5) to the more acidic extreme (pH 2.5). The conformation and morphology changes of the proteins were studied using Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) imaging techniques. This marks the first recapitulation of the self-assembly nature of chemically extracted human hair keratin proteins, which comprises a mixture of 17 subtypes. Attempts to reassemble purified keratin fractions are also presented at the end of this chapter.

Keywords: Self-assembly; Nanofilaments; Crude keratin proteins; TEM; AFM

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4.1 Introduction

In the last three decades, significant effort has been devoted to functionalize and utilize human hair keratins. Understanding the intrinsic behavior and the interactions of the solubilized human hair keratins therefore becomes pivotal in advancing their innovative applications. To that end, a top-down approach is adopted to extract the cysteine-rich intermediate filament keratin proteins from human hair and allow the reassembly of these filamentous keratin proteins. A range of pH conditions, from the isoelectric point of the keratin proteins (pH 5.5) to the more acidic extreme (pH 2.5) are explored and the conformations and morphology changes of the proteins are studied using Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) imaging techniques. Self-assembled filamentous mesh-like networks from human hair keratins is demonstrated at pH below 3.3. In contrast, conventional self-assembly (SA) protocol (neutral to basic conditions) result in the formation of sporadic random clusters. The biological properties and hydrolytic stability of the self-assembled keratin proteins coating are tested *in vitro* using primary human dermal fibroblast cells and primary human keratinocytes. The self-assembled coating remains stable up to 5 days in serum supplemented cell culture media and are demonstrated to be non-cytotoxic and support cells proliferation.

Keratins, the hidden gems confined in the abundance and readily available bio-wastes such as human hair, were produced at around 750 million kg annually [1]. These cysteine-rich proteins emerged as a novel nature-derived biomaterial and has been widely utilized in the biomedical field as sponges, 3D hydrogels, coating, thin film, particulates, hydrolyzed form, scaffold and fibers [2-6]. Although much effort has been dedicated to understand the behavior of solubilized keratin fractions from human hair, little is known about its subtype expression profiles and the interaction mechanisms among the 17 subtypes [7, 8]. The self-assembly potential of keratins have been previously reported for both epidermal and recombinant hair keratins, demonstrating the importance of crosslinking and hierarchy construction mechanism of intermediate filament proteins [9-11]. A recent study also revealed the potential for extracted hair keratins to self-assemble into fibers, although these nanostructures were much thicker than regular keratin intermediate filaments and only a

few micrometers long [12]. Unlike the extent of achievement in the microfilaments and microtubules, substantial development was stowed into the intermediate filaments groups to reveal the complex expression pattern and clinical relevant subtypes [13-16]. Herein, we report a method to induce the conformation change of keratins fiber by controlling its self-assembly process. This was demonstrably achieved through a unique combination of pH and ionic control coupled with step down dialysis process. Keratins obtained *via* Shindai method [17], without going through additional purification steps, were observed to self-assemble into filamentous meshes. This is the first successful *in vitro* attempt to recapitulate back the native assembly properties of keratin intermediate filaments from its soluble monomeric forms.

4.2 Result and Discussion

4.2.1 Assembly of Crude Human Hair Keratin Proteins Solution

Previous reports utilizing conventional self-assembly buffer (phosphate buffer and Tris buffer) have documented the reassembly and elongation process of hair recombinant and epidermal keratin proteins fiber to be governed by their different subtypes [9, 11, 18, 19]. In contrast, it was observed that the naturally extracted human hair keratins to give raise to either irregular beads (diameter > 20 nm), sparse bundle filaments (diameter ~7 nm) and/or spheroidal globular meshes (diameter < 6 nm) when subjected to Tris buffer (pH 9.0 and 7.3) and phosphate buffer (pH 7.5) respectively (Figure 4.1). Herein, this study reports the first finding in the formation of uniform self-assembled (SA) human hair keratin nanofibers achieved *via* step down dialysis in acidic condition. Two concentrations of KCl salt (2.5 mM and 20 mM) were then added to the dialyzed proteins solution for 1 h to further enhance the self-assembly activity.

The morphology and conformation changes of keratins solution are shown in Figure 4.2. Irregular beads mesh features were slowly transited into regular and elongated self-assembled nano filaments when the pH is lowered, illustrated in the schematic diagram in Figure 4.2A. Filamentous structure can be observed in the acidic condition (pH 2.5 – 3.3) prepared in 0.7 – 50 mM citric acid solution supplemented with 1 mM DTT. It is worth

noting that step down dialysis in acidic condition could directly induce the self-assembled fiber even without addition of self-assembly (SA) solution, indicating that protonation of keratins proteins plays a major role to achieve in thermodynamically favorable condition for the self-assembly process.

Figure 4. 1 Representative TEM images of SA-keratin at neutral to basic buffer condition, A) Low Tris buffer (LT) consists of 2 mM Tris HCl, 1m M DTT, pH 9.0; B) Elongation Buffer (EB) consists of 10 mM Tris-HCl, pH 7.3 and C) 0.7 mM sodium phosphate buffer consist of 1 mM DTT.

To further affirm such self-assembly event was mainly pH dependent, 1.25 mM hydrochloric acid (HCl) and 55.3 mM acetic acid were used to adjust the dialysis buffer to pH 2.9 in lieu of 2.5 mM citric acid. Prominently, HCl showed much lower buffering ability against high molarity urea as compared to citric acid and acetic acid, resulting in the precipitation of keratin proteins during dialysis and the absence of any self-assembled fibers. In contrary, the homogenous filamentous structures were observed in 55.3 mM acetic acid buffer group (Figure 4.3), showing that the similar self-assembled effect as the 2.5 mM citric acid buffer.

In this study, pH of the buffer was adjusted mainly by altering the concentration of citric acid. Among the acidic condition (pH 2.5, 2.9, 3.3 and 4.5), keratin solutions were found precipitated at pH 4.5 condition due to the pH change in the dialysis solution across its pI (Table 4.1). The precipitation issue also happened erratically at pH 3.3 during the 4 M urea dialysis step, possibly due to the unstable keratin molecules given its pI. Particularly, when the buffer pH fluttered during the step-down dialysis between pH 4.5 – 5, precipitation of the protein solution will be induced. This underlines the crucial role of monitoring the pH

changes during the gradual urea removal dialysis step in this reported self-assembly protocol.

Figure 4. 2: A) Schematic of the conformation changes of the human hair keratin proteins from irregular beads like mesh into regular and elongated self-assembled nano filaments when the pH is lowered. B) Representative TEM images of SA-keratin at different pH and concentration of KCl salt. All pH stated above were prepared with Citric acid buffer except pH 7.5, which was prepared

in 0.7 mM phosphate buffer. All SA-keratin were left for 1 h before fixation with 0.1% glutaraldehyde (scale bar: 50 nm).

Figure 4. 3 Representative TEM images of SA-keratin reconstituted in 55.3 mM acetic acid buffer (pH 2.9) at different concentrations of KCl salt.

Table 4. 1 Compilation of the acidic buffer composition, pH condition and pH changes during the step-down dialysis in this study. The stability of the dialyzing and assembled keratins solution was described either as Soluble (S), Precipitated (P) or Not Available “N/A”.

pH & Buffer	8M Urea	4M Urea	2M Urea	0M Urea	with 2.5mM KCl	with 20mM KCl
pH 2.5 (50mM Citric acid)	S (pH 4.5)	S (pH 3.5)	S (pH 3)	S (pH 2.5)	S	S
pH 2.96 (2.5mM Citric acid)	S (pH 4.6)	S (pH 3.9)	S (pH 3.3)	S (pH 2.9)	S	S
pH 3.3 (0.7mM Citric acid)	S (pH 5.5)	S/P (pH 4.5)	P (pH 4)	P (pH 3.3)	N/A	N/A
pH 4.5 (0.7mM Citric acid + NaOH)	S (pH 6.5)	S (pH 5.5)	P (pH 5)	P (pH 4.5)	N/A	N/A
pH 5.5 (0.7mM Citric acid + NaOH)	S (pH 7.5)	S (pH 6.5)	S (pH 6)	S (pH 5.5)	S	P
pH 2.98 (1.25mM Hydrochloric acid)	S (pH 6.3)	P (pH 4.5)	P (pH 3.7)	P (pH 2.9)	N/A	N/A
pH 2.98 (55.3mM Acetic Acid)	S (pH 4.2)	S (pH 3.8)	S (pH 3.44)	S (pH 2.9)	S	S

a) All buffer stated in table above consists of 1 mM DTT

4.2.2 Morphology and Conformation Changes of the Assembled Keratin Proteins

The filament average diameters derived from multiple transmission electron microscope (TEM) images, using ImageJ ($n = 200$), was illustrated in Figure 4.4. Filamentous structures' average diameters around 6 – 10 nm can be observed in the acidic condition prepared in 0.7 – 50 mM citric acid solution supplemented with 1 mM DTT. In acidic condition (without SA solution), the fiber diameters were observed to be decreased proportionally with the buffer pH, as evidenced from the average fibers' diameter shift from 9.1 nm to 6.6 nm when the buffer solution was changed from pH 3.3 to pH 2.5 (Figure 4.5). Moreover, increasing of salts concentration was most significantly observed to induce increment of fiber diameter in 0.7 mM citric acid condition (pH 3.3). This was evidenced from change of diameter observed from 9.1 nm to 10.7 nm when salt concentration was changed from 0 mM to 20 mM (Figure 4.5C). Increasing ionic strength through the introduction of salts was reported to induce lateral association of keratins filaments *in vitro* [20], albeit such bundling effect is interplayed with the filament elongation process [21]. Assembled and elongated hair keratin filaments formed using our proposed protocol are analogous to the previously reported self-assembled recombinant or purified epidermal keratin subtypes [22, 23]. Under high pH conditions, the “locking” of basic head domains in cytokeratins was previously described to inhibit proper filament assembly process [23]. Therefore, the assembly dynamic of our hair keratins was presumed to be enhanced under protonation condition at acidic environment. Furthermore, the average diameters of the keratin proteins filament subjected to both 2.5 mM citric acid and 55.3 mM acetic acid (pH 2.9) were approximately identical, at 8.6 nm and 8.9 nm respectively at the present of SA solution.

Thermodynamically stable keratin nanofilaments were able to form in acidic conditions at pH 3.3, pH 2.9 and pH 2.5, without the addition of KCl salt. This further emphasized that the self-assembly event in the current study are pH driven. A recent study by Cera, L *et al* reported the self-assembled nanostructure of recombinant K31 and K81 (in pH 8 condition) into 10 nm IFs [12]. Under the same assembly condition, this study revealed the formation

of extracted keratin into 70 nm to 100 nm wide, and a few micrometers long fibers [12]. The authors further characterized and compared the topology and cell adhesion of the coating formed by the recombinant and extracted keratins and have highlighted the better homogeneity and cellular activity supported by the former coating. In contrast, this study highlights the spontaneous and pH-dependent assembly properties of crude human hair keratins into elongated, 10 nm IFs. The morphology of the SA-keratins formed in this study is also comparable to epithelial (K8/K18) and epidermal keratins (K5/K14) subjected to pH 9 and pH 7.5 Tris-HCl buffers [22].

Figure 4. 4 Fiber diameters obtained using ImageJ software via scale bar calibration. 200 data points were taken randomly from 5 – 6 TEM images of different replicates to yield the average fiber diameters.

Bead-like structures with average diameter of 19.5 nm was observed when keratins were dialyzed at pH 5.5 possibly due to isoelectric point (keratin pI: 4.5 - 5.5) precipitation. Improved connectivity between the beads was observed upon the introduction of 2.5 mM KCl, resulting in the network fibers of flattened structure. This could be attributed to bridging salts which has been widely reported to promote protein assembly event. High ionic charge, however, have been demonstrated to perturb the charges of the protein molecules and trigger aggregation [24, 25]. This effect specifically apparent when the environments pH nearing the protein pI, in which the protein becomes more susceptible to precipitation with the reduction of the stabilizing electrostatic repulsion forces. Indeed, apparent agglomeration was observed upon addition of keratins solutions (at pH 5.5) to

high salt content SA solutions (20 mM KCl), albeit they still maintain the organized network structures.

Figure 4. 5 Measured fiber diameter of SA-keratin (n=200) in different buffer condition. (A-D) keratins self-assembled in Citric acid buffer at different pH condition, and E) in acetic acid buffer. The minimum and maximum boundary lines of each colored box indicate the 25th and 75th percentile values, respectively. The line within the box marks the mean. Whiskers (above and below each box) indicate the range within 1.5IQR. F) The effect of pH and salt condition to fiber diameter is summarized. Data presented as mean ± standard deviation (SD).

Moreover, the SA-keratin prepared in above acidic conditions were mainly composed of the alpha-helix conformed keratin proteins based on the CD profiles (Figure 4.6), which represented by two characteristic dips at 208 nm and 222 nm. Despite the ionic strength in the buffer, which manipulated by the salt concentration, the keratin proteins were dominated by alpha-helix secondary structure. It has been previously discussed that keratin proteins at pH 5.5 where highly unstable hence precipitated when subjected to a high salt condition. This was further evidenced from the CD profile (Figure 4.6D), at which no

peaks/dips were detected (20 mM KCl) indicating the absence or extremely low amount of proteins present in the solution due to the precipitation.

Figure 4. 6 Circular Dichroism profile of 0.5 mg/ml SA-keratin in different buffer conditions and salt concentrations. (A-D) Keratin self-assembled in Citric acid buffer at different pH condition, and (E) in acetic acid buffer.

4.2.3 Formation of Self-assembled 2D Coating Construct

Among the acidic condition in this study, the SA-keratin prepared in 2.5 mM citric acid (pH 2.9) showed the greatest homogeneity and consistency in term of its fiber's morphology and diameters. Hence, this condition was selected for the subsequent coating deposition and *in vitro* study. The filamentous SA-keratin formed at this condition were tantamount and translatable into network form as observed under the atomic force microscopy (AFM) in enhanced color and normal mode (Figure 4.7A). The Z-heights of the SA-keratin coating (demarked with red line) were obtained using the AFM mapping analysis. At a macroscopic scale, a rather heterogeneous distribution of the deposited coating can be observed on the silicon wafer. To ensure good observation and quantification

of fiber morphology and diameter, the images presented in Figures 4.7 were intentionally captured at sparsely coated areas. The fewer layers regions were made up of 30 nm thick SA-keratin coating, while the thickness of heavier clustered region could reach up to 200 nm (Figure 4.7B). The thickness of the SA-keratin coating was further examined using a surface profilometer, which showed comparable trend (Figure 4.7C).

Figure 4. 7 A) Representative TEM and AFM images of SA-keratin prepared in 2.5 mM citric acid buffer (pH 2.9) after initiation of self-assembly for 1 h. AFM images were presented in enhanced color mode and normal mode using XEI Software. B) Z-height profile obtained from the AFM line scan were labelled in black, blue and red, corresponded to the different KCl concentration. C) Thickness of the SA-keratin coating at varied KCl concentration, obtained from Surface Profilometer.

Nevertheless, with the kind favor of our collaborators, Miss K. Divakarla and Prof. W. Chrzanowski from the University of Sydney, the morphological details of individual fibers were captured and observed under high resolution AFM (Figure 4.8). The fibers' diameters

appeared to be wider as compared to the TEM images. SA-keratin formed without KCl salt gave the smallest fibers diameter at around 16 nm (Figure 4.8A), while 2.5 mM KCl and 20 mM KCl assembled fibers displayed fibers diameter at around 18 nm and 25 nm (Figure 4.8: B & C), respectively. The roughness and chunky features of the fibers can further be visualized from the 3D view (Figure 4.8: D-F), revealing up to 13 nm thickness at the joint region. The larger fiber diameters observed in the coating format were expected due to tip broadening effect [26] of AFM imaging and relatively longer deposition duration (1 h) during the coating preparation. This led to a greater cluster of the self-assembled filaments as compared to the fibers examined under TEM (8 – 10 nm).

Figure 4. 8 Representative AFM images of SA-keratin showing individual fibers in (A-C) normal view and (D-E) 3D view, prepared in 2.5 mM citric acid buffer (pH 2.9) after initiation of self-assembly for 1 h. High-resolution AFM analysis was carried out by Miss K. Divakarla and Prof. W. Chrzanowski from the University of Sydney.

A simple illustration is shown in Figure 4.9 (A & B) to explain the significant effect of loading duration on the deposited protein clusters (emphasized in red font). When the self-assembly was initiated for 1 h prior to TEM sample preparation, the assembled keratins

were only deposited on the TEM grids for 30 s, thus yielding a thin layer of proteins (Figure 4.9A). In contrast, to mimic the deposition of the SA-keratin coating on a Silicon wafer, the assembly procedure was conducted by mixing the keratin solution with the SA solution directly on a TEM grid (Figure 4.9B). This led to a heavily clustered fiber morphology (Figure 4.9D), which was relatable to the AFM images (Figure 4.7A)D).

Figure 4. 9 (A-B) Illustration of self-assembly procedure and duration leading to different fiber morphology. The modification was emphasized in red font. (C-D) Representative TEM images of keratin assembled via the two different methods. The SA-keratin were dialyzed in 2.5 mM citric acid buffer (pH 2.9) at the present of 20 mM KCl.

4.2.4 Kinetics of the Deposition of Self-assembled Keratin Proteins

To further convert the self-assembled keratin proteins solution into a 2D coating keratin network, the SA-keratin was deposited in 24 well plates in a static manner for 1 h (Figure 4.10A). The adsorption kinetics of the SA-keratin coating (Figure 3A) was monitored *via* Localized Surface Plasmon Resonance (LSPR) system on a 0.9 cm² Silica fused sensor chip. Keratins introduced to SA solution at pH 2.9 and pH 5.5 were observed to achieve pseudo-saturation [27, 28] by the ~22nd min (Figure 4.10: B & D), after the flow was paused.

The deposition SA-keratin coating was noted by the detected peak shift signal, which is proportional to the change in refractive index contributed by the adsorbed protein. A higher initial absorption rate was observed in SA-keratin formed at pH 2.9 in contrast to the keratin at pH 5.5, as evidenced from the steeper gradient (0.499 over 0.036, Figure A.1) captured from 10th minute onward. Such observation could be attributed to the stronger electrostatic interaction between the positively charged SA-keratin (pH 2.9) and the sensor chip surface, in contrary to the neutral charged keratin at pI condition (pH 5.5). Moreover, an increment of 0.8 nm peak shifted was observed at the saturated point, and further declined to 0.3 nm following rinsing steps which removed the loosely bound keratin. Close to 40% of the coating remained on the sensor chip at the end of the extensive flushing, suggesting its great adherent property (Figure 4.10C).

In contrast, under the same LSPR experimental set up, keratin at pH 5.5 showed a smaller peak shift upon saturation point (0.3 nm) and minimal attachment (11.1%) on the sensor chip after urea rinse (Figure 4.10E), conceivably due to the charge effect and beads-like morphology allowing lesser molecules to be bounded on the surface (complete and triplicated LSPR profiles can be found in Figure A.2). As self-assembled keratin nanofilaments formed at 2.5 mM citric acid (pH 2.9) condition showed the greatest homogeneity and consistency in terms of fiber diameter and morphology, this condition was selected as the optimum SA-keratin.

Figure 4. 10 (A) Schematic diagram showing the formation of SA-keratin coating network. (B-E) Localized Surface Plasmon Resonance (LSPR) analysis to simulate the coating deposition using (B-C) pH 2.9 SA-keratin solution and (D-E) pH 5.5 keratin solution in the absence of salt. The red arrow indicates the injection of keratins solution into the LSPR sensor region, while the blue arrows indicate the multiple rinsing steps.

4.2.5 Stability of the Deposited Self-Assembled Keratin Proteins Network

To realize the potential utilization of this SA-keratin coating in biomedical application, its stability study was conducted in DMEM cell culture media at physiological temperature (37 °C). Thus, SA-keratin coating was prepared by mixing the dialyzed keratin and SA

solution, casting the mixture in the 24-well (for 1 h) and washing it to remove salt residues (Figure 4.10A). The successful formation of this uniform and good coverage SA-keratin coating could be observed by the brown color denoting DAB positive (DAB⁺) staining of human hair cytokeratin protein marker. The DAB⁺ signals were measured to be relatively constant (Figure 4.11A) up to 5 days of incubation in DMEM cell culture media, suggesting good stability of SA-keratin coating. Significant reduction in the keratin coating stability was noted at day 8, in which the coating was found to be detached from the deposited surface, registering close to 50% reduction in the DAB⁺ signals (Figure 3D). The bright field images revealed that coating deposited at the center of the culture wells were virtually faded at day 15, as confirmed by the significant reduction in DAB⁺ signal from the mapping analysis (Figure 4.11A-B). It is worth noting that, a self-assembled 2D nanofibrous platform was fabricated using a low keratin concentration (0.5 mg/mL), demonstrating good adhesion and stability in physiological conditions. Lastly, the cytotoxicity of different cell lines on the SA-keratin coating will be presented in Section 6.3.

Figure 4. 11 A) Immunoperoxidase staining profile of SA-keratin coating in DMEM media over 15 days and B) the corresponding time course stability of coating based on absorbance mapping at 468 nm. Data presented are means $\pm$ SD at $n=3$. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, $*p<0.05$, compared to Day 0 of the corresponding sample group.

4.2.6 Self-assembly of Purified Human Hair Keratin Protein Fractions

After identifying 2.5 mM citric acid (pH 2.9) as the optimized buffer condition to assemble the human hair keratin proteins, the human hair keratin fractions purified via ion exchange HPLC (Section 5.4) were concentrated and subjected to the identical step-down dialysis. Epithelium and human hair keratin, such as K1/K10, K3/K12, K4/K13 and K31/K85, were revealed to show heterodimerization with the opposite subtypes (type I / II) for structural functionality expression [16, 29]. Although homodimerization of these keratin subtypes were also reported, there are no current evidence proving they possess distinct functionality [30]. Hence, it is encouraging to investigate the self-assembly ability of the purified keratin, which consists of different enriched keratin fractions.

Among the five individual keratin fractions, fraction 1 (F1) appeared to be a type II keratin enriched fraction, as evidence from the single band (type II) observed on the Silver stained gel and Western blot profile (see Chapter 5, Figure 5.10 & Figure 5.11), while fractions 2 – 5 consist of both type I and II keratin bands. Contrary to expectations, all purified fractions occurred as clusters of thick fibers or globules instead of the uniform nanofilamentous fibers (Figure 4.12). The diameters of these protein clusters range from 20 nm to 40 nm, albeit few ~10 nm diameter size fibers were identified, most obviously in fraction 4 (Figure 4.12: F4, indicated by red arrow). This could be due to the loss of self-assembly activity after purification, agglomeration of protein cluster after concentrating, or inadequate subtypes to induce assembly. All in all, it is intriguing and motivating to study and compare the structure and self-assembly differences of the crude human hair keratin solution and the purified fractions despite the erratic outcome.

Figure 4. 12 Representative TEM images of the five main fractions (F1 – F5) eluted from a weak-anion exchange column, after undergoing step-down dialysis in 2.5 mM citric acid buffer (pH 2.9).

More details will be discussed in the next chapter (Section 5.5) regarding an optimized protocol to acquire partially purified human hair keratin protein fractions.

4.3 Conclusion

In summary, the first self-assembly of naturally extracted human hair keratins into regular nanosized intermediate filaments network were achieved in acidic conditions ($\leq$ pH 3.3) using weak acids. A gradual decrease in the fiber's diameters from approximate 10 nm to 6 nm can be observed when the pH further reduced to pH 2.5. The SA-keratin at its optimum condition (2.5 mM citric acid, pH 2.9) were realized as biocompatible coating by depositing the proteins solution in static manner for 1 h and the coating remains stable in physiological condition up to 5 days. Furthermore, the enriched keratin fractions appeared as irregular clusters after being subjected to the identical self-assembly procedure. Altogether, this finding provides more insights into the intrinsic self-assembly potential of crude total human hair keratin extracts, and helps to pave the way for future mechanistic studies and functional advanced explorations.

References

- [1] A. Gupta, "Human hair "waste" and its utilization: gaps and possibilities," *Journal of waste management*, vol. 2014, 2014.
- [2] A. Vasconcelos and A. Cavaco-Paulo, "The use of keratin in biomedical applications," *Curr. Drug Targets*, vol. 14, no. 5, pp. 612-619, 2013.
- [3] P. Hartrianti *et al.*, "Fabrication and characterization of a novel crosslinked human keratin-alginate sponge," *J. Tissue Eng. Regen. Med.*, vol. 11, no. 9, pp. 2590-2602, 2017.
- [4] F. Taraballi *et al.*, "Understanding the nano-topography changes and cellular influences resulting from the surface adsorption of human hair keratins," *Adv. Healthcare Mater.*, vol. 1, no. 4, pp. 513-519, 2012.
- [5] 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.
- [6] X. Zhao *et al.*, "Calcium phosphate coated Keratin-PCL scaffolds for potential bone tissue regeneration," *Materials Science and Engineering: C*, vol. 49, pp. 746-753, 2015.
- [7] S. S. Adav *et al.*, "Studies on the proteome of human hair-identification of histones and deamidated keratins," *Sci. Rep.*, vol. 8, no. 1, pp. 1-11, 2018.
- [8] L. Langbein, M. A. Rogers, H. Winter, S. Praetzel, and J. Schweizer, "The catalog of human hair keratins II. Expression of the six type II members in the hair follicle and the combined catalog of human type I and II keratins," *Journal of Biological Chemistry*, vol. 276, no. 37, pp. 35123-35132, 2001.
- [9] R. Eichner, T.-T. Sun, and U. Aebi, "The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments," *Int. J. Biochem. Cell Biol.*, vol. 102, no. 5, pp. 1767-1777, 1986.
- [10] P. A. Coulombe and E. Fuchs, "Elucidating the early stages of keratin filament assembly," *Int. J. Biochem. Cell Biol.*, vol. 111, no. 1, pp. 153-169, 1990.

- [11] R. N. Parker, K. L. Roth, C. Kim, J. P. McCord, M. E. Van Dyke, and T. Z. Grove, "Homo-and heteropolymer self-assembly of recombinant trichocytic keratins," *Biopolymers*, 2017.
- [12] L. Cera *et al.*, "A bioinspired and hierarchically structured shape-memory material," *Nature Materials*, pp. 1-8, 2020.
- [13] I. Hofmann, H. Herrmann, and W. W. Franke, "Assembly and structure of calcium-induced thick vimentin filaments," *Eur. J. Cell Biol.*, vol. 56, no. 2, pp. 328-341, 1991.
- [14] L. Kreplak, U. Aebi, and H. Herrmann, "Molecular mechanisms underlying the assembly of intermediate filaments," *Exp. Cell Res.*, vol. 301, no. 1, pp. 77-83, 2004.
- [15] D. Chrétien, F. Metoz, F. Verde, E. Karsenti, and R. Wade, "Lattice defects in microtubules: protofilament numbers vary within individual microtubules," *Int. J. Biochem. Cell Biol.*, vol. 117, no. 5, pp. 1031-1040, 1992.
- [16] R. Moll, M. Divo, and L. Langbein, "The human keratins: biology and pathology," *Histochemistry and cell biology*, vol. 129, no. 6, pp. 705-733, 2008.
- [17] T. Fujii, S. Takayama, and Y. Ito, "A novel purification procedure for keratin-associated proteins and keratin from human hair," *J. Biol. Macromol.*, vol. 13, no. 3, 2013.
- [18] J. Schweizer *et al.*, "New consensus nomenclature for mammalian keratins," *Int. J. Biochem. Cell Biol.*, vol. 174, no. 2, pp. 169-174, 2006.
- [19] J. Deek, F. Hecht, L. Rossetti, K. Wißmiller, and A. R. Bausch, "Mechanics of soft epithelial keratin networks depend on modular filament assembly kinetics," *Acta Biomater.*, vol. 43, pp. 218-229, 2016.
- [20] P. A. Coulombe, O. Bousquet, L. Ma, S. Yamada, and D. Wirtz, "The 'ins' and 'outs' of intermediate filament organization," *Trends Cell Biol.*, vol. 10, no. 10, pp. 420-428, 2000.
- [21] J. Kayser, H. Grabmayr, M. Harasim, H. Herrmann, and A. R. Bausch, "Assembly kinetics determine the structure of keratin networks," *Soft Matter*, vol. 8, no. 34, pp. 8873-8879, 2012.

- [22] H. Herrmann, T. Wedig, R. M. Porter, E. B. Lane, and U. Aebi, "Characterization of early assembly intermediates of recombinant human keratins," *J. Struct. Biol.*, vol. 137, no. 1-2, pp. 82-96, 2002.
- [23] H. Herrmann, M. Häner, M. Brettel, N.-O. Ku, and U. Aebi, "Characterization of distinct early assembly units of different intermediate filament proteins," *J. Mol. Biol.*, vol. 286, no. 5, pp. 1403-1420, 1999.
- [24] B. Ozbas, J. Kretsinger, K. Rajagopal, J. P. Schneider, and D. J. Pochan, "Salt-triggered peptide folding and consequent self-assembly into hydrogels with tunable modulus," *Macromolecules*, vol. 37, no. 19, pp. 7331-7337, 2004.
- [25] L. M. Carrick, A. Aggeli, N. Boden, J. Fisher, E. Ingham, and T. A. Waigh, "Effect of ionic strength on the self-assembly, morphology and gelation of pH responsive β -sheet tape-forming peptides," *Tetrahedron*, vol. 63, no. 31, pp. 7457-7467, 2007.
- [26] D. Keller, "Reconstruction of STM and AFM images distorted by finite-size tips," *Surf. Sci.*, vol. 253, no. 1-3, pp. 353-364, 1991.
- [27] J.-P. Simonin, "On the comparison of pseudo-first order and pseudo-second order rate laws in the modeling of adsorption kinetics," *Chem. Eng. J.*, vol. 300, pp. 254-263, 2016.
- [28] Y. Liu, "New insights into pseudo-second-order kinetic equation for adsorption," *Colloids Surf. Physicochem. Eng. Aspects*, vol. 320, no. 1-3, pp. 275-278, 2008.
- [29] D. P. Harland and J. E. Plowman, "Development of hair fibres," in *The Hair Fibre: Proteins, Structure and Development*: Springer, 2018, pp. 109-154.
- [30] T. A. Smith and D. A. Parry, "Sequence analyses of Type I and Type II chains in human hair and epithelial keratin intermediate filaments: promiscuous obligate heterodimers, Type II template for molecule formation and a rationale for heterodimer formation," *J. Struct. Biol.*, vol. 158, no. 3, pp. 344-357, 2007.

Chapter 5

Purification of Crude Human Hair Keratin Proteins

In this chapter, methods to purify the human hair keratin protein subtypes are described and discussed. Three chromatography approaches, namely Asymmetrical field flow fractionation (AFFF), Gel permeation chromatography (GPC) and Reverse Phase/Ion exchange chromatography (RP/IEX) HPLC, were utilized to attain enriched keratin subtype fractions. Although AFFF emerged as a versatile and gentle fractionating technique, the separation resolution was obscured by the manifold keratin populations. Substantially, IEX HPLC technique demonstrated the greatest potential in obtaining partially enriched Type II keratin fractions. The total yield of the collected fractions was quantified using MicroBCA assay, while the identification of protein subtypes was ascertained with SDS PAGE, immunoblotting and MALDI-TOF mass spectrometry analyses.

Keywords: Asymmetrical field flow fractionation; Gel permeation chromatography; Ion exchange chromatography

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5.1 Introduction

Crude human hair keratin protein extract comprises of a mixture of 17 keratin subtypes. As cell adhesion motif, LDV sequence is present in all the type I keratins and one type II keratin (KRT82), it is intriguing to compare the property of the two keratin subtypes. Moreover, several advantages of top-down approach over recombinant technique include the abundance of starting material, low cost, and the consideration of posttranslational modification (PTM) process. Therefore, to actualize the second objective of the study, three liquid chromatography approaches, namely Asymmetrical field flow fractionation (AFFF), Gel permeation chromatography (GPC) and Reverse Phase/Ion exchange chromatography (RP/IEC) HPLC were employed to fractionate the different keratin subtypes based on the proteins' differences in molecular masses, polarity and isoelectric point, respectively. This chapter briefly describes the optimization procedure, obstacles encountered and outcome from these selected techniques. Subsequently, the most pertinent fractionation strategy and outcomes will be further discussed along with the thorough proteins' identification of the enriched keratin fractions.

5.2 Purification of Human Hair Keratin Proteins using AFFF Technique

AFFF emerges as a versatile and gentle separation technique which fractionates the analytes based on their diffusion coefficient [1, 2]. In brief, analytes encounter a parabolic flow profile after being injected into the AFFF channel in a constant laminar flow. The analytes or particles of varied size/mass will reach equilibrium along the streamline and form different diffusion layers based on their diffusion coefficients, creating velocities gradients along the channel. Smaller or lighter particles with a greater diffusion ability and/or smaller frictional force against the membrane wall will be eluted earlier as compared to the heavier analytes.

5.2.1 Optimization of Fractionating Variables on AFFF

The AFFF system's parameters control during a typical separation process include the focusing, elution and rinsing steps. A method set up window (NovaFFF AF2000 software)

showing the variables during a fractionation is shown in Figure 5.1. During the focusing step (Figure 5.1a), a backflow stream was activated to converge analyte particles at the beginning of the channel. Duration taken in this step was adjusted to ensure fully injection of analytes into the separating channel. A constant crossflow (Figure 5.1b) was then applied to generate a force that drove the analyte particles against the membrane wall. During the elution step, the focus flow was switched off and the analyte was then subjected to the crossflow and tip flow (injection flow). The crossflow strength was reduced following either linear (depicted in the slope region c in Figure 5.1) or exponential power gradients. Following that, the separation events were initiated with the course of crossflow reduction. Eventually, the crossflow was halted (Figure 5.1d) and the remaining analytes were eluted from the channel, while the system entered the final rinsing step.

Figure 5. 1: A snapshot of the method and parameters window in the NovaFFF software showing the three main steps during a fractionation, a) focus step, b) constant crossflow step, c) crossflow reduction step and d) rinse step.

Mobile phase consisted of 0.001% sodium dodecyl sulfate (SDS), 1 M sodium chloride (NaCl) and 0.2% sodium azide (NaN_3) was utilized in this study to denature and solubilized the human hair keratin proteins. Four different variables, including the injection time, crossflow rate, crossflow decline slope and crossflow decline power were studied to improve the peaks resolution detected by UV detector at 280 nm. Two distinct peaks were observed on the fractogram during the crossflow reduction phases and the final rinsing step, respectively (Figure 5.2). As it was previously discussed the separation event occurs in the crossflow reduction phases, multiple shoulders and asymmetry tailing effect were also observed in the first peak region. This indicates that the fractionated keratin proteins could be eluted within the first peak region with apparent unresolved shoulders and tailing. A simple nomenclature on the constant crossflow, crossflow reduction and rinsing duration were respectively expressed as “b, c, d” for direct interpretation purpose. The samples concentration in this study was fixed at 4 mg/ml throughout the entire study.

Figure 5. 2 Representative AFFF fractogram showing the effect of A) injection time, B) crossflow rate, C) crossflow decline slope and D) crossflow power slope on the peak resolution.

A typical AFFF fractogram includes a small void peak at the beginning of the elution phase (5 min or ~10 min in Figure 5.2), which is usually negligible. Increasing the injection time from 4 min to 8 min resulted in the significant decrease of the void peak and a relatively flat baseline at the beginning of the elution step (Figure 5.2A). However, increasing injection time did not improve resolution of the first peak. Moreover, the peak resolution was observed to be most significantly affected by the crossflow rate. The shoulders in the first peak were resolved and spread out greater when the crossflow rate was increased from 2 ml/min to 5 ml/min (Figure 5.2B). Nonetheless, longer separation duration, higher buffer consumption and peak broadening effect were experienced with the increase of crossflow rate.

The reduction in linear crossflow duration resulted in the delayed appearance of the second peak with no improvement on the resolution of the first peak observed (Figure 5.2C). In addition to the linear reduction of the crossflow, exponential functions were also tested in which the least broadening effect on the first peak (Figure 5.2D) was noted when the highest power was used (0.5). However, the first eluted fraction still appeared as an asymmetric peak without much denoting improvement. This could be due to the limitation of the AFFF technique when dealing with analytes with wide distribution and overlap of Molecular Weights (MWs), resulting in imperfect resolution of the protein fractions despite the optimized purification process.

In addition, BSA proteins were used as a comparator to estimate the mass distribution of the keratin proteins. Figure 5.3 shows two distinct peaks representing the monomeric (peak 1) and dimeric (peak 2) forms of BSA, indicating the AFFF great separation performance for analytes with discrete MW. It is worth noting that there are inherent structural differences between the globular BSA proteins and filamentous keratin proteins, and as such mass distribution comparison between the two proteins could not be achieved in a precise manner. Nonetheless, it could be approximated that the most right shoulder (16 min onward – shown by green arrowhead) of the first peak contains dimeric form of keratin

proteins (~120 kDa), which corresponds well with the second peak of the BSA proteins. This suggests that both types I and II keratin were eluted within the most left shoulder (11 – 15 min – shown by blue arrowhead) of the first peak.

Overall, increasing the crossflow rate resulted only in a slight improvement in the resolution of the first peak, and full resolved peaks were yet to be achieved. For practical purpose, 4 mg/ml crossflow rate with duration of “40, 10, 5” flow setting was utilized to collect the keratin fractions for further analysis.

Figure 5. 3 BSA proteins were used as a comparator to estimate the mass distribution of the unresolved human hair keratin proteins. Peaks 1 and 2 on the BSA proteins profile (red) corresponds to its monomer and dimer, respectively.

5.2.2 Separation Profile Stained with Coomassie Blue

In order to validate the prediction of the keratin proteins distribution, the region of interest labelled in the fractogram (Figure 5.4A) were collected from multiple runs and resolved based on their molecular weight through SDS PAGE. The resolved gel thereafter was stained with Coomassie blue to visualize the distribution of the type I and II keratin bands within the eluted fractions (Figure 5.4B). A void peak (denoted as V), which is also known as a system peak and typically appears when the flow change triggers the UV detector

signal, were detected with no protein bands on the gel (Figure 5.4B). In contrary, no visible bands were observed in fractions 1.1 to 1.3, which correspond to the left shoulder region in the first peak.

Positively stained bands at around 40 kDa were observed in fractions 1.4 to 1.7, suggesting the presence of type I keratins. Among these four fractions, fraction 1.6 was found to contain the most type I keratins band, as evidenced by the strongest intensity detected in the gel. In addition, a faint staining was noted at higher protein mass (~60 kDa), suggesting the presence of type II keratins in the fraction 1.6. Beside this sole fraction, no clear bands corresponded to the type II keratin were detected. This could probably attribute to the lower concentration of the type II keratins as compared to their type I counterpart. Notably, the detected fractions were eluted ascendingly in their molar masses. In addition to the AFFF's resolution limitation in separating a complex mixture of biological samples, the severe dilution effect after the separation should also be addressed for downstream analysis purpose [3]. AFFF technique will be beneficial in applications such as proteomic studies, protein conformation, self-dissociation and degradation of protein complexes integration studies, when coupled with mass spectrometry (MS), multi-angle light scattering detector (MALS) or MALDI-TOF-MS [4, 5].

Figure 5. 4 Fractions under the region of interest A) were collected during the separation and were desalted using protein concentrator (7000 rcf, 15 min) and subjected to B) SDS PAGE for Coomassie blue staining. RK represents the raw keratin proteins before purification, while the fractionated type I (40 kDa) and type II (55 kDa) keratin bands were demarked with the red boxes. STD represents the protein molecular weight standard.

5.3 Purification of Human Hair Keratin Proteins using GPC Technique

In addition to the AFFF, GPC technique was utilized to separate the human hair keratin proteins based on their molecular mass differences. Eluent used in this study consisted of 100 mM NaCl in 100 mM sodium phosphate buffer (pH 7.2). The keratin proteins solution was prepared in three different buffer condition, including DI water, MES buffer and 6 M urea in 50 mM sodium acetate (denotes as BA). The detected chromatogram profiles of each samples are shown in Figure 5.5 (A - C). The first peak was observed to be eluted at around 8 min, followed by a tail trailing between 10 – 12 min. Subsequently, the collected spectrums were evaluated using ASTRA software to calculate the molar mass under the peak of interest.

Figure 5. 5 Human hair keratin proteins (10 mg/ml) were dissolved in three different solvent, including A) DI water, B) MES buffer and C) BA buffer (50 mM acetate acid, 6 M urea), respectively. The molar masses of the peak of interest detected using refractive index (RI) and UV detectors were computed using ASTRA software and shown on the right of each graph. All run was conducted at room temperature and the flow rate was maintained at 0.8 ml/min.

Based on the software computation, which exploits the refractive index (RI) information of the protein samples, all peak analyzed were estimated to have mass more than 113 kDa, indicating the agglomeration of the keratin proteins. To further validate the result, comparative run was performed utilizing known protein standard, BSA and polymer standard, PEG mixture (Figure 5.6). In the standard runs, column oven was heated to 60 °C and 1.0 ml/min flow rate was applied. For BSA proteins, three regions of interested selected for data evaluation displayed molar masses of 110 kDa $\pm$ 8%, 86 kDa $\pm$ 6% and 73.8 kDa $\pm$ 2%, respectively (Figure 5.6A). The BSA proteins dimers were eluted in the first peak; however, the BSA monomeric form (60 kDa) could not be ascertained with absolute surety as both peaks 2 and 3 showed close mass approximation to the BSA monomer. Negative signal picked up by the RI detector after 10.5 min, indicating samples were fully ejected from the column, thus the subsequent peaks were disregarded.

Figure 5. 6 A) BCA proteins and B) PEG polymers were used as calibration for molar mass distribution and validation for ASTRA computation arithmetic.

In addition, a mixture of equal mass of 10 kDa and 40 kDa PEG (10 mg/ml) were used as the second reference. Three regions of interested selected for data evaluation showed molar masses of 62.5 kDa $\pm$ 2%, 33 kDa $\pm$ 1% and 10 kDa $\pm$ 3%, respectively. Notably, the first peak indicated the PEG aggregates followed by the 40 kDa PEG (second peak) and the 10 kDa PEG (third peak). The computational algorithm in ASTRA software was demonstrated to be more accurate for polymeric compound (*i.e.*, PEG) as compared to protein samples (*i.e.*, BSA or keratin). This might be due to the complexity of protein samples that involve folding and interaction among themselves and/or with the column material. A similar study by Eichner *et al.* have reported that only keratin complex of certain epithelial keratin pairs was obtained using Sephacryl S-400 column, further indicating the limitation of GPC technique in resolving complex biological samples [6].

5.4 Purification of Human Hair Keratin Proteins using RP HPLC Technique

The next separation technique in this study utilized a reverse phase (RP) column which exploits the hydrophobic interaction between the analytes and the stationary phase (alkyl chain). Less polar molecules would interact stronger with the stationary phase, resulting a longer retention time in the column. Although the both type I and II keratins share a high conservation degree of their amino acid sequence, type II keratin contains more non-polar amino acids [7]. Therefore, it is interesting to find out the extent of separation using a RP column.

A solvent peak can be observed before 0.5 min in Figure 5.7, followed by a sample peak eluted from 1.8 to 2.8 min. Fractions 8 - 10 dissected the sample peak were found to contain two visible protein bands at ~40 kDa and ~55 kDa molar mass with the strongest band intensity was noted in fraction 9 which contained the majority of the eluted protein samples. This indicates that no distinct separation was occurred. Based on this rapid assessment, it could be concluded that RP column was unable to fully separate the keratin subtypes using the current mobile phase. Moving forward, denaturant and reducing agents such as Guanidine HCl, dithiothreitol (DTT), and tris(2-carboxyethyl)phosphine (TCEP) can be added to the sample to ensure a stable keratin proteins throughout the separation [5]. Alkylation or thiol blocking agent could also improve the condition by stabilizing the highly reactive keratin proteins [8]. In separate attempt, Ion exchange chromatography (IEX) HPLC showed greater potential in fractionating the human hair keratin proteins, as such the focus and effort were shifted to this purification method.

Figure 5. 7 Twelve fractions from the separation profile A) were collected from three runs, dried using Eppendorf Concentrator Plus (20 mbar, 1,400 rpm, room temperature, overnight) and subjected to B) Coomassie blue stained after SDS PAGE. The elution buffer used in this study was 30-70% Acetonitrile with 0.1% Trifluoroacetic acid (ACN/ 10% TFA) with HPLC grade water (0.1% TFA).

5.5 Purification of Human Hair Keratin Proteins using IEX HPLC Technique

The pI range of type I and II keratin were previously presented in Section 2.3, Table 2.2. Type I keratins, also named as acidic keratins due to its relatively acidic pI ($pI < 4.8$); while type II keratins are relatively basic, possessing pI values > 5 . Thus, ion exchange chromatography was utilized to fractionate the two keratin subtypes at their pI boundary.

5.5.1 Optimization of Elution Gradient on IEX HPLC

An analytical weak anion exchange (WAX) column packed with positively charge beads which attract the negatively charge molecules was used in this study. Buffer solution consisted of 8 M urea and 50 mM sodium acetate was prepared at pH 5, which served as the isoelectric point boundary between the two keratin subtypes. Theoretically, type I keratin would be negatively charged at pH 5, while type II keratins would carry positive charge. When the salt concentration was slowly increased during the elution step, type II keratins would exit the column first due to a stronger repulsion force by the column material; while type I keratins would be eluted at a longer retention time, as a higher salt concentration was needed to disrupt the protein-column interaction.

Under the designed experimental setup, human hair keratin proteins were first eluted with a linear NaCl increment from 0 to 0.2 M concentration over the duration of 60 min. An asymmetrical peak (First peak) with a right tailing was observed from 10 - 22 min followed with two unresolved peaks (second peak) at 25 - 27 min (Figure 5.8A). Moreover, a small shoulder (third peak) was observed at 28 – 30 min. To further resolve the detected peaks, a steeper salt increment gradient (0 - 0.1M) was applied over the same period (Figure 5.8B). Forthwith, two peaks with improved resolution albeit incomplete separation were observed in the previous first peak. Moreover, the second peak appeared as an indistinct peak and the third peak remained as a small shoulder. A close-up dissection on the salt concentration in relation to the peak's appearance was conducted to further optimize the elution step. It was observed that, at salt concentration range of 37.5 - 41 mM, the first peak was eluted

(10 – 12 min) followed closely with the adjacent peak and shoulder (12 – 20 min), which corresponded to 41 - 48 mM NaCl. The third peak appeared at the NaCl concentration range of 48 - 56 mM. Lastly, the signal fell to the baseline indicating a fully elution of samples, above 60 mM NaCl (35 min onwards).

Figure 5. 8 Chromatogram of human hair keratin proteins eluted in a gradient of A) 0 – 0.2 M NaCl and B) 0 – 0.1 M NaCl over 60 min duration. The NaCl concentration was presented in red line while the red asterisk indicates the salt concentration in relation to the appearance of peaks.

Based on the previous analysis, a two-step gradient method was applied to further improve the peak profile. NaCl salt concentration was increased gradually from 0 – 17.5 mM in the first 15 min, followed by an rapid increase from 32.5 to 100 mM for the next 15 min. Lastly, 500 mM of NaCl was constantly flushed through the column to fully rinse off any remaining samples. By applying the above method, five peaks were obtained at approximately 10, 14, 27, 33 and 45 min (Figure 5.9A). This separation profile was highly reproducible and stable across different sample batches, as shown in Figure 5.9B. In order to scale up the yield of the fractions, a semi preparative WAX column was used following the optimized run method. The separation profile was found to be applicable for the semi preparative column. The five constant peaks were retained (Figure 5.9C) with slight delayed in the peaks' elution at around 11, 16, 33, 38 and 47 min. In addition, the peak 0, which was originally small and negligible in the analytical column run became magnified during the semi preparative column run. Moving on, the fractions of interest were collected

from multiple runs and concentrated for SDS PAGE, immunoblotting analysis and quantification purpose.

Figure 5.9 Chromatogram of human hair keratin proteins eluted in a A) two-step gradient method, which the salt concentration was indicated by the red line. Three representative batches of separation profile using an B) analytical column and C) semi preparative column via the two-step gradient method and the peaks were labelled from 0 – 5, respectively.

5.5.2 Silver Staining and Western Blotting

Six fractions of interest, labelled from 0 to 5 were collected from multiple runs, dialyzed in DI water, concentrated with a CentriVac device, and subjected to SDS PAGE. Two visible bands at approximately 40 kDa and 55 kDa could be observed in the silver stained profile, which represents the type I (red arrow) and type II (black arrow) keratins, respectively (Figure 5.10). However, sole type II band were visible in fraction 0 and 1, at which the band's intensity appeared the strongest among the other fractions. Both type I

and II bands were detected in fractions 2 to 5, though the fractions were collected from well resolved peaks. In addition, the intensity of the type I band present in fraction 2 was relatively weak as compare to fractions 3 and 4, while both types I and II bands in fraction 5 were extremely faint. Few visible bands were detected at molar mass lower than 28 kDa, which were corresponded to the KAP traces. To further define the detected bands, immunoblotting was performed using anti-type I and II specific antibodies, KRT34 and AE3, respectively.

Figure 5.10 Chromatograph of human hair keratin proteins separated using a semi preparative column via a two-step gradient method at pH 5. A) Six regions of interest were highlighted and labelled from 0 to 5, respectively. B) Silver stained profile of the corresponded six fractions and the type I and II bands were indicated by the red and black arrow, respectively.

Immunoblotting analysis was performed by sequentially incubating the electro-transferred membrane with AE3 antibody and followed by the KR34 antibody. Positively stained bands at around 55 kDa were observed on fractions 0 - 4 after the AE3 antibody incubation (Figure 5.11B). Few weak bands at around 45 kDa were visible in fractions 2 - 4, indicating the non-specific binding of the AE3 antibody. After introducing K34 antibody, which detects the type I keratins on the same blot, positively stained bands at around 45 kDa was only detected in fractions 3 and 4. The strongest signal of type I keratins was noted in fraction 4, while the type II keratin band showed highest intensity in fractions 0 and 2. Consistent with the silver staining profile (Figure 5.10B), fraction 5 showed no visible bands during immunoblotting, suggesting low protein concentration. Considering the

results from silver staining and the immunoblotting analysis, it can be concluded that fractions 0, 1 and 2 predominantly comprised of the type II keratins protein, while fractions 3 to 4 consisted of a mixture of both type I and II keratins. The strong affinity of the dimerized keratins was further visualized by the coexisting pairs in fractions 3 and 4. Albeit, three type II enriched fractions were obtained by utilizing the optimized two-step gradient method using a WAX column.

Figure 5. 11 Immunoblotting analysis of the six fractions, which were collected in the previous chromatogram (Figure 5.10A), using A) KRT34 and B) AE3 antibodies. The antibodies recognize the type I (yellow box) and II keratins (red box), respectively. The molecular mass of standard was indicated at the left of the panel.

5.5.3 Yield Quantification

The yield of the purified fractions obtained using analytical and semi preparative WAX columns were compared and quantified using MicroBCA assay. As fraction 0 were not collected at the usage of the analytical column, the average masses of fractions 1 to 5 were determined from three representative runs, ranging between 1.67 μg to 5.35 μg (Table 5.1A). The analytical column's bed was designed to be narrower, hence, a smaller protein amount approximately 500 μg was injected during the run to maximize the separation efficiency and to avoid blockage of the column. The yield percentage of each run was

obtained by dividing the total purified keratin mass with the injected mass. The average yield percentage obtained using the analytical column was determined to be 3.5%.

On the other hand, the average masses of fractions 0 to 5 obtained from four representative runs *via* a semi preparative column were determined to be ranging between 7.41 μg to 96.54 μg (Table 5.1B). A four times higher amount of proteins at around 2000 μg were injected in the semi preparative WAX column, providing an average yield percentage close to 11%, which was approximately 3.2 times greater than the analytical column. However, to further realize these enriched and purified keratin fractions into functional applications, an extensive up-scaling is needed.

Table 5. 1 The yield percentage of the fractions obtained via an A) analytical and B) semi preparative WAX column were compiled and compared.

A)	Frac #		0	1	2	3	4	5	Total purified keratin (μg)	Injected keratin (μg)	Yield %
	Run #										
Yield per fraction (ug)	Run 1		7.73	2.07	0.69	2.11	0.99	13.60	480	2.83	
	Run 2		2.74	2.68	0.69	1.10	1.53	8.74	480	1.82	
	Run 3		5.58	2.79	17.08	2.89	2.49	30.82	525	5.87	
Average			5.35	2.51	6.15	2.03	1.67	17.72	495.00	3.51	
Standard Deviation			2.50	0.38	9.46	0.89	0.76	11.60	25.98	2.11	

B)	Frac #		0	1	2	3	4	5	Total purified keratin (μg)	Injected keratin (μg)	Yield %
	Run #										
Yield per fraction (ug)	Run 1		8.17	22.36	47.69	28.32	41.40	29.58	177.53	1925	9.22
	Run 2		-1.58	20.70	32.19	34.39	230.79	71.23	389.30	1955	19.91
	Run 3		15.65	15.45	57.27	21.09	21.57	12.16	143.19	1955	7.32
	Run 4		0.00	1.23	7.87	8.71	92.39	11.84	122.04	1400	8.72
Average			7.41	14.94	36.26	23.13	96.54	31.20	208.01	1808.75	11.29
Standard Deviation			7.97	9.60	21.56	11.04	94.34	27.94	123.00	272.87	5.80

5.5.4 MALDI-TOF-MS Analysis

To verify the identity of the purified fractions, Coomassie blue stained bands were excised and further analyzed with MALDI-TOF-MS. To obtain sufficient sample amount for the MALDI-TOF-MS analysis, fractions collected from three individual runs were combined and 30 μg of proteins were resolved with gel electrophoresis (Figure 5.12A). The type I

(red arrow) and type II bands (black arrow) were respectively designated as “T1” and “T2”, (Figure 5.12B-C), while the different eluted fractions were denoted as F1- F5.

Figure 5. 12 A) Chromatogram showing fraction of interested collected from three different run and B) 30 μ g of each fractions were subjected to SDS PAGE and Coomassie blue staining. The red and black arrow on the right of the gel indicated the type I and II bands, respectively, while RK denoted to the unpurified raw keratins protein. C) Ten visible bands were incised and labelled accordingly before sending out for MALDI-TOF-MS analysis.

The simplified MALDI-TOF-MS data were presented in a matrix diagram (Figure 5.13) in which the proteins scores distribution of the detected human hair keratin subtypes was indicated on the right most labelling. In performing the protein identification (ID) matching *via* Swissprot database, protein score values below 30 (grey) were not be considered due to low abundancy and signal. The abundancy of the detected subtypes was presented in heat map diagram with color gradient ranged from yellow (lowest) to red (highest). Interestingly, among the 11 type I human hair keratins, only KRT31, KRT32, KRT33A, KRT33N, KRT34, and KRT35 were detected during the protein ID matching. On the other hand, all 6 type II keratins (KRT81 – KRT86) were detected. The absence of numerous type I hair keratins might be due to the low protein concentration in the fractions/bands. Samples F3T2, F4T2, F5T2 and F6T2 (black font), which correspond to the type II keratin band in the gel, displayed relatively high protein score values in the type II keratins matches. However, F1T2 and F2T2 showed none or extremely low protein scores during the analysis

due to low protein content. It is worth noting that limited resolution during gel electrophoresis process, the resolved band might be cross contaminated with the similar isotopes from the two keratins subtypes. Such cross-detection artefact was noted in samples F3T1, F4T2, F5T1, F5T2 and F6T2, in which both keratin subtypes were detected.

Figure 5. 13 Matrix diagram showing the proteins score distribution of the detected keratin subtypes (red – type II; black – type I) in the purified fractions. The lowest protein score threshold was set at 30 to ensure statistical confidence.

Several cytoskeletal keratins, which mostly originated from the shredded skin or dust contaminant, were also detected in the full MALDI-TOF-MS spectra with low score values (Table 5.2). Despite considerable differences on the abundance of the same keratin group in various fractions were not observed, contingency of different keratin complexes/pairs were eluted during the separation process should not be renounced. As MALDI-TOF-MS technique might not be adequate in analyzing complex and minute samples [9]. In addition,

the repeatability and quality of these MALDI-TOF-MS data would need to be validated before any clear conclusion could be made. Additionally, liquid chromatography mass spectrometry (LCMS) analysis could also be utilized in conjunction with immunoblot analysis to further resolve the fractions ID prior to the MALDI-TOF-MS analysis.

Table 5. 2 MALDI-TOF analysis of the purified keratin proteins fractions. The protein identification was performed by Swissprot database search and the protein scores were compile as below.

Samples/ Protein Name (MW)	F1T2	F2T2	F3T1	F3T2	F4.1	F4T2	F5T1	F5T2	F6T1	F6T2
Keratin, type I cuticular Ha1/ KRT31 (48.632 kDa)			240		724	84	342	18	576	204
Keratin, type I cuticular Ha2/ KRT32 (51.79 kDa)			75		105	71	72		95	88
Keratin, type I cuticular Ha3-I/ KRT33A (47.17 kDa)			177		486	104	341	27	340	183
Keratin, type I cuticular Ha3-II/ KRT33B (47.33 kDa)			221		595	82	308	22	513	205
Keratin, type I cuticular Ha4/ KRT34 (50.82 kDa)			94		191	71	119		145	89
Keratin, type I cuticular Ha5/ KRT35 (51.64 kDa)			89		101	66	78		85	83
Keratin, type II cuticular Hb1/ KRT81 (56.83 kDa)			32	420		430	22	78		330
Keratin, type II cuticular Hb2/ KRT82 (57.984 kDa)				40		53				
Keratin, type II cuticular Hb3/ KRT83 (55.92 kDa)		24	38	424		409	19	90		301
Keratin, type II cuticular Hb4/ KRT84 (65.941kDa)				43		53		18		
Keratin, type II cuticular Hb5/ KRT85 (57.31 kDa)		19	21	206		184	17	64		112
Keratin, type II cuticular Hb6/ KRT86 (55.12 kDa)		24	40	433		433	23	91		322
Keratin, type II cytoskeletal 1/ KRT1 (66.17 kDa)			27							
Keratin, type II cytoskeletal 2/ KRT2 (65.67 kDa)				25						
Keratin, type II cytoskeletal 8/ KRT8 (53.671 kDa)		18								
Keratin, type II cytoskeletal 9/ KRT9 (62.25 kDa)										19
Keratin, type I cytoskeletal 13/ KRT13 (49.90 kDa)					44		29		45	

Keratin, type I cytoskeletal 15/ KRT15 (49.41 kDa)					45		29			
Keratin, type I cytoskeletal 20/ KRT20 (48.51kDa)									11	
Keratin, type I cytoskeletal 28/ KRT28 (51.16kDa)	13									
Keratin, type II cytoskeletal 79/ KRT28 (58.08kDa)										19
Keratin-associated protein 21-2/ KRTAP21-2 (9.58kDa)	18									
DNA replication factor Cdt1 (60.92 kDa)		19								
WAP four-disulphide core domain protein 1 (24.76 kDa)		21	19	48			34	41		30
T-complex protein 1 subunit gamma (61.07 kDa)										
Putative keratin-87 protein/ KRT87P (29.55 kDa)			25	98		78	26	44		60
Putative zinc finger protein 826 (20.851kDa)	22									
KH domain-containing, RNA-binding, signal transduction-associated protein (48.31 kDa)					22	19	28		36	22
Mitochondrial coiled-coil domain protein 1 OS=Homo (13.3kDa)	25	32								
Intermediate filament family orphan 2 (57.805kDa)	25	31						24		
Kinesin-like protein KIF7 OS=Homo sapiens GN=KIF (151.4kDa)	14									
Blood vessel epicardial substance OS=Homo sapiens (41.937kDa)	17	29						19		
Serine/threonine-protein kinase RIO2 OS=Homo (63.69kDa)							17	18		

5.5 Conclusion

In conclusion, fractionation with pI differences were demonstrated to be the most effective approach to purify chemically extracted human hair keratin proteins that possess 17 keratin subtypes that are highly complex and similar in nature. Adopting a weak ion exchange column at pH 5 condition, type II keratins enriched fractions and several complex fractions were acquired with overall yield of 11.3%. Although distinct clear separation of the two keratin subtypes could not be achieved due to the immensely dimerized state of the keratin proteins, the enriched fractions obtained using the proposed protocol were anticipated to resemble the bioactivity and structural properties of the native human hair keratin proteins.

References

- [1] J. C. Giddings, F. J. Yang, and M. N. Myers, "Flow field-flow fractionation as a methodology for protein separation and characterization," *Anal. Biochem.*, vol. 81, no. 2, pp. 395-407, 1977.
- [2] J. C. Giddings, F. J. Yang, and M. N. Myers, "Application of sedimentation field-flow fractionation to biological particles: molecular weights and separation," *Separation Science*, vol. 10, no. 2, pp. 133-149, 1975.
- [3] G. Yohannes, S. K. Wiedmer, E. K. Tuominen, P. K. Kinnunen, and M.-L. Riekkola, "Cytochrome c-dimyristoylphosphatidylglycerol interactions studied by asymmetrical flow field-flow fractionation," *Anal. Bioanal. Chem.*, vol. 380, no. 5-6, pp. 757-766, 2004.
- [4] P. Staswick, M. Hermodson, and N. Nielsen, "Identification of the cystines which link the acidic and basic components of the glycinin subunits," *J. Biol. Chem.*, vol. 259, no. 21, pp. 13431-13435, 1984.
- [5] G. Bobe, D. C. Beitz, A. E. Freeman, and G. L. Lindberg, "Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography," *J. Agric. Food Chem.*, vol. 46, no. 2, pp. 458-463, 1998.

- [6] R. Eichner, T.-T. Sun, and U. Aebi, "The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments," *Int. J. Biochem. Cell Biol.*, vol. 102, no. 5, pp. 1767-1777, 1986.
- [7] I. Szeverenyi *et al.*, "The Human Intermediate Filament Database: comprehensive information on a gene family involved in many human diseases," *Human mutation*, vol. 29, no. 3, pp. 351-360, 2008.
- [8] J. A. Bietz, "Separation of cereal proteins by reversed-phase high-performance liquid chromatography," 1983.
- [9] M. Hamdan and P. G. Righetti, "Modern strategies for protein quantification in proteome analysis: advantages and limitations," *Mass Spectrom. Rev.*, vol. 21, no. 4, pp. 287-302, 2002.

Chapter 6

Cellular Responses to Human Hair Proteins

Antioxidative properties of total human hair proteins, keratins and keratin associated proteins (KAPs) as free radical scavengers are investigated using DPPH and H₂DCFDA assays. Thereafter, the effect of a thiol blocking agent, N-Ethylmaleimide (NEM), on keratins is further discussed. Furthermore, the radical scavenging ability of the purified keratin fractions are explored using DPPH assay. The biocompatibility and toxicity of the self-assembled keratin coating were tested with primary human dermal fibroblasts (HDFs) and human epidermal keratinocytes (HEKs). Lastly, the protective effect of HDFs from hydrogen peroxide induced oxidative stress are demonstrated in human hair keratin supplemented media.

Keywords: Antioxidant; Free radical scavengers; Thiols; Cell toxicity; Oxidative stress

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6.1 Introduction

Human hair keratins and KAPs are well recognized for their intrinsically high cysteine content, registering close to 9-10% in the extracted human hair proteins [1, 2]. This high cysteine content results in formation of strong intermolecular disulfide bonds that confer hair its outstanding mechanical strength. More importantly, the presence of cysteine residues, which are highly reductive and able to scavenge free radicals [3], have been attributed to antioxidative properties of keratin proteins. In separate studies, chicken feather keratin hydrolysates have been reported to possess antioxidant and antibacterial activities [4, 5]. Additionally, Wan *et al.* demonstrated the free radical scavenging and Fe^{2+} chelation properties of novel antioxidant peptides obtained from chicken feather keratin [6]. Most recently, our group has successfully demonstrated the antioxidative potential of human hair proteins that is capable of effecting bioactive influences through its antioxidizing capacity [7]. However, little is known about the contribution of the various human hair proteins to the antioxidizing ability. In this chapter free radical scavenging abilities of total human hair proteins (THP), keratin proteins and keratin associated proteins (KAPs) is demonstrated in a comparative study utilizing DPPH and H_2DCFDA assays. Additionally, the antioxidant properties of the previous purified keratin fractions are presented. Cellular responses to these proteins as well as the keratin potential utilization as media supplement is discussed in the final section of this chapter.

The cysteine content of the extracted keratins and KAPs were analyzed using Ellman's assay and correlate to the radical scavenging power. The DPPH radical scavenging ability of the human hair proteins were determined *via* discoloration of the dark violet DPPH reagent (Abs: 517 nm). The scavenging capacities on hydroxyl radical, produced in the present of HRP and hydrogen peroxide (H_2O_2), were investigated using chemically de-esterified dichlorofluorescein (DCFH) reagent through the formation of fluorescent dichlorofluorescein (DCF) adducts (Ex: 490 nm and Em: 520 nm). Comparison of the three human hair proteins is first discussed, followed by the discussion on the purified fractions.

6.2 Evaluation of Antioxidant Properties of Human Hair Proteins

6.2.1 Radical Scavenging Ability of Different Human Hair Proteins

Based on the amino acid analysis, KAPs were classified as high sulfur (contains 20 mol% cysteines residues) and ultra-high sulfur proteins (contains >30 mol% of cysteines residues) [8, 9]. In comparison, keratin was reported to contain approximately 9.3 mol% of cysteine residues, registering half of the amount being detected in KAPs [10, 11]. In this study, the THP (Na₂S extracted) was found to contain merely 1.86 mol% of free thiols content, while free thiols content in keratin and KAP (Shindai method extracted) were determined to be 19.37 and 17.33 mol%, respectively (Table 6.1). The low cysteines amount in THP samples could be attributed to the harsh extraction procedure using strong reducing agent, Na₂S, at high pH condition, which could cause the proteins chain hydrolysis and oxidation during the downstream dialysis step [12]. The hydrolyzed fragments of the THP could be observed in the smeary and higher background signal on the SDS PAGE resolved gel (Figure 6.1) as opposed to the keratin lane at the same protein loading. Moreover, the discrepancy of the KAP cysteine content obtained in our study could be attributed to the long extraction duration (72 h), extensive dialysis and handling procedures, which could inevitably oxidize the active thiol groups. Nevertheless, when excessive NEM was introduced to the keratin solution (denotes as Keratin-NEM), the free thiol percentage of the keratin was significantly reduced to 1.11mol%, indicating successful thiol capping process.

Table 6. 1 Protein and thiol concentration of THP, keratin and KAPs solutions obtained using BCA assay and Ellman assay. The mole percent of free thiols available in these samples were calculated by normalizing the thiol content with the protein content.

Assays (unit) Samples	Total keratin concentration (μ M)	Total free thiol concentration (mM)	Mole percentage of free thiol available in keratin (mol%)	Average Mole percentage of free thiol available in keratin (mol%)
THP 1	446.47	0.88	1.96	1.86 $\pm$ 0.11
THP 2	551.55	1.03	1.86	
THP 3	641.95	1.12	1.74	
Keratin 1	506.66	10.04	19.81	19.37 $\pm$ 0.47
Keratin 2	506.78	9.81	19.36	
Keratin 3	504.80	9.52	18.87	
KAP 1	365.50	7.24	19.80	17.33 $\pm$ 2.41

KAP 2	390.50	6.73	17.23	
KAP 3	445.00	6.66	14.97	
Keratin -NEM 1	247.990	0.340	1.3707	1.11 ± 0.23
Keratin -NEM 2	239.619	0.239	0.9978	
Keratin -NEM 3	211.877	0.200	0.9458

Figure 6. 1 SDS PAGE profile of keratin associated proteins (KAP), keratins and total hair proteins (THP) stained with Coomassie Blue Dye.

L-cysteine (L-cys) was used as an antioxidant comparator to the THP, keratin, and KAPs. Figure 6.2 shows the percentage of DPPH scavenging activity of each sample groups at concentration ranging from 1.18 μM to 200 μM . The average half maximal inhibitory concentrations (IC_{50}), which measure the potency of a substance in inhibiting free radicals, were summarized in Figure 6.2F. The IC_{50} values for L-cys and THP were determined to be 573.3 μM and 488.9 μM , respectively. While keratin and KAP exhibited lower IC_{50} values of 112.6 μM and 168.8 μM , respectively, indicating a higher DPPH scavenging efficiency as compared to L-cys and THP. In comparison, Shirwaikar *et al.* measured IC_{50} values of 354 μM and 126.94 μM for the antioxidant effects of ascorbic acid and berberine,

which is an isoquinoline alkaloid present in clinically medicinal plants [13]. This indicates the superiority of our chemically extracted keratin as stronger free radical scavengers. To further prove the cysteine groups role in scavenging the free radicals, NEM was used to chemically block the free thiols present in keratin (keratin-NEM group). The scavenging effect of keratin-NEM were not detected in the designed concentration range, giving strong evidence to the cysteines' pivotal role in scavenging the DPPH radical.

Figure 6.2 DPPH radical scavenging activity of A) L-cysteine, B) total hair proteins, C) keratin, D) KAP and E) keratin-NEM. F) The IC₅₀ values of these samples were estimated with DoseResp model.

To further analyze and compare the antioxidant capacity of human hair proteins as free radical scavengers, a fluorescent probe, DCFH, was introduced to the Horseradish Peroxidase (HRP) - H₂O₂ mixtures and the quenching effect of the oxidation of DCFH was studied (Figure 6.2). HRP catalyze the oxidation of DCFH probes into fluorescent DCF adduct by facilitating the electron transfer with H₂O₂ [14]. Keratin, keratin-NEM and KAP solutions at respective concentration of 3.6 μM, 9 μM and 20 μM were introduced to the DCFH-HRP-H₂O₂ mixtures with H₂O₂ concentration ranging from 0.65 μM to 1000 μM.

The signal obtained when water/buffer was added in the DCFH-HRP-H₂O₂ mixtures (denotes as vehicle control) were set to be 100% for a better data visualization purpose. The concentration dependent scavenging effect was observed in all three proteins groups, as evidence from the reduction of DCF signal in concomitant with increase of proteins concentration. Among the tested groups, keratin showed the most significant free radical quenching effect at 3.6 μ M when tested against the increasing H₂O₂ concentrations. This was evidenced from the lowest DCF signal being detected (9.9% - 21.8%) in keratin group as compared to KAP (20% - 34.5%) and keratin-NEM (38.6% - 47.8%). Notably, 20 μ M keratin dampened 92.6% of DCF signal in the present of 1000 μ M H₂O₂, while the same concentration of KAP and keratin-NEM treatment only yielded 90.5% and 80.5% reduction, respectively. Interestingly, it was noted that the keratin-NEM at 3.6 μ M, 9 μ M and 20 μ M tested against the highest H₂O₂ concentration (1000 μ M) could reduce the DCF signals by 52.2%, 65% and up to 80%, respectively. This suggests H₂O₂ scavenging activity of the human keratin proteins could not be solely attributed to their cysteine residues. Highly oxidizable amino acid containing aromatic (Trp, Tyr, Phe) or nucleophilic sulfur (Met) groups could contribute to the scavenging effect as they could facilitate hydrogen radical transferred [3, 7].

Figure 6. 3 The quenching effect of keratin (blue), keratin-NEM (green) and KAP (orange) proteins on DCF signal induced by the present of 0.2 μM HRP and elevated H_2O_2 concentration. Three protein concentrations at 3.6 μM , 9 μM and 20 μM were introduced in this study. Data presented are means $\pm$ SD at $n=3$. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, $*p<0.05$, compared between the different concentration groups.

6.2.2 Radical Scavenging Ability of Purified Human Hair Keratin Fractions

As reported in the previous chapter (Section 5.5), a two-elution steps method was established to resolve six individual peaks from the crude human hair keratin extracts. Immunoblotting analysis revealed the presence of type II keratins in fractions 0, 1 and 2, (Figure 6.4). Considering the potential application of these purified fractions, the antioxidant activity of the fractions at concentration of 18.18 μM (equivalent to 0.9 mg/ml) were examined using DPPH assay. The DPPH signal reduction is comparable to the strength of radical scavenging reactions attributed to the previously discussed antioxidants, *i.e.*, keratin, and KAP (Table 6.2).

Figure 6. 4 A) Chromatogram of human hair keratins showing the six fractions of interested, labelled from 0 to 5. B) Immunoblot of the collected fractions against KRT34 and AE3 proteins markers, which indicate the type I and II keratin bands, respectively. Panel A and B have been presented in the previous chapter as Figure 5.10 & 5.11 and are presented here to facilitate discussion in this chapter.

Comparison between the various human hair proteins and L-cys at the same concentration revealed that keratin produced the greatest signal reduction at 13.42%, while KAP, THP and L-cys showed 3.21%, 1.69%, and 2.86% of DPPH signal reduction, respectively (Table 6.2). This keratin radical scavenging capacity was being maintained even after the purification process, as evidenced by similar level of DPPH signal reduction (ranging between 10% to 15.7%) being detected across the 5 fractions. Although early evidence indicated the preservation of the keratins protein's bioactivity after fractionation, it is worth noting that the purified fractions data presented were obtained from one sample group due to limited amount. As such further validation would need to be done to strengthen the claim.

Table 6. 2 DPPH signal reduction induced by L-Cys (n=3), human hair proteins (n=3) and purification keratins (n=1) at fixed concentration of 18.18 μ M.

Category	Samples	Reduction %
Standard	L-Cys	2.86 ± 0.33
Human Hair Proteins	Keratin	13.42 ± 2.29
	KAP	3.21 ± 0.26
	THP	1.69 ± 4.23

Purified Fractions (keratin)	Frac 1	15.75
	Frac 2	15.51
	Frac 3	11.35
	Frac 4	10.67
	Frac 5	14.99

6.3 Cellular Response to Self-assembled 2D human hair keratin proteins network

The formation of coating using SA-keratin was previously discussed in Chapter 4.3.3. The cytotoxicity of the SA-keratin coating was tested on HDF and HEK primary cells over the course of 5 days. The metabolic activity (Figure 6.5: A & C) of both the cell types grown on the coating was found to be comparable to the control group (cells grown in non-coated surface). The bright field images showing morphology of the HDF and HEK adhering on the SA-keratin coating at day 5 (Figure 6.5:B & D), indicating positive cell-material interaction. Moreover, extracellular matrix (ECM) proteins, fibronectin was found to be expressed in both HDF and HEK, as evidenced from the strong green signal near the cell membranes (Figure 6.6). Although the fibronectin expression of the HDF and HEK cells did not show any significant differences (Figure 6.6C), an increase in the vinculin intensity (Figure 6.6D) was noted between the control and the coating groups. This indicated the cell adhesion of both HDF and HEK were enhanced on the SA-keratin coatings in comparison to the control. Keratin-derived biomaterials have been reported to facilitate wound healing and in more recent studies have been demonstrated to possess antioxidant properties [6, 7, 15-18]. Improved performance of keratin platform such as thin film or coating fabricated with such homogeneous and regular fibers network is hypothesized due to a better adhesion property (Chapter 4.3.4). These preliminary results indicate the casted coating have great potential to be applied in biomedical or cosmetic related applications.

Figure 6. 5 Biocompatibility of SA-keratin coating (pH 2.9) was demonstrated for (A-B) HDF and (C-D) HEK cells. Metabolic activity of A) HDF and C) HEK over the course of 5 days obtained using PrestoBlue Assay. Bright field images showing the respective cell morphology of B) HDF and D) HEK grown on SA-keratin coating on day 5.

Figure 6. 6 Representative immunofluorescent images of A) HDF and B) HEK grown on SA-keratin coating (pH 2.9). ECM protein fibronectin (green) and focal adhesion protein vinculin (green) was visualized with immunofluorescence. The actin network and nuclei were stained red and blue, respectively. The staining intensities of C) fibronectin and D) vinculin expressed by HDF and HEK were quantified using ImageJ. Scale bar: 25μm.

6.4 Cellular Response to Human Hair Proteins

The antioxidant properties of human hair proteins, keratin and KAP, as free radical scavengers have been previously demonstrated. Moving on, the potential of human hair proteins as antioxidant for *in vitro* application was investigated using primary HDF cells. The cytotoxicity of human hair proteins at a range of concentrations was first tested over three time points, before adopting them as media supplement to protect the HDF cells from H₂O₂ induced oxidative stress.

6.4.1 Proliferation and Viability Test

Keratin and KAP solutions were diluted to 9 μ M, 20 μ M and 40 μ M concentration with culture media and were incubated with HDF cell lines for 1, 4 and 24 h. PrestoBlue and PicoGreen assays were used to analyze the metabolic activity and cellular number of the cells, respectively. A significant increment of metabolic activities was observed in 20 μ M and 40 μ M keratin treatment groups within 1 h of incubation (Figure 6.7A), while significant reduction in metabolic activity was noted following 4 and 24 h treatment of 40 μ M KAP. The reduction in the metabolic activity was not reflected in the cell quantification with PicoGreen assay in which both treatment groups across all concentrations and time points showed no significant difference as compared to control (Figure 6.7B). Phase contrast images (Figure 6.7C) showed the morphology of the HDF was not affected by the treatment. However, precipitation of KAP in the cell culture medium was noted to be significant, as more dark color patches appeared with the increased of KAP concentration. This precipitation could potentially interfere with the cellular activity and contribute to the reduction in metabolic activity detected within this treatment group (Figure 6.7A). In contrast, keratin proteins did not demonstrate the same extent of precipitation at the same concentration range. This could be owing to the relatively lower molecular weight (~10 kDa) of KAP thus constituting more protein molecules at the same concentration, as compared to keratin (~50 kDa). The dialyzed keratin also presented a more stable suspension form when interacting with the cultured cells, as compared to KAP. Thus, keratin treatment groups were further evaluated in the following sections.

Figure 6.7 A) Metabolic activity and B) cell number of HDF treated with keratin and KAP at varied concentration over 1h, 4h and 24h. C) Representative phase contrast images showing morphology of HDF cells after 24 h treatment. Data presented are means $\pm$ SD at $n=3$. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, $*p<0.05$, compared to 0 μM of the corresponding time point groups.

6.4.2 Oxidative Stress Induction and Protection Effect of Keratin treatment

To assess the keratin protective effect against the oxidative stress, HDF cells were co-treated with 500 μM H_2O_2 and keratin solution (9 μM , 20 μM and 40 μM) up to 24 h. This H_2O_2 concentration were chosen based on previous optimization [7], at which the treatment groups protective effect was most significantly demonstrated. In this study, antioxidant NAC (1 mM) was utilized as a positive control. After the introduction of 500 μM H_2O_2 for 1 h, the metabolic activity of the HDF without any treatment dropped drastically to 60% (Figure 6.8A). However, cotreatment of keratin and H_2O_2 resulted in significant recovery

of proliferation rate that was consistently observed in all three keratin concentration treatment groups. Among the tested concentration groups, 40 μM keratin demonstrated the greatest protective effect against oxidative stress, as evidenced from the recovery of metabolism rate that is comparable to the negative control and positive control (1 mM NAC). Consistent with previous result (Figure 6.7), keratin-treated group exhibited elevated metabolism rate as the keratin concentration increased, indicated the ability in improving proliferation. This observation was persisted up to 24 h and was not detected in the NAC treatment group.

The protective effect of keratin was gradually diminished after 4 h and completely vanished by 24 h. When treated with keratin, the HDF metabolic activity declined to 50% subjected to the prolonged treatment of 500 μM H_2O_2 , though PicoGreen assay data revealed similar cell counts at 4 h (Figure 6.8B). The cells condition could be extremely unhealthy at this point and not able to sustain normal biological activity thus showing the reduction in metabolic signal. This was evidenced from the rounded and contracted cell morphology as observed in the negative control, 9 μM and 20 μM keratin groups (Figure 6.9) under exposure of 500 μM H_2O_2 . The rounded cells were eventually detached, leading to a significant decrease in cell numbers at 24 h. In contrast, NAC-treated group (positive control) showed great protective effect on the cells up to 24 h, as observed by the proliferation rate and cell numbers that were comparable to the negative control group.

Figure 6.8 A) Metabolic activity and B) cell numbers of HDF in response to 500 μM H_2O_2 and designed treatment groups. Data presented are means $\pm$ SD at $n=4$. Comparison of means was done using one-way ANOVA with Tukey's HSD post hoc test, * $p < 0.05$, compared to Ctr corresponded to 0 μM and 500 μM H_2O_2 at each timing point, respectively.

Figure 6. 9 Representative bright field images of HDF cell morphology after introduction to various treatments for 4 h.

Furthermore, the ROS levels induced in the HDFs were evaluated using the CellROX reagent which exhibits bright green photostable fluorescence upon oxidation by ROS and subsequently bind to DNA. After 2 h of H₂O₂ exposure, the normalized intracellular ROS level increased by 1.5-fold as compared to the non-treatment group (Figure 6.10A). It was

observed that the ROS signal induced by 500 μM H_2O_2 was most significantly reduced in 40 μM keratin treatment group. This further strengthen the dose dependent effect of keratin solution in exerting its protective and antioxidant properties. The cell number of this study was quantified using Hoechst 33342 dyes, as presented in Figure 6.10B. A similar increased in the cell quantity can be observed in the 40 μM keratin treatment group, which in good agreement with the PicoGreen analysis (Figure 6.9B). Albeit, some basal level of intracellular ROS signal was captured in the negative control (non- treatment group) as well as the keratin and NAC treatment groups. This was evidenced from the ROS intensity collected from 100 cells signals analyzed with ImageJ “Color Threshold” function (Figure 6.11A) [19]. HDF cells treated with 1 mM NAC and 40 μM keratins were stained with CellROX and Hoechst 33342 fluorescent probes, and the representative images (merged channel) are presented in Figure 6.11B. When HDF cells were exposed to 500 μM H_2O_2 without any treatment, sharp bluish-green signal localized in the cell nucleus were observed, indicating the elevated oxidative stress in the cells. In contrast, the ROS signal were quenched significantly when the cells were cotreated with 1 mM NAC and 40 μM keratin. This further supports the effectiveness of 40 μM keratin in protecting the cells from oxidative stress in the form of media supplement.

Figure 6. 10 A) Normalized ROS signal and B) cell numbers of HDF in response to 500 μM H_2O_2 and designed treatment groups. Data presented are means $\pm$ SD at $n=4$. Comparison of means was done using one-way ANOVA with Tukey’s HSD post hoc test, *# $p<0.05$, compared to Ctr (no keratin treatment) corresponded to 0 μM and 500 μM H_2O_2 , respectively.

Figure 6. 11 A) ROS intensity collected from 100 HDF cells using ImageJ software “Color Threshold” function. The intensity reading was gathered from 5 - 6 frames of images. B) Live HDF cells were stained with CellROX (green) and Hoechst 33342 (blue) fluorescent dyes and fixed for imaging purpose. Images from two channels were merged using ImageJ software (See Figure A.3 for complete set of images). Data presented are means $\pm$ SD at $n=100$. Comparison of means was done using one-way ANOVA with Tukey’s HSD post hoc test, *# $p<0.05$, compared to Ctr corresponded to 0 μM and 500 μM H_2O_2 , respectively.

6.5 Conclusion

In summary, the antioxidant properties of human hair proteins, keratin and KAP were investigated using various *in vitro* approaches. The DPPH radical scavenging capacity of keratin was revealed to be slightly greater than KAP, giving an IC_{50} value of 122.6 μM compared to 168.8 μM for KAP. Similarly, keratin showed superior effectiveness in quenching DCF signal in comparison to KAP. This outcome was unanticipated due to the intrinsically higher cysteine content in KAP (~30mol%) than keratin proteins (~10mol%). Regardless, KAP was not adopted in the eventual cellular study due to its high aggregation in cell culture medium. Moreover, the free radical scavenging ability of the enriched keratin fractions were found to be preserved in spite of the extensive purification and concentrating procedures. Biocompatibility of the novel SA-keratin coating was also reported, showing minimal cytotoxic effects and expression of the ECM protein, fibronectin. Subsequently, the antioxidant effect of keratin as media supplements was demonstrated on HDFs exposed

to H₂O₂ induced oxidative stress. It was revealed that 40 µM keratin was able to sustain high cell proliferation and survival rate in an acute oxidative stress environment.

References

- [1] P. Hill, H. Brantley, and M. Van Dyke, "Some properties of keratin biomaterials: kerateines," *Biomaterials*, vol. 31, no. 4, pp. 585-593, 2010.
- [2] A. Vasconcelos, G. Freddi, and A. Cavaco-Paulo, "Biodegradable materials based on silk fibroin and keratin," *Biomacromolecules*, vol. 9, no. 4, pp. 1299-1305, 2008.
- [3] R. J. Elias, S. S. Kellerby, and E. A. Decker, "Antioxidant activity of proteins and peptides," *Crit. Rev. Food Sci. Nutr.*, vol. 48, no. 5, pp. 430-441, 2008.
- [4] R. B. Fraser and D. A. Parry, "Molecular packing in the feather keratin filament," *Journal of structural biology*, vol. 162, no. 1, pp. 1-13, 2008.
- [5] A. L. V. Villa *et al.*, "Feather keratin hydrolysates obtained from microbial keratinases: effect on hair fiber," *BMC Biotechnol.*, vol. 13, no. 1, p. 1, 2013.
- [6] M.-Y. Wan, G. Dong, B.-Q. Yang, and H. Feng, "Identification and characterization of a novel antioxidant peptide from feather keratin hydrolysate," *Biotechnol. Lett.*, vol. 38, no. 4, pp. 643-649, 2016.
- [7] H. Y. Lai, S. Wang, V. Singh, L. T. Nguyen, and K. W. Ng, "Evaluating the antioxidant effects of human hair protein extracts," *J. Biomater. Sci. Polym. Ed.*, vol. 29, no. 7-9, pp. 1081-1093, 2018.
- [8] M. A. Rogers *et al.*, "Characterization of a cluster of human high/ultrahigh sulfur keratin-associated protein genes embedded in the type I keratin gene domain on chromosome 17q12-21," *J. Biol. Chem.*, vol. 276, no. 22, pp. 19440-19451, 2001.
- [9] C. R. Robbins and C. R. Robbins, *Chemical and physical behavior of human hair*. Springer, 2002.
- [10] T. Fujii, S. Takayama, and Y. Ito, "A novel purification procedure for keratin-associated proteins and keratin from human hair," *J. Biol. Macromol.*, vol. 13, no. 3, 2013.

- [11] T. Inoue, M. Ito, and K. Kizawa, "Labile proteins accumulated in damaged hair upon permanent waving and bleaching treatments," *Journal of cosmetic science*, vol. 53, no. 6, pp. 337-344, 2002.
- [12] B. D. Hames, *Gel electrophoresis of proteins: a practical approach*. OUP Oxford, 1998.
- [13] A. Shirwaikar, A. Shirwaikar, K. Rajendran, and I. S. R. Punitha, "In vitro antioxidant studies on the benzyl tetra isoquinoline alkaloid berberine," *Biol. Pharm. Bull.*, vol. 29, no. 9, pp. 1906-1910, 2006.
- [14] C. P. LeBel, H. Ischiropoulos, and S. C. Bondy, "Evaluation of the probe 2', 7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress," *Chem. Res. Toxicol.*, vol. 5, no. 2, pp. 227-231, 1992.
- [15] 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.
- [16] 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, 2015.
- [17] R. Kelly *et al.*, "Keratin and soluble derivatives thereof for a nutraceutical and to reduce oxidative stress and to reduce inflammation and to promote skin health," ed: Google Patents, 2006.
- [18] S. Xu, L. Sang, Y. Zhang, X. Wang, and X. Li, "Biological evaluation of human hair keratin scaffolds for skin wound repair and regeneration," *Mater. Sci. Eng., C*, vol. 33, no. 2, pp. 648-655, 2013.
- [19] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "NIH Image to ImageJ: 25 years of image analysis," *Nat. Methods*, vol. 9, no. 7, pp. 671-675, 2012.

Chapter 7

Conclusions and Recommendations

This final chapter summarizes all findings with regards to the interaction and behavior study on human hair keratin proteins which were discussed in this dissertation. The proposed hypotheses and objectives are recapitulated in correlation to the reported findings. Future opportunities and strategies to reinforce the research is proposed at the end of this chapter.

7.1 Conclusion

The overall aim of this dissertation is to conduct fundamental studies to understand human hair keratin properties that would allow us to fully exploit its benefits as biomaterials across a wide discipline of applications. The greatest mystery in hair keratin assembly remains the highly complicated yet conserved hair fiber forming event, which involves 17 keratin subtypes assembling through a cascade of interactions. Despite this lack of understanding, the propensity of epithelial keratins to self-assemble is real and has been demonstrated before. Therefore, it is hypothesized that hair keratins can perform similar self-assembly to form unique material platforms, under favorable conditions *in vitro*, albeit such conditions have yet to be reported. Additionally, it was hypothesized that fractionation of specific keratin subtypes can be achieved using established protein separation techniques, facilitating the understanding of the role of specific keratin fractions in the self-assembly process and potential application as antioxidants. The major findings of this study are enumerated into three phases following the objectives set in this study, which are to (1) develop and optimize a protocol for inducing self-assembly of hair keratins into fibrous networks, (2) develop and optimize a protocol for separation of Types I and II hair keratins, and (3) evaluate the potential of hair keratin extracts as antioxidizing supplements for *in vitro* cell culture applications. The substantial findings of this study are presented, and the limitations are elaborated. Ultimately, recommendations for future studies are proposed (see section 7.2).

The first hypothesis has been supported by establishing the first and foremost self-assembled regular, continuous and nanosized intermediate filaments network using chemically extracted human hair keratins. The self-assembly process was found to be a pH dependent event, which could be achieved at weak acidic condition ($\leq$ pH 3.3). The first objective was achieved by performing systematic study and observation on the self-assembled condition, fibers' morphology, and structures. Based on TEM analysis, the assembled fiber diameters reduced from ~ 10 nm to 6 nm when the environmental pH was reduced to pH 2.5. In addition, a biocompatible coating constituted of the SA-keratin at its optimum condition (2.5 mM citric acid, pH 2.9) was fabricated via deposition of the SA-

keratin solution in static manner for 1 h. Observation under AFM revealed the coating thickness to be 30 – 200 nm. More importantly, the coating retained the nanofilaments network feature of native human hair keratin and remained stable in physiological condition up to 5 days. Future work on more in-depth study and potential application of this assembled keratin will be discussed in the following section.

Regrettably, the second proposed objective was not completely achieved, although a partially enriched type II keratin fraction was obtained with WAX chromatography after extensive optimization and troubleshooting using diverse chromatography techniques including a versatile yet underrated AFFF method. Eventually, pI differences between the keratin subtypes were identified as the criterion with the greatest potential to enable type I and II keratin separation. Despite being subjected to high denaturant and reducing conditions (8M urea and 5 mM TCEP) at pH 5, the strongly dimerized keratins were mostly eluted in a complex form together with the type II enriched fractions. The total yield per fractionation using a semi preparative column was approximately 11.3%, which allowed fundamental studies to be carried out but will pose a challenge for large scale applications. Recommendations to further this work are elaborated in the next section.

The third objective was achieved by elucidating the antioxidant properties and cellular responses of human hair keratins, in comparison to THP and KAP, *via in vitro* approach. Among all, keratin was demonstrated to possess a superior DPPH and H₂O₂ free radical scavenging capacity. Furthermore, the antioxidant effect of keratin as media supplemented was further validated on HDF cells exposed to elongated H₂O₂ induced oxidative stress, up to 24 h. High cell proliferation, survival rate and low ROS level were observed when HDF were cotreated with 40 µM keratin in the presence of 500 µM H₂O₂, for 1 h duration. Concurrently, the DPPH radical scavenging ability of the enriched keratins fractions were found to be preserved despite the extensive purification and concentrating procedures.

Altogether, these findings provide new perspectives into the self-assembly and antioxidant capability of crude and purified human hair keratins, to further reinforce the future fundamental study and development of human hair keratin-derived biomaterials.

7.2 Future Perspective and Recommendation

7.2.1 In-depth Mechanistic Study of Self-assembly of Crude/Purified Keratins

This work has reported the morphology changes of self-assembled keratins subjected to basic, neutral, and acidic condition *via* step down dialysis. It was revealed that keratin proteins appeared majority in irregular and clustered form at high/neutral pH, and adopted the widen, beads-like aggregate shape at isoelectric point (pH 5.5), and eventually transformed as regular nanofilaments at around 10 nm diameter in low pH condition. However, the exact frame of view when the morphology transformation occurred remains unknown. Previous study has described the assembly of K5/K14 in extremely high denaturing condition, consisting 9.5 M urea [1]. In fact, the assembly of human hair keratins into ~10 nm diameter was noticeable in 8M urea, at pH 2.9 (Figure 7.1). This further alludes to the doubt of the mechanism and defined initiation of fiber assembly event of human hair keratins. The highly sensitive LSPR, incorporated with the customized ligands which bind to the assembled filaments, could be potentially used to explore the fiber forming mechanism. Albeit, the immerse sensitivity of LSPR is susceptible to minor interferences, including the sensor chip condition, manual handling of buffer/sample injection, ambient temperature, *etc.* The interpretation of LSPR data could also be challenging and tedious as it requires iterative processing and parameters fitting [2]. Another powerful tool that could be adopted is Cryo TEM, which is often applied to observe topology changes of soft molecules or capture the conformation changes of proteins samples [3].

Figure 7. 1 TEM images of human hair keratin dialyzed in 8 M urea, 1 mM, and 2.5 mM citric acid at pH 2.9. (Image credit to Marin Yee)

In addition, more optimization on the downstream concentrating and dialyzing steps will be required to thoroughly address the loss of self-assembly ability of the purified keratin fractions. As the obtained mass per fractions was less than ~50 µg, fractions from multiple runs have to be combined and further concentrated to acquire the minimum 0.2 mg/ml threshold before initiation of dialysis process. The current concentrating method is by using a vacuum concentrator, CentriVac, at 20 °C over 3 days. The extensive and long duration of centrifuging during the drying process could induce agglomeration of proteins, leading to the clustered features as observed under TEM (Figure 4.12). Alternative concentrating method, such as protein concentrator, could be attempted by taking into consideration the sample or salt blockage during the spinning process.

7.2.2 Translatable Properties and Potential Applications of the SA-keratin

The regular and continuous nanofilaments network was formed by using low concentration of crude human hair keratins, at 0.5 mg/ml. The effect of keratins concentration on the assembled topology is shown in Figure 7.2. The cross-sectional morphology of freeze-dried 1 mg/ml SA-keratin (pH 2.9) sponges consisted of fibrous and interconnected pores. As the concentration increased to 4 mg/ml, the fibrous structure was disappeared, and in its replacement the sheet-like structure was noted. Furthermore, casting duration was previously described to affect the network cluster (Figure 4.9), indicating the importance of concentration and casting time to retain the fibrous network of the SA-keratin formed platform. These preliminary data suggested the crucial variables to translate such assembled nanofilaments into 3D platform, such as sponges, thin film, coating, etc.

The biocompatibility of SA-keratin coating was demonstrated in Section 6.3, as evidenced from the good proliferation activity and fibronectin expression in HDF and HEK cells. It is recommended to further investigated the adhesion molecules produced by specific cell types, for instances, vinculin, integrin expression of HEK, *etc.* This could expedite the

understanding of SA-keratin formed construct, in comparison to the normal keratin or other fibrillar proteins constructed platform.

Figure 7. 2 Cross-sectional SEM images of freeze-dried SA-keratin (pH 2.9) sponges, fabricated at A) 1 mg/ml and B) 4 mg/ml.

7.2.3 Keratin Enriched Fractions and Potential Application

The advantages of purifying keratin subtypes from crude human hair extract over the recombinant technique is the readily available material and to conserve the native functionality and structure of the keratin's molecules. Albeit, it could be arduous and laborious to obtain supremely purified fractions from the 17 keratin subtypes mixture. This dissertation has presented a two elution-step method to obtain partially purified keratin fractions and further characterized them using SDS PAGE, western blotting, MALDI-TOF-MS, TEM and DPPH assay. Functionalization of these enriched fractions is restricted due to considerable low yield despite a semi preparative column was adopted. In view of resources and time factors, the extent of fractionation can be further improved by highly modulated and sequential chromatography processing flow. A preparative weak anion exchange column could also be adopted to further scale up the yield per fractions, after optimization of the sequential purification procedure. Nevertheless, self-assembly of these

enrich fractions could be performed using interfacial polyelectrolyte complexation (IPC) platform as slight amount of sample volume is required. Fiber formation, live cells encapsulation or miniature laboratory designed incorporated the antioxidant properties of the purified keratins can be explored [4].

References

- [1] P. A. Coulombe and E. Fuchs, "Elucidating the early stages of keratin filament assembly," *Int. J. Biochem. Cell Biol.*, vol. 111, no. 1, pp. 153-169, 1990.
- [2] J. A. Jackman, A. R. Ferhan, and N.-J. Cho, "Nanoplasmonic sensors for biointerfacial science," *Chem. Soc. Rev.*, vol. 46, no. 12, pp. 3615-3660, 2017.
- [3] L. Norlén, S. Masich, K. N. Goldie, and A. Hoenger, "Structural analysis of vimentin and keratin intermediate filaments by cryo-electron tomography," *Exp. Cell Res.*, vol. 313, no. 10, pp. 2217-2227, 2007.
- [4] A. C. Wan, M. F. Cutiongco, B. C. Tai, M. F. Leong, H. F. Lu, and E. K. Yim, "Fibers by interfacial polyelectrolyte complexation—processes, materials and applications," *Mater. Today*, vol. 19, no. 8, pp. 437-450, 2016.

Publications

Journal Articles

- Lai, H. Y., Wang, S., Singh, V., Nguyen, L. T., & Ng, K. W. (2018). Evaluating the antioxidant effects of human hair protein extracts. *Journal of Biomaterials science, Polymer edition*, 29(7-9), 1081-1093.
- Kerk, S. K., Lai, H. Y., Sze, S. K., Ng, K. W., Schmidtchen, A., & Adav, S. S. (2018). Bacteria display differential growth and adhesion characteristics on human hair shafts. *Frontiers in microbiology*, 9, 2145.
- Adav, S. S., Subbaiah, R. S., Kerk, S. K., Lee, A. Y., Lai, H. Y., Ng, K. W., ... & Schmidtchen, A. (2018). Studies on the proteome of human hair-Identification of histones and deamidated keratins. *Scientific reports*, 8(1), 1599.
- Rakshit, M., Gautam, A., Toh, L. Z., Lee, Y. S., Lai, H. Y., Wong, T. T., Ng, K. W. (2020). Hydroxyapatite Particles Induced Modulation of Collagen Expression and Secretion in Primary Human Dermal Fibroblasts. *International Journal of Nanomedicine*, 15, 494-4956.
- Zhao, Z. T., Moay, Z. K., Lai, H. Y., Goh, H. R., Chua, H. M., Setyawati, M. I. and Ng, K. W. (2020). Characterization of Anisotropic Human Hair Keratin Scaffolds Fabricated via Directed Ice Templating. *Macromolecular Bioscience*. DOI: 10.1002/mabi.202000314.
- Lai, H. Y., Setyawati, M. I., Ferhan, A. R., Divakarla, K., Chua, H. M., W. Chrzanowski, N. J. Cho, and Ng, K. W. (2020). Self-assembly of Solubilized Human Hair Proteins. *ACS Biomaterials Science & Engineering*. DOI: 10.1021/acsbmaterials.0c01507.
- Lai, H. Y., Nguyen, L. T., Adav S. S., Chua, H. M., Loke, J. J., Miserez, A., Schmidtchen, A., Ng, K. W. (2021). Top-down Approach: Purification of Enriched Human Hair Keratins for Behavior Study. Manuscript in Preparation.

- Lai, H. Y., Setyawati, M. I., Vizetto-Duarte C., Chua, H. M., Low, C. T., Ng, K. W. (2021) Dissection of human hair extracts' antioxidant capacity: ROS scavengers for *in vitro* application. Manuscript in Preparation.

Patent

- Ng Kee Woei, Lai Hui Ying. (2020). *Self-Assembly of Solubilized Human Hair Proteins into Intermediate Filament Networks* (SG Patent Application No. 10202003536S). Singapore Patent.

Conference Presentations

- Lai, H. Y., Andrew C.A. Wan, & Kee Woei Ng (2019, December). *Investigating the Self-assembly of Naturally Extracted Human Hair Keratin Intermediate Filament Proteins*. Poster session presented at the 16th Pacific Polymer Conference (PPC16), Suntec Singapore Convention and Exhibition Centre, Singapore.
- Lai, H. Y., Adav. S.S, Vizetto-Duarte C & Ng, K.W. (2019, October). *Purification of Human Hair Keratin Subtypes for Functional Studies*. Oral presentation at TERMIS-AP19 Conference, Brisbane, Australia.
- Lai, H. Y., Luong T. H. Nguyen & Kee Woei Ng (2017, November). *Separation of Human Hair Keratin Subtypes for Interaction and Behavioural Studies*. Poster session presented at Separation Science 2017 Conference, Biopolis, Singapore.

APPENDIX A: Supplementary Information

Figure A. 1 Initiate adsorption rate of keratins solution (pH 2.9 and pH 5.5) during Localized Surface Plasmon Resonance (LSPR) analysis.

Figure A. 2 Localized Surface Plasmon Resonance (LSPR) analysis in triplicated run to stimulate the coating deposition using SA-keratin solution at A) pH 2.9 and B) pH 5.5 in the absence of KCl salt.

Figure A. 3 Live HDF cells were fluorescently stained with CellROX (green) and Hoechst 33342 dyes (blue) and fixed with 4% PFA for imaging purpose. Images from two channels were merged using ImageJ software.