Human hair keratin electrospun matrices for tissue regeneration

Sow, Wan Ting

2016

Sow, W. T. (2016). Human hair keratin electrospun matrices for tissue regeneration.
Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/68533>

<https://doi.org/10.32657/10356/68533>

NANYANG
TECHNOLOGICAL
UNIVERSITY

Human Hair Keratin Electrospun Matrices for Tissue Regeneration

SOW WAN TING

School of Materials Science and Engineering

2015

Human Hair Keratin Electrospun Matrices for Tissue Regeneration

School of Materials Science and Engineering

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

2015

Abstract

Keratins present themselves as potential autologous alternatives to current natural proteins used as matrices in regenerative medicine applications, because they are abundant, bioactive and easily extracted from human hair. The aim of this work is to study the feasibility of electrospinning human hair keratin and evaluate the potential of electrospun keratin fibers as random and aligned templates for tissue regeneration. Keratins extracted from human hair using sodium sulfide were blended with poly (ethylene oxide) (PEO) at various concentrations and electrospun. The fiber morphology and diameter distribution of the optimised fibrous platforms were analysed by scanning electron microscopy (SEM). Material properties of the resulting keratin fibrous platforms were characterized using fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA) to understand their chemical, thermal and mechanical properties respectively. Biodegradation profiles of keratin fibers were also studied under hydrolytic and enzymatic conditions. Cytotoxicity of the keratin fibers were examined by assessing cell viability and morphology of L929 murine fibroblasts cultured on the fibers through Live/Dead staining assay and SEM. In addition, the potential of keratin fibers for wound healing was evaluated by culturing primary human dermal fibroblasts (HDFs) on them. Cell viability of cultured HDFs was quantified using PicoGreen and AlamarBlue assays while the cell morphology and distribution was observed using Live/Dead and F-actin staining. Immunostaining was also done to assess the ability of cultured HDFs in secreting extracellular matrix (ECM) proteins such as fibronectin and collagen III. The interaction between HDFs and keratin was then elucidated by cell-material interaction study through integrin inhibition assay and Western blotting. Results showed that keratin fibers were successfully electrospun using a solution of 30 wt% keratin and 0.5 wt% PEO. The resulting fibrous platforms consisted of well-formed sub-micron fibers with mean fiber diameter of $0.70 \pm 0.09 \mu\text{m}$ and $0.74 \pm 0.10 \mu\text{m}$ for random and aligned keratin fibers

respectively. Chemical analysis indicated that keratin retained its chemical conformation after electrospinning. L929 murine fibroblasts cultured on keratin fibers showed healthy cell growth, demonstrating no cytotoxicity issues. Results from HDFs studies showed that proliferation and metabolic activity of HDFs on keratin fibers were comparable to the positive control and significantly higher on aligned keratin fibers compared to random keratin fibers on day 3 and day 5 after cell seeding. Live/dead staining indicated that HDFs cultured on the keratin fibers could form cellular networks according to the architecture of the keratin fibrous platforms. Besides, F-actin staining revealed that the HDFs exhibited their typical spindle morphology and responded well to the topography of the keratin fibers. It was also shown that keratin fibers could stimulate the HDFs to secrete and deposit ECM proteins essential for early stage of wound healing. Cell-material interaction study demonstrated that $\alpha_4\beta_1$ integrin played a significantly role in HDFs attachment to keratin. In this work, it is revealed that keratin fibrous platforms provide a conducive environment to facilitate the growth of primary HDFs and promote ECM production. From these results, it is concluded that electrospun human hair keratin fibers have the potential to be developed into templates for tissue regeneration.

Acknowledgements

I would like to thank my supervisor, Assistant Professor Ng Kee Woei for the opportunities to work on the research topic of this dissertation. I would also like to thank my thesis advisory committee members, Associate Professor Tan Lay Poh and Associate Professor Cho Nam Joon for their advices on this work.

I wish to extend my gratitude to many people who have helped me along the way especially:

Patrick Lai and Wilson Lim for their guidance on the usage of equipment for materials characterization and the immediate help rendered whenever I find them.

Alan Lim, Derrick Ang and Yee Yan for their guidance on electron microscopy techniques and help in facilitating my experiments in the most convenient way.

Ingridd Setyawati and Shahrouz Amini for their unconditional help in experimental trials and suggestions to improve the spectroscopic analysis in my work.

Raphael Ortiz for his patience in helping me understand the fundamental theory of FFT analysis.

Xin Xin and Yuan Siang for their detailed guidance for cell culture practices and immunostaining.

Monica Suryana, Ivan Lam and Ajay Tijore for sharing their experience and insightful discussion on cell-material interaction studies.

Zhao Yun and Yang Fei for their valuable advice and wise recommendations whenever I encountered problems in Western blotting.

Last but not least, I very much want to thank my lovely final year project students, Germaine and Sendy for their dedication and hard work in contributing to this keratin electrospinning work.

Table of Contents

Abstract.....	i
Acknowledgements	iii
Table of Contents	v
Figure Captions.....	ix
Table Captions	xix
Abbreviations	xxi
Chapter 1: Introduction.....	1
1.1 Background.....	2
1.2 Motivation.....	3
1.3 Research Work: Objectives and Scope	4
1.4 Thesis Outline	6
Chapter 2: Literature Review	9
2.1 Keratin.....	10
2.1.1 Human Hair Keratin.....	10
2.1.2 Bioactivity of Keratin	13
2.1.3 Extraction of Keratin.....	14
2.1.4 Keratin as a Biomaterial.....	15
2.2 Electrospinning	17
2.2.1 Fundamental Theory	18
2.2.2 Solution Parameters	19

2.2.3	Controlling Parameters	21
2.2.4	Alignment of Fibers	22
2.3	Current Research on Keratin-based Electrospun Fibers	27
2.3.1	Composite Fibers from Keratin Blends	27
2.3.2	Applications of Keratin-based Electrospun Fibers in Tissue Engineering ..	31
Chapter 3:	Experimental Methodology	37
3.1	Extraction of Human Hair Protein	38
3.2	Materials Fabrication	38
3.2.1	Preparation of Keratin Solutions.....	38
3.2.2	Fibers Fabrication	39
3.2.3	Film Formation	39
3.2.4	Coating Preparation	40
3.3	Materials Characterization	40
3.3.1	Western Blotting of Extracted Keratin Solution.....	40
3.3.2	Viscosity of Keratin Solution.....	41
3.3.3	Morphology of the Fabricated Materials	42
3.3.4	Chemical Conformation of Keratin Materials	43
3.3.5	Thermal Properties of Keratin Materials	44
3.3.6	Mechanical Properties of Fibers	44
3.3.7	Biodegradation Studies of Fabricated Materials.....	45
3.3.8	Chemical Assays on Keratin Films.....	46
3.4	Cell Culture	46
3.4.1	Cell Seeding	46
3.4.2	Cell Quantification.....	47
3.4.3	Cell Morphology Analysis.....	47
3.4.4	Immunocytochemistry	48
3.4.5	Integrin Inhibition Assay	49
3.4.6	Western Blotting for Expression of $\alpha_4\beta_1$ integrins.....	49
3.5	Statistical Analysis.....	50

Chapter 4: Keratin Extraction and Fiber Fabrication	53
4.1 Results.....	55
4.1.1 Characterization of Extracted Keratin	55
4.1.2 Optimization of Formulation for Electrospinning of Keratin	57
4.2 Discussion	63
4.3 Conclusion	66
 Chapter 5: Characterisation of Electrospun Keratin Fibers	 67
5.1 Results.....	69
5.1.1 Chemical Conformation.....	69
5.1.2 Thermal Properties.....	71
5.1.3 Mechanical Properties.....	73
5.1.4 Biodegradation Profiles	74
5.1.5 Cell Compliance.....	77
5.2 Discussion	79
5.3 Conclusion	82
 Chapter 6: Tuning Alignment of Keratin Fibers and Their Potential for Wound Healing	 85
6.1 Results.....	88
6.1.1 Optimization of Alignment via Controlling Parameters of Electrospinning	88
6.1.2 Mechanical Properties of Optimized Aligned Keratin Fibers.....	92
6.1.3 Human Dermal Fibroblasts Viability.....	94
6.2 Discussion	98
6.3 Conclusion	101
 Chapter 7: Involvement of Keratin and Electrospinning in Cell-Material Interaction	 103
7.1 Results.....	105
7.1.1 Preliminary Cell Study on Keratin Films.....	105

7.1.2	Characterization of Vacuum-Dried and Air-Dried Keratin Films	106
7.1.3	Cell-Material Interaction Study between Keratin and Human Fibroblasts	109
7.2	Discussion	111
7.3	Conclusion	114
 Chapter 8: Conclusion and Recommendations		117
8.1	Conclusion	118
8.2	Recommendations.....	121
8.2.1	Modeling of Electrospinning Parameters to Develop Multi-scale Scaffolds via Multimodal Electrospinning	121
8.2.2	In-depth Evaluation on the Potential of Keratin Fibers for Skin Wound Healing.....	122
8.2.3	Development of Purified Keratin for Fundamental Structural Studies.....	122
 References		 125
 Publications		 141

Figure Captions

Figure 2-1 Structure and assembly of intermediate filaments. A and B: helical domains; L: non-helical linkers; N and C: N and C terminus. Reproduced with permission from [37].....	11
Figure 2-2 Chemical interactions between keratin protein chains. Reproduced with permission from [38].....	11
Figure 2-3 Schematic model of hair. The hair consists of three main parts: cuticle, cortex and medulla. Reproduced with permission from [37]	12
Figure 2-4 Human hair keratin subtypes. A) Illustration of their expression sites and B) summarizing table of their location in human hair. Adapted with permission from [42]	13
Figure 2-5 Electrospinning of polymeric solution into continuous fibers	18
Figure 2-6 Polycaprolactone electrospun fibers with a) beads at concentration of 0.1g/mL and b) no beads at 0.12g/mL. Reproduced with permission from [99]	20
Figure 2-7 Schematic diagram of a rotating drum. Reproduced with permission from [109].....	23
Figure 2-8 Schematic diagram of a rotating disk. Reproduced with permission from [109]	24
Figure 2-9 Schematic diagram of a parallel plate setup and the corresponding electric field involve. Reproduced with permission from [108].....	24
Figure 2-10 Light microscopy images of fibers (A, D, G). FFT frequency image (B, E, H) and FFT alignment graphs (C, F, I) for the corresponding light microscopy images. Peak height and shape in the FFT alignment graphs report the relative alignment degree while peak positions report the principle axis of orientation of the fibers. Reproduced with permission from [110].....	25

Figure 2-11 X ray diffraction intensity versus Azimuthal angles measured from the preferred axis of polypropylene reinforced with various amounts of partially aligned VGCF. (a) 2.5% (b) 7% fiber volume fraction. Reproduced with permission from [111]26

Figure 2-12 Angular distribution of the aligned collagen fibers ($AD = 10.7^\circ$). Reproduced with permission from [112] 27

Figure 2-13 Study on wool keratin/PLLA electrospun fibers. A) Quantification of cell viability by MTT assay, B) Live/Dead staining of cells cultured samples and C) FTIR spectra of samples. Reproduced with permission from [141]..... 33

Figure 2-14 Study on wool keratin/PLLA electrospun fibers. A) Quantification of cell viability by MTT assay, B) Live/Dead staining of cells cultured samples and C) FTIR spectra of samples. Reproduced with permission from [138]..... 34

Figure 4-1 A) Comassie blue stain and B) Western Blot of human hair extract. Comassie blue stain showed the presence of proteins with keratin's typical molecular weight of 45kDa for acidic keratin and 50kDa for basic keratin in the human hair extracts. Western blot further confirmed the presence of keratin in the extracts. 55

Figure 4-2 Representative FTIR spectra of lyophilized keratin, consisting of the general absorption peaks of proteins, i.e. Amide A (2800-4000 cm^{-1}), Amide I (1600-1700 cm^{-1}), Amide II (1480-1580 cm^{-1}) and Amide III (1220-1330 cm^{-1})..... 56

Figure 4-3 Keratin in solutions with different pH environment. A transparent solution was obtained for NaOH solvent while cloudy solutions were obtained for H_2O and HCl solvents 57

Figure 4-4 Results of electrospinning 10wt% keratin in NaOH indicating unsuccessful formation of keratin fibers. The microscopic image on the right showed the structures of the residues formed after electrospinning 58

Figure 4-5 Keratin mixture consisting of blending A) PLC and b) PEO with keratin solution. Precipitate was formed when keratin solution was blended with PLC while a good solution mixture was produced when keratin solution was blended with PEO..... 58

Figure 4-6 SEM images of electrospun fibers with 1 wt% PEO and A) 10wt% keratin, B) 20wt% keratin, C) 25wt% keratin and D) 30wt% keratin. Accumulation of beadles fibers was observed when the keratin concentration reached 30wt% although some of the fibers were no fully well-formed. 59

Figure 4-7 SEM images of electrospun fibers with 30wt% keratin and A) no PEO, B) 0.3wt% PEO, C) 0.5wt% PEO and D) 1wt% PEO. Accumulation of majority well formed fibers with negligible wet drips or fibers was observed when 0.5wt% PEO was used in combination with 30wt% keratin 60

Figure 4-8 A) Table of viscosities of different formulation with their corresponding B) Pictures of the electrospun fibers except for formulation 40wt% keratin and 0.5wt% PEO due to C) gelation of solution. Viscosities at which the formulation allows successful fabrication of well-formed fibers fell in the range of 30.29 to 41.58 mPa 61

Figure 4-9 Graphs comparing viscosities of formulations with varying concentration of A) PEO with keratin concentration fixed at 30wt%, B) keratin with PEO concentration fixed at 0.5wt% and C) keratin with PEO concentration fixed at 1wt%. The gradients of the linear trendline fitted to the graph are 8.862, 6.777 and 4.750 for A), B) and C) respectively, indicating that PEO played a more dominant role in manipulating the viscosity of the formulation. 62

Figure 4-10 Results from the optimized formulation (i.e. 30wt% Keratin and 0.5wt% PEO in Na₂S solvent) for electrospinning keratin showing A) scaffold spun out from the formulation, B) the viscosity of the formulation, C) the SEM image of the scaffold and D) fiber diameter measurement of the scaffold 63

Figure 5-1 Representative Fourier Transform Infrared spectra of an A) electrospun keratin fibers, B) lyophilized keratin, C) Na₂S hydrates and D) PEO. Electrospun keratin

matrix and lyophilized keratin have similar spectra consisting of the general absorption peaks of proteins, i.e. Amide A ($2800-4000\text{ cm}^{-1}$), Amide I ($1600-1700\text{ cm}^{-1}$), Amide II ($1480-1580\text{ cm}^{-1}$) and Amide III ($1220-1330\text{ cm}^{-1}$). Compared to the lyophilized keratin's spectrum, the keratin fibers' spectrum has two additional peaks at 1114 cm^{-1} and 994 cm^{-1} originating from $\text{S}_2\text{O}_3^{2-}$ group of Na_2S hydrates. The PEO spectrum has its typical fingerprint region containing the prominent triplet peaks due to C-O-C stretching but these peaks were not found in the keratin matrix's spectrum due to low content of PEO.

Reproduced with permission from [124] 69

Figure 5-2 Representative Fourier Transform Infrared spectra of an A) electrospun keratin fibers after PBS washing B) lyophilized keratin. The two peaks at 1114 cm^{-1} and 994 cm^{-1} originating from $\text{S}_2\text{O}_3^{2-}$ group of Na_2S hydrates disappear on the spectra electrospun keratin fibers after PBS washing and the spectra of the electrospun keratin fibers after PBS washing resembles closely to the spectra of the lyophilized keratin..... 71

Figure 5-3 DSC curve of the extracted keratin showing 3 endothermic peaks which indicate water evaporation at 99°C , α helix denaturation at 248°C and β sheet denaturation at 299°C 72

Figure 5-4 DSC curve of 30wt% keratin and 0.5 wt% PEO electrospun fibers showing 3 distinct peaks which indicate water evaporation at 108°C , α helix denaturation at 239°C and β sheet denaturation at 273°C 72

Figure 5-5 Stress strain curve of keratin and gelatin fibers showing distinctively that keratin had higher strain at break while gelatin was stiffer. 73

Figure 5-6 A) Keratin Fibers, B) keratin film and C) collagen film of the same shape and size (i.e. 4 cm^2). The films were created as controls in the degradation studies of keratin fibers. 74

Figure 5-7 Biodegradation profiles of keratin fibers, keratin films and collagen films over 30 days in four different environments namely A) PBS according to ASTM standard, B) Tris buffer which is the solvent for enzymatic digestion in degrading keratin fibers, C)

chymotrypsin in Tris buffer and D) Proteinase K in Tris buffer. * $p < 0.01$, significance difference in weight percentages between keratin film and collagen film 75

Figure 5-8 Degradation profiles of keratin fibers in four different environments (i.e. PBS, tris buffer, chymotrypsin and proteinase K). Hydrolytic degradation of keratin fibers was faster in Tris buffer compared to PBS while enzymatic degradation of keratin fibers was faster in Proteinase K compared to chymotrypsin. * $p < 0.01$, significance difference in weight percentages of between keratin fibers in chymotrypsin and keratin fibers in proteinase K presence 76

Figure 5-9 Fluorescence microscopy images of cell cultured samples over 7 days. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). Cells on the samples started to spread by day3 and proliferated significantly by day7. Reproduced with permission from [124] 77

Figure 5-10 Z-stack confocal microscopy image of the cross section of a 7-day cell cultured keratin fibers, showing even cell penetration across the depth of the matrix. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). Reproduced with permission from [124] 78

Figure 5-11 Representative scanning electron microscopy images of cells cultured for 7 days on the samples. Low magnification images (top row) depict the overview of cell distribution and high magnification images (bottom row) depict the cell morphologies. Reproduced with permission from [124] 78

Figure 6-1 Representative SEM images of the 14 possible combinations to obtain aligned keratin fibers and their corresponding FFT peak unit. Combination #13 produced fibers with the best alignment while combination #1 produced fibers with the worst alignment 88

Figure 6-2 Plot of FFT quantification (left) of the 14 combinations and their corresponding average FFT peak unit in descending order from the top. The highest peak unit is 0.238 while the lowest peak unit is 0.089 89

Figure 6-3 The role of tip to collector distance on the degree of alignment of keratin electrospun fibers. A) Summary plot, B) SEM images and C) FFT quantifications of combinations #5, #2 and #6 showing that increasing tip-to-collector distance gave better alignment with higher average FFT peak value..... 89

Figure 6-4 The role of drum rotation speed on the degree of alignment. A) Summary plot of increasing rotating drum speeds and their corresponding FFT peak value and B) combinations with their respective rotating drum speed and corresponding surface velocities 90

Figure 6-5 The role of drum rotation speed on the degree of alignment before the threshold rotating speed. A) SEM images and B) FFT quantifications of combinations #9, #10, #11 and #12. C) Summary plot showing that increasing rotating speed gave better alignment before the threshold rotating drum speed..... 91

Figure 6-6: The role of drum rotation speed on the degree of alignment after the threshold rotating speed. A) SEM images and C) FFT quantifications of combinations #13, #12 and #14. B) Summary plot showing that increasing rotating speed gave poorer alignment after the threshold rotating drum speed..... 91

Figure 6-7 A) The SEM image of aligned fibers produced from the optimized combination followed by its corresponding FFT image and FFT quantification. B) High magnification (left) and low magnification (right) SEM images of the optimized aligned keratin fibers 92

Figure 6-8 Stress-strain curves of aligned keratin, random keratin and random gelatin fibers showing distinctively that aligned keratin had higher Young's modulus and ultimate tensile strength but lower strain at break than random keratin. 93

Figure 6-9 Cell viability and proliferation of human dermal fibroblasts cultured on the electrospun fibers. A) PicoGreen assay results and B) Alamar Blue assay results showing

healthy cell growth on aligned keratin, random keratin and random gelatin electrospun fibers over a period of 10 days..... 94

Figure 6-10 Fluorescence microscopy images of cell cultured samples over 7 days. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). HDFs on the samples exhibited their typical spindle morphology by Day 3..... 95

Figure 6-11 Fluorescence microscopy images of cell cultured samples over 7 days. F-actin (red) of the cells was stained by Phalloidin and the nucleus (blue) of the cells was stained by DAPI. HDFs on the keratin fibers arranged themselves according to the architecture of the fibrous samples by Day 3..... 96

Figure 6-12 Immunofluorescence staining of fibronectin on cell cultured samples over 7 days. Samples were stained for fibronectin (green) and nuclei (blue). Expression of fibronectin were found on keratin samples but not gelatin samples. 97

Figure 6-13 Immunofluorescence staining of collagen III on cell cultured samples over 7 days. Samples were stained for collagen III (green) and nuclei (blue). Expression of collagen III were found on all samples 97

Figure 6-14 Illustrative summary of the human tissues. Reproduced with permission from [169]..... 98

Figure 6-15 Illustration of the structure of the skin (epidermis and dermis) which consisted of A) multiple cell types in which keratinocytes and fibroblasts are the most critical cells for dermal wound healing. SEM images of B) collagen bundles at the dermis tissue and C) fibroblasts aligning themselves according to the architecture of the dermis tissue. Adapted with permission from [170, 171]..... 99

Figure 7-1 Fluorescence microscopy images of cell cultured samples over 7 days. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used

to stain the nuclei of non-viable cells (red). L929 (top) showed healthy growth on AD films while HDF (bottom) barely proliferated on AD film. 105

Figure 7-2 Comparison of HDF compliance on AD film and VD film. (A) PicoGreen assay results and (B) fluorescence microscopy images of cell cultured samples over 7 days. HDFs showed healthy cell growth on VD films but barely proliferated on AD films. 106

Figure 7-3 Surface morphologies of AD and VD keratin films. (A) AFM 3D Height and 2D Amplitude images and (B) surface roughness calculated from 2D Amplitude images of AD and VD films. There were no significant differences on the surface morphologies between AD and VD keratin films..... 106

Figure 7-4 XPS N1s narrow scans with deconvolutions of 3 components which are amide, amine and protonated amine of A) VD keratin film, B) AD keratin film and C) keratin fibers. D) A comparison plot on the amide bond percentage in the nitrogen species of keratin fibers, VD and AD keratin films..... 107

Figure 7-5 Comparison on the amount of amine groups in VD and AD film from Ninhydrin assay. VD film contained significantly higher amount of amine groups compared to AD film 108

Figure 7-6 Comparison on the amount of free thiol groups in VD and AD films from Ellman Reagent's assay. VD film contained significantly higher amount of thiol groups compared to AD film 109

Figure 7-7 Western blot of human hair extracts showing the presence of acidic keratin subtype, K31, K33 and K34 with a molecular weight of 45kDa..... 109

Figure 7-8 Effect of A) α_4 integrin-blocking antibody and B) β_1 integrin-blocking antibody on the cell attachment on keratin film, fibronectin coating, polystyrene and untreated polystyrene. Significant decrease in cell attachment was observed on keratin films when HDFs were blocked with α_4 and β_1 integrin-blocking antibodies. * $p < 0.05$. 110

Figure 7-9 Expression of integrins by HDFs cultured on fibronectin coating (FC), keratin film (KF) and untreated (uPS). A) Ponceau red staining of transfer membrane loaded with cell lysates harvested 4 hours after HDF seeding on the samples and B) Western Blot showing expression of β -actin, α_4 and β_1 integrins from cell lysates harvested 7 days after HDF seeding on the samples..... 111

Table Captions

Table 2-1 Processing parameters in electrospinning	19
Table 2-2 Table of publications on keratin-based electrospun fibers	31
Table 4-1 Protein concentration of 5 different batches of keratin extracts and their percentage yield. The average yield is 26.25% of protein extracted out from 0.05g/mL of human hair	55
Table 5-1 General Absorbance Bands of Proteins	70
Table 5-2 Collated results of the mechanical properties of keratin fibers and gelatin fibers, showing values of the Young's modulus, ultimate tensile strength and strain at break determined from the stress-strain curve	74
Table 6-1 Optimization table of controlling parameters involving applied voltage, tip to collector distance, feedrate and rotating speed of the drum collecting the fibers to find the best combination to fabricate aligned keratin fibers. The initial recommended combination was 10kV applied voltage, 15cm tip to collector distance, 0.5ml/h feedrate and 600 rpm of drum rotating speed.	85
Table 6-2 Collated results of mechanical properties of aligned keratin, random keratin and random gelatin fibers, showing values of Young's modulus, ultimate tensile strength and elongation at break determined from the stress-strain curves	93

Abbreviations

2-ME	β -mercaptoethanol
AD film	Air Dried film
AFM	Atomic Force Microscopy
ANOVA	One-way Analysis of Variance
BMP- 2	Bone Morphogenetic Protein-2
BSA	Bovine Serum Albumin
DMA	Dynamic Mechanical Analysis
DMEM	Dulbecco's Modified Eagle Medium
DMF	N,N-dimethylformamide
DSC	Differential Scanning Calorimetry
dsDNA	Double Stranded Deoxyribonucleic Acid
DTT	Dithiothreitol
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FTIR-ATR	Fourier Transform Infrared Spectroscopy- Attenuated Total Reflectance
H ₂ O	Water
HCl	Hydrochloride Acid
HDF	Human Dermal Fibroblast
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
LDV	Leu-Asp-Val
Na ₂ S	Sodium Sulphide
NaOH	Sodium Hydroxide
PA6	Polyamide 6
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline-Tween 20
PCL	Poly (ϵ -caprolactone)
PEO	Polyethylene oxide
PFA	Paraformaldehyde
PHBV	Poly(hydroxybutylate-co-hydroxyvalerate)
PHMA	Poly2-hydroxyethylmethacrylate
PLA	Poly lactide
PLC	Poly (L-lactide/ ϵ -caprolactone)
PLLA	Poly (L-lactic acid)
RGD	Arg-Gly-Asp
SEM	Scanning Electron Microscope
TCEP	Tris (2-carboxyethyl) Phosphine
TFE	2,2,2-trifluoroethanol
VCAM-1	Vascular Cell Adhesion Protein 1
VD film	Vacuum Dried Film
XPS	X-ray Photon Spectrometry

Chapter 1:

Introduction

This chapter introduces the current state of the art of tissue engineering and limitations of the technology. It also briefly summarizes the evolution of tissue regeneration research in developing solutions to address these limitations. Realization of how electrospun keratin fibers maybe a potential alternative platform for tissue regeneration is discussed as part of the motivation of this dissertation. With a clear agenda in place, the research work in this dissertation was divided into 3 phrases; each with a hypothesis. Key activities of each phase are listed out to achieve a specific objective and therefore prove the hypotheses. Finally, the outline of this dissertation is presented in the last section of the chapter.

1.1 Background

One of the most common, costly and potentially fatal healthcare problem is tissue damage [1]. Major tissue damage results in the need for transplantation whereby autografting is usually the standard preference. However, limitations of autografting include donor sites morbidity and limited supplies of autografts [2, 3]. Therefore, much focus has been put in improving the state-of-the-art of tissue engineering and regenerative medicine to counter the shortage of transplantable tissues and organs [4].

The main focus of tissue engineering is to use a scaffold to guide cellular behavior via physical and biochemical cue and hence act as an effective platform that mimics physiological conditions for wound healing. Natural polymers such as proteins and polysaccharides are known to be naturally bio-inductive and hence good potential templates to improve cell-material interactions. Examples include collagen, fibrin, chitosan, gelatin, elastin and hyaluronic acid [3, 5]. However, most of these materials are of animal origins which may lead to risks of pathogen transfer and issues of immunological rejections [6-8]. To avoid these issues, human adipose tissue-derived collagen [9] and recombinant artificial proteins [10] have been investigated. Human fibrin-based platforms are also available for clinical use [11]. Nevertheless, these human proteins are either costly or limited in supply.

From this point of view, keratin which can be extracted from the unlimited supply of human hair easily becomes an attractive protein of human origin for tissue regeneration. Moreover, using keratin from hair offers the possibility that it can be entirely personalized to individual patients specifically, thus acting as an autologous template to eliminate any risks of pathogen transfers. In addition, as a protein, keratin contains the cell adhesion sequence, Leu-Asp-Val (LDV) [12], which is specifically recognized by cell receptor, $\alpha_4\beta_1$ integrin [13]. It has been discussed that proteins such as vascular cell adhesion protein 1 (VCAM-1) and fibronectin can promote $\alpha_4\beta_1$ mediated adhesion of human dermal fibroblasts [14], endothelial cells [15, 16] and leukocytes [17-19], which are cells critical for tissue healing and it has been shown that these proteins contain LDV

cell adhesion motif [13, 20]. These characteristics led to the postulation that platforms derived from human hair keratin could be advantageous in encouraging tissue regeneration.

Electrospinning has been selected as a effective and scalable way to generate fibrous platforms that mimic the architecture of the native extracellular matrix (ECM) [21]. Moreover, electrospinning help to enhance the purpose of keratin as a biological material by fully utilizing the bio-activeness of keratin via the generation of high surface area to volume ratio [22, 23] for cell adhesion, proliferation and migration on keratin fibrous platform. Hence, it will be interesting and useful to explore the potential of human hair keratin as a fibrous platform for tissue regeneration.

1.2 Motivation

As mentioned earlier, human hair keratin is a potential material for tissue engineering due to its abundance, bio-inductive abilities and anti-allergic actions. Hence, it is important to study the biological behavior of extracted keratin as a material. Electrospun fibrous platform serves as a good template to study cellular interaction with keratin due to its microporous nature which will enhance physical interaction between the cell and keratin material.

A handful of research groups have tried to create keratin fibrous platforms through electrospinning [24-33]. However, due to the low molecular weight of the extracted keratin, most have resorted to fabricate fibrous platforms using keratin in combination with other natural or synthetic polymers [34]. Hence, the development of fibrous platform with relatively high content of keratin or even pure keratin through electrospinning remains a major challenge. In addition, there are limited reports describing cellular interaction with electrospun keratin.

This research therefore proposes to fill this knowledge gap by fabricating fibrous matrices with relatively high content of keratin extracted from human hair via

electrospinning. These fibrous platforms will then be used in cell culture studies to understand and evaluate the potential of electrospun human hair keratin as an alternative material for tissue engineering.

Currently, there are limited papers that explored the cellular viability of primary human cell lines on electrospun keratin fibrous platforms. As this proposed work aims to contribute knowledge of keratin fibrous platform as potential tissue regenerative material in human, it is therefore of interest to investigate the cell response of human cell lines to evaluate better the ability of keratin fibrous platforms for tissue engineering.

1.3 Research Work: Objectives and Scope

The general objective of this proposed work is to evaluate the feasibility of fabricating fibrous platforms using keratin extracted from human hair, study the viability of using these platforms as templates for culturing human cells and elucidate the cell-material interaction between human hair keratin and human cells.

The scope of this proposed research work can be sub-divided based on three hypotheses.

***Hypothesis 1:** Human hair extracted keratin can be electrospun into random and aligned fibers via the dissolution of high content keratin into the solvent of the electrospinning formulation.*

***Specific objective:** Evaluate the correlation between fabrication parameters and the physical properties of random and aligned electrospun keratin fibers.*

Key activities:

- Fabricate random fibrous keratin platforms by optimizing the solution parameters which include the keratin concentration and synthetic polymer (used to aid the electrospinnability of keratin) concentration.
- Fabricate aligned fibrous keratin platforms by optimizing the electrospinning parameters which include applied voltage, feedrate, spinneret-collector distance and speed of the drum collector.
- Characterize the physical and chemical properties of the electrospun keratin platforms.

Hypothesis 2: *Electrospun keratin platforms can support the attachment, proliferation and function of human dermal fibroblasts via topographical cues from the architecture of the platforms and biochemical cues from the inherent protein characteristics of the keratin.*

Specific objective: Evaluate the ability of random and aligned electrospun keratin platforms to support human fibroblast attachment, proliferation and function.

Key activities:

- Carry out basic cell studies by observing cell response using Live/Dead staining assay and cell morphology using scanning electron microscopy.
- Quantify cell viability and proliferation using metabolic and DNA quantification assays.
- Analyze cell behavior as a function of shape, cytoskeletal development, focal adhesion and cell distribution using immunocytochemistry.
- Assess the extent of ECM production using immunocytochemistry.

Hypothesis 3: *Keratin plays a bioactive role to enhance the growth of human dermal fibroblasts via the affinity of $\alpha_4\beta_1$ integrin to the LDV cell adhesion motif.*

Specific objective: Elucidate the expression of $\alpha_4\beta_1$ integrin in human dermal fibroblasts cultured on human hair keratin templates.

Key activities:

- Fabricate keratin films to eliminate the topographical cues for cell-material interaction study.
- Carry out Western blotting on keratin extracts to demonstrate the presence of LDV containing keratin subtypes.
- Characterize the physical and chemical properties of the keratin films.
- Assess the degree of α_4 and β_1 integrins dependency of cell attachment between human cells and human hair keratin via integrins inhibition assay.
- Observe the expression of α_4 and β_1 integrins from human cells cultured on keratin films via Western blotting.

1.4 Thesis Outline

To realize the potential wound healing application of keratin electrospun fibers, it is imperative to look into what have been discussed about keratin and how the electrospinning technique can enhance keratin's role in tissue regeneration. In Chapter 2, a review on keratin as a biomaterial and how electrospinning can be employed to create a viable platform from keratin for wound healing were discussed. Besides, existing researches on electrospun fibers with keratin as part of the component were collated into a comprehensive summary in Chapter 2. Elaboration of materials and methods utilized in this work will be presented in Chapter 3. In Chapter 4, feasibility of extracting keratin from human hair and processing it into a fibrous platform via electrospinning were explored. Afterwhich, the materials properties and cell compliance of the optimized fibrous platform were analyzed in Chapter 5. Content presented in Chapter 6 seek to develop tenability in the alignment of the keratin fibrous platform and examine the

potential of keratin fibrous platform for wound healing. Chapter 7 focused on elucidating the possible underlying involvement of keratin and electrospinning in cell-material interaction between human hair keratin and human cells. Finally, Chapter 8 concluded the work in this thesis with recommendations on future work.

Chapter 2:

Literature Review

This chapter is split into 3 sections, namely; 1) Keratin, 2) Electrospinning and 3) Current research of keratin-based electrospun fibers. The first section summarizes the theory of human hair keratin and discusses the state of the art in keratin extraction, keratin material fabrications and applications. The second section summarizes the theory of electrospinning and explains how the solution parameters and controlling parameters affect the morphology of the resulting fibrous platforms produced from electrospinning. It also discusses various ways established to quantify the degree of alignment of the resulting fibrous platforms. The last section summarizes all the on-going research of composite fibers from keratin blends and reviews on the current research trends and applications for keratin-based electrospun fibers.

2.1 Keratin

Keratin is a class of cytoskeletal proteins which can be extracted out of many natural resources such as hair, feathers, wools and nails and horns of mammals. It distinguishes itself from the other proteins by its high content of cysteine residues. Cysteines are sulphur-containing amino acids which accounts for the intermolecular disulfide bridges that contribute to the keratin's stability and superior mechanical strength [35].

2.1.1 Human Hair Keratin

Human keratin can be broadly classified into soft keratins and hard keratins. Soft keratins can be found in the skin epithelia while hard keratins can be found in more resilient structures like nails and hairs [36]. The human keratin family consists of 54 subtypes which are split into acidic keratin or basic keratin subtypes. There are 28 acidic keratin subtype (~45 kDa); of which 17 are epithelial and 11 are hair keratins while there are 26 basic keratin subtypes (~50 kDa); of which 20 are epithelial and 6 are hair keratins [36].

As a member of the cytoskeletal component, keratins present themselves as intermediate filament proteins ranging from 8 to 10 nm in diameter and organized in the manner in which 2 parallel helical chains dimerize into a coiled-coil fashion as shown in Figure 2-1 [37].

Figure 2-1 Structure and assembly of intermediate filaments. A and B: helical domains; L: non-helical linkers; N and C: N and C terminus. Reproduced with permission from [37]

Polymerized keratin are held together by inter and intra molecular bonding of polar and non-polar amino acids and disulfide cysteines bonds, giving keratin its highly stable supermolecular structure [38] as shown in Figure 2-2.

Figure 2-2 Chemical interactions between keratin protein chains. Reproduced with permission from [38]

It is noted that keratins from hair contain higher amount of cysteine compared to the other keratin sources [34] and hence form a tougher and more durable structure through the disulfide bonds.

The human hair consists of three major components which are the cuticle, cortex and medulla [37] as shown in Figure 2-3. The outmost layer of the hair is the cuticle. It is a thin layer of overlapping flattened cells which give the hair a scaly appearance at the microscopic view. The cuticle serves to protect the hair from physical and chemical damages as it contains β -keratins [34]. The next layer is the cortex which makes up a major area of the hair. The cortex is where keratin intermediate filaments are found. The

core of the hair is the medulla which can sometimes contain a column of loosely connected keratinized cells [39].

Figure 2-3 Schematic model of hair. The hair consists of three main parts: cuticle, cortex and medulla. Reproduced with permission from [37]

Human hair keratins consisted of three categories; the α , β and γ keratins. α -keratins can be found in the cortex of the human hair. They are low in sulfur content and have a molecular weight ranging from 40-60 kDa. α -keratins act as a structural support with their α -helical tertiary structures. β -keratins reside in the cuticle, offering a protective function against chemical and physical damages for the human hair [40]. Besides forming a protective layer, β -keratins do not form any useful reconstituted structures and are hard to extract [39, 41]. γ -keratins are high in sulfur content and have a relatively lower molecular weight of 10-25 kDa. They act as disulfide cross-linkers to hold the keratin's supermolecular structure together.

Expression of specific keratin subtypes can be found in different locations in the human hair [42-44] as shown in Figure 2-4

Figure 2-4 Human hair keratin subtypes. A) Illustration of their expression sites and B) summarizing table of their location in human hair. Adapted with permission from [42]

Certain keratin subtypes in the human hair keratin contains Leu-Asp-Val (LDV) as part of their protein sequence. These keratin subtypes are mainly acidic keratin; K31, K32, K33a, K33b, K34, K35, K37 and K38 while K82 is the only basic keratin subtype in human hair that contains LDV.

2.1.2 Bioactivity of Keratin

Generally, protein possesses various biological properties such as biodegradability and bioactivity. Compared to biocompatible synthetic polymers available currently, proteins offer an advantage of inducing cell response and ECM remodeling by carrying cell adhesion motifs which have high affinity with cell receptors. Materials constructed from these proteins could significantly enhance cell response [45]. Likewise, keratin as a protein therefore emerges as an attractive source for constructing potential bioactive materials for tissue engineering.

As mentioned earlier, it has been documented that human hair keratin contains LDV as part of its protein sequence [12]. LDV is a cell-binding amino acid sequence which is specifically recognized by $\alpha_4\beta_1$ integrin [13] and can commonly be found on proteins such as fibronectin and vascular cell adhesion protein 1 (VCAM-1) [13, 20]. It has been widely discussed that these LDV-containing proteins can promote $\alpha_4\beta_1$ mediated

adhesion of human dermal fibroblasts [14], endothelial cells [15, 16] and leukocytes [17-19], which are cells critical for tissue healing.

Besides human hair keratin, it is also noteworthy that keratin extracted from animal wool contains different cell adhesion motif from human hair keratin. Wool keratin contains amino acid motif Arg-Gly-Asp (RGD) which are common cell adhesion motifs found on various ECM proteins such as collagen and fibronectin. It has been demonstrated that wool keratin can facilitate osteoblasts differentiation due to the amino acid motif RGD found on keratin [46].

2.1.3 Extraction of Keratin

The first patent to be issued on extraction of keratin was the technique of using lime to extract keratins from animal horns by John Hoffmeier in 1905. In three decades that followed, more techniques using oxidative and reductive chemistries [41] were developed to extract keratins, not only from horns and hooves but also from wool and human hair eventually [47-49]. These extraction methods involve using reducing or oxidizing chemicals to cleave the disulphide bonds of γ -keratins such that α -keratins are partially hydrolyze, making extracted keratins soluble [41]. Reduction methods were more commonly used due to resulting keratin extracts being more stable and allowing re-crosslinking easily [41].

There were many combinations of different reductants used in keratin extraction. One example is the Shindai method which includes an extraction buffer of 25 mM Tris HCl, 2.6 M thiourea, 5M urea and 5% β -mercaptoethanol (2-ME) and incubating the mixture at 50 °C for a period of 1 to 3 days [50]. Urea aids in breaking down the hydrogen bonds to increase the accessibility of the disulphide bonds while 2-ME reduces the disulphide bonds. Presence of thiourea helped to increase the yield and it was reported by Nakamura et al. that Shindai method gave a maximum yield of 65% in the extraction of keratin from human hair. It was discussed that 2-ME was required to increase the efficiency of extraction because the extraction buffer without 2-ME decreased the yield by about 5%

[50]. Schrooyen et al. reported a simpler version by using an aqueous mixture of just urea and 2-ME with a much shorter incubation time of 2 hours [51]. The exact recipe was a minimum concentration of urea was 5M and at a pH of more than 7.0 was required for solubilizing the keratins to a maximum value of 75% within the 2 hours of incubation time. The group also discussed that any lower concentration of urea would then require a higher pH of the solution to obtain an equal extraction yield but it was noted that increase in pH would bring about degradation of the extracted keratin. Despite the high yield, the group however found out that extensive protein aggregation was observed upon dialysis. The group therefore changed its keratin extraction method to using just sodium sulphide (Na_2S) as the reductant and the extraction yield was 60%. Tosik et al. also reported possible keratin extraction using Na_2S [52]. The group utilised an aqueous mixture of 5% NaOH and 0.1 M of Na_2S . The process of keratin extraction was carried out at a temperature of 40 °C for 2 hours and the maximal yield was 40 %.

Besides Na_2S , other possible chemicals used in reductive keratin extraction used were dithiothreitol (DTT) and tris (2-carboxyethyl) phosphine (TCEP). Notably, TCEP was discussed to be a better alternative for 2-ME as it offers a more stable extraction due to the oxidation-resistant nature of TCEP solutions [53]. In addition, TCEP is water soluble, odourless and works over a wider range of pH than reductants like DTT [54].

2.1.4 Keratin as a Biomaterial

After the first appearance of the word “keratin” in the literature at around 1850, many researches on keratin extractions had been done, leading to the attention in developing keratins for medical purposes due to the biological properties found in the keratin extract. Early inventions include keratin powders for cosmetics and coatings for drugs. Thereafter, keratin research ranges from the structure and function of the protein to its biochemistry and biocompatibility. Over the past few decades, there are positive reviews on the prospect of using keratin as a biomaterial for clinical applications [34].

Keratin can be processed into various types of biomaterials such as gels, films, sponges and fibrous scaffolds. Research on keratin-related biomaterial started as early as 1972 in the literature [55-57], followed by the first known in-vivo study in 1982 appeared whereby implantation of a vascular graft coated with denatured wool keratin derivatives in a dog for more than 200 days without thrombosis, was successful. Since then, keratin finds its application in neural repair [58, 59], bone regeneration [46], hemostasis [60] and wound healing [61-64].

It has been demonstrated that keratin act as a useful base for good adhesion and rapid proliferation of fibroblasts [48, 65]. In addition, keratin has been proven to be effective in mediating Schwann cells so as to facilitate functional nerve regeneration [59] and also promising cultivation of corneal epithelial cells to stimulate cornea reconstruction [66, 67]. Much focus has also been put on keratin's potential for bone regeneration following the ability of keratin to allow strong calcium ions (Ca^{2+}) chelating Schiff base groups onto scaffolds and hence enhancing formation of calcium phosphate [68]. The formation of uniform calcium phosphate on keratin scaffolds will induce apatite-forming ability, resulting in an osteoconductive scaffold. Such keratin scaffolds have been reported to support healthy growth of bone-related cells such as sarcoma osteogenic cells [69, 70] and osteoblasts [46] for bone regeneration. Besides, another area on keratin's potential for bone regeneration was also looked into, by using keratin as a vehicle for delivering bone morphogenetic protein-2 (BMP-2) to enhance bone regeneration. Results showed support for preosteoblasts differentiation and growth by various groups [71-73]. In-vivo work has also been done by implanting keratin matrices in the distal femur of the sheep to study osteointegration. Results showed implant being infiltrated by granulation tissue followed by new bone formation [74].

In the area of wound healing, there are numerous patents proving keratin's ability to promote wound healing [61-64]. Results reported in these patents ranges from demonstrating keratin as wound healing agents in enhancing the proliferation of human dermal fibroblast [61] to showing accelerated wound healing in burn wounds using animal and human models. Promotion of skin healing by releasing keratin powder from

an absorbent wound dressing to the wound has been reported [75]. Keratin materials applied to wounds on rats and humans have shown not only faster healing of wounds but also reduced pain [62]. In addition, when keratin materials were applied to bleeding wounds, they provided a porous environment for cell infiltration and granulation tissue formation when forming a physical seal of the wound site [64], deeming them desirable compared to blood clot.

Keratin has also been widely blended with other polymers to form novel composites for anticipated synergistic effect on cell response and wound healing. Cell proliferation and vascularization were stimulated by keratin-collagen composite in rat, demonstrating accelerated wound healing and tissue compatibility [76]. Moreover, keratin-collagen composite containing poly2-hydroxyethylmethacrylate (PHMA) allowed in vivo construction of tissue engineered epidermis when applied to burn wounds [77]. Keratin-gelatin composite has also been studied on full-thickness wounds in dogs, reporting presence of sebaceous gland and hair follicles with normal thickness of epidermis grown at an early stage [78, 79]. In depth study was also done to prove synergistic effect of keratin composite when Lee et al. first reported keratin-silk fibroin blend composite which improved biocompatibility and anti-thrombogenicity compared to pure silk fibroin and keratin films [80].

Overall, it is concluded that there is a good potential for research on keratin so as to understand, control and exploit the use of keratin to solve healthcare problems.

2.2 Electrospinning

Electrospinning is a simple and efficient technique to produce fibrous material. Compared to other processes, electrospinning is more cost effective and scalable. As a result, electrospinning has been widely used and some applications have gone further to the industrial level from laboratory research and development stage [81]. Many patents on biomedical applications of electrospun fibers have been published and the areas

include drug delivery, tissue engineering, wound dressing and cosmetics. Figure 2-5 below shows a diagram of an electrospinning setup.

Figure 2-5 Electrospinning of polymeric solution into continuous fibers

2.2.1 Fundamental Theory

In electrospinning, a voltage is applied to a polymer liquid, resulting in like charges accumulating on the liquid surface. When the pressure due to the electrostatic repulsion of like charges exceeds the surface tension of the liquid, the liquid surface becomes unstable and erupts into a liquid jet with the formation of a Taylor cone. Meanwhile, the polymer chain entanglements within the solution will prevent the liquid jet from breaking up into droplets. During the process, the polymer liquid jet gets stretched from the needle tip of high electric potential to the collector's platform of low electric potential. As the liquid jet stretches, the solvent evaporates and solid fibers will be formed and accumulate on to the collector.

Although the setup for electrospinning is simple, the process is highly complex and optimizing of parameters is very important [81]. Factors such as the electrospinnability of a polymer solution, the presence of bead defects, the diameter and various different interesting morphologies of the fibers depend greatly on the processing parameters. The processing parameters can be categorized into solution properties, electrospinner parameters and ambient parameters as shown in Table 2-1 below.

Table 2-1 Processing parameters in electrospinning

Category	Processing Parameters
Solution Properties	Molecular Weight and Solution Viscosity Surface Tension Solution Conductivity Dielectric Effect of the Solvent Solution Concentration
Electrospinner Parameters	Voltage Feedrate Distance between the needle tip and collector Needle diameter
Ambient Parameters	Temperature Humidity Air Velocity

The most important categories of parameters in this work are the solution parameters to derive an optimized formulation and the electrospinner parameters to derive high degree of alignment to electrospin keratin fibers.

2.2.2 Solution Parameters

The most important solution parameters in this work to get an optimized formulation to electrospin keratin are molecular weight of the polymers, solution viscosity and solution concentration. These three parameters also correlate with each other.

Whether there are fibers being produced continuously throughout the electrospinning process depends on the molecular weight of the polymer in the solution. As mentioned earlier, the presence of the polymer chain entanglements in the solution is important as it help to maintain the liquid jet which will be stretched into fibers. Hence, the molecular weight of the polymer in the solution should be above a critical molecular weight so as to achieve the minimum polymer chain length needed for the formation of stable entanglements. Therefore, studies facing the problem of electrospinning low molecular weight polymer, often had to blend the polymer with another ultra high molecular weight polymer which will aid the electrospinning process [82-85]. Besides, chain entanglements can enhance solution viscosity, ensuring sufficient degree of solution viscosity to restrain effects of surface tension and hence allow uniform jet formation during electrospinning [86]. Many studies [25, 87-89] have reported that solution viscosity played a dominant role in altering the morphology and the fiber diameter of the electrospun fibers. Low

viscosity will result in electrospinning as solution would tend to form droplets rather than continuous fibers. However, solutions that are too viscous could result in difficulty in the ejection of jets from polymer solution [90]. Therefore, to find an optimal viscosity range is required in order to ensure feasible electrospinning from the solution.

The optimal viscosity range in which feasible electrospinning is allowed, differs from system to system. Maximal solution viscosities for electrospinning of various polymer solutions may range from 1 to 215 mPa [91-94]. For pure polyethylene oxide (PEO) system, it was reported that the viscosity range should be between 1 to 20 mPa for PEO solutions to be spun into well-formed PEO fibers by electrospinning [95]. However, no similar study has been reported for any keratin system.

The dependency on solution viscosity for electrospinnability could be explained by the need to balance the surface tension. Sufficient solution viscosity is needed to allow longer stress relaxation times which could avoid the fracturing of the ejected jets during electrospinning and prevent surface tension to become a dominant factor which will result in beaded fibers otherwise [96, 97] .

To achieve sufficient viscosity, a certain critical polymer concentration was to be reached [98] to obtain well-formed fibers, hence concentration of the polymer in the solution plays an important role in the system's electrospinnability. When concentration of the polymer solution is too low, it will yield fibers with more bead defects [99] as shown in Figure 2-6A. This is because lower polymer concentration will mean that there are fewer chain entanglements. With fewer chain entanglements, surface tension of the solution has a dominant influence along the liquid jet and this causes the beads to form along the fiber due to the solvent molecules coming together.

Figure 2-6 Polycaprolactone electrospun fibers with a) beads at concentration of 0.1g/mL and b) no beads at 0.12g/mL. Reproduced with permission from [99]

On the contrary, if the polymer concentration used is too high, it would lead to the solution having the tendency to gel up, resulting in difficulty in the ejection of jets from the polymer during electrospinning.

2.2.3 Controlling Parameters

The most important electrospinner parameters to alter the formation and morphology of keratin fibers in this work are the voltage, feedrate and tip to collector distance.

Voltage is crucial to initiate the electrospinning process by providing a electrostatic force to overcome the solution's surface tension [96]. However, it should not reach beyond the threshold voltage needed to induce the necessary charges in the solution to start electrospinning [90]. It is reported that voltage that is too high can cause formation of beads in fibers [21]. The effect of voltage on resulting fiber diameter is widely discussed and there is variation reported. It was commonly reported that increased in applied voltage would result in thinner fibers due to increase in the electrostatic repulsive force that stretches the polymer jet [100]. In contrast, there are also reports that indicated that higher applied voltage resulted in thicker fibers due to more polymer ejection induced by the stronger electrostatic force pulling the solution out [101].

Feedrate determines the amount of solution dispersed, the jet velocity and the travelling time of the jet from the needle tip to the fiber collector. Lower feedrate allows sufficient time for solvent evaporation and hence result in drier fibers being produced with no destructive issues brought about by wet fibers [102]. Feedrates that are too high would result in beaded fibers being produced due to insufficient proper dry time before the jet reaches the collector [103]. The effect of feedrate on the resulting fiber diameter is straightforward. It is widely reported that fiber diameter increases with feedrate as the solution would be drawn in higher amounts from the tip to the collector, forming thicker fibers at higher feedrate [103].

Tip to collector distance also affect the fiber diameter and morphology by having a direct influence on the electric field strength and the flight time of the polymer jet. Distance between the tip to collector indicates the amount of time for the solvent to evaporate from the solution before reaching the collector as solid fibers. Short tip to collector distance would cause insufficient time for multiple bead formations at various points of the fiber due to inadequate drying before the fiber could reach the collector [96, 104]. On the contrary, if the distance arranged was too far for the applied voltage to maintain a constant electric field, unstable droplets will fall straight to the collector before it could be stretched. It is therefore important to identify the optimal distance that allows the solvent to evaporate with sufficient time to form continuous fibers [101, 105, 106].

2.2.4 Alignment of Fibers

Exploring broader into the potential of electrospinning, there are many interesting areas such as creating hollow fibers, porous fibers, ribbon-like fibers and aligned fibers. Of all areas, the subject of getting aligned fibers has been extensively researched upon in tissue engineering and special collectors such as rotating drums, parallel plates, disc collector, water bath system and other innovative collectors were designed to allow control over fiber alignment, yield and deposition area [107]. It has been discussed by many studies the importance of aligned fibers in modulating cell behaviour [108] through topographical cues and electrospinning emerges as the most common technique to fabricate aligned fibrous scaffolds due to its simplicity.

Random distributions of non-woven fibers are commonly observed via a general electrospinning process. These fibers which orient isotropically will usually be formed on a grounded stationary collector. Even though the applications of these random fibers are vastly employed, there are still others whereby aligned fibers are required as mentioned earlier. The change in geometry of collector can help to meet this demand. Collectors specially setup to collect fibers include a rotating drum, rotating disk and parallel plate.

A rotating drum is one of the most common collectors used to replace a grounded stationary collector in hope to fabricate parallel aligned fibers. Alignment of the fibers is made possible under the influence of high velocities and mechanical tensile force of the rotating collector. In order to improve the degree of alignment, tangential velocity at the edge of rotating drum has to coincide with electrospinning jet velocity. Figure 2-7 is an illustration of a rotating drum [109].

Figure 2-7 Schematic diagram of a rotating drum. Reproduced with permission from [109]

In of highest possible degree of alignment, there is a threshold velocity which is characteristic for different systems. Generally, the degree of alignment is proportional to tangential velocity after the initiation of fiber alignment. This is because, with increasing drum velocity, fibers will experience mechanical extension and with further increment in drum rotation speed, the crystal structure of fibers will be highly orientated as a result of deformation. However, fracturing of fiber can take place when drum velocity exceed the threshold of the system. This will then dampen fiber quality and fiber alignment [91, 108].

In the process of electrospinning, the spinning jet will experience bending instability and distributes over a wide area. To eliminate this bending instability as much as possible, a rotating disk which is similar to rotating drum but with a much smaller collecting area is used as a collector to get highly aligned fibers. With the use of such knife-edge disk as shown in Figure 2-8, electric field lines will converge towards the edge of the disk as it rotates and guide the deposition of highly aligned fibers [109].

Figure 2-8 Schematic diagram of a rotating disk. Reproduced with permission from [109]

However, the disadvantage of the rotating disk compared to rotating drum is that electrospinning with the disk collector limits the option of scaling up in production because of small area of deposition [91].

Unlike rotating mandrels, parallel plate collector is stationary and makes alignment of fiber possible by the manipulating the electrostatic force on the conductive area. This technique takes advantage of the sensitivity of jet to surrounding electric field, and electrical properties of polymer becomes critical as electrostatic forces can guide the alignment of fibers [108]. Figure 2-9 shows an illustration [108] of a parallel plate setup.

Figure 2-9 Schematic diagram of a parallel plate setup and the corresponding electric field involve. Reproduced with permission from [108]

Both grounded plates will form an electric field which induces the incoming jet to deposit perpendicularly to the conducting plates across the air gap in between. Stacking density of the deposited nano fibers can be regulated by changing the width of the gap in between the conducting plates. However, not all polymer solutions suit this method of alignment because its solution electrical properties do not lie within the critical range for operative deposition across parallel plates. Electrostatic force will be inadequate to direct solution

with depressed conductivity, uncontrolled whipping of jet will occur with solution conductivity that is too high [91, 108].

In of tuning the alignment of electrospun fibers, various ways have been developed to quantify alignment for systematic studies. Approaches include 2 dimensional Fast Fourier Transform (FFT), X-ray diffraction techniques and image analysis in terms of individual orientation and angular deviation.

Figure 2-10 shows an illustration of images that can be generated from 2D FFT function for quantification of alignment.

Figure 2-10 Light microscopy images of fibers (A, D, G). FFT frequency image (B, E, H) and FFT alignment graphs (C, F, I) for the corresponding light microscopy images. Peak height and shape in the FFT alignment graphs report the relative alignment degree while peak positions report the principle axis of orientation of the fibers. Reproduced with permission from [110]

A frequency plot with pixels accumulated along a specific axis will be observed for image with aligned fibers. Alignment density for the image will be represented by the shape and significant peaks in the alignment plot. Uniform degree of fiber alignment will have tall and narrow peak while various degree of alignment will have wide and broad peak [110]. The alignment plot can be generated with the aid of MATLAB.

Besides, X-ray diffraction had been documented to be able to quantify the alignment of fiber. The alignment will be measured with respect to a desired plane azimuthal diffraction at one-half of the maximum intensity peak of the diffraction profile. The measurements will be in degrees and represented by “Z”. In order to provide a 2D measurement of fiber alignment, this value is to be halved so that fiber angles of deviation from the reference axis can be provided. High intensity of diffraction pattern at greater angles will be observed with randomly aligned fibers while intensity at desired orientation (i.e. 0°) will be seen with aligned fibers [111]. Figure 2-11A below represents the graph of higher degree of alignment compared to Figure 2-11b.

Figure 2-11 X ray diffraction intensity versus Azimuthal angles measured from the preferred axis of polypropylene reinforced with various amounts of partially aligned VGCF. (a) 2.5% (b) 7% fiber volume fraction. Reproduced with permission from [111]

Lastly, direct image analysis in terms of individual fiber orientation and angular deviation enables the analysis of fiber orientation distribution. The angle deviation of fibers will vary from -90° to $+90^\circ$ with 0° as no deviation i.e. desired alignment. Image-pro express software will be used to measure the angular deviation of fibers from its respective SEM images. These values will then be collated for the quantification of fiber alignment with the aid of circular statistics. A histogram of the collated data as shown in Figure 2-12 is then constructed for analysis of the degree of alignment [112].

Figure 2-12 Angular distribution of the aligned collagen fibers ($AD = 10.7^\circ$). Reproduced with permission from [112]

2.3 Current Research on Keratin-based Electrospun Fibers

In exploring the material and biological properties of the keratin, much research work has been done through preparation of films [66, 113-117], gels [59, 60, 118, 119], and sponges [12, 48, 73, 120-122] from extracted keratin. In recent years, fibrous biomaterial has been identified as the potential for abundant biomedical applications because almost all of the human tissues are in fibrous form [81]. Besides, it would be interesting to observe how electrospun keratin differs from other forms of keratin. Hence, the preparation of extracted keratin has also been extended to creating fibrous structure through electrospinning.

2.3.1 Composite Fibers from Keratin Blends

Due to the low molecular weight of the extracted keratin, many researchers have resorted to fabricate fibrous matrices using keratin in combination with other natural or synthetic polymers.

2.3.1.1 Electrospinning of Keratin from Other Sources Besides Human Hair

Li et al. [27] created keratin/poly (L-lactic acid) (PLLA) electrospun fibrous matrix by dissolving 1 wt% of PLLA and keratin from wool into an organic mixture of chloroform

and N,N-dimethylformamide (DMF) such that the blend ratio was 1:1 (w/w). It was concluded that keratin particles were embedded into the PLLA fibers separately and homogeneously. Cell culture was done on the matrix and it was shown that keratin provided continuous osteoblast cell growth.

One research group from Institute for Macromolecular Studies, Italy and Politecnico di Torino, Italy has been actively doing research work on electrospun keratin-based material derived from wool. This group focuses more on the general material science of keratin blend electrospun fibrous matrix through investigating the basic properties such as mechanical and thermal properties of keratin blend fibers, how the other polymer in the blend affects the bonding of the keratin and vice versa. In addition, films from the keratin blends were also prepared to make comparisons with the electrospun fibrous matrices on the keratin properties. Their research includes electrospinning of the following blends:

- 1) Keratin/ Poly(ethylene oxide) (PEO) [25, 26]
- 2) Keratin/ Polyamide 6 (PA6) [30, 123]
- 3) Keratin/ Fibroin [31]

For keratin/PEO, the blend was prepared by adding PEO into keratin aqueous solution. The first attempt reported in 2006 was done by preparing 50:50 blends with total polymer concentrations of 5, 7 and 10wt%. Fibers with bead defects were generated from blends with 5 wt% polymer concentration. Subsequently, reported in 2008, keratin/PEO blends were prepared in different ratios of 90/10, 70/30, 50/50, 30/70 and 10/90 with keratin concentration fixed at 2.7%. The solution with 90/10 keratin/PEO blend produced droplets and fibers with bead-like defects during electrospinning. Electrospinning of pure keratin aqueous solution was not done.

However, for the keratin/PA6 and keratin/fibroin research, pure keratins dissolved in formic acid were being electrospun. In the work of keratin/PA6 blend, 28 wt% solutions were being prepared in different ratios of 100/0, 90/10, 70/30, 50/50, 30/70, 10/90 and 0/100 using formic acid as the solvent. Fibers with distinct bead defects started to form in the electrospinning of 70/30 keratin/PA6 blend. As for the work on keratin/fibroin blend, 15wt% of fibroin in formic acid and 15wt% of keratin in formic acid were blended in

different ratios of 100/0, 90/10, 70/30, 50/50, 30/70, 10/90 and 0/100. Well-formed, beadless fibers were electrospun from 90/10 keratin/fibroin solution but bead defects were obvious in pure keratin (100/0) fibers.

Various attempts have also been done to increase the solubility of the keratin in protein compatible electrospinning solvents such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in order to generate good fibers. Extracted keratin are hard to dissolve because their SH groups are easily oxidized to form disulfide bonds which restrict the ease of solubility of keratin [33]. To protect the SH group from oxidation, Yuan et al. treated the keratin derived from human epidermis with iodoacetic acid and the modified keratin was termed as m-keratin. A 15wt% final concentration of polymer solution was prepared with polylactide (PLA)/ m-keratin dissolved in HFIP at a ratio of 8/2 [29]. The ratio was fixed at 8/2 because preliminary results from their research showed that electrospun matrices with high keratin content were rough and had defects on them. It was noted that the solubility of m-keratin was very low even in HFIP. Subsequently, the group did a study in preparing m-keratin/ poly(hydroxybutylate-co-hydroxyvalerate) (PHBV) [33] dissolved in HFIP. 6wt% solutions were prepared in different ratios of 0/10, 3/7, 7/3 and 10/0. It was reported that pure electrospun m-keratin (10/0) using HFIP as the solvent resulted in fibers containing many beads. The group explained that this result was due to the broad molecular weight distribution and low solubility of m-keratin. Recently, the group attempted and managed to electrospin keratin/PEO blends with high weight ratio of 90:10 at a total polymer concentration of 2 wt% using 2,2,2-trifluoroethanol (TFE) as the solvent. However, for further characterization, the resulting matrix had to be cross-linked to prevent dissolution but fiber morphology was compromised after cross-linking.

Baek et al. [32] managed to electrospin well-formed and beadless pure keratin fibers by dissolving the soluble fraction of keratin derived from wool into formic acid. It was found that the pure keratin solutions prepared could be electrospun in the range of 20% to 40% concentration but good fibers without bead defects were generated only at the keratin concentration not less than 30%. Despite getting the results of good fibers with pure keratin solution, it was reported that the electrospinnability of keratin was so poor

that stable spinning did not maintain throughout the electrospinning process. Hence, to improve the electrospinnability, the group blended keratin with fibroin to generate fibrous matrix for further characterization to show that the blended matrix is a good metal ion adsorption filter.

2.3.1.2 Electrospinning of Keratin Derived from Human Hair

Research work using keratin extracted from animal wool has been more prominent and active due to huge investments from the wool industry. Likewise, for groups that did electrospinning of keratin, majority has used keratin derived from wool. However, it is noted that human hair keratin might be a more compatible protein for human healthcare application due to its autologous possibility. Besides, the bioactive cell adhesion motif found in human hair keratin is different from that found in wool keratin, indicating possibility of different cell response on keratin platforms from different sources. Hence, it is also worthwhile to explore the feasibility of electrospinning keratin from human hair.

It was only until recently that electrospinning using keratin extracted from human hair was reported. In 2013, Sow et al. [124] first reported the feasibility of electrospinning human hair keratin by dissolving 30 wt% keratin and 0.5 wt% PEO into Na₂S solution. This work is part of the entire work in this dissertation. In 2014, Hsuel et al. [125] reported that well-formed human hair keratin fibers could be spun by mixing gelatin solution and keratin solution at different volume ratios of 1:2, 1:1 and 2:1 (keratin : gelatin). The keratin solution was prepared from 10 wt% keratin in 98% formic acid while the gelatin solution was prepared from 20 wt% gelatin in 98% formic acid.

In 2015, three additional studies on electrospinning using human hair keratin were reported. Edwards et al. [126] reported that keratin and poly (ϵ -caprolactone) (PCL) were prepared individually and mixed at different ratios of 90:10, 80:20, 70:30 and 60:40 (PCL : keratin). The keratin solution was prepared from 10 wt% keratin in DI water while the PCL solution was prepared from 10 wt% PCL in trifluoroethanol. Zhao et al. [68] has also electrospun human hair keratin with PCL. The electrospinning solution was prepared by

mixing PCL and keratin to a final concentration of 10 wt% at a weight ratio of 7:3 (PCL : keratin) in hexafluoroisopropanol. Deniz et al. [127] reported that electrospinning solutions were prepared by adding 1 wt%, 2 wt% and 4 wt% of keratin into 10 wt% polyvinyl alcohol (PVA) in DI water.

Although electrospinning of human hair keratin was only reported recently, it is worthwhile to note that research focusing on using keratin-based electrospun fibers for tissue engineering has shifted from using keratin derived from various sources to just focusing on using keratin derived from human hair as summarized by Table 2-2 in the next section. The next section discusses the evolution of using keratin-based electrospun fibers as tissue regeneration platforms.

2.3.2 Applications of Keratin-based Electrospun Fibers in Tissue Engineering

Keratin-based electrospun fibers have been studied for a wide variety of applications including a metal adsorption filter for water treatment [32, 123, 128-132], vehicle of high surface area to volume ratio for anti-bacterial effects [133, 134] and ECM-mimicking scaffolds for tissue regeneration. In tissue engineering, many have evaluated the biocompatibility of keratin-based electrospun fibers through basic cell studies using NIH 3T3 fibroblasts. Generally, better cell viability and proliferation were observed on electrospun fibers which contained keratin as the additional component. Table 2-2 summarizes the existing reports on electrospun keratin fibers for tissue regeneration up till now.

Table 2-2 Table of publications on keratin-based electrospun fibers

Keratin Source	Other Components	Cell Line	Publications
Human Epidermis	Polylactide	NIH 3T3 Cells	Yuan et al. (2008) [29]
Wool	Poly (L-lactic acid)	MC 3T3 Osteoblasts	Li et al. (2009) [135]

Human Epidermis	Poly(hydroxybutylate-co-hydroxyvalerate)	NIH 3T3 Cells	Yuan et al. (2009) [136]
Human Epidermis	Polyethylene Oxide	NIH 3T3 Cells	Xing et al. (2011) [137]
Human Epidermis	Poly(hydroxybutylate-co-hydroxyvalerate)	In-vivo (Mouse)	Yuan et al. (2012) [138]
Wool	Poly (lactic-co-glycolic) Acid	Bone Mesenchymal Stem Cells	Zhang et al. (2013) [139]
Wool	Poly (L-lactic acid)	Sarcoma Osteogenic Cells	Li et al. (2013) [69]
Hagfish Thread	Poly lactide	MG-63 Cells	Kim et al. (2013) [140]
Wool	Poly (L-lactic acid)	Human Foreskin Fibroblasts	Zhang et al. (2013) [141]
Human Hair	Polyethylene Oxide	L929 Murine Cells Human Dermal Fibroblasts	Sow et al. (2013) [124]
Human Hair	Gelatin	Schwann Cells	Hsueh et al. (2014) [125]
Human Hair	Poly (ϵ -caprolactone)	NIH 3T3 Cells	Edwards et al. (2015) [126]
Human Hair	Poly (ϵ -caprolactone)	Human Mesenchymal Stem Cells	Zhao et al. (2015) [68]
Human Hair	Polyvinyl Alcohol	Sarcoma Osteogenic Cells	Deniz et al. (2015) [127]

Most of the in-depth tissue engineering studies focused on bone regeneration and evaluation of keratin-based electrospin fibers was done using various bone-related cells

such as sarcoma osteogenic cells, MG-63 cells, MC 3T3 osteoblasts and bone marrow mesenchymal stem cells. Recently, one study evaluated the potential of keratin-based fibers for neural regeneration using Schwann cells [125]. Only two research groups focused on wound healing and evaluated the biological properties of the keratin-based fibers using human foreskin fibroblasts and in-vivo work.

Firstly, Zhang et al. introduced a keratin-based scaffold by electrospinning wool keratin/ Poly (L-lactic acid) (PLLA) (wool keratin: PLLA = 1:9 w/w) fibers [141] and showed that cell viability of keratin /PLLA fibers tend to be higher than that of pure PLLA fibers as shown in Figure 2-13A. It was also indicated by the study that human foreskin fibroblasts exhibited spread morphology of keratin/PLLA fibers as shown in Figure 2-13B. However, it is noted that the FTIR spectra of the keratin /PLLA fibers reported in this study, as shown in Figure 2-13C, showed insignificant amide I (1630 cm^{-1}) and amide II (1550 cm^{-1}) bands, suggesting that keratin quality could have been compromised.

Figure 2-13 Study on wool keratin/PLLA electrospun fibers. A) Quantification of cell viability by MTT assay, B) Live/Dead staining of cells cultured samples and C) FTIR spectra of samples. Reproduced with permission from [141]

Another study focusing on wound healing; Yuan et al. introduced Poly(hydroxybutylate-co-hydroxyvalerate) (PHBV)/m-keratin (PHBV: m-keratin = 7:3, w/w) at a total polymer concentration of 6 wt% [138] to be the platform for further cell study after showing that PLA/m-keratin (PLA: m-keratin = 8:2) [29], PEO/m-keratin (PEO: m-keratin = 1:9) [24] and PHBV/ m-keratin (PHBV: m-keratin = 7:3) [33] and 6 % m-keratin fibers could be

electrospun but were not optimized platforms suitable for cell studies. It was demonstrated that NIH 3T3 cells integrated well with the electrospun fibers and that cell proliferation was significantly higher for NIH 3T3 cells cultured on keratin/PHBV fibers compared to pure PHBV fibers as shown in Figure 2-14A. Animal testing was also done using Athymic nude mice (n=3 for each experimental group) and results showed that wound size was significantly reduced for keratin/PHBV fibers compared to the control and pure PHBV fibers by Day 4 and this significant trend continued at Day 7 and Day 9, as shown in Figure 2-14B. The group concluded that keratin/PHBV keratin fibers could promote wound healing through results from cell proliferation study and animal testing. However, it is noted that the FTIR spectra of the keratin /PHBV fibers reported in this study, as shown in Figure 2-14C, showed insignificant amide I (1650 cm^{-1}) and amide II (1545 cm^{-1}) bands, suggesting that keratin quality could have been compromised.

Figure 2-14 Study on wool keratin/PLLA electrospun fibers. A) Quantification of cell viability by MTT assay, B) Live/Dead staining of cells cultured samples and C) FTIR spectra of samples. Reproduced with permission from [138]

A critical remark on the current understanding of how electrospun keratin fibers support cell growth for wound healing is that the studies were limited in terms of the keratin platforms used for evaluation. It is noted that the chemical conformation of these platforms did not significantly reflect the protein characteristics of keratin as mentioned

earlier in both wound healing studies and hence the effect of the keratin platforms on cell response is uncertain.

All in all, from this coverage of the on-going studies on electrospun keratin fibers by other research groups up till now, it is concluded that there is still significant knowledge gap in the know-how of electrospinning keratin as the major subject and subsequent understanding of the cell compliance of keratin-based electrospun fibers for wound healing.

Chapter 3:

Experimental Methodology

This chapter summarizes the materials and methods used in this work and it is split into five sections. The first section starts by revealing the technique used in extracting keratin from human hair followed by processing it into a biomaterial as discussed in the second section of materials fabrication. The resulting fibrous material was then characterized for its material properties using methods described in the third section on materials characterization. To evaluate the cell compliance and potential of the keratin fibrous platform for wound healing, a series of cell culture studies were done and the techniques used were indicated in details for the forth section on cell culture. Statistical analysis was done whenever necessary in this work to demonstrate whether there were significant differences among and between groups of specimens. The methods used for statistical analysis were described in the last section.

3.1 Extraction of Human Hair Protein

Human hair obtained from random local salons, were washed with ethanol and dried at room temperature. The hair was then soaked into a mixture of chloroform and methanol (2:1, v/v) for 24 h to delipidize it. Keratin extraction was done by adding the delipidized hair into 0.125M of sodium sulfide (Na_2S) (Sigma Aldrich) aqueous solution and incubating the mixture for 4 h at 40 °C. The supernatant was then dialyzed against 5 L of deionized water in cellulose tubing (Sigma Aldrich, MWCO: 12400). The dialyzed solution was finally lyophilized to obtain keratin powder.

3.2 Materials Fabrication

3.2.1 Preparation of Keratin Solutions

Commonly used electrospinning solvents such as hexafluoroisopropanol (HFIP) and acetic acid were used. In addition, solvents with different pH values were also tried out. Common solvents with various pHs were used and they include 1M of hydrochloride acid (HCl) at pH 0, distilled water (H_2O) at pH 7 and 1M of sodium hydroxide (NaOH) at pH 14.

Keratin solutions were also prepared in combination with a synthetic polymer. 70/30 poly (L-lactide/ ϵ -caprolactone) (PLC) from PURAC was used by dissolving it into HFIP from Sigma Aldrich. The solution was then blended with keratin in Na_2S solution. The mixture was prepared such that it contains 1wt% of PLC and 10wt% of keratin.

Another synthetic polymer used was polyethylene oxide (PEO) from Sigma Aldrich. The keratin/PEO solution was prepared by dissolving keratin into Na_2S solution and then by simply adding PEO into the keratin solution.

The final optimized formulation was used for electrospinning keratin fibers by mixing 30 wt% keratin with 0.5 wt% PEO in Na_2S solution.

3.2.2 Fibers Fabrication

Electrospinning was done by dispensing the optimally formulated keratin solution from a single spinneret at a constant feedrate of 0.5 mL/h, and an accelerating voltage of 10 kV. A grounded, metallic collector fixed at 15 cm away from the tip of the spinneret was used to collect the fibers to obtain random keratin fibers.

Aligned keratin fibers for downstream studies were fabricated by dispensing the same optimally formulated keratin solution from a single spinneret at a constant feedrate of 0.5 mL/h, and an accelerating voltage of 10 kV. A grounded rotating drum fixed at 12 cm away from the tip of the spinneret was used to collect the aligned keratin fibers at a surface velocity of 119.69 m/min.

As a control in the cell study for keratin fibers, electrospinning of gelatin was done by dispensing 50wt% gelatin (Gelatin powder from Bovine Skin Type B, Sigma Aldrich) in acetic acid (Sigma Aldrich) solution from a single spinneret at a constant feedrate of 0.5 mL/h, and an accelerating voltage of 16 kV. A grounded, flat metallic platform fixed at 12 cm away from the tip of the spinneret was used to collect random gelatin fibers. The gelatin fibers were subsequently crosslinked overnight with 2.5% of glutaraldehyde (Grade I, Sigma Aldrich) vapour.

3.2.3 Film Formation

Keratin air-dried films were produced by casting the optimally formulated keratin solution for electrospinning keratin on to 4 cm² circular glass coverslips and air-drying overnight at room temperature before peeling.

Keratin vacuum-dried films were produced by casting the optimally formulated keratin solution for electrospinning keratin on to 4 cm² circular glass coverslips and vacuum-drying for 30 min at room temperature before peeling.

Collagen films were fabricated as a control for biodegradation study. They were produced by casting collagen solution (Biocoat Rat Tail Collagen I, Corning) on to 4 cm² circular glass coverslips and air-drying overnight at room temperature before peeling.

3.2.4 Coating Preparation

Fibronectin coating was fabricated as a control for cell-material interaction study. They were produced by dissolving fibronectin powder (Human Fibronectin, Corning) into H₂O at a concentration of 1mg/mL and subsequently casting of the fibronectin solution on to 4 cm² circular glass coverslips and air-drying for 2h at room temperature.

3.3 Materials Characterization

3.3.1 Western Blotting of Extracted Keratin Solution

Protein electrophoresis separation was done using the XCell SureLock[®] Mini-Cell System with NuPAGE[®] 4-12% Bis-Tris Gels (Invitrogen). Extracted keratin solution was mixed with 2µl of sample reducing agent (DTT, 10X) and 5µl of LDS Sample Buffer (4X). A total volume of 20µl sample mixture containing 15µg of protein was loaded into each lane. The SeeBlue[®] Plus2 Prestained Standard (1X) was used to identify the molecular weight ranges of the samples' bands. Electrophoresis was performed at 120V for 90 minutes (Bio-rad) in 0.05% NuPAGE[®] MOPS SDS Running Buffer (20X, Invitrogen). The separated proteins in the gel were then stained with SimplyBlue[™] Coomassie SafeStain (Invitrogen) for 1 hour, and destained in ultrapure water on the belly dancer overnight.

Extracted proteins separated on SDS-polyacrylamide gel were transblotted onto a nitrocellulose membrane using the iBlot[®] Dry Blotting System (Invitrogen). Afterwhich, the membranes were blocked with 5% non-fat milk powder in Phosphate Buffered Saline (PBS, pH=7.4) with 0.5% for 1 hour at room temperature.

For total keratin analysis, the membranes were incubated with AE13 (Abcam) primary antibody in 1:2000, for an overnight at 4°C. Subsequently, the membranes were incubated with anti-mouse secondary HRP-conjugated antibody (Abcam) in dilution factor 1:4000, for 1h at room temperature. Both primary and secondary antibodies are diluted with 5% non-fat milk solution.

For acidic sub-type keratin analysis, the membranes were incubated with 3 different primary antibodies diluted to 1:2000 with 5% BSA (Sigma Aldrich) in PBST, for an overnight at 4°C. For keratin subtype K31 primary antibody (Epitomics), anti-rabbit secondary HRP antibody (Dako) in dilution factor 1:4000 with 5% non-fat milk solution was used. For keratin subtype K33 and K34 primary antibodies, anti-guinea pig secondary HRP antibodies (Dako) in dilution 1:4000 with 5% non-fat milk solution were used. All secondary antibodies incubation was conducted for 1h at room temperature. The blots were visualized with the SuperSignal West Femto Chemiluminescent Substrate System (Thermo Scientific) and imaged with ChemiDoc (Bio-rad).

3.3.2 Viscosity of Keratin Solution

A rheometer (Anton Paar Physica MCR 301), coupled with PTD 200 Peltier temperature controller at 25.0 °C, was used to measure shear rate-dependent viscosity measurements. The test was done using a cone-plate geometry (CP-60-0.5, Diameter: 59.972µm, Angle: 0.483°, Truncation: 56µm) in controlled shear rate mode, with logarithmical increment from 0.1 to 10,000 s⁻¹ and by using data point gradient of 10. The acquired data were analyzed with Rheoplus v2.66 software. The settings for the rheometer have been optimized beforehand with data point gradients, different cone-plate geometries and shear-rate ranges to ensure consistency of viscosity results over three different samples (n = 3). Finally, the viscosity value of the keratin solution was determined by extrapolation from the linear region of the graph obtained.

3.3.3 Morphology of the Fabricated Materials

Electrospun fibers morphology was observed under a scanning electron microscope (SEM) (JSM-6360F, JEOL Co.). The SEM helps to generate images of the samples' topography by bombarding the surface of the samples with its electrons and detecting the resulting secondary electrons generated by the samples' surface due to the bombardment. It is noted that resolution is affected by charging of samples especially biological specimens. Hence dried samples were sputtered with platinum at 20 mA for 60 sec and analyzed at an accelerating voltage of 5 kV so as to avoid charging issues that will lead to poor quality images. SEM images were taken at different magnifications. 5 random SEM images (2000x) were taken for fiber diameter measurement using Image J and a total of 150 data points were used to determine the average diameter. OriginPro 8.5 was used to plot histogram of fiber diameter distribution for further analysis.

2-D FFT analysis was done to determine the degree of fiber alignment as a function of electrospinning parameters. FFT works by the principle that it produced information of the spatial frequency of the original image. 3 random SEM images were converted into 8-bit grayscale TIF files, cropped to 704 x 704 pixels and were subsequently processed with ImageJ software (NIH) using an oval profile plug-in (developed by Bill O'Connell) [142] in the software. To correct for mathematical transformation inherent to this type of analysis, the FFT output image were rotated 90° so as to allow the correct principal axis of orientation to be directly determined from the position of the peak in the final FFT alignment plot. This FFT alignment plot is a graphical depiction of FFT frequency distribution generated by placing circular projection on the FFT output image and performing radial summation of the pixel intensities between 0° to 360°, in 1° increments. The degree of alignment is reflected by the overall shape and height of the peaks present in the plot. For direct comparison across different data sets, all FFT data were normalized to a baseline value of 0 before being plotted in arbitrary units, called FFT Peak units.

Keratin films were analyzed using the atomic force microscopy (AFM) and 3D height and 2D amplitude images of the films were collected. AFM measures the roughness of the surface by detecting the interaction of forces between the sample surface and the

cantilever. Similar to SEM, AFM is able to characterize sample morphology but at a smaller depth of field, slower scanning speed and the possibility of image artifacts. The analysis was done in tapping mode by Nanoscope IIIa (Veeco Instruments) using single-beam silicon cantilever probes (Veeco RTESP: resonance frequency 300KHz, nominal tip radius of curvature 10nm, force constant 40N/m). Data sets were subjected to a first-order flattening when necessary. The Nanoscope 6.13R1 software (Digital Instruments) was used to calculate Roughness (Ra), which is an arithmetic value that expressed the absolute height of a surface in comparison to a two-dimensional plane represented by the average sample height. Mean Ra values were extracted from 2D amplitude images taken in 6 independent experiments.

3.3.4 Chemical Conformation of Keratin Materials

Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) was done to determine the chemical composition of the lyophilized keratin, PEO, Na₂S hydrates and electrospun keratin fibers. FTIR is an absorption technique whereby IR radiation which passes through the sample will get absorbed by the functional group possessed by the sample. Thus, it determines the chemical composition of the sample via analysis of the acquired adsorption bands in the samples' spectra. It is noted that moisture can interfere with the absorption band causing broadening of band, thus samples were ensured to be as dry as possible before FTIR characterization. Absorbance spectra of the samples were recorded with a resolution of 2 cm⁻¹ and at wavelengths from 600 to 4000 cm⁻¹ using a FTIR spectrometer (Perkin Elmer).

X-ray photon spectrometry (XPS) was done to determine the nitrogen compositions of the electrospun keratin fibers and keratin films. XPS is a technique that helps to quantify the elemental composition of a sample via the irradiation of X-rays beam on the sample while detecting the number of electrons and their kinetic energy resulting from the irradiation. However, XPS is a surface technique and hence only information about the surface chemistry of the material can be extracted. XPS was measured on a Kratos AXIS Ultra spectrometer (Kratos Analytical Ltd) using a mono Al K α radiation source ($h\nu =$

1486.71 eV) operating at 5 mA and 15 kV. Deconvolution of the XPS peaks was done using XPSPEAK41 application software. N1s peak from the high resolution XPS spectra ($n=5$) of keratin films and fibers were deconvoluted into 3 peaks which represent amine group (-C-NH-) at binding energy of 399.4eV, amide group (-C=O-NH-) at binding energy of 400.5 eV and protonated amine group (-C-NH₃⁺) at binding energy of 401.4 eV. The percentage of the amide bonds in the nitrogen species were derived by calculating the area of the amide group peak as a function of the broad area of N1s peak and compared among the keratin fibers and films.

3.3.5 Thermal Properties of Keratin Materials

The thermal properties of the fibers and extracted keratin were assessed and compared through the results from the differential scanning calorimeter (TA Instruments). In differential scanning calorimetry (DSC), the difference in the amount of heat needed to increase the temperature of a sample and a reference is measured. It is a common technique to measure the glass transition temperature and melting temperature of synthetic polymers. As for proteins, DSC can help to detect their denaturation temperature because the unfolding of the protein structure is related to the protein's thermal stability. Hence DSC was done to identify the denaturation temperatures of the extracted keratin and electrospun fibers by ramping the temperature from 25 °C to 400 °C at a rate of 10 °C/min. Results were analyzed using TA Universal Analysis software. It is noted that DSC results of samples with different mixture of materials are hard to interpret due to overlapping transitions.

3.3.6 Mechanical Properties of Fibers

The mechanical properties of keratin fibers and gelatin fibers were measured using dynamic mechanical analyzer (DMA) Q800 (Research Instruments). DMA in film tensile mode measures the mechanical properties of the sample by pulling the sample with two clamps until it breaks. It is note that results with false accuracy may be generated if the sample did not break properly during the pulling process. Samples were tested in tensile mode by ramping the strain at a rate of 12%/min until they break and

using a preload force of 0.001N. The ultimate tensile strength and strain at break were determined from the stress-strain curves generated from 3 independent experiments for each sample type. The Young's modulus was determined by calculating the gradient of the initial linear slope of the stress-strain curve.

3.3.7 Biodegradation Studies of Fabricated Materials

Degradation behaviours of keratin fibers, keratin films and collagen films were studied under four different conditions, which were in PBS (pH 7.2), Tris buffer (pH 8), proteinase-K (Sigma Aldrich) in Tris buffer and chymotrypsin (Sigma Aldrich) in Tris buffer. Samples were created in circular shape with a size of exactly 4 cm² and mass of at least 40 mg.

For hydrolytic degradation studies, the samples were soaked in PBS and Tris buffer and incubated at 37 °C. Three samples of each type were recovered at each degradation time points and dried before measuring their weight. The degradation time points were set to be day 1, 3, 7, 14, 21 and 30. At the degradation time point, the weight of the degraded sample (w_f) was measured and weight loss percentage of the sample was calculated ($\% \text{weight loss} = (w_f - w_i)/w_i \times 100\%$), based on the initial mass of each sample (w_i) before incubation. This protocol is in accordance to the ASTM standard (ASTM F1635-11: Standard Test Method for in-vitro Degradation Testing of Hydrolytically Degradable Polymer Resins and Fabricated Forms for Surgical Implants).

For enzymatic degradation studies, two enzymes which were chymotrypsin and proteinase K) were used. Each enzyme was dissolved and activated in 0.05M tris buffer (pH 8.0) with a concentration of 1.0 IU/mL. The degradation time points were set to be day 1, 3, 7, 14, 21 and 30 with changing of fresh medium every 3 days because the enzyme activity could only last for 3 days as suggested by the supplier's protocol. Three samples of each type were recovered at each degradation time points and dried before measuring their weight. The weight of the degraded sample (w_f) was measured and

weight loss percentage of the sample was calculated ($\% \text{weight loss} = (w_f - w_i)/w_i \times 100\%$), based on the initial mass of each sample (w_i) before incubation.

3.3.8 Chemical Assays on Keratin Films

The ninhydrin detects free amine groups by producing purple colour, known as Ruhemann's purple when reacting with primary and secondary amines. The amount of free amine groups in the keratin films were quantified by ninhydrin assay in which the samples were immersed into 0.4wt% of ninhydrin solution (4mg of ninhydrin powder from Sigma Aldrich in 1mL of H₂O) and incubated at 100 °C for 20 min. The supernatant were then cooled to room temperature prior to their absorbance measurement at a wavelength of 570nm.

The Ellman's reagent detects free thiols by producing yellow colour in the presence of the thiol chemical group. The amount of free thiol groups in the keratin films were quantified by Ellman's reagent assay in which the samples were immersed into 0.4wt% of Ellman's reagent solution (4mg of Ellman's reagent powder from Thermo Scientific in 1mL of reaction buffer; 0.1 M sodium phosphate containing 1mM EDTA, pH8) and incubated at room temperature for 20 min. The absorbance of the supernatant were measured at a wavelength of 412nm.

3.4 Cell Culture

3.4.1 Cell Seeding

In vitro cell culture studies were done by culturing L929 murine cells (CCL-1™; ATCC, VA, USA) and primary HDFs (PCS-201-010™; ATCC) to assess the cell compliance of keratin fibers and films. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (PAA Laboratory) supplemented with 10% Fetal Bovine Serum (FBS) (PAA Laboratory), 1% Penicillin-Streptomycin (Gibco) and 1% L-Glutamine (200 mM, PAA Laboratory), at 37 °C and 5% CO₂.

Prior to cell seeding, samples were UV-sterilized for 10 min and rinsed twice with phosphate buffer saline (PBS). Subsequently, cells were trypsinized, resuspended in complete DMEM, counted and pipetted onto the different samples in 12 well-plates at a density of 5000 cells per cm^2 . The cell-seeded samples were then incubated for 4 h at 37 °C and 5% CO₂ to allow for cell attachment. Afterwhich, each well was filled with 1 mL of complete DMEM. Characterization of the samples was mostly carried out at 1, 3 and 7 days after seeding. Fresh medium were changed every 3 days within experiments.

3.4.2 Cell Quantification

PicoGreen (Invitrogen) and AlamarBlue (Invitrogen) assays were used to measure the proliferation and metabolic activity of the cells respectively. PicoGreen assay quantified the amount of double stranded deoxyribonucleic acid present within cell lysates. Cell lysates were obtained by transferring and immersing the samples into a mixture of 0.1% Triton X-100 and 0.25% trypsin. The mixture were then agitated on a shaker for 1h before thorough pipetting to obtain a homogeneous cell suspension. The cell suspension was then mixed with equal amount of PicoGreen working solution and the fluorescence of the mixture was determined using 480nm and 520 nm as the excitation and emission wavelengths respectively.

To measure the metabolic activity, culture medium was replaced by a modified medium which contained 10% AlamarBlue reagent and no FBS. Subsequently, the cells were incubated in the dark for 4 h at 37°C, 5% CO₂. Afterwhich, absorbance of the supernatant was measured at wavelengths of 570 and 600nm.

3.4.3 Cell Morphology Analysis

Cell viability and distribution were evaluated using the Live/Dead staining assay. Cell cultured samples were washed with PBS and incubated in a solution of 2 μM Calcein-AM (Invitrogen) and 4 μM ethidium homodimer-1 (Invitrogen) in PBS at 37 °C for 15 min. Samples were then washed again in PBS and transferred onto microscope slides for imaging under a fluorescence (Nikon 80i) or confocal laser microscope (LeicaSP5) using a 10x objective.

Prior to using a scanning electron microscope (SEM) for analysis, cell cultured samples were fixed using 4% paraformaldehyde (PFA) in PBS, for 15 min at room temperature. Dehydration was carried out in an ascending ethanol series (50%, 75%, 80%, 95% and 100%) for 10 min each at room temperature. Dried samples were subsequently sputtered with platinum at 20 mA for 60 sec and analyzed under a SEM (JSM-5410, JEOL Co.) at an accelerating voltage of 5 kV. SEM images were taken at magnifications of 2000x and 750x

Cell morphology was further observed in detail by F-actin and nuclei staining. Cells were fixed using 4% PFA in PBS, for 15 min at room temperature and permeabilized with 0.1% TritonX-100 in PBS for 5 min at room temperature. The cytoskeleton of the cells were stained with phalloidin (Invitrogen) with 1:400 dilution in PBS for 1h at room temperature followed by staining of the nuclei with DAPI (Invitrogen) for 5 min at room temperature. Samples were rinsed 3 times with PBST (PBS with 0.05% tween 20) after every incubation step. Finally, samples were transferred onto microscope slides and mounted with fluorescent mounting medium (Dako) for imaging under a fluorescence (Nikon 80i) or confocal laser microscope (LeicaSP5) using a 20x objective.

3.4.4 Immunocytochemistry

The presence of the extracellular proteins which were fibronectin and collagen III were detected via immunostaining. Cells were fixed using 4% PFA in PBS, for 15 min at room temperature and permeabilized with 0.1% TritonX-100 in PBS for 5 min at room temperature. Subsequently, blocking of samples were done by adding 10% goat serum (Dako) in PBS for 1h at room temperature, followed by incubation of primary antibody solution for overnight shaking at 4°C. The primary antibody solutions were prepared by diluting anti-fibronectin primary antibody (Abcam) at 1:1000 in blocking solution and diluting anti-collagen III primary antibody (Abcam) at 1:200 in blocking solution for detection of fibronectin and collagen III respectively. Detection of both primary antibodies were done by adding goat anti rabbit Alexa Fluor 488 (Invitrogen) diluted at

1:400 in blocking solution for 1h of incubation time at room temperature, followed staining of the nuclei with DAPI (Invitrogen) for 5 min at room temperature. Samples were rinsed 3 times with PBST (PBS with 0.05% tween 20) after every incubation step. Finally, samples were transferred onto glass coverslips and mounted with fluorescent mounting medium (Dako) for imaging under a fluorescence (Nikon 80i) or confocal laser microscope (LeicaSP5) using a 20x objective.

3.4.5 Integrin Inhibition Assay

Integrin inhibition assay were done on keratin VD film, fibronectin coating and polystyrene.

Samples (except for one of samples labeled as untreated polystyrene) were blocked with heat-inactivated BSA at a concentration of 2mg/mL in PBS for 1h at room temperature and rinsed twice with PBS thereafter. Cells were trypsinized and held in suspension in DMEM without FBS. The cell suspensions were pre-incubated with antibodies to the integrin receptors (Abcam); α_4 integrin and β_1 integrin for 30 min at 37°C, 5% CO₂ prior to cell seeding. Addition cell suspensions as negative controls were also prepared by pre-incubating with antibodies with the same isotype (Santa Cruz) as the antibodies to the integrin receptors. The cell suspensions were seeded on the samples for 4 h before cell counting to determine cell attachment. Cell counting was done by flushing the samples twice, gently with PBS before obtaining cell lysates for PicoGreen assay quantification.

3.4.6 Western Blotting for Expression of $\alpha_4\beta_1$ integrins

Protein electrophoresis separation was done using the XCell SureLock[®] Mini-Cell System with NuPAGE[®] 4-12% Bis-Tris Gels (Invitrogen). Cell lysates obtained from 4h and Day 7 of cell culture were mixed with 2 μ l of sample reducing agent (DTT, 10X) and 5 μ l of LDS Sample Buffer (4X). A total volume of 20 μ l sample mixture containing 35 μ g of protein was loaded into each lane. The SeeBlue[®] Plus2 Prestained Standard (1X) was used to identify the molecular weight ranges of the samples' bands. Electrophoresis was

performed at 120V for 120 minutes (Bio-rad) in 0.05% NuPAGE[®] MOPS SDS Running Buffer (20X, Invitrogen).

Extracted proteins separated on SDS-polyacrylamide gel were transblotted onto a nitrocellulose membrane using the iBlot[®] Dry Blotting System (Invitrogen). Ponceau S Red (Sigma Aldrich) staining was conducted for membrane obtained from cell lysates harvested 4h post cell seeding. Membranes from cell lysates harvested 7 days post cell seeding were blocked with 5% non-fat milk powder in Phosphate Buffered Saline (PBS, pH=7.4) with 0.5% for 1 hour at room temperature.

For loading control, the membranes were incubated with β -actin (Abcam) primary antibody in dilution factor 1:5000, for an overnight at 4°C. Subsequently, the membranes were incubated with anti-mouse secondary HRP-conjugated antibody (Abcam) in dilution factor 1:4000, for 1h at room temperature. Both primary and secondary antibodies are diluted with 5% non-fat milk solution.

For α_4 integrin and β_1 integrin expression, the membranes were incubated with 2 different primary antibodies; α_4 integrin primary antibody (Abcam) and β_1 integrin primary antibody (Abcam) diluted to 1:200, for an overnight at 4°C. Subsequently, the membranes were incubated with anti-mouse secondary HRP-conjugated antibody (Abcam) in dilution factor 1:4000, for 1h at room temperature. Both primary and secondary antibodies are diluted with 5% non-fat milk solution.

The blots were visualized with the SuperSignal West Femto Chemiluminescent Substrate System (Thermo Scientific) and imaged with ChemiDoc (Bio-rad).

3.5 Statistical Analysis

All data of representative experiments carried out in triplicate are presented as mean $\pm$ standard deviations. One-way analysis of variance (ANOVA), which tests the null

hypothesis that "samples having the equal means in population", is a statistical technique used to determine if the means are different between three or more samples (using F distribution). If the variance between groups is larger than the variance within the group, it's likely that the mean differs. Generally the samples are considered to be independent, the variances of populations should be equal and the response variable residuals are normally distributed while one-way ANOVA is executed. One-way ANOVA was employed followed by Student t-test (two samples assuming equal variance). Bonferroni correction was done on the two-tail P value from the t-test to determine statistical significance differences between experimental groups. Corrected P-value <0.05 was considered as statistically significant. Analysis ToolPak was installed in Microsoft Excel software to conduct all statistical analysis.

Chapter 4:

Keratin Extraction and Fiber Fabrication

Keratin was extracted in-house from the raw material, human hair by using sodium sulfide (Na_2S) which allows keratin dissolution by the cleavage of disulfide bond. The human hair extracts were then characterized using 660nm assay, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot to determine the protein yield and confirm the presence of keratin in the extracts. Extracted keratin in solid form via lyophilization was also characterized using the Fourier Transform Infrared Spectroscopy- Attenuated Total Reflectance (FTIR-ATR) to determine the chemical conformation of the keratin extracted from human hair. After keratin extraction, the keratin extracts were processed into keratin fibers as a tissue engineering platform. As mentioned in Chapter 2, electrospinning of keratin itself is a challenge among researchers due to relatively low molecular weight of the extracted keratin, hence, in this work, polyethylene oxide (PEO) was added in small amount to facilitate the electrospinning. In addition, to produce high concentration of keratin solution for electrospinning, Na_2S was added into the electrospinning mixture to cleave the disulphide bonds of the keratin, in order to allow high dissolution of keratin. Therefore, the formulation for electrospinning keratin in this work involves high concentration of keratin, minimal amount of PEO and Na_2S aqueous solution. Optimization was done to develop a formulation that allowed successful fiber formation via electrospinning and viscosities of the tested formulations were measured to further understand the importance of the formulation's viscosity in electrospinning keratin with PEO. Scanning

electron microscope (SEM) was used to evaluate the best formulation to produce well-formed, beadless keratin fibers. The optimized formulation was subsequently used for electrospinning keratin fibers for further materials characterization and cell compliance evaluation.

4.1 Results

4.1.1 Characterization of Extracted Keratin

Prior to confirmation of the presence of keratin in the human hair extracts, quantification of proteins were done using 660nm assay and results of the protein concentration from 5 different batches of keratin extracts were shown in Table 4-1.

Table 4-1 Protein concentration of 5 different batches of keratin extracts and their percentage yield. The average yield is 26.25% of protein extracted out from 0.05g/mL of human hair

Sample	1	2	3	4	5
Protein Concentration (µg/ml)	14125	10780	13920	12856	13954
Yield (%)	28.25	21.56	27.84	25.71	27.91

The yield as shown in Table 4-1 was calculated by the amount of protein per mL of the extraction solution produced from 0.05g of hair. The average yield of the keratin extraction using Na₂S is 26.25%.

4.1.1.1 Western Blotting of Extracted Keratin Solution

The extracts were then diluted to give 15µg protein in each 20ul aliquot that was loaded into each well of the pre-cast gels for separation by electrophoresis. Coomassie blue staining and Western blotting of the gels produced results as shown in Figure 4-1.

Figure 4-1 A) Coomassie blue stain and B) Western Blot of human hair extract. Coomassie blue stain showed the presence of proteins with keratin's typical molecular weight of 45kDa for acidic keratin and 50kDa for basic keratin in the human hair extracts. Western blot further confirmed the presence of keratin in the extracts.

Comassie blue staining of the gel after electrophoresis showed that the proteins in the human hair extracts have molecular weights of 45 kDa and 50 kDa as indicated by the distinct bands. These two bands were likely to be acidic keratin with a molecular weight of 45 kDa and basic keratin with a molecular weight of 50 kDa. Western blot further confirmed that these two bands were indeed an indication of the presence of keratin.

4.1.1.2 Fourier Transform Infrared Spectroscopy Characterization of Lyophilized Keratin

After which the keratin solution was lyophilized and characterized using FTIR-ATR and results are shown in Figure 4-2.

Figure 4-2 Representative FTIR spectra of lyophilized keratin, consisting of the general absorption peaks of proteins, i.e. Amide A (2800-4000 cm^{-1}), Amide I (1600-1700 cm^{-1}), Amide II (1480-1580 cm^{-1}) and Amide III (1220-1330 cm^{-1})

The spectra of the lyophilized keratin consisted of the characteristic absorbance peaks of proteins namely the Amide A (2800-4000 cm^{-1}), Amide I (1600-1700 cm^{-1}), Amide II (1480-1580 cm^{-1}) and Amide III (1220-1330 cm^{-1}) peaks.

4.1.2 Optimization of Formulation for Electrospinning of Keratin

It is essential to optimize the electrospinning solution such that the mixture allows electrospinning of keratin into reproducible fibers. Keratin dissolution and behavior of keratin interaction with suitable synthetic polymers were looked into. The concentration of the chosen components was then varied in order to derive an optimized formulation for electrospinning keratin fibers.

4.1.2.1 Keratin in Various Solvents

Keratin was dissolved in different pH environment using hydrochloride acid (HCl, pH0), water (H₂O, pH7) and sodium hydroxide (NaOH, pH14) solvents. Results of this pH variation are shown in Figure 4-3.

Figure 4-3 Keratin in solutions with different pH environment. A transparent solution was obtained for NaOH solvent while cloudy solutions were obtained for H₂O and HCl solvents

It was found that keratin is well dissolved in alkaline solution because NaOH solvent produced a transparent solution while H₂O and HCl solvents produced cloudy solutions after keratin were added to them.

Since keratin dissolved well in alkaline solution, 5 to 10wt % of keratin in NaOH solvent were electrospun. However, no fibers were formed during electrospinning and the solution was sprayed out instead, forming a large pool of solution and dried droplets as shown in Figure 4-4.

Figure 4-4 Results of electrospinning 10wt% keratin in NaOH indicating unsuccessful formation of keratin fibers. The microscopic image on the right showed the structures of the residues formed after electrospinning

Subsequently another alkaline solution with lower pH was used to dissolve the keratin. The solvent was Na_2S solution and 5 to 25wt% of keratin in Na_2S solution were spun. However, similar to electrospinning of keratin in NaOH solvent, droplets were sprayed out instead.

4.1.2.2 Blending Synthetic Polymers with Keratin

In order to facilitate electrospinning of keratin, a synthetic polymer was added in combination with keratin in Na_2S solution.

The first synthetic biocompatible polymer selected was poly(L-lactide/ ϵ -caprolactone) (PLC) and keratin mixture was prepared by blending PLC dissolved in hexafluoroisopropanol (HFIP) solvent with keratin dissolved in Na_2S solution. However, large precipitate was formed immediately after the two polymers were mixed together as shown in Figure 4-5A.

Figure 4-5 Keratin mixture consisting of blending A) PLC and b) PEO with keratin solution. Precipitate was formed when keratin solution was blended with PLC while a good solution mixture was produced when keratin solution was blended with PEO.

Another biocompatible polymer selected was PEO which formed a good mixture of solution with keratin as shown in Figure 4-5B.

4.1.2.3 Optimization of Formulation via Electrospinning Solution Parameters

After preliminary trials in determining the components for the formulation, it was found that keratin solution blended with PEO as an additive facilitates electrospinning.

Na₂S solvent is used to allow dissolution of high amount of keratin. Subsequently, solution parameters were varied to optimize the formulation for electrospinning through varying the keratin and PEO concentration.

First attempt to electrospin keratin/PEO solution was done by varying the keratin concentrations while fixing the PEO concentration at 1 wt%. Scanning electron microscopy was done to examine the morphology of the spun fibers. Results shown in Figure 4-6 revealed that fibers started to form when the keratin concentration reached 25wt% but higher keratin concentration of 30wt% was needed to accumulate fibers into a viable scaffold.

Figure 4-6 SEM images of electrospun fibers with 1 wt% PEO and A) 10wt% keratin, B) 20wt% keratin, C) 25wt% keratin and D) 30wt%keratin. Accumulation of beadles fibers was observed when the keratin concentration reached 30wt% although some of the fibers were no fully well-formed.

Fixing the keratin concentration at 30wt% further attempts were done to electrospin keratin/PEO solutions by varying the PEO concentration and the results were shown in Figure 4-7.

Figure 4-7 SEM images of electrospun fibers with 30wt% keratin and A) no PEO, B) 0.3wt% PEO, C) 0.5wt% PEO and D) 1wt% PEO. Accumulation of majority well formed fibers with negligible wet drips or fibers was observed when 0.5wt% PEO was used in combination with 30wt% keratin

Results revealed that PEO was needed for fiber formation as no fibers can be spun out of pure 30wt% keratin itself. By adding 0.5wt% PEO to 30wt% keratin solution, well-formed fibers were spun with negligible wet drips or fibers. However, too little PEO (i.e. 0.3 wt%) or too much PEO (i.e. 1 wt%) resulted in fibers with beads and drips.

4.1.2.4 Viscosity of Different Formulations

Viscosity of the solution with potential formulation for electrospinning keratin was determined using the rheometer as shown in Figure 4-8.

Figure 4-8 A) Table of viscosities of different formulation with their corresponding B) Pictures of the electrospun fibers except for formulation 40wt% keratin and 0.5wt% PEO due to C) gelation of solution. Viscosities at which the formulation allows successful fabrication of well-formed fibers fell in the range of 30.29 to 41.58 mPa

From Figure 4-8, it was found that the viscosities at which the formulation allows successful fabrication of well-formed fibers (i.e. Formulation #7, 10 and 11) fell in the range of 30.29 to 41.58 mPa. However, formulation #8 produced no fibers and formulation #9 produced fibers with frequent drips, even though their viscosities fell in this identified range of 30.29 to 41.58 mPa. This indicates that a certain amount of PEO was needed to electrospin keratin fibers. It was also needed that viscosities that were too low resulted in drips and very few fibers while viscosities that were too high resulted in thick wet fibers. Extremely high viscosities led to gelation of solution which could not be electrospun as show in Figure 4-8C.

The viscosity results were analyze by fixing the concentration of one of the components in the formulation and ranging the concentration of the other as shown in Figure 4-9.

Figure 4-9 Graphs comparing viscosities of formulations with varying concentration of A) PEO with keratin concentration fixed at 30wt%, B) keratin with PEO concentration fixed at 0.5wt% and C) keratin with PEO concentration fixed at 1wt%. The gradients of the linear trendline fitted to the graph are 8.862, 6.777 and 4.750 for A), B) and C) respectively, indicating that PEO played a more dominant role in manipulating the viscosity of the formulation.

Viscosities were compared with increasing concentration of PEO while fixing the keratin concentration to 30wt% (Figure 4-9A) and increasing concentration of keratin while fixing the PEO concentration (Figure 4-9B and C). All the graphs showed that viscosity of the formulation increased by increasing the concentration of one of the components. Besides, the gradient of the trendline fitted to the graph is the highest at 8.862 for varying PEO concentration, indicating that PEO played a more dominant role in manipulating the viscosity of the formulation.

4.1.2.5 Optimized Formulation for Electrospinning for Electrospinning of Keratin

The optimized formulation for electrospinning keratin was concluded to be 30wt% keratin and 0.5wt % PEO in Na_2S solution which resulted in well-formed fibers accumulated into a viable scaffold as show in Figure 4-10A.

Figure 4-10 Results from the optimized formulation (i.e. 30wt% Keratin and 0.5wt% PEO in Na_2S solvent) for electrospinning keratin showing A) scaffold spun out from the formulation, B) the viscosity of the formulation, C) the SEM image of the scaffold and D) fiber diameter measurement of the scaffold

The viscosity of this formulation was 36.63 ± 1.53 mPa (Figure 4-10B) and with a normal distribution tending towards a fiber diameter of 700 ± 90 nm as measured and shown by the SEM image in Figure 4-10C and D.

4.2 Discussion

Keratins are intermediate filament proteins that differentiates from the other proteins by their high content of cysteine residues [143]. Cysteines are sulphur containing amino acids which account for the intermolecular disulfide bridges that contribute to hair's resistance against protease action and stability [144]. Many techniques had been developed to extract keratin via destructing the tightly packed structure of hair; primarily by breaking down the disulfide bridges [34]. In this work, Na_2S was selected to extract keratin from human hair because the extraction is inexpensive, simple, fast and consistent in dissociating keratin filaments within the hair cortex [119, 145]. Coomassie blue staining and Western blotting showed that keratin was indeed successfully extracted from

human hair using Na_2S with an average yield of 26.25%. Tosik et al. also reported successful extraction using similar protocol which was utilizing an aqueous mixture of 5% NaOH and 0.1 M of Na_2S [52] and the maximal yield was 40% which was slightly higher due to NaOH involved to aid the hydrolysis of intact keratin during the extraction. The keratin extracts consisted of keratin with the typical molecular weight of keratin subtypes which are acidic keratins at 45kDa and basic keratins at 50kDa. Besides, keratin extracts lyophilized into solid form had chemical conformations which are the amide bonds similar to those of the general proteins. These indicated that the extracts contained intact keratin with no destruction of important bonds that gave proteins their characteristics.

In order to process the extracted keratin into a biomaterial for tissue engineering, electrospinning was selected as a scalable and efficient technique to create a scaffold out of keratin. Dissolution behavior of keratin was evaluated to find the most suitable solvent for electrospinning keratin. Solubility of the keratin is an issue due to its strong disulphide bonds which act as linker to hold the superstructure of the protein. HFIP and acetic acid which are both common electrospinning solvents were not able to dissolve keratin due to low pH of the solvents. It was found that flocculation and precipitates were formed when keratin interacted with solvents with low pH environment. In contrast, keratin dissolved in solvents in high pH environment and hence alkaline solutions such as NaOH and Na_2S aqueous solution were considered. Na_2S was selected over NaOH to dissolve high amount of keratin because it does not hydrolyze keratin and significantly destruct the keratin's protein structure [146], unlike NaOH . Keratin was dissolved in Na_2S through the reduction of disulfide bonds in cysteine by hydrosulfide (HS^-) ions as shown by the following equations [147]:

As mentioned in the literature review, research on electrospinning of keratin have resorted to using keratin in combination with other natural or synthetic polymer [34] due to molecular weight of the extracted keratin too low to form polymer entanglements to facilitate electrospinning. To overcome this issue, a synthetic polymer was added in combination with keratin in Na_2S solution. Two biocompatible synthetic polymers which were PLC and PEO were considered and PEO was selected because it could dissolve easily in the Na_2S aqueous solution due to its hydrophilic nature, making the solution system simpler. Moreover, high molecular weight PEO was proven to enhance electrospinnability, following reports that this could be achieved with natural polysaccharides such as chitosan and sodium alginate [82-85].

With keratin and PEO in Na_2S solvent as part of the formulation to electrospin keratin-based scaffold, optimization of the solution parameters in the formulation was done by varying keratin and PEO concentrations such that a reproducible well-formed keratin fibrous scaffold could be fabricated using an optimized formulation. The viscosities of possible formulation were also assessed.

Observations revealed that with a small amount of PEO added in, the concentration of keratin has to reach 25 wt% to be sufficient enough for fiber formation but keratin has to be increased to 27.5wt% in order to electrospin well-formed fibers. However, when the concentration of keratin went up to 40 wt%, the solution started gelling up. Likewise for PEO, a minimum amount of 0.5 wt% was needed to facilitate electrospinning but higher amount of PEO would cause the solution to be too viscous for electrospinning. It was evaluated that formulations with low viscosities could not be electrospun due to inadequate amount of polymers in the solution and formulation with high viscosities also posed a problem in which the difficulty in ejection of jets from viscous polymer solution may result in fibers with numerous bead defects [148]. An optimal range of 30.29 to 41.58 mPa solution viscosity was required to electrospin well-formed fibers. However, it should be noted that keratin solutions with absence of PEO or too small amount of PEO were not feasible to be electrospun into fibers even though their viscosities were in the optimal range. The high molecular weight PEO not only played a dominant role in

manipulating the viscosity of the electrospinning solution, it was the key component to provide polymer entanglements to facilitate electrospinning of keratin.

Hence, it was evaluated that minimum amounts of 0.5 wt% PEO and 30wt% keratin were needed in the formulation to electrospin keratin.

With this formulation in which the solution viscosity was 0.0363Pa, a well-formed scaffold of fibers was produced from electrospinning. The consistency and homogeneity of fiber morphology of the optimized keratin fibers and normal distribution of fiber diameters ($R^2 = 0.978$) over a reasonably narrow range confirmed the stability of electrospinning. The scaffold consisted of a normal population of 700 nm fibers, giving a homogeneous sub-micron architecture.

4.3 Conclusion

Intact keratin was successfully extracted from human hair using Na_2S with a yield of 26.25%. In order to process keratin into a biomaterial via electrospinning, viscosity of the electrospinning solution and the addition of a certain amount of PEO into the solution were keys to enhance the feasibility of getting reproducible well-formed scaffold of fibers. The optimized formulation was 30 wt% keratin and 0.5 wt% PEO in Na_2S solvent, with a viscosity value of 0.363 Pa. The scaffold produced consisted of homogeneous sub-micron architecture of fibers with a normal distribution of fiber diameters tending towards an average of 700 nm.

Chapter 5:

Characterisation of Electrospun Keratin Fibers

Randomly aligned fibers spun from 30 wt% Keratin and 0.5 wt% PEO were characterized to further understand the properties of electrospun keratin fibers. Basic materials characterizations such as chemical conformation was determined by Fourier Transform Infrared (FTIR) spectroscopy, thermal properties was determined by Differential Scanning Calorimetry (DSC) and mechanical properties was determined by Dynamic Mechanical Analysis (DMA). The potential of the keratin fibers tissue engineering platforms was also evaluated by observing their degradation profiles and cell compliance. For biodegradation studies, experiments were designed base on ASTM standard (ASTM F1635-11) in which Phosphate Buffer Saline (PBS) solution was used to soak the specimens and the specimen weight loss over a period of time was determined. However it was noted the standard does not take into account that enzymes in the human body could degrade proteins. Thus, more studies were performed by adding enzymes such as chymotrypsin and proteinase K to determine the degradation profile of keratin fibers. Proteinase K was selected as the positive control because it is known for its keratin digestion activity as suggested by its name. Chymotrypsin was selected because not only is it produced by the human body, it is also widely prescribed as medicine to counter wound infections. For cell compliance studies, immortalized L929 mouse cells were used as recommended by ASTM standard (ASTM F813-07), to evaluate the biocompatibility of keratin electrospun fibers. Cell studies were done by culturing L929 mouse cells on keratin fibers and the cell viability of the fibers was evaluated using

Live/Dead staining and SEM imaging at various time points. This chapter will give a better understanding of the material characteristics of the electrospun keratin fibers and its potential for tissue engineering.

5.1 Results

5.1.1 Chemical Conformation

The infrared absorbance spectra of the electrospun keratin matrices, lyophilized keratin, Na₂S hydrates and PEO are shown in Figure 5-1.

Figure 5-1 Representative Fourier Transform Infrared spectra of an A) electrospun keratin fibers, B) lyophilized keratin, C) Na₂S hydrates and D) PEO. Electrospun keratin matrix and lyophilized keratin have similar spectra consisting of the general absorption peaks of proteins, i.e. Amide A (2800-4000 cm⁻¹), Amide I (1600-1700 cm⁻¹), Amide II (1480-1580 cm⁻¹) and Amide III (1220-1330 cm⁻¹). Compared to the lyophilized keratin's spectrum, the keratin fibers' spectrum has two additional peaks at 1114 cm⁻¹ and 994 cm⁻¹ originating from S₂O₃²⁻ group of Na₂S hydrates. The PEO spectrum has its typical fingerprint region containing the prominent triplet peaks due to C-O-C stretching but these peaks were not found in the keratin matrix's spectrum due to low content of PEO. Reproduced with permission from [124]

The spectra of the keratin fibers and lyophilized keratin consisted of the characteristic absorbance peaks of proteins, namely the Amide A (2800-4000 cm⁻¹), Amide I (1600-1700 cm⁻¹), Amide II (1480-1580 cm⁻¹) and Amide III (1220-1330 cm⁻¹) peaks. These general absorbance peaks of proteins are summarized in Table 5-1 below.

Table 5-1 General Absorbance Bands of Proteins

Type of Absorption Band	Frequencies (Wavenumber/ cm^{-1})	From Figure 5-1	Description
Amide A	2800 – 4000	3277	Wide absorption band containing 9 vibrational overlapped modes [149]
Amide I	1600 – 1700	1636	Arises mainly from C=O stretching of the amide and is very sensitive to the protein secondary structure [150]
Amide II	1480 – 1580	1521	Arises from NH bending and CN stretching [151]
Amide III	1220 - 1330	1238	Arises from NH bending and CN stretching [151]

The two additional peaks at 1114 cm^{-1} and 994 cm^{-1} in the spectrum of the keratin matrices compared to that of the lyophilized keratin belonged to the thiosulfate ($\text{S}_2\text{O}_3^{2-}$) group which has two characteristic peaks at 995 cm^{-1} and 1115 cm^{-1} [152]. The spectrum of the Na_2S hydrates also consisted of the peaks of $\text{S}_2\text{O}_3^{2-}$ group and had prominent O-H stretching peaks at 3218 cm^{-1} and 1635 cm^{-1} [153, 154]. The PEO's spectrum contained its typical fingerprint region, which is the spectrum below 1500 cm^{-1} , with a complex pattern of numerous sharp distinct peaks. Among these peaks, the most prominent peak is the triplet shape of peaks at 1144 cm^{-1} , 1096 cm^{-1} and 1059 cm^{-1} , representing C-O-C stretching of the PEO chains [155].

In order to remove the solvent residues which may implicate cytotoxic issues, the keratin fibers were washed with PBS prior to cell culture studies. Figure 5-2 shows the FTIR spectra of the keratin fibers after PBS washing.

Figure 5-2 Representative Fourier Transform Infrared spectra of an A) electrospun keratin fibers after PBS washing B) lyophilized keratin. The two peaks at 1114 cm⁻¹ and 994 cm⁻¹ originating from $S_2O_3^{2-}$ group of Na_2S hydrates disappear on the spectra electrospun keratin fibers after PBS washing and the spectra of the electrospun keratin fibers after PBS washing resembles closely to the spectra of the lyophilized keratin

The $S_2O_3^{2-}$ peaks are shown to be absent from the spectra of the keratin fibers which has been washed with PBS and the spectra of the keratin fibers (after PBS washing) resembled closely to the spectra of the lyophilized keratin.

5.1.2 Thermal Properties

DSC was used to identify the denaturation temperature of the keratin structures and thermal stability of the protein was compared between the extracted keratin and the electrospun fibers.

Figure 5-3 shows the DSC results of the extracted keratin.

Figure 5-3 DSC curve of the extracted keratin showing 3 endothermic peaks which indicate water evaporation at 99 °C, α helix denaturation at 248 °C and β sheet denaturation at 299 °C

It was observed that there were three major endothermic peaks. The first peak at 99 °C was due to water evaporation [156], the second peak was due to α helix denaturation at 248 °C [157, 158] and the third peak at 299 °C might be due to the denaturation of β sheet structure [26, 159]. It was also noted that these denaturation peaks were due to irreversible unfolding of keratin secondary structure superimposed by various decomposition reactions [160].

Compared to the DSC results of keratin, the results of the fibers as shown in Figure 5-4 also revealed the three major endothermic peaks as described earlier.

Figure 5-4 DSC curve of 30wt% keratin and 0.5 wt% PEO electrospun fibers showing 3 distinct peaks which indicate water evaporation at 108 °C, α helix denaturation at 239 °C and β sheet denaturation at 273 °C

It was observed that the denaturation temperatures of α helix and β sheet in the fibers were lower at 239 °C and 273 °C respectively compared to those in the extracted keratin. In addition, the endothermic peak representing α helix denaturation was much gentler and not so obvious in Figure 5-4. Since the area under the DSC curve is a measure of the α helix content [161], it could be concluded that there were less α helix structures in the fibers than in the extracted keratin.

5.1.3 Mechanical Properties

The stress-strain curve of random keratin fibers was obtained from DMA as shown in Figure 5-5 with random gelatin fibers as the control.

Figure 5-5 Stress strain curve of keratin and gelatin fibers showing distinctively that keratin had higher strain at break while gelatin was stiffer.

Young's modulus, ultimate tensile strength and strain at break were obtained from Figure 5-5 and collated into a Table 5-2.

Table 5-2 Collated results of the mechanical properties of keratin fibers and gelatin fibers, showing values of the Young's modulus, ultimate tensile strength and strain at break determined from the stress-strain curve

Sample	Fiber diameter (μm)	Young's Modulus (MPa)	Ultimate Tensile Strength (MPa)	Strain at Break (%)
Keratin Fibers	0.70 ± 0.09	11.06 ± 1.58	1.8 ± 0.1	78.0 ± 7.2
Gelatin Fibers	0.70 ± 0.04	85.15 ± 4.34	2.5 ± 0.3	4.0 ± 0.5

It was ensured the random gelatin fibers used have similar fiber diameter with random keratin as fiber diameter is also one of the factors that can affect the mechanical properties of the electrospun material as a whole [162]. From Table 5-2, it is shown that gelatin fibers which had a Young's modulus of 85.15 ± 4.34 MPa were much stiffer than keratin fibers which had a Young's modulus of 11.06 ± 1.58 MPa. Besides, gelatin fibers had higher ultimate tensile strength of 2.5 ± 0.3 MPa as compared to keratin fibers with ultimate tensile strength of 1.8 ± 0.1 MPa. Gelatin fibers tend to be a lot more brittle [163] with a low strain at break of $4.0 \pm 0.5\%$ compared to keratin fibers which had strain at break at $78.0 \pm 7.2\%$.

5.1.4 Biodegradation Profiles

For degradation studies, two controls, keratin film and collagen film as shown in Figure 5-6, were used for comparison.

Figure 5-6 A) Keratin Fibers, B) keratin film and C) collagen film of the same shape and size (i.e. 4 cm^2). The films were created as controls in the degradation studies of keratin fibers.

It was ensured that the keratin fibers and controls are of the same shape, size (i.e. 4 cm^2) and similar mass of 40 mg for the degradation studies.

Biodegradation profiles of keratin fibers were obtained by soaking into different environments; A) PBS according to ASTM standard, B) Tris buffer which is the solvent for enzymatic digestion in degrading keratin fibers, C) chymotrypsin in Tris buffer and D) Proteinase K in Tris buffer. These profiles were compared with the degradation profiles of keratin film and collagen film as shown in Figure 5-7.

Figure 5-7 Biodegradation profiles of keratin fibers, keratin films and collagen films over 30 days in four different environments namely A) PBS according to ASTM standard, B) Tris buffer which is the solvent for enzymatic digestion in degrading keratin fibers, C) chymotrypsin in Tris buffer and D) Proteinase K in Tris buffer. * $p < 0.01$, significance difference in weight percentages between keratin film and collagen film

From Figure 5-7A and B, all natural polymers involved showed trends of hydrolytic degradation but minimally near a period of 30 days. Although insignificant, it was noted that collagen tend to degrade faster than keratin in PBS environment while keratin tend to degrade faster than collagen in Tris buffer which had an environment of pH8.

Regarding enzymatic degradation, it was observed from Figure 5-7C and D that these natural polymers degraded significantly with the presence of chymotrypsin (Figure 5-7C)

and proteinase K (Figure 5-7D) over a period of 30 days. From Figure 5-7C, it was noted that keratin tend to degrade faster than collagen in the environment with presence of chymotrypsin but the differences were not significant. From Figure 5-7D, collagen degraded much significantly faster than keratin in the environment with the presence of proteinase K.

It was also observed that keratin fibers tend to degrade faster than keratin film in all the environments in the study. Evaluation of the effects of the four different environments on degradation of keratin fibers was also done as shown in Figure 5-8.

Figure 5-8 Degradation profiles of keratin fibers in four different environments (i.e. PBS, tris buffer, chymotrypsin and proteinase K). Hydrolytic degradation of keratin fibers was faster in Tris buffer compared to PBS while enzymatic degradation of keratin fibers was faster in Proteinase K compared to chymotrypsin. * $p < 0.01$, significance difference in weight percentages of between keratin fibers in chymotrypsin and keratin fibers in proteinase K presence

Figure 5-8 shows that keratin fibers consistently degraded faster in Tris buffer environment than in PBS environment, although the differences were insignificant. For environments with the presence of enzymes, keratin fibers degraded significantly faster in the presence of proteinase K compared to in the presence of chymotrypsin.

5.1.5 Cell Compliance

Cell viability of the keratin fibers and keratin films were evaluated using the Live/Dead staining assay with cell culture insert as the control. Fluorescence images from Live/Dead staining of L929 cultured samples are shown in Figure 5-9.

Figure 5-9 Fluorescence microscopy images of cell cultured samples over 7 days. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). Cells on the samples started to spread by day3 and proliferated significantly by day7. Reproduced with permission from [124]

On day 1 of culture, viable cells were evident on the cell culture inserts, keratin fibers and keratin films. On day 3 of culture, a small proportion of cells started to spread into their typical spindle morphology on all the samples. By day 7, cells on the cell culture inserts and keratin films had become highly confluent but were mostly rounded. Cells on the keratin fibers were not as confluent but they exhibited a high degree of spreading. From the maximum projection of cross sectional confocal images, cells on the keratin fibers

were able to penetrate into the pores of the fibers to produce relatively semi three-dimensional cell-matrix constructs (Figure 5-10).

Figure 5-10 Z-stack confocal microscopy image of the cross section of a 7-day cell cultured keratin fibers, showing even cell penetration across the depth of the matrix. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). Reproduced with permission from [124]

Cell morphology on the different samples at day 7 of culture was more apparent on the SEM images shown in Figure 5-11.

Figure 5-11 Representative scanning electron microscopy images of cells cultured for 7 days on the samples. Low magnification images (top row) depict the overview of cell distribution and high magnification images (bottom row) depict the cell morphologies. Reproduced with permission from [124]

Cells on the cell culture inserts generally presented two different morphologies. A proportion of these were flat and largely polygonal in shape but the majority was round

and bulging. Similar morphologies could be observed on cells which were cultured on the keratin films. A proportion of cells on the films were round and bulging, stacking on top of a layer of cells that were in highly spread shape. Cells cultured on the keratin fibers adopted highly spread, elongated or spindle morphologies on the fibers. They were mostly flattened on the keratin fibers.

5.2 Discussion

Development of keratin fibers via electrospinning had been a challenge due to difficulty in dissolution of high concentration of keratin and relatively low molecular weight [34]. In addition, to the synthesis of keratin fibers, reports on cellular interaction with electrospun keratin are limited. Hence, with the formulation of 30 wt% keratin and 0.5wt% PEO in Na₂S solvent, a novel electrospun keratin-based fibers was developed in this work and deemed suitable to act as a platform for materials characterization and cell interaction studies.

Keratin retained its protein conformation and chemical structure after electrospinning, as shown by the FTIR comparison between the electrospun keratin fibers and the lyophilized keratin. There was clear indication of signature peaks originating from the vibrations of the peptide groups in a protein, with the Amide I and Amide II peaks being the most prominent vibrational peaks of the protein backbone [151]. Compared to the lyophilized keratin, the keratin fibers contained the S₂O₃²⁻ group, as a result of the reaction of Na₂S in H₂O during the preparation of electrospinning solution. This reaction is shown by the following equation [152]:

Presence of the S₂O₃²⁻ group, which has its characteristic peaks at 1115 cm⁻¹ and 995 cm⁻¹ [152], was indicated by the absorption peaks in the infrared spectra of the Na₂S hydrates and keratin fibers at 1114 cm⁻¹ and 994 cm⁻¹. The presence of the two peaks from Na₂S

hydrates could be eliminated by washing the keratin fibers with PBS, indicated that the Na_2S residues could be easily remove from the keratin fibers.

In addition, the characteristic infrared absorption bands of PEO, which occurs in the fingerprint region of wavelengths below 1500 cm^{-1} , did not appear in the keratin fibers' spectrum. This was due to the relatively small amount of PEO present in the keratin fibers. FTIR analyses further suggest that the interaction between PEO and keratin, which improved the electrospinnability of the keratin solution, was purely physical.

Therefore, as a summary of the keratin fibers' chemical properties, the keratin fibers developed had chemical conformation resembled those of the extracted keratin, indicating the electrospinning did not significantly affect the protein quality and the fibers produced retained their general protein characteristics which are the amide bonds.

However, keratin fibers were less thermally stable than the extracted keratin after electrospinning as their secondary structures denatured at lower temperatures. It could be due to the high speed formation of the fibers and the quick solvent evaporation during electrospinning that hindered the keratin self-assembly [164]. This led to a less thermally stable and less complex supermolecular organisation in the keratin of the fibers.

In addition, electrospinning might had also caused less α helix structures and more β sheet structures to be present in the keratin fibers as indicated by the DSC curve of the keratin fibers. This might be due to the stretching of keratin during electrospinning that caused the α helix structures to transform into β sheet structures since the extension of keratin could lead to α - β transformation [165].

The mechanical properties of the keratin fibers were understood to be that keratin fibers were less stiff and brittle compared to gelatin fibers [26, 163]. The presence of PEO in the keratin fibers might also had contributed to the lower Young's modulus and higher strain at break compared to pure gelatin fibers [26].

Both hydrolytic degradation and enzymatic degradation of keratin fibers were assessed to evaluate their potential as a biodegradable material for tissue engineering.

Hydrolytic degradation was based on the standard ASTM (ASTM F1635-11: Standard Test Method for in-vitro Degradation Testing of Hydrolytically Degradable Polymer Resins and Fabricated Forms for Surgical Implants). However, protein-based materials degrade faster in the human body than predicted by the standard test because they can undergo accelerated degradation due to presence of enzymes in the human body. Therefore, looking into the enzymatic degradation profile of keratin is necessary.

Keratin fibers were degradable minimally under hydrolytic environment and significantly under enzymatic environment. Higher pH of the hydrolytic environment played a role in keratin being degraded faster hydrolytically as a result of reducing condition which catalyzed the degradation process. As for enzymatic degradation, keratin fibers degraded significantly faster in the presence of proteinase K than in the presence of chymotrypsin as a result of differences in the mechanism of degradation activity between these two enzymes. Proteinase K are very well known to digest keratin, as suggested by its name, because the enzyme is specific against hydrophobic amino acid residues primarily [166]. In contrast, chymotrypsin is highly selective over bonds involving aromatic amino acids with their carboxyl group and hence showing preference in degrading esters much more rapidly than proteins [167].

This difference in enzymatic degradation mechanism might also explain the trend in which keratin tends to degrade faster than collagen under enzymatic digestion by chymotrypsin but it degraded significantly slower than collagen under enzymatic digestion by proteinase K. Keratin is a very rigid protein which is less prone to enzymatic degradation because of the high amount cysteine holding keratin's intact structure [34], hence it would be natural that collagen showed faster enzymatic degradation behavior under the broad spectrum proteinase K. However, since chymotrypsin is weak in digesting proteins because of its preference for ester bonds, hydrolysis process by the tris buffer used as the medium for these enzymatic degradation might have become relatively

more dominant in causing keratin's tendency to degrade faster than collagen in the degradation study involving chymotrypsin.

Overall it was observed that keratin fibers tend to degrade faster than keratin film given its higher surface area to volume ratio, indicating the potential tunability of degradation profiles of the keratin fibers via alteration of the fiber diameters and architecture of the electrospun material.

Immortalized L929 mouse cells were used as recommended by ASTM (ASTM F813-07: Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices). Live/Dead staining from day 1 showed that the different samples used, i.e. cell culture inserts, electrospun keratin fibers and keratin films, were not cytotoxic. All samples induced healthy cellular response, with a few cells starting to spread from day 3 and proliferating to confluency by day 7. Both cell culture inserts and keratin films did not support significant cell spreading, although cell proliferation on cell culture inserts was clearly more significant. In the environment provided by the keratin fibers, even though cell proliferation was less significant than on the cell culture inserts, cell spreading was significantly enhanced, likely due to the combined physical and biochemical cues provided by the keratin fibers. In addition to the high surface area to volume ratio provided by the fibrous structure of the keratin matrices also allowed higher degree of cell-material interactions [23, 168].

5.3 Conclusion

Keratin fibers were successfully electrospun without compromising amide bonds. However it was revealed that keratin fibers were thermally less stable than the extracted keratin, attributed to keratin structures being less organized due to the process of electrospinning. The stretching process of electrospinning was also speculated to be playing a potential role in α - β transformation of protein structures when the extracted keratin was electrospun into fibers. Keratin fibers were less brittle and stiff compared to gelatin fibers mainly due to the presence of PEO in keratin fibers. These keratin fibers

were evaluated to be suitable for tissue engineering as they are biodegradable and biocompatible, given their degradation profiles and the results from cell studies that keratin fibers posed no cytotoxic issues.

Chapter 6:

Tuning Alignment of Keratin Fibers and Their Potential for Wound Healing

Fabricating of aligned keratin fibers were attempted in this work by varying the controlling parameters of the electrospinning process. These controlling parameters include applied voltage, tip to collector distance, feedrate and rotating speed of the drum collecting the fibers. The variation in the controlling parameters was done by following the optimization table shown in Table 6-1.

Table 6-1 Optimization table of controlling parameters involving applied voltage, tip to collector distance, feedrate and rotating speed of the drum collecting the fibers to find the best combination to fabricate aligned keratin fibers. The initial recommended combination was 10kV applied voltage, 15cm tip to collector distance, 0.5ml/h feedrate and 600 rpm of drum rotating speed.

Exp (#)	Applied Voltage (kV)				Distance (cm)			Feedrate (ml/h)			Rotating Speed (rpm)						
	8	10	12	15	12	15	20	0.5	0.75	1	100	200	300	400	500	600	700
1	*					*		*								*	
2		*				*		*								*	
3			*			*		*								*	
4				*		*		*								*	
5		*			*			*								*	
6		*					*	*								*	
7		*				*			*							*	
8		*				*				*						*	
9		*				*		*			*						
10		*				*		*				*					
11		*				*		*					*				
12		*				*		*						*			
13		*				*		*							*		
14		*				*		*									*

The optimization table is a systematic guideline to follow for a new method to work optimally with minimal effort and time. It works on the basis of N +

(N-1) rule. Hence, instead of carrying out $N_1 \times N_2 = 4 \times 2 = 8$ experiments to vary one variable at a time, the optimization table above organizes the experiments such that only $N_1 + (N_2 - 1) = 4 + (2 - 1) = 5$ experiments were required in changing one variable at a time.

The morphologies of the fibers produced from the 14 experimental combinations were observed under the SEM. In order to evaluate the degree of alignment of the fibers from each combination, the corresponding SEM images were transformed into Fast Fourier Transform (FFT) images using Image J. Subsequently, the FFT images were quantified using a program developed by Bill O'Connell to determine the FFT peak unit. The FFT peak units were compared among all 14 experimental combinations with the highest FFT peak unit indicating the best degree of alignment. The combination of controlling parameters which gave the best alignment was subsequently chosen to be the optimized aligned fibers for further characterization in this work. Mechanical properties of the optimized aligned keratin fibers were done using Dynamic Mechanical Analysis (DMA) and compared with random keratin fibers. The potential of both aligned and random keratin fibers as platforms for wound healing were evaluated by culturing human dermal fibroblasts (HDFs) on the keratin fibers for 1, 3 and 7 days. Cell viability of HDFs on these keratin fibers was quantified using PicoGreen and AlamarBlue assay. The PicoGreen assay gave an indication of the cell numbers by quantifying the amount of DNA present while the AlamarBlue assay gave an indication of the metabolic activities of cells by quantifying the chemical reduction of the culture medium resulting from cell growth. HDFs morphology and distribution on both random and aligned keratin fibers were assessed using Live/Dead staining. Further detail of HDFs morphology on keratin fibers were

evaluated by Phalloidin staining. In anticipation to give a broader picture on the behavior of HDFs on keratin fibers, immunostaining to detect fibronectin and collagen III was done to evaluate whether the HDFs were able to execute their role in extracellular matrix (ECM) production during early stage of wound healing, when cultured on keratin fibers.

6.1 Results

6.1.1 Optimization of Alignment via Controlling Parameters of Electrospinning

Morphology of fibers produced from each combination of controlling parameters was observed and analyzed on the degree of alignment with the SEM images as shown in Figure 6-1.

Figure 6-1 Representative SEM images of the 14 possible combinations to obtain aligned keratin fibers and their corresponding FFT peak unit. Combination #13 produced fibers with the best alignment while combination #1 produced fibers with the worst alignment

The SEM images were used to determine the degree of alignment of fibers produced from the 14 potential combinations. Figure 6-1 shows that combination #13 produced fibers with the best alignment while combination #1 produced fibers with the worst alignment. Figure 6-2 gives a summary of the FFT peak units of all 14 combinations in descending order from the top.

Figure 6-2 Plot of FFT quantification (left) of the 14 combinations and their corresponding average FFT peak unit in descending order from the top. The highest peak unit is 0.238 while the lowest peak unit is 0.089

From Figure 6-2, the best alignment has a FFT peak unit of 0.238 and the worst alignment has a FFT peak unit of 0.089.

Further analysis was done on how the controlling parameters affect the alignment of fibers. The two controlling parameters which are the tip to collector distance as shown in Figure 6-3 and the drum rotating speed as shown in Figure 6-4 were identified to play a role in affecting the degree of fiber alignment.

Figure 6-3 The role of tip to collector distance on the degree of alignment of keratin electrospun fibers. A) Summary plot, B) SEM images and C) FFT quantifications of combinations #5, #2 and #6 showing that increasing tip-to-collector distance gave better alignment with higher average FFT peak value

From Figure 6-3, by decreasing the tip to collector distance from 20 cm (combination #6) to 15 cm (combination #2) to 12 cm (combination #5), the alignment of the fibers increased from FFT peak value of 0.163 (combination #6) to 0.192 (combination #2) to 0.210 (combination #5).

Figure 6-4 The role of drum rotation speed on the degree of alignment. A) Summary plot of increasing rotating drum speeds and their corresponding FFT peak value and B) combinations with their respective rotating drum speed and corresponding surface velocities

For universal comparisons, the drum rotation speed is converted to their corresponding surface velocity by taking the size of the rotating drum into account. The surface velocity was determined by multiplying the speed of the rotating drum in rpm with πD in which D is the diameter of the rotating drum (i.e. 76.2 mm). Figure 6-4A showed that there was a threshold surface velocity needed to get best alignment of keratin fibers. This surface velocity was 119.69 m/min with the highest average FFT peak value of 0.238 from combination #13. It was further analyzed how increasing rotating speed caused better alignment before the threshold rotating speed in Figure 6-5 and how increasing the rotating speed caused poorer alignment after the threshold rotating speed in Figure 6-6.

Figure 6-5 The role of drum rotation speed on the degree of alignment before the threshold rotating speed. A) SEM images and B) FFT quantifications of combinations #9, #10, #11 and #12. C) Summary plot showing that increasing rotating speed gave better alignment before the threshold rotating drum speed

Figure 6-5 showed that increasing the rotating drum speed from 100 rpm to 400 rpm, the alignment of the fibers increased with FFT peak value increasing from 0.110 to 0.188.

Figure 6-6: The role of drum rotation speed on the degree of alignment after the threshold rotating speed. A) SEM images and C) FFT quantifications of combinations #13, #12 and #14. B) Summary plot showing that increasing rotating speed gave poorer alignment after the threshold rotating drum speed

Figure 6-6 showed that by increasing the rotating drum speed from 500 rpm to 700 rpm, the alignment of the fibers decreased with FFT peak value decreasing from 0.238 to 0.177. It was also observed that there were broken fibers under the SEM when the rotating drum speed reached 600 rpm and 700 rpm.

Overall it was concluded that the optimized combination of 10kV applied voltage, 12 cm tip to collector distance, 0.5 mL/h of feedrate and surface velocity of 119.69 m/min gave the best alignment with average FFT peak of 0.24 as shown in Figure 6-7.

Figure 6-7 A) The SEM image of aligned fibers produced from the optimized combination followed by its corresponding FFT image and FFT quantification. B) High magnification (left) and low magnification (right) SEM images of the optimized aligned keratin fibers

6.1.2 Mechanical Properties of Optimized Aligned Keratin Fibers

The stress-strain curve of aligned keratin fibers as shown in Figure 6-8, was obtained from DMA by applying tensile strain parallel to the orientation of the aligned fibers, with random keratin fibers and random gelatin fibers as the controls.

Figure 6-8 Stress-strain curves of aligned keratin, random keratin and random gelatin fibers showing distinctively that aligned keratin had higher Young's modulus and ultimate tensile strength but lower strain at break than random keratin.

Values of the Young's modulus, ultimate tensile strength and strain at break were obtained from Figure 6-8 and collated in Table 6-2.

Table 6-2 Collated results of mechanical properties of aligned keratin, random keratin and random gelatin fibers, showing values of Young's modulus, ultimate tensile strength and elongation at break determined from the stress-strain curves

Sample	Fiber diameter (μm)	Young's Modulus (MPa)	Ultimate Tensile Strength (MPa)	Strain at Break (%)
Aligned Keratin	0.74 ± 0.10	39.92 ± 6.44	3.8 ± 0.3	42.5 ± 4.1
Random Keratin	0.70 ± 0.09	11.06 ± 1.58	1.8 ± 0.1	78.0 ± 7.2
Random Gelatin	0.70 ± 0.04	85.15 ± 4.34	2.5 ± 0.3	4.0 ± 0.5

It was ensured that all types of fibers used have similar fiber diameter. From Table 6-2, it is shown that the aligned keratin fibers which had a Young's modulus of 39.92 ± 6.44 MPa were much stiffer than random keratin fibers which had a Young's modulus of 11.06 ± 1.58 MPa.

Besides, aligned keratin fibers had higher ultimate tensile strength of 3.8 ± 0.3 MPa as compared to random keratin fibers with ultimate tensile strength of 1.8 ± 0.1 MPa.

However, aligned keratin fibers tend to be more brittle with lower strain at break of $42.5 \pm 4.1\%$ compared to keratin fibers which had strain at break at $78.0 \pm 7.2\%$.

6.1.3 Human Dermal Fibroblasts Viability

Cell compliance of the aligned keratin and random keratin fibers were evaluated with gelatin random keratin as the control, by culturing human dermal fibroblasts on them.

6.1.3.1 Human Dermal Fibroblasts Viability

Quantifications of the cell viability were done using PicoGreen assay which measures the amount of double stranded deoxyribonucleic acid (dsDNA) and AlamarBlue assay which detects the level of metabolic activity of the cells. Results from the PicoGreen assay and AlamarBlue assay are shown in Figure 6-9.

Figure 6-9 Cell viability and proliferation of human dermal fibroblasts cultured on the electrospin fibers. A) PicoGreen assay results and B) Alamar Blue assay results showing healthy cell growth on aligned keratin, random keratin and random gelatin electrospun fibers over a period of 10 days.

PicoGreen results indicated that the number of HDFs increased over time until Day 7 and started to become constant from Day 7 to Day 10 while the AlamarBlue results indicated

that the metabolic activity of the HDF population on all the samples increased over time, even up to Day 10. Both results also showed that the cell number and metabolic activity of the HDF population on aligned keratin matrices were significantly higher than random keratin matrices at Day 3 and Day 5.

Live/Dead staining was done to observe the cell morphology and distribution of HDF on aligned keratin, random keratin and random gelatin as shown in Figure 6-10.

Figure 6-10 Fluorescence microscopy images of cell cultured samples over 7 days. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). HDFs on the samples exhibited their typical spindle morphology by Day 3

F-actin staining was also done on HDFs cultured on aligned keratin, random keratin and random gelatin fibers and observed at higher magnification as shown in Figure 6-11.

Figure 6-11 Fluorescence microscopy images of cell cultured samples over 7 days. F-actin (red) of the cells was stained by Phalloidin and the nucleus (blue) of the cells was stained by DAPI. HDFs on the keratin fibers arranged themselves according to the architecture of the fibrous samples by Day 3.

Results from Figure 6-10 and Figure 6-11 showed that most of the HDFs exhibited their typical spindle morphology by Day 3 of cell culture. In addition, the HDFs formed network of cells according to the topography of the fibrous samples.

6.1.3.2 Extracellular Matrix Production

Expression of fibronectin and collagen III were checked via immunostaining as shown in Figure 6-12 and Figure 6-13 respectively.

Figure 6-12 Immunofluorescence staining of fibronectin on cell cultured samples over 7 days. Samples were stained for fibronectin (green) and nuclei (blue). Expression of fibronectin were found on keratin samples but not gelatin samples.

Figure 6-13 Immunofluorescence staining of collagen III on cell cultured samples over 7 days. Samples were stained for collagen III (green) and nuclei (blue). Expression of collagen III were found on all samples

Figure 6-12 and Figure 6-13 showed that HDFs cultured on both aligned and random keratin fibers were capable of secreting fibronectin and collagen III. Some of the HDFs cultured secreted fibronectin and collagen II since 1 day after cell seeding and by 3 days, most cells were shown to be capable to secrete these extracellular proteins. Likewise, HDFs on random fibers were shown to secrete collagen III. However, it is shown in Figure 6-12 that HDFs cultured gelatin fibers did not secrete any fibronectin, even up till 7 days after cell seeding.

6.2 Discussion

Human tissues are intricate matrices housing many different cells in their complex and well-defined organization of extracellular proteins. The architecture and environment of the tissues in the human body are two important features for considerations when designing a viable scaffold for tissue engineering. Figure 6-14 provides an illustrative summary of the structures of different human tissues [169].

Figure 6-14 Illustrative summary of the human tissues. Reproduced with permission from [169]

From Figure 6-14, many human tissues such as the muscle, partial regions of the brain and heart, blood vessels, tendon, ligament and skin dermis have aligned architectures. Focusing on skin dermis, it is a well-organized ensemble of multi cell types such as fibroblasts, macrophages and adipocytes embedded in an aligned extracellular matrix with predominantly collagen bundles [170, 171] as shown in Figure 6-15.

Figure 6-15 Illustration of the structure of the skin (epidermis and dermis) which consisted of A) multiple cell types in which keratinocytes and fibroblasts are the most critical cells for dermal wound healing. SEM images of B) collagen bundles at the dermis tissue and C) fibroblasts aligning themselves according to the architecture of the dermis tissue. Adapted with permission from [170, 171]

Figure 6-15B) is an SEM image of the collagen bundles observed in the dermis of the rat skin and Figure 6-15C) is an SEM image of aligned dermal fibroblast in the same tissue of the rat [170], suggesting that aligned architecture are needed to mimic the environment for fibroblasts in dermal wound healing. Besides, Many studies have discussed that matrices with aligned fibers improve cell proliferation [112, 172-174], migration [172, 175] and ECM production [176-178]. However, whether this advantage of aligned fibrous matrix over random fibrous matrix is significant varies across different studies using different types of scaffolds and cell types [108]. Hence, it is worthwhile to evaluate whether aligned architecture can enhance the biological function of keratin in this work which has not been reported before.

To get highly aligned fibers, variation of controlling parameters involving the applied voltage, feedrate, tip to collector distance and surface velocity in keratin fiber collection were done with the initial recommendation of 10 kV applied voltage, 15 cm tip to

collector distance, 0.5mL/h feedrate and surface velocity of 143.63 m/min. However, it was found that a shorter tip to collector distance of 12 cm gave better alignment. This can be attributed to the whipping instability of the fibers and the decrease in directional electric field at longer distance [179].

It was widely discussed that higher surface velocity would result in better alignment [95, 180] due to more effective drawing of the polymer jet resulting from higher tangential force exerted on the jet for mechanical alignment. However, it was found that a lower surface velocity of 119.69 m/min gave better alignment. It is speculated that breakage of fibers due to too high the surface velocity used to collect the fibers, had led to broken fibers being deposited randomly on the rotating drum. Hence, there is a threshold surface velocity which is 119.69 m/min to electrospin well-aligned keratin fibers.

Therefore it was concluded that the combination of 10kV applied voltage, 12cm tip to collector distance, 0.5mL/h feedrate and a surface velocity of 119.69 m/min would produce keratin fibers with the best alignment.

Human dermal fibroblasts which are cells found mainly in the dermis and critical for wound healing were used. Fibroblast activity on the matrices is relevant as they play an important role of producing new ECM necessary to support cell and vascular in-growth [181, 182]. PicoGreen assay showed that the cell number of the human fibroblast cultured on both random and aligned keratin fibers increased over time. Likewise, result from the AlamarBlue assay was consistent with the PicoGreen assay indicating that the metabolic activity of the HDF population increased over time. Both quantifications of HDF viability implied that the keratin fibers could support healthy cell growth comparable to the positive control.

Live/Dead staining and F-actin staining showed that keratin fibers induced healthy cellular response with most of the HDFs exhibiting their typical spindle shape morphology by Day 3. It is also interesting to see that these HDFs could respond to the topographical cues given by the random and aligned keratin fibers and formed network of cells according to the architecture of the keratin fibers.

During wound healing, HDFs' most important role is to produce new extracellular proteins to repair the damage on the injury site [183, 184]. Two of these essential extracellular proteins are fibronectin and collagen III especially in the early stage of wound healing [185]. Immunostaining showed that there were expression of fibronectin and collagen III on the cultured random and aligned keratin fibers, indicating that the keratin fiber could induce these HDFs to secrete extracellular matrix proteins essential for wound healing.

6.3 Conclusion

Well-aligned keratin fibers were successfully electrospun from an optimized combination of controlling parameters which were 10kV applied voltage, 12 cm tip to collector distance, 0.5 mL/h of feedrate and surface velocity of 119.69 m/min in the collection of fibers using a rotating drum. The resulting aligned keratin fibers had higher tensile strength and stiffness than random keratin fibers. Both random and aligned keratin fibers supported cell growth of primary HDF. Besides, HDFs were able to respond and arrange themselves according to the architecture of the keratin fibers. It was evaluated that these keratin fibers have good potential for wound healing as they are capable of inducing the HDFs to secrete extracellular proteins.

Chapter 7:

Involvement of Keratin and Electrospinning in Cell-Material Interaction

To look further into keratin potential as a bioactive material, keratin films were used in the study instead of keratin fibers, in order to eliminate the possibilities of topographical cues in influencing the growth of human dermal fibroblasts (HDFs) on keratin. The keratin films were cast from the formulated solution used in this work to electrospin keratin fibers. Two different drying methods were used in the casting of films. Quick drying of keratin films was done by vacuum drying while slow drying of keratin films was done by air drying. These films were labeled as vacuum-dried (VD) films and air-dried (AD) films. The cell compliance of both AD and VD films were evaluated by culturing HDFs on them for 1, 3 and 7 days. Cell viability was observed using Live/Dead staining and quantified using PicoGreen assay. The films were also characterized for their differences in their material properties such as morphology using AFM, nitrogen bonding using XPS and availability of free functional groups such as amines and thiols using chemical assays like Ninhydrin and Ellman's reagent respectively. Potential cell-material interaction between keratin and HDFs was elucidated and demonstrated. The keratin extracts were further analyzed by Western blotting to determine the presence of keratin subtypes which contain the following cell adhesion motif, Leu-Asp-Val (LDV). To test the role of $\alpha_4\beta_1$ integrin in HDFs attachment on keratin, inhibition assay was done by seeding HDFs blocked separately with α_4 and β_1 integrins antibodies. Expression of α_4 and β_1 integrins by HDFs was also checked via

Western blotting. The cell-material interaction study was done to give an evaluation on whether interaction between keratin and HDFs could be mediated by the affinity of $\alpha_4\beta_1$ integrin expressed by HDFs to LDV protein sequence found in human hair keratin.

7.1 Results

7.1.1 Preliminary Cell Study on Keratin Films

Cell viability of L929 mouse cells and HDFs cultured on AD films was evaluated using Live/Dead staining assay as shown in Figure 7-1.

Figure 7-1 Fluorescence microscopy images of cell cultured samples over 7 days. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). L929 (top) showed healthy growth on AD films while HDF (bottom) barely proliferated on AD film.

From Figure 7-1, L929 proliferated well on AD films and became highly confluent at Day 7. However, HDFs cultured on AD films did not proliferate much and they did not seem to attach well on AD films.

Afterwhich, a comparison of HDF compliance on AD film and VD film was attempted with Live/Dead staining assay to observe cell morphology and PicoGreen assay to quantify cell viability of HDFs cultured on the keratin films. Results are shown in Figure 7-2.

Figure 7-2 Comparison of HDF compliance on AD film and VD film. (A) PicoGreen assay results and (B) fluorescence microscopy images of cell cultured samples over 7 days. HDFs showed healthy cell growth on VD films but barely proliferated on AD films.

Figure 7-2 showed that compared to the poor growth of HDFs on AD films, HDFs could grow very well on VD films with most of the HDFs exhibiting their typical spindle shape morphology by Day 1 and becoming highly confluent by Day 7.

7.1.2 Characterization of Vacuum-Dried and Air-Dried Keratin Films

Differences in film morphology between VD and AD keratin film was checked using AFM and the surface roughness was calculated from the AFM 2D amplitude images for comparison as shown in Figure 7-3.

Figure 7-3 Surface morphologies of AD and VD keratin films. (A) AFM 3D Height and 2D Amplitude images and (B) surface roughness calculated from 2D Amplitude images of AD and VD films. There were no significant differences on the surface morphologies between AD and VD keratin films

Results from Figure 7-3 indicated that the surface roughness of AD films and VD films were almost the same with AD film having a average roughness of $6.52 \pm 1.07 \text{ nm}$ and VD film having a average roughness of $6.37 \pm 0.65 \text{ nm}$. This implies that surface morphologies of the AD and VD films are very similar.

Differences in surface chemistry between AD and VD keratin films was analyzed using XPS. N1s peak from the high resolution XPS spectra of keratin films and fibers were deconvoluted to 3 peaks which represent amine group ($-\text{C}-\text{NH}-$) at binding energy of 399.4 eV, amide group ($-\text{C}=\text{O}-\text{NH}-$) at binding energy of 400.5 eV and protonated amine group ($-\text{C}-\text{NH}_3^+$) at binding energy of 401.4 eV [186, 187]. The percentage of the amide bonds in the nitrogen species were derived by calculating the area of the amide group peak as a function of the broad area of N1s peak and compared among the keratin fibers, AD and VD keratin films as shown in Figure 7-4.

Figure 7-4 XPS N1s narrow scans with deconvolutions of 3 components which are amide, amine and protonated amine of A) VD keratin film, B) AD keratin film and C) keratin fibers. D) A comparison plot on the amide bond percentage in the nitrogen species of keratin fibers, VD and AD keratin films.

XPS result indicated that most nitrogen species in the keratin specimens were found in amide bonds which are the signature protein bonds. However, there were minority of the nitrogen species which were in the form of amine and protonated amine groups. From Figure 7-4D, the percentage of amide bonds in the nitrogen species of the keratin specimens were 70%, 79.19% and 93.1% for keratin fibers, VD and AD keratin films respectively. It was therefore determined that AD film contained the least percentage of amine and protonated amine groups as compared to VD film and keratin fibers.

Differences in the amount of free amine groups between VD and AD films were further confirmed by Ninhydrin assay as shown in Figure 7-5.

Figure 7-5 Comparison on the amount of amine groups in VD and AD film from Ninhydrin assay. VD film contained significantly higher amount of amine groups compared to AD film

Results from Ninhydrin assay (Figure 7-5) showed that VD film contained significantly higher amount of amine groups compared to AD film.

Besides the detection of free amine groups, the amount of free thiol groups in VD and AD films was also determined using Ellman reagent assay as shown in Figure 7-6.

Figure 7-6 Comparison on the amount of free thiol groups in VD and AD films from Ellman Reagent's assay. VD film contained significantly higher amount of thiol groups compared to AD film

Results from Ellman reagent assay (Figure 7-6) showed that VD film contained significantly higher amount of thiol groups compared to AD film.

7.1.3 Cell-Material Interaction Study between Keratin and Human Fibroblasts

For cell-material interaction study, further Western blotting was done on the keratin extracts to confirm the presence of keratin subtypes as shown in Figure 7-7.

Figure 7-7 Western blot of human hair extracts showing the presence of acidic keratin subtype, K31, K33 and K34 with a molecular weight of 45kDa

The bands at 45 kDa in Figure 7-7 showed that the human hair extracts contained acidic keratin subtypes, namely K31, K33 and K34.

Cell interaction on keratin material was then evaluated by culturing HDFs on keratin VD films. The role of $\alpha_4\beta_1$ integrin of HDFs in enhancing HDFs attachment to keratin VD film was analyzed by blocking HDFs with α_4 antibody and β_1 antibody prior to HDFs seeding on the keratin VD film. Figure 7-8 showed the comparisons in the degree of cell

attachment between blocked HDFs with keratin VD film and unblocked HDFs with keratin VD film. This inhibition assay was quantified with PicoGreen assay and on additional controls such as fibronectin coating, polystyrene and untreated polystyrene.

Figure 7-8 Effect of A) α_4 integrin-blocking antibody and B) β_1 integrin-blocking antibody on the cell attachment on keratin film, fibronectin coating, polystyrene and untreated polystyrene. Significant decrease in cell attachment was observed on keratin films when HDFs were blocked with α_4 and β_1 integrin-blocking antibodies. * $p < 0.05$

Results from the inhibition assay showed that cell attachment decreased on keratin films when they were seeded with HDFs blocked with α_4 integrin-blocking antibody and HDFs blocked with β_1 integrin-blocking antibody. Cell attachment on fibronectin films decreased significantly when they were seeded with HDFs blocked with β_1 integrin-blocking antibody but not HDFs blocked with α_4 integrin-blocking antibody. As for both polystyrene and untreated polystyrene, the integrin-blocking antibodies did not significantly affect cell attachment of HDFs on both substrates.

Expression of α_4 integrin and β_1 integrin by the HDFs were checked using Western blotting. Results are shown in Figure 7-9.

Figure 7-9 Expression of integrins by HDFs cultured on fibronectin coating (FC), keratin film (KF) and untreated (uPS). A) Ponceau red staining of transfer membrane loaded with cell lysates harvested 4 hours after HDF seeding on the samples and B) Western Blot showing expression of β -actin, α_4 and β_1 integrins from cell lysates harvested 7 days after HDF seeding on the samples

Figure 7-9A showed the possibility of integrins of HDFs being expressed when cultured on keratin film (KF) and fibronectin coating (FC) but not on untreated polystyrene (uPS) after 4 hours of seeding. Figure 7-9B showed that α_4 integrin (~115kDa) and β_1 integrin (~130kDa) of HDFs were expressed when cultured all samples for 7 days.

7.2 Discussion

With advances in tissue engineering and better understanding of cell biology, tissue scaffolds are being developed to act as a platform to promote tissue regeneration, so as to overcome the limited supply of suitable transplant available. Tissue scaffolds can come in forms of natural polymers, synthetic polymers or a combination of both to overcome their individual shortcomings. The biggest advantage of proteins over synthetic polymers is that they can induce biological recognition via cell adhesion motifs. Hence, the potential of keratin as an alternative bioactive material was examined in this work.

Verma *et al.* reported that human hair keratins contained the cell adhesion motif which is Leucine-Aspartic Acid-Valine (LDV) [12] and these motifs mainly come from acidic keratin subtypes. Further Western blotting on human hair extracts in this work confirmed that these extracts contained K31, K33 and K34 acidic keratin subtypes which originates from cortex of the human hair [42]. These acid keratin subtypes contains LDV as part of their protein sequence. Therefore, it was confirmed that the keratin extracts used in this work to create platforms for cell studies, contained LDV.

LDV is a cell adhesion motif which has been widely discussed to be part of the protein sequence of common proteins such as fibronectin and vascular cell adhesion molecule-1 (VCAM-1) and it is recognized by the cell adhesion molecule, $\alpha_4\beta_1$ integrin [188, 189]. Besides, it was also found out that HDFs expressed functional $\alpha_4\beta_1$ integrin and evaluated that $\alpha_4\beta_1$ integrin facilitates fibroblasts attachment to fibronectin [190]. These proven concepts led to speculation in this work that cell-material interaction between keratin and HDFs may be mediated by the affinity of $\alpha_4\beta_1$ integrin to the LDV protein sequence.

Cell study was carried out between HDFs and keratin by casting of keratin films to eliminate the possibilities of topographical cues in affecting cell attachment, growth and function [191, 192]. Preliminary cell study on air-dried keratin films showed that these films were not cytotoxic as indicated by healthy growth of L929 mouse cells. However, when HDFs were cultured on the air-dried keratin films, they did not seem to attach well on the films at Day 1 and subsequently resulting in poor growth of HDFs on the films over Day 3 and Day 7.

With this stark difference in HDFs response on air-dried keratin film compared to HDFs response on electrospun keratin fibers as previously discussed, the difference between these two processing methods; casting of films via air drying and formation of fibers via electrospinning were analyzed. It was evaluated that compared to air-dried film casting process, besides the generation of material with fibrous topography which is negligible in casted films, electrospinning is a relatively quick drying process. It was discussed in Chapter 5 that high speed formation of the keratin material and the quick solvent

evaporation during electrospinning had altered the keratin assembly to a less complex supermolecular organization [164]. Moreover, there are reports indicating that morphologically different protein derivatives from the same parent protein exhibit different cell response. For example, Zako *et al.* reported that reducing the disulphide bonds of insulin in the presence of tris(2-carboxyethyl)phosphine (TCEP) resulted in formation of insulin filaments which exhibited significant increase in PC12 cell viability compared to intact insulin fibrils [193]. In addition, Zako *et al.* also showed that the secondary structures of insulin filaments were different from intact insulin fibrils. Another example is a report by Midwood *et al.* on the cell interactions with fibronectin matrices [194]. Midwood *et al.* reported that cells expressing $\alpha_4\beta_1$ integrin showed poor cell attachment and were unable to spread on or contract a 3D fibrin-fibronectin matrix when the fibronectin was intact while cells expressing $\alpha_4\beta_1$ integrin were able to adhere, spread and induce matrix contraction when interacting with 3D fibrin- fragmented fibronectin matrix. It was concluded that proteolytic cleavage of fibronectin from intact fibronectin was necessary for cell interactions via $\alpha_4\beta_1$ integrins.

With this frame of references, the possibility that the quick drying process due to electrospinning might have altered keratin's organization in favour for HDF's response compared to the slow drying process during the casting of air-dried keratin films. In view of this perspective, two different drying methods were used in the casting of keratin. Quick drying of keratin films was done by vacuum drying while slow drying of keratin films was done by air drying. These films were labeled vacuumed-dried (VD) films and air-dried (AD) films. Comparison of HDF response on both films revealed that HDFs were able to adhere, spread and proliferate well on VD keratin film but not on AD keratin film. This distinct phenomenon partially supported the previous discussion on the occurrence in which morphologically different protein derivatives from the same parent protein exhibiting different cell response.

Morphological differences between keratin in VD film and AD film were evaluated by AFM and indirectly by characterizations of chemical bonds. The similar roughness in

nanoscale between AD and VD film as detected by AFM, indicated that the AD and VD films had similar nanotopography despite being processed differently.

Examination of chemical bonds revealed that keratin in VD film had significantly more free amines than AD film although keratin in VD film was still dominated by amide bonds. This might be due to the rapid drying condition which reduced the time significantly for amide bonds development during VD film formation. Besides, it was also revealed that keratin in VD film had significantly higher amount of free thiol groups compared to keratin in AD film, It was inferred that although keratin was reduced by Na_2S from its disulphide bonds to thiol bonds before the film formation process, free thiol groups were unstable [195] and formed back into disulphide bonds via oxidation during the slow air-drying process. Hence, there were less thiol groups in keratin AD film compared to keratin VD film. Recalling the phenomenon from HDF study between keratin VD and AD films, it was further speculated that keratin in VD film was partially fragmented with more free amine groups and denatured with relatively higher amount of thiol groups compared to keratin in AD film and thus resulting in matricryptic sites which become exposed after structural or conformational alterations of proteins [193, 194, 196], allowing $\alpha_4\beta_1$ dependent adhesion via the V-region exposure.

Dependency of $\alpha_4\beta_1$ integrin in HDF adhesion on keratin VD film was evaluated. Results showed that α_4 integrin and β_1 integrin played a significant role in HDF attachment to keratin VD film while only β_1 integrin played a significant role in HDF attachment to fibronectin coating. This was further explained that fibronectin contained more than one type of cell adhesion motif. Other than LDV, fibronectin also contains RGD which allows cell attachment via $\alpha_5\beta_1$ and $\alpha_8\beta_1$ integrins [197], hence the dependency of α_4 integrin for HDF attachment on fibronectin was not as significant as on keratin.

7.3 Conclusion

Two methods which were slow drying and quick drying of keratin, were done in casting of keratin films for cell-material interaction study. The resulting keratin VD films and

AD films elicited significantly different HDF behavior. This phenomenon drew forth the possibility that morphologically different protein derivatives from the same parent protein could exhibit different cell response. It was found that keratin in VD film had more free amine groups and thiol groups and hence concluded to be partially fragmented and denatured while keratin in AD film was more organized. With regards to the phenomenon that VD film supported HDF attachment and growth while AD film did not, this prompted the postulation that the keratin conformation in VD film had allowed $\alpha_4\beta_1$ dependent adhesion of HDF via the V-region exposure from the LDV cell adhesion motif. It was proven that $\alpha_4\beta_1$ integrin played a significant role in HDF attachment of keratin VD film, hence elucidating the cell-material interaction between keratin VD film and HDF being mediated by the affinity of $\alpha_4\beta_1$ integrin expressed by HDFs to the LDV protein sequence found in human hair keratin.

Chapter 8:

Conclusion and Recommendations

This chapter consists of a section which relates the three hypothesis to the findings in this work and discusses on how objectives were thus achieved. This section is followed by recommendations on the possible future work as a continuation to the various findings of this work.

8.1 Conclusion

The aim of this dissertation was to create a fibrous ECM mimicking platform from human hair keratin as an evaluation of keratin's potential for wound healing and understand the underlying mechanism of cell-material interaction between human hair keratin and human cells which are critical for wound healing.

Exploring human hair keratin as an alternative natural polymer for tissue regeneration has grabbed much attention in recent years due to its potential as an autologous material. In order to create a cell-viable platform from human hair keratin, electrospinning emerges as a scalable and simple technique to generate ECM-mimicking nanofibrous scaffolds. However, due to low molecular weight of keratin, generation of feasible fibrous platform consisting of keratin as the majority for cell culture became a challenge and hence current understating of how keratin electrospun platforms affect cell behavior is limited. Therefore the main objective of this study is to create a novel fibrous platform with keratin as the major component via electrospinning. This study is split into 3 phases with different specific objectives as discussed in the introduction chapter.

The 1st objective was achieved by developing an optimized formulation which allow the formation of well-formed keratin-based fibers and also highly aligned keratin fibers derived from an optimized combination of electrospinning parameters. Possible formulations with keratin and PEO in Na_2S solvent have been developed and tested for their viscosities and feasibilities in formation of keratin fibers. Observations indicated that a certain but minimal amount of PEO was essential for successful formation of well-formed keratin fibers due to the need for PEO's long chain entanglement to facilitate electrospinning of keratin. However, when PEO exceeds a critical concentration, the solution became too viscous to generate well-formed fibers during electrospinning. The optimized formulation was 30 wt% keratin and 0.5 wt% PEO in Na_2S solvent, with a viscosity value of 0.363 Pa. In addition to random keratin fibers, attempts to fabricate aligned keratin fibers were done to evaluate the tunability of keratin fibers alignment via electrospinning parameters and the effect of topographical cues on cell behavior on keratin fibers. The degree of alignment of keratin fibers were manipulated dominantly by

the tip-to-collector distance and the surface velocity of the rotating drum. These support the dissertation's 1st hypothesis that human hair extracted keratin can be electrospun into random and aligned fibers via the dissolution of high content keratin into the solvent of the electrospinning formulation. The creation process of optimized formulation with high content keratin and optimized combination of electrospinning parameters to fabricate high degree of alignment of keratin fibers further suggests that a systematic model may be developed to realize more possible formulations and combinations to successfully spin keratin fibers with the desired morphology and architecture. Future work on modeling the electrospinning parameters is discussed in the next section.

The 2nd objective was achieved by evaluating primary HDF behavior on both types of keratin fibers in terms of metabolic activity, proliferation, cellular distribution and protein expression in-vitro. Keratin fibers could induce healthy cell growth with HDFs exhibiting their typical spindle shape morphology on them. Besides, HDFs could respond to the topographical cues given by the keratin fibers and arrange themselves to form network of cells according to the different architecture of the keratin fibers platform. Keratin fibers also stimulated HDFs to express fibronectin and collagen III which are ECM proteins essential in the early stage of wound healing. In addition, the keratin fibrous platforms used in this work were found to possess significant amide conformations, demonstrating their strong protein characteristics. This supports the 2nd hypothesis that electrospun keratin platforms can support the attachment, proliferation and function of human dermal fibroblasts via topographical cues from the architecture of the platforms and biochemical cues from the inherent protein characteristics of the keratin. For further evaluation on cell compliance of electrospun keratin platforms for wound healing, human epidermal keratinocytes are important candidates to consider for cell studies, besides HDFs. Both HDFs and keratinocytes studies should be evaluated in unison such that a robust system may be developed to evaluate keratin fibrous platforms for wound healing by co-culturing both cells. The importance of keratinocytes for in-depth future work on evaluating keratin fibers for wound healing is discussed in the next section.

The 3rd objective was achieved by carrying out experiments focusing on elucidating cell-material interaction using keratin films. Cell-material interaction study between keratin VD film and HDF was designed to investigate the degree of dependency on $\alpha_4\beta_1$ integrin in HDFs adherence to keratin VD film. It was proven that $\alpha_4\beta_1$ integrin played a significant role in HDF attachment of keratin VD film, hence elucidating the cell-material interaction between keratin VD film and HDF being mediated by the affinity of $\alpha_4\beta_1$ integrin expressed by HDFs to the LDV protein sequence found in human hair keratin. This proves the 3rd hypothesis that keratin plays a bioactive role to enhance the growth of human dermal fibroblasts cultured on human hair keratin templates.

There were additional findings from Phase 3 of the study which focused on elucidating the involvement of keratin and electrospinning in cell material interaction with HDFs. Experiments in phase 3 was carried out by casting keratin films to eliminate the effect of topographical cues on cell-material interaction. Two different methods were used in casting of keratin films; quick drying which is in parallel with the rapid formation of solid fibers from the solution during electrospinning to form VD films and slow drying to form AD films as a comparison with the former method. HDFs showed distinctively different behavior on VD film compared to AD film, the phenomenon that VD film supported HDF attachment and growth while AD film did not. This prompted the possibility that morphologically different protein derivatives from the same parent protein could exhibit different cell response. Existence of significantly higher amount of free groups such as amine groups and thiol groups implied that keratin in VD film was less intact than keratin in AD film. This led to the postulation that the keratin conformation in VD film had allowed $\alpha_4\beta_1$ integrin dependent adhesion of HDF via the exposed V-region of the LDV cell adhesion motif. Possible future work for the realization of this postulation is discussed in the next section.

As a summary, a viable keratin platform to facilitate cell growth for tissue regeneration has been developed in this work and provided insights that cell-material interaction between keratin and HDFs may be mediated by the affinity of $\alpha_4\beta_1$ integrin to the LDV cell adhesion motif.

8.2 Recommendations

8.2.1 Modeling of Electrospinning Parameters to Develop Multi-scale Scaffolds via Multimodal Electrospinning

The architecture of the electrospun scaffold determines the performance of cell behavior with the fiber diameter as the prime controllable design parameter [198]. Nanoscale fibers reportedly improve cell response by sustaining stem cell differentiation induced by growth factors [94, 199-202] and accelerating cell attachment and proliferation [203, 204] with respect to micro-scale fibers. Moreover, nanofibers offered kinetic boost to cell performance via higher specific surface area [205, 206]. However, nanofibers result in dense network of fibers, impeding cell migration through the thickness. Therefore, microscale fibers emerged as solutions to outperform nanofibers in terms of cell ingrowth and viability [207]. In addition, statistical model was designed in providing a theoretical insight of the relationship between porosity and fiber diameter of the electrospun architecture [208, 209], predicting that increase in pore size could be done by increasing fiber diameter at fixed porosity. This prediction were consistent with many experiments [207, 209, 210] and hence supporting the addition advantage of microscale fibers for providing larger pore size, crucial for perfusion of cell catabolites and metabolites through the electrospun scaffold and ECM formation [211]. This discussion on the need for optimal trade-off between microscale fibers and nanoscale fibers [207, 212] explains the pursuit for multi-scale scaffolds via multimodal electrospinning to achieve fiber distributions with combination of two or more fiber populations [200, 205, 210, 213]. Hence it will be worthwhile to apply this multi-scale scaffold concept to develop the keratin fibers in this work into a more robust fibrous platform which inherit the advantages of both nanoscale fibers and microscale fibers to enhance cell functionality. In pursuit for such system using multimodal electrospinning, a model specific to the formulated electrospinning solution used in this work is essential in order to ensure reproducibility and predictability of the ultimate fibrous platform with multiple fiber populations. Therefore, further effort in relating the electrospinning parameters to the resulting architecture of the fibrous platform from electrospinning keratin in this work should be placed to develop a systematic model.

8.2.2 In-depth Evaluation on the Potential of Keratin Fibers for Skin Wound Healing

Besides HDFs, human epidermal keratinocytes are also cells critical for skin wound healing as they are the main cells of the epidermis. During the end of inflammation phase in wound healing, keratinocytes migrate from the edges to the injury site to cover the wound bed. This wound healing stage is known as re-epithelialization which is critical for re-establishing the role of the skin as a barrier against the external environment [214, 215]. In addition, like HDFs, keratinocytes secrete fibronectin [216] which plays an important function in promoting cell attachment, proliferation and migration during wound healing [217-219]. Hence, it is worthwhile to investigate how keratin fibers can promote the cell response of keratinocytes in wound healing, especially in terms of keratinocytes migration to the injury site to cover the wound as quickly as possible to prevent fatal infections from external environment. Likewise, migration of HDFs should also be further investigated to evaluate the potential of keratin fibers in accelerating wound healing.

Another important wound healing stage is the ECM remodeling stage in which cells in the wound site start to secrete matrix metalloproteinases which are capable of degrading extracellular matrix proteins. In view of this, it is important to study how these matrix metalloproteinases can affect the structural integrity of the keratin fibers over time. Finally, it is crucial to test the keratin fibrous platform in-vivo for its degradation profile, biocompatibility and immunocompatibility as a more realistic reflection on the potential of keratin fibers for wound healing.

8.2.3 Development of Purified Keratin for Fundamental Structural Studies

In this study, crude human hair extracts were used in the synthesis of keratin platforms. For a more comprehensive systematic study on how LDV in keratin enhance the cell attachment via $\alpha_4\beta_1$ integrin and whether the quantity of LDV would yield significant effect on cell attachment and growth, it is recommended that further effort could be placed on purifying the crude keratin extracts into individual keratin subtypes such that

retrieving of specific keratin subtypes for material synthesis could be done in a controllable manner. Besides, materials produced from unpurified pool of proteins tend to yield results with low signal to noise ratio in certain spectroscopic techniques [220] and hence pose a limitation to fundamental studies on the characteristic of the protein itself. With purified keratins, more analysis could be done to investigate the effect of different processing methods on the morphology and structural organization of keratin using techniques like transmission electron microscope (TEM) and Raman spectroscopy. While TEM provides a direct way to view the morphology of different protein organization, Raman spectroscopy offers useful explanations of morphological changes via chemical bonding. Results from Raman spectroscopy could give deductions on whether proteins are in their folded intact structures or unfolded flexible structures by quantifying changes in the amount of disulphide (S-S) bonds in the protein specimen through analyzing the S-S stretching frequencies at 514 cm^{-1} and thiol (S-H) stretching frequencies at 2547 cm^{-1} [193]. Hence, by using Raman spectroscopy could further verify the morphological difference between keratin in AD film and keratin in VD film.

References

- [1] R. Langer and J. P. Vacanti, "Tissue engineering," *Science*, vol. 260, pp. 920-926, 1993.
- [2] S. J. Hollister, "Porous scaffold design for tissue engineering," *Nature materials*, vol. 4, pp. 518-524, 2005.
- [3] S. Zhong, Y. Zhang, and C. Lim, "Tissue scaffolds for skin wound healing and dermal reconstruction," *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, vol. 2, pp. 510-525, 2010.
- [4] L. G. Griffith and G. Naughton, "Tissue engineering--current challenges and expanding opportunities," *Science*, vol. 295, pp. 1009-1014, 2002.
- [5] J. S. Boateng, K. H. Matthews, H. N. E. Stevens, and G. M. Eccleston, "Wound healing dressings and drug delivery systems: a review," *Journal of pharmaceutical sciences*, vol. 97, pp. 2892-2923, 2008.
- [6] X. Liu and P. X. Ma, "Polymeric Scaffolds for Bone Tissue Engineering: on Musculoskeletal Bioengineering. Guest Editor: Kyriacos A. Athanasiou," *Annals of biomedical engineering*, vol. 32, pp. 477-486, 2004.
- [7] T. C. Holmes, "Novel peptide-based biomaterial scaffolds for tissue engineering," *TRENDS in Biotechnology*, vol. 20, pp. 16-21, 2002.
- [8] A. Lynn, I. Yannas, and W. Bonfield, "Antigenicity and immunogenicity of collagen," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 71, pp. 343-354, 2004.
- [9] J. S. Choi, H. J. Yang, B. S. Kim, J. D. Kim, S. H. Lee, E. K. Lee, *et al.*, "Fabrication of porous extracellular matrix scaffolds from human adipose tissue," *Tissue Engineering Part C: Methods*, vol. 16, pp. 387-396, 2009.
- [10] C. Yang, P. J. Hillas, J. A. Baez, M. Nokelainen, J. Balan, J. Tang, *et al.*, "The application of recombinant human collagen in tissue engineering," *Biodrugs*, vol. 18, pp. 103-119, 2004.
- [11] A. Meana, J. Iglesias, M. Del Rio, F. Larcher, B. Madrigal, M. Fresno, *et al.*, "Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels," *Burns*, vol. 24, pp. 621-630, 1998.
- [12] V. Verma, P. Verma, P. Ray, and A. R. Ray, "Preparation of scaffolds from human hair proteins for tissue-engineering applications," *Biomedical Materials*, vol. 3, p. 025007, 2008.
- [13] R. MAKAREM and M. J. HUMPHRIES, "LDV: a novel cell adhesion motif recognized by the integrin $\alpha 4 \beta 1$," *Biochemical Society Transactions*, vol. 19, 1991.
- [14] J. Gailit, M. Pierschbacher, and R. A. F. Clark, "Expression of Functional $\alpha 4 \beta 1$ Integrin by Human Dermal Fibroblasts," *Journal of investigative dermatology*, vol. 100, pp. 323-328, 1993.
- [15] B. Garmy-Susini, H. Jin, Y. Zhu, R. J. Sung, R. Hwang, and J. Varner, "Integrin $\alpha 4 \beta 1$ -VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation," *J Clin Invest*, vol. 115, pp. 1542-1551, 2005.
- [16] S. Massia and J. Hubbell, "Vascular endothelial cell adhesion and spreading promoted by the peptide REDV of the IIICS region of plasma fibronectin is

- mediated by integrin alpha 4 beta 1," *Journal of Biological Chemistry*, vol. 267, pp. 14019-14026, 1992.
- [17] J. L. Guan and R. O. Hynes, "Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha 4 \beta 1$," *Cell*, vol. 60, pp. 53-61, 1990.
 - [18] U. H. von Andrian and B. Engelhardt, " $\alpha 4$ integrins as therapeutic targets in autoimmune disease," *New England Journal of Medicine*, vol. 348, pp. 68-72, 2003.
 - [19] O. Barreiro, M. Yáñez-Mó, J. M. Serrador, M. C. Montoya, M. Vicente-Manzanares, R. Tejedor, *et al.*, "Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes," *The Journal of cell biology*, vol. 157, pp. 1233-1245, 2002.
 - [20] J. M. Clements, P. Newham, M. Shepherd, R. Gilbert, T. J. Dudgeon, L. A. Needham, *et al.*, "Identification of a key integrin-binding sequence in VCAM-1 homologous to the LDV active site in fibronectin," *Journal of cell science*, vol. 107, pp. 2127-2135, 1994.
 - [21] T. J. Sill and H. A. von Recum, "Electrospinning: applications in drug delivery and tissue engineering," *Biomaterials*, vol. 29, pp. 1989-2006, 2008.
 - [22] J. Doshi and D. H. Reneker, "Electrospinning process and applications of electrospun fibers," *Journal of electrostatics*, vol. 35, pp. 151-160, 1995.
 - [23] W. J. Li, C. T. Laurencin, E. J. Caterson, R. S. Tuan, and F. K. Ko, "Electrospun nanofibrous structure: a novel scaffold for tissue engineering," *Journal of biomedical materials research*, vol. 60, pp. 613-621, 2002.
 - [24] Z. C. Xing, J. Yuan, W. P. Chae, I. K. Kang, and S. Y. Kim, "Keratin Nanofibers as a Biomaterial," in *2010 International Conference on Nanotechnology and Biosensors IPCBEE*, 2011, pp. 120-124.
 - [25] A. Aluigi, A. Varesano, A. Montarsolo, C. Vineis, F. Ferrero, G. Mazzuchetti, *et al.*, "Electrospinning of keratin/poly (ethylene oxide) blend nanofibers," *Journal of Applied Polymer Science*, vol. 104, pp. 863-870, 2007.
 - [26] A. Aluigi, C. Vineis, A. Varesano, G. Mazzuchetti, F. Ferrero, and C. Tonin, "Structure and properties of keratin/PEO blend nanofibres," *European Polymer Journal*, vol. 44, pp. 2465-2475, 2008.
 - [27] J. Li, Y. Li, L. Li, A. F. T. Mak, F. Ko, and L. Qin, "Preparation and biodegradation of electrospun PLLA/keratin nonwoven fibrous membrane," *Polymer Degradation and Stability*, vol. 94, pp. 1800-1807, 2009.
 - [28] L. Li, Y. Li, J. Li, A. F. T. Mak, F. Ko, and L. Qin, "Cytotoxicity and Cell Adhesion of PLLA/keratin Composite Fibrous Membranes," in *13th International Conference on Biomedical Engineering*, 2009, pp. 1492-1495.
 - [29] J. Yuan, J. Shen, and I. K. Kang, "Fabrication of protein- doped PLA composite nanofibrous scaffolds for tissue engineering," *Polymer International*, vol. 57, pp. 1188-1193, 2008.
 - [30] M. Zoccola, A. Montarsolo, A. Aluigi, A. Varesano, C. Vineis, and C. Tonin, "Electrospinning of polyamide 6/modified-keratin blends," *E-Polymers*, vol. 27, pp. 1433-1436, 2007.

-
- [31] 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, pp. 2819-2825, 2008.
- [32] D. H. Baek, C. S. Ki, I. C. Um, and Y. H. Park, "Metal ion adsorbability of electrospun wool keratose/silk fibroin blend nanofiber mats," *Fibers and Polymers*, vol. 8, pp. 271-277, 2007.
- [33] J. Yuan, Z. C. Xing, S. W. Park, J. Geng, I. K. Kang, J. Shen, *et al.*, "Fabrication of PHBV/keratin composite nanofibrous mats for biomedical applications," *Macromolecular Research*, vol. 17, pp. 850-855, 2009.
- [34] J. G. Rouse and M. E. Van Dyke, "A review of keratin-based biomaterials for biomedical applications," *Materials*, vol. 3, pp. 999-1014, 2010.
- [35] Y. Hirao, K. Ohkawa, H. Yamamoto, and T. Fujii, "A novel human hair protein fiber prepared by watery hybridization spinning," *Macromolecular Materials and Engineering*, vol. 290, pp. 165-171, 2005.
- [36] L. Langbein, M. A. Rogers, H. Winter, S. Praetzel, U. Beckhaus, H.-R. Rackwitz, *et al.*, "The catalog of human hair keratins I. Expression of the nine type I members in the hair follicle," *Journal of Biological Chemistry*, vol. 274, pp. 19874-19884, 1999.
- [37] C. Popescu and H. Höcker, "Hair—the most sophisticated biological composite material," *Chemical Society Reviews*, vol. 36, pp. 1282-1291, 2007.
- [38] J. A. Rippon, "The structure of wool," *Wool dyeing*, pp. 1-51, 2013.
- [39] W. Crewther, R. Fraser, F. Lennox, and H. Lindley, "The chemistry of keratins," *Advances in protein chemistry*, vol. 20, pp. 191-346, 1965.
- [40] J. Rouse and M. Van Dyke, "A Review of Keratin-Based Biomaterials for Biomedical Applications," *Materials*, vol. 3, pp. 999-1014, 2010.
- [41] P. Hill, H. Brantley, and M. Van Dyke, "Some properties of keratin biomaterials: kerateines," *Biomaterials*, vol. 31, pp. 585-593, 2010.
- [42] 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, pp. 35123-35132, 2001.
- [43] J. Schweizer, P. E. Bowden, P. A. Coulombe, L. Langbein, E. B. Lane, T. M. Magin, *et al.*, "New consensus nomenclature for mammalian keratins," *The Journal of cell biology*, vol. 174, pp. 169-174, 2006.
- [44] J. Schweizer, L. Langbein, M. A. Rogers, and H. Winter, "Hair follicle-specific keratins and their diseases," *Experimental cell research*, vol. 313, pp. 2010-2020, 2007.
- [45] J. A. Burdick and R. L. Mauck, *Biomaterials for tissue engineering applications: a review of the past and future trends*: Springer Science & Business Media, 2010.
- [46] A. Tachibana, S. Kaneko, T. Tanabe, and K. Yamauchi, "Rapid fabrication of keratin-hydroxyapatite hybrid sponges toward osteoblast cultivation and differentiation," *Biomaterials*, vol. 26, pp. 297-302, 2005.
- [47] A. Belcarz, G. Ginalska, J. Zalewska, W. Rzeski, A. Ślósarczyk, D. Kowalczyk, *et al.*, "Covalent coating of hydroxyapatite by keratin stabilizes gentamicin release," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 89, pp. 102-113, 2009.

-
- [48] A. Tachibana, Y. Furuta, H. Takeshima, T. Tanabe, and K. Yamauchi, "Fabrication of wool keratin sponge scaffolds for long-term cell cultivation," *Journal of biotechnology*, vol. 93, pp. 165-170, 2002.
- [49] H. Thomas, A. Conrads, K.-H. Phan, M. van de Löcht, and H. Zahn, "In vitro reconstitution of wool intermediate filaments," *International Journal of Biological Macromolecules*, vol. 8, pp. 258-264, 1986.
- [50] A. Nakamura, M. Arimoto, K. Takeuchi, and T. Fujii, "A rapid extraction procedure of human hair proteins and identification of phosphorylated species," *Biological and Pharmaceutical Bulletin*, vol. 25, pp. 569-572, 2002.
- [51] P. M. Schrooyen, P. J. Dijkstra, R. C. Oberthür, A. Bantjes, and J. Feijen, "Partially carboxymethylated feather keratins. 1. Properties in aqueous systems," *Journal of agricultural and Food Chemistry*, vol. 48, pp. 4326-4334, 2000.
- [52] K. Wrześniewska-Tosik and J. Adamiec, "Biocomposites with a content of keratin from chicken feathers," *Fibres & Textiles in Eastern Europe*, vol. 15, p. 60, 2007.
- [53] J. A. Burns, J. C. Butler, J. Moran, and G. M. Whitesides, "Selective reduction of disulfides by tris (2-carboxyethyl) phosphine," *The Journal of Organic Chemistry*, vol. 56, pp. 2648-2650, 1991.
- [54] J. E. Plowman, S. Deb- Choudhury, A. Thomas, S. Clerens, C. D. Cornellison, A. J. Grosvenor, *et al.*, "Characterisation of low abundance wool proteins through novel differential extraction techniques," *Electrophoresis*, vol. 31, pp. 1937-1946, 2010.
- [55] "Method of preparing keratin-containing films and coatings," ed: Google Patents, 1972.
- [56] Y. Kawano and S. Okamoto, "Film and gel of keratins," *Kagaku To Seibutsu*, vol. 13, pp. 291-2, 1975.
- [57] S. Okamoto, "On the formation of films from some proteins," *Journal of Food Science and Technology*, 1977.
- [58] P. J. Apel, J. P. Garrett, P. Sierpinski, J. Ma, A. Atala, T. L. Smith, *et al.*, "Peripheral nerve regeneration using a keratin-based scaffold: long-term functional and histological outcomes in a mouse model," *The Journal of hand surgery*, vol. 33, pp. 1541-1547, 2008.
- [59] P. Sierpinski, J. Garrett, J. Ma, P. Apel, D. Klorig, T. Smith, *et al.*, "The use of keratin biomaterials derived from human hair for the promotion of rapid regeneration of peripheral nerves," *Biomaterials*, vol. 29, pp. 118-128, 2008.
- [60] T. Aboushwareb, D. Eberli, C. Ward, C. Broda, J. Holcomb, A. Atala, *et al.*, "A keratin biomaterial gel hemostat derived from human hair: Evaluation in a rabbit model of lethal liver injury," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 90, pp. 45-54, 2009.
- [61] M. Van Dyke, C. Blanchard, S. Timmons, A. Siller-Jackson, and R. Smith, "Soluble keratin peptide," ed: Google Patents, 2001.
- [62] C. R. Blanchard, S. F. Timmons, and R. A. Smith, "Keratin-based hydrogel for biomedical applications and method of production," ed: Google Patents, 2000.
- [63] D. R. Cowsar, "Bioactive keratin peptides," ed: Google Patents, 2012.
- [64] M. E. Van Dyke, "Wound healing compositions containing keratin biomaterials," ed: Google Patents, 2012.

-
- [65] K. Yamauchi, M. Maniwa, and T. Mori, "Cultivation of fibroblast cells on keratin-coated substrata," *Journal of Biomaterials Science, Polymer Edition*, vol. 9, pp. 259-270, 1998.
 - [66] S. Reichl, "Films based on human hair keratin as substrates for cell culture and tissue engineering," *Biomaterials*, vol. 30, pp. 6854-6866, 2009.
 - [67] S. Reichl, M. Borrelli, and G. Geerling, "Keratin films for ocular surface reconstruction," *Biomaterials*, vol. 32, pp. 3375-3386, 2011.
 - [68] X. Zhao, Y. S. Lui, C. K. C. Choo, W. T. Sow, C. L. Huang, K. W. Ng, *et al.*, "Calcium phosphate coated Keratin-PCL scaffolds for potential bone tissue regeneration," *Materials Science and Engineering: C*, vol. 49, pp. 746-753, 2015.
 - [69] J.-S. Li, Y. Li, X. Liu, J. Zhang, and Y. Zhang, "Strategy to introduce an hydroxyapatite-keratin nanocomposite into a fibrous membrane for bone tissue engineering," *Journal of Materials Chemistry B*, vol. 1, pp. 432-437, 2013.
 - [70] J. Li, X. Liu, J. Zhang, Y. Zhang, Y. Han, J. Hu, *et al.*, "Synthesis and characterization of wool keratin/hydroxyapatite nanocomposite," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 100, pp. 896-902, 2012.
 - [71] R. C. de Guzman, J. M. Saul, M. D. Ellenburg, M. R. Merrill, H. B. Coan, T. L. Smith, *et al.*, "Bone regeneration with BMP-2 delivered from keratose scaffolds," *Biomaterials*, vol. 34, pp. 1644-1656, 2013.
 - [72] C. J. Kowalczewski, S. Tombyln, D. C. Wasnick, M. R. Hughes, M. D. Ellenburg, M. F. Callahan, *et al.*, "Reduction of ectopic bone growth in critically-sized rat mandible defects by delivery of rhBMP-2 from keratine biomaterials," *Biomaterials*, vol. 35, pp. 3220-3228, 2014.
 - [73] A. Tachibana, Y. Nishikawa, M. Nishino, S. Kaneko, T. Tanabe, and K. Yamauchi, "Modified keratin sponge: binding of bone morphogenetic protein-2 and osteoblast differentiation," *Journal of bioscience and bioengineering*, vol. 102, pp. 425-429, 2006.
 - [74] G. J. Dias, P. V. Peplow, A. McLaughlin, F. Teixeira, and R. J. Kelly, "Biocompatibility and osseointegration of reconstituted keratin in an ovine model," *Journal of Biomedical Materials Research Part A*, vol. 92, pp. 513-520, 2010.
 - [75] M. E. Van Dyke, S. F. Timmons, C. R. Blanchard, A. J. Siller-Jackson, and R. A. Smith, "As absorbent with skin healing properties when incorporated into diapers, feminine hygiene products, wound dressings, including both human and veterinary uses, as soft tissue augmentation medium when used in subdermal implants; cosmetics," ed: Google Patents, 2001.
 - [76] Y.-H. Chen, W.-R. Dong, Y.-Q. Xiao, B.-L. Zhao, G.-D. Hu, and L.-B. An, "Preparation and bioactivity of human hair keratin-collagen sponge, a new type of dermal analogue," *Nan Fang Yi Ke Da Xue Xue Bao*, vol. 26, pp. 131-138, 2006.
 - [77] Y. Chen, W. Dong, Q. Chen, B. Zhao, Z. Zou, Y. Xiao, *et al.*, "[Biological dressing with human hair keratin-collagen sponge-poly 2-hydroxyethyl methacrylate composite promotes burn wound healing in SD rats]," *Nan fang yi ke da xue xue bao = Journal of Southern Medical University*, vol. 27, pp. 1621-1626, 2007.

- [78] S. Thilagar, N. A. Jothi, A. Omar, M. Kamaruddin, and S. Ganabadi, "Effect of keratin-gelatin and bFGF-gelatin composite film as a sandwich layer for full-thickness skin mesh graft in experimental dogs," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 88, pp. 12-16, 2009.
- [79] N. A. Jothi, S. Thilagar, A. Omar, M. Kamaruddin, and G. Shanthi, "Effect of keratin-gelatin and bFGF-gelatin composite film on wound healing in dogs: A histopathological study," *Indian Journal of Veterinary Surgery*, vol. 29, pp. 100-102, 2008.
- [80] K. Lee, S. Kong, W. Park, W. Ha, and I. Kwon, "Effect of surface properties on the antithrombogenicity of silk fibroin/S-carboxymethyl kerateine blend films," *Journal of Biomaterials Science, Polymer Edition*, vol. 9, pp. 905-914, 1998.
- [81] P. Lu and B. Ding, "Applications of electrospun fibers," *Recent patents on nanotechnology*, vol. 2, pp. 169-182, 2008.
- [82] Y. Zhang, B. Su, S. Ramakrishna, and C. Lim, "Chitosan nanofibers from an easily electrospinnable UHMWPEO-doped chitosan solution system," *Biomacromolecules*, vol. 9, pp. 136-141, 2007.
- [83] H. Nie, A. He, W. Wu, J. Zheng, S. Xu, J. Li, *et al.*, "Effect of poly (ethylene oxide) with different molecular weights on the electrospinnability of sodium alginate," *Polymer*, vol. 50, pp. 4926-4934, 2009.
- [84] M. Pakravan, M. C. Heuzey, and A. Ajji, "A fundamental study of chitosan/PEO electrospinning," *Polymer*, vol. 52, pp. 4813-4824, 2011.
- [85] H. Nie, A. He, J. Zheng, S. Xu, J. Li, and C. C. Han, "Effects of chain conformation and entanglement on the electrospinning of pure alginate," *Biomacromolecules*, vol. 9, pp. 1362-1365, 2008.
- [86] J. Doshi and D. H. Reneker, "Electrospinning process and applications of electrospun fibers," in *Industry Applications Society Annual Meeting, 1993., Conference Record of the 1993 IEEE*, 1993, pp. 1698-1703.
- [87] H. Fong, I. Chun, and D. Reneker, "Beaded nanofibers formed during electrospinning," *Polymer*, vol. 40, pp. 4585-4592, 1999.
- [88] X. Geng, O.-H. Kwon, and J. Jang, "Electrospinning of chitosan dissolved in concentrated acetic acid solution," *Biomaterials*, vol. 26, pp. 5427-5432, 2005.
- [89] P. Gupta, C. Elkins, T. E. Long, and G. L. Wilkes, "Electrospinning of linear homopolymers of poly (methyl methacrylate): exploring relationships between fiber formation, viscosity, molecular weight and concentration in a good solvent," *Polymer*, vol. 46, pp. 4799-4810, 2005.
- [90] N. Bhardwaj and S. C. Kundu, "Electrospinning: a fascinating fiber fabrication technique," *Biotechnology advances*, vol. 28, pp. 325-347, 2010.
- [91] D. Han and K. C. Cheung, "Biodegradable cell-seeded nanofiber scaffolds for neural repair," *Polymers*, vol. 3, pp. 1684-1733, 2011.
- [92] P. K. Baumgarten, "Electrostatic spinning of acrylic microfibers," *Journal of colloid and interface science*, vol. 36, pp. 71-79, 1971.
- [93] S. N. Khan, *Electrospinning polymer nanofibers-electrical and optical Characterization*: ProQuest, 2007.
- [94] F. Yang, R. Murugan, S. Wang, and S. Ramakrishna, "Electrospinning of nano/micro scale poly (L-lactic acid) aligned fibers and their potential in neural tissue engineering," *Biomaterials*, vol. 26, pp. 2603-2610, 2005.

-
- [95] V. Beachley, E. Katsanevakis, N. Zhang, and X. Wen, "Highly aligned polymer nanofiber structures: fabrication and applications in tissue engineering," in *Biomedical Applications of Polymeric Nanofibers*, ed: Springer, 2012, pp. 171-212.
- [96] S. Ramakrishna, K. Fujihara, W. Teo, T.-C. Lim, and Z. Ma, "An introduction to electrospinning and nanofibers. 2005," *Singapura: World Scientific Publishing Company*.
- [97] L. Buttafoco, N. Kolkman, P. Engbers-Buijtenhuijs, A. Poot, P. Dijkstra, I. Vermes, *et al.*, "Electrospinning of collagen and elastin for tissue engineering applications," *Biomaterials*, vol. 27, pp. 724-734, 2006.
- [98] K. J. Choi, J. E. Spruiell, and J. L. White, "Orientation and morphology of high-density polyethylene film produced by the tubular blowing method and its relationship to process conditions," *Journal of Polymer Science: Polymer Physics Edition*, vol. 20, pp. 27-47, 1982.
- [99] S. Ramakrishna, *An introduction to electrospinning and nanofibers*: World Scientific Publishing Company Incorporated, 2005.
- [100] J. Deitzel, J. Kleinmeyer, D. Harris, and N. B. Tan, "The effect of processing variables on the morphology of electrospun nanofibers and textiles," *Polymer*, vol. 42, pp. 261-272, 2001.
- [101] P. B. Malafaya, G. A. Silva, and R. L. Reis, "Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications," *Advanced drug delivery reviews*, vol. 59, pp. 207-233, 2007.
- [102] X. Yuan, Y. Zhang, C. Dong, and J. Sheng, "Morphology of ultrafine polysulfone fibers prepared by electrospinning," *Polymer International*, vol. 53, pp. 1704-1710, 2004.
- [103] L. Wannatong, A. Sirivat, and P. Supaphol, "Effects of solvents on electrospun polymeric fibers: preliminary study on polystyrene," *Polymer International*, vol. 53, pp. 1851-1859, 2004.
- [104] A. Frenot and I. S. Chronakis, "Polymer nanofibers assembled by electrospinning," *Current opinion in colloid & interface science*, vol. 8, pp. 64-75, 2003.
- [105] C. S. Ki, J. W. Kim, J. H. Hyun, K. H. Lee, M. Hattori, D. K. Rah, *et al.*, "Electrospun three-dimensional silk fibroin nanofibrous scaffold," *Journal of applied polymer science*, vol. 106, pp. 3922-3928, 2007.
- [106] K. Lee, H. Kim, H. Bang, Y. Jung, and S. Lee, "The change of bead morphology formed on electrospun polystyrene fibers," *Polymer*, vol. 44, pp. 4029-4034, 2003.
- [107] W. Teo and S. Ramakrishna, "A review on electrospinning design and nanofibre assemblies," *Nanotechnology*, vol. 17, p. R89, 2006.
- [108] V. Beachley, E. Katsanevakis, N. Zhang, and X. Wen, "Highly Aligned Polymer Nanofiber Structures: Fabrication and Applications in Tissue Engineering," *[Without Title]*, pp. 1-42, 2011.
- [109] W. E. T. a. S. Ramakrishna, "A review on electrospinning design and nanofibre assemblies," *Nanotechnology*, vol. Volume 17, 2006.
- [110] C. E. Ayres, B. S. Jha, H. Meredith, J. R. Bowman, G. L. Bowlin, S. C. Henderson, *et al.*, "Measuring fiber alignment in electrospun scaffolds: a user's

- guide to the 2D fast Fourier transform approach," *Journal of Biomaterials Science, Polymer Edition*, vol. 19, pp. 603-621, 2008.
- [111] R. J. Kuriger, M. K. Alam, D. P. Anderson, and R. L. Jacobsen, "Processing and characterization of aligned vapor grown carbon fiber reinforced polypropylene," *Composites Part A: Applied Science and Manufacturing*, vol. 33, pp. 53-62, 2002.
 - [112] S. Zhong, W. E. Teo, X. Zhu, R. W. Beuerman, S. Ramakrishna, and L. Y. L. Yung, "An aligned nanofibrous collagen scaffold by electrospinning and its effects on in vitro fibroblast culture," *Journal of Biomedical Materials Research Part A*, vol. 79, pp. 456-463, 2006.
 - [113] K. Yamauchi, A. Yamauchi, T. Kusunoki, A. Kohda, and Y. Konishi, "Preparation of stable aqueous solution of keratins, and physiochemical and biodegradational properties of films," *Journal of biomedical materials research*, vol. 31, pp. 439-444, 1998.
 - [114] K. Katoh, M. Shibayama, T. Tanabe, and K. Yamauchi, "Preparation and physicochemical properties of compression-molded keratin films," *Biomaterials*, vol. 25, pp. 2265-2272, 2004.
 - [115] T. Tanabe, N. Okitsu, and K. Yamauchi, "Fabrication and characterization of chemically crosslinked keratin films," *Materials Science and Engineering: C*, vol. 24, pp. 441-446, 2004.
 - [116] T. Fujii, D. Ogiwara, and M. Arimoto, "Convenient procedures for human hair protein films and properties of alkaline phosphatase incorporated in the film," *Biological and Pharmaceutical Bulletin*, vol. 27, pp. 89-93, 2004.
 - [117] T. Fujii and Y. Ide, "Preparation of translucent and flexible human hair protein films and their properties," *Biological and Pharmaceutical Bulletin*, vol. 27, pp. 1433-1436, 2004.
 - [118] Y. C. Lin, M. Ramadan, M. Van Dyke, L. E. Kokai, B. J. Philips, J. P. Rubin, *et al.*, "Keratin gel filler for peripheral nerve repair in a rodent sciatic nerve injury model," *Plastic and reconstructive surgery*, vol. 129, p. 67, 2012.
 - [119] 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*, pp. 1-8, 2012.
 - [120] K. Katoh, T. Tanabe, and K. Yamauchi, "Novel approach to fabricate keratin sponge scaffolds with controlled pore size and porosity," *Biomaterials*, vol. 25, pp. 4255-4262, 2004.
 - [121] A. Kurimoto, T. Tanabe, A. Tachibana, and K. Yamauchi, "Keratin sponge: Immobilization of lysozyme," *Journal of bioscience and bioengineering*, vol. 96, pp. 307-309, 2003.
 - [122] 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, pp. 1250-1254, 2008.
 - [123] A. Aluigi, C. Vineis, C. Tonin, C. Tonetti, A. Varesano, and G. Mazzuchetti, "Wool keratin-based nanofibres for active filtration of air and water," *Journal of Biobased Materials and Bioenergy*, vol. 3, pp. 311-319, 2009.
 - [124] W. T. Sow, Y. S. Lui, and K. W. Ng, "Electrospun human keratin matrices as templates for tissue regeneration," *Nanomedicine*, vol. 8, pp. 531-541, 2013.

- [125] Y. Hsueh, Y. Chen, K. Yen, C. Wen, and O. Mestak, "Evaluation of Electrospinning Keratin Membrane with Gelatin Addition in Relation to Nerve Regeneration," *J Bioengineer & Biomedical Sci*, vol. 4, p. 2, 2014.
- [126] A. Edwards, D. Jarvis, T. Hopkins, S. Pixley, and N. Bhattarai, "Poly (ϵ -caprolactone)/keratin-based composite nanofibers for biomedical applications," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 103, pp. 21-30, 2015.
- [127] D. Y. Deniz, M. V. Kahraman, and S. E. Kuruca, "UV-reactive electrospinning of keratin/4-vinyl benzene boronic acid-hydroxyapatite/poly (vinyl alcohol) composite nanofibers," *Polymer Composites*, 2015.
- [128] C. S. Ki, E. H. Gang, I. C. Um, and Y. H. Park, "Nanofibrous membrane of wool keratose/silk fibroin blend for heavy metal ion adsorption," *Journal of Membrane Science*, vol. 302, pp. 20-26, 2007.
- [129] A. Aluigi, C. Tonetti, C. Vineis, C. Tonin, R. Casasola, and F. Ferrero, "Wool keratin nanofibres for copper (II) adsorption," *Journal of Biobased Materials and Bioenergy*, vol. 6, pp. 230-236, 2012.
- [130] A. Aluigi, C. Tonetti, C. Vineis, C. Tonin, and G. Mazzuchetti, "Adsorption of copper (II) ions by keratin/PA6 blend nanofibres," *European Polymer Journal*, vol. 47, pp. 1756-1764, 2011.
- [131] A. Aluigi, A. Corbellini, F. Rombaldoni, and G. Mazzuchetti, "Wool-derived keratin nanofiber membranes for dynamic adsorption of heavy-metal ions from aqueous solutions," *Textile research journal*, vol. 83, pp. 1574-1586, 2013.
- [132] A. Aluigi, C. Tonetti, C. Vineis, A. Varesano, C. Tonin, and R. Casasola, "Study on the adsorption of chromium (VI) by hydrolyzed keratin/polyamide 6 blend nanofibres," *Journal of nanoscience and nanotechnology*, vol. 12, pp. 7250-7259, 2012.
- [133] M. B. Dickerson, A. A. Sierra, N. M. Bedford, W. J. Lyon, W. E. Gruner, P. A. Mirau, *et al.*, "Keratin-based antimicrobial textiles, films, and nanofibers," *Journal of Materials Chemistry B*, vol. 1, pp. 5505-5514, 2013.
- [134] M. Hajmaleki, R. Khajavi, and T. Toliyat, "Cytotoxicity and antibacterial properties of nano fibrous scaffolds based on extracted keratin from human hair waste and silver nano particles," *Medical Science Journal of Islamic Azad Univesity-Tehran Medical Branch*, vol. 25, pp. 46-54, 2015.
- [135] L. Li, Y. Li, J. Li, A. F. Mak, F. Ko, and L. Qin, "Cytotoxicity and cell adhesion of PLLA/keratin composite fibrous membranes," in *13th International Conference on Biomedical Engineering*, 2009, pp. 1492-1495.
- [136] J. Yuan, Z.-C. Xing, S.-W. Park, J. Geng, I.-K. Kang, J. Shen, *et al.*, "Fabrication of PHBV/keratin composite nanofibrous mats for biomedical applications," *Macromolecular Research*, vol. 17, pp. 850-855, 2009.
- [137] Z.-C. Xing, J. Yuan, W.-P. Chae, I.-K. Kang, and S.-Y. Kim, "Keratin nanofibers as a biomaterial," in *International Conference on Nanotechnology and Biosensors IPCBEE*, 2011, p. 120.
- [138] J. Yuan, J. Geng, Z. Xing, K. J. Shim, I. Han, J. C. Kim, *et al.*, "Novel wound dressing based on nanofibrous PHBV-keratin mats," *Journal of tissue engineering and regenerative medicine*, 2012.

-
- [139] H. Zhang and J. Liu, "Electrospun poly (lactic-co-glycolic acid)/wool keratin fibrous composite scaffolds potential for bone tissue engineering applications," *Journal of Bioactive and Compatible Polymers*, vol. 28, pp. 141-153, 2013.
- [140] B.-S. Kim, K. E. Park, W. H. Park, and J. Lee, "Fabrication of nanofibrous scaffold using a PLA and hagfish thread keratin composite; its effect on cell adherence, growth, and osteoblast differentiation," *Biomedical Materials*, vol. 8, p. 045006, 2013.
- [141] J. Zhang, Y. Li, J. Li, Z. Zhao, X. Liu, Y. Zhang, *et al.*, "Generation of biofunctional and biodegradable electrospun nanofibers composed of poly (L-lactic acid) and wool isoelectric precipitate," *Textile Research Journal*, p. 0040517513499438, 2013.
- [142] C. Ayres, G. L. Bowlin, S. C. Henderson, L. Taylor, J. Shultz, J. Alexander, *et al.*, "Modulation of anisotropy in electrospun tissue-engineering scaffolds: analysis of fiber alignment by the fast Fourier transform," *Biomaterials*, vol. 27, pp. 5524-5534, 2006.
- [143] R. Moll, M. Divo, and L. Langbein, "The human keratins: biology and pathology," *Histochemistry and cell biology*, vol. 129, pp. 705-733, 2008.
- [144] C. Popescu and H. Höcker, "Hair—the most sophisticated biological composite material," *Chem. Soc. Rev.*, vol. 36, pp. 1282-1291, 2007.
- [145] F. Taraballi, S. Wang, J. Li, F. Y. Y. Lee, S. S. Venkatraman, W. R. Birch, *et al.*, "Understanding the Nano-topography Changes and Cellular Influences Resulting from the Surface Adsorption of Human Hair Keratins," *Advanced Healthcare Materials*, 2012.
- [146] D. Goddard and L. Michaelis, "A study on keratin," *Journal of Biological Chemistry*, vol. 106, p. 605, 1934.
- [147] A. J. Poole, R. E. Lyons, and J. S. Church, "Dissolving Feather Keratin Using Sodium Sulfide for Bio-Polymer Applications," *Journal of Polymers and the Environment*, vol. 19, pp. 995-1004, 2011.
- [148] Z. Li and C. Wang, "Effects of working parameters on electrospinning," in *One-Dimensional Nanostructures*, ed: Springer, 2013, pp. 15-28.
- [149] C. Tonin, A. Aluigi, C. Vineis, A. Varesano, A. Montarsolo, and F. Ferrero, "Thermal and structural characterization of poly(ethylene-oxide)/keratin blend films," *Journal of Thermal Analysis and Calorimetry*, vol. 89, pp. 601-608, 2007.
- [150] W. Gallagher, "FTIR analysis of protein structure," 2007.
- [151] J. Kong and S. Yu, "Fourier transform infrared spectroscopic analysis of protein secondary structures," *Acta biochimica et biophysica Sinica*, vol. 39, pp. 549-559, 2007.
- [152] J. Bandekar, R. Sethna, and M. Kirschner, "Quantitative determination of sulfur oxide species in white liquor by FT-IR," *Applied spectroscopy*, vol. 49, pp. 1577-1582, 1995.
- [153] C. Contreras, S. Sugita, and E. Ramos, "Preparation of sodium aluminate from basic aluminum sulfate," *Advances in Technology of Materials and Materials Processing Journal*, vol. 8, p. 122, 2006.
- [154] D. M. Byler and H. Susi, "Examination of the secondary structure of proteins by deconvolved FTIR spectra," *Biopolymers*, vol. 25, pp. 469-487, 2004.

-
- [155] C. Tonin, A. Aluigi, C. Vineis, A. Varesano, A. Montarsolo, and F. Ferrero, "Thermal and structural characterization of poly (ethylene-oxide)/keratin blend films," *Journal of thermal analysis and calorimetry*, vol. 89, pp. 601-608, 2007.
- [156] A. Aluigi, M. Zoccola, C. Vineis, C. Tonin, F. Ferrero, and M. Canetti, "Study on the structure and properties of wool keratin regenerated from formic acid," *International Journal of Biological Macromolecules*, vol. 41, pp. 266-273, 2007.
- [157] K. Katoh, M. Shibayama, T. Tanabe, and K. Yamauchi, "Preparation and properties of keratin-poly (vinyl alcohol) blend fiber," *Journal of Applied Polymer Science*, vol. 91, pp. 756-762, 2004.
- [158] F. J. Wortmann and H. Deutz, "Characterizing keratins using high pressure differential scanning calorimetry (HPDSC)," *Journal of Applied Polymer Science*, vol. 48, pp. 137-150, 1993.
- [159] A. YAMAUCHI and K. YAMAUCHI, "Formation and Properties of Wool Keratin Films and Coatings," *Protein-Based Films and Coatings*, pp. 253-74, 2002.
- [160] M. Spei and R. Holzem, "Thermoanalytical investigations of extended and annealed keratins," *Colloid and Polymer Science*, vol. 265, pp. 965-970, 1987.
- [161] M. Spei and R. Holzem, "Further thermoanalytical investigations of annealed keratins: The time and temperature dependence," *Colloid & Polymer Science*, vol. 268, pp. 630-635, 1990.
- [162] S.-C. Wong, A. Baji, and S. Leng, "Effect of fiber diameter on tensile properties of electrospun poly (ϵ -caprolactone)," *Polymer*, vol. 49, pp. 4713-4722, 2008.
- [163] Z.-M. Huang, Y. Zhang, S. Ramakrishna, and C. Lim, "Electrospinning and mechanical characterization of gelatin nanofibers," *Polymer*, vol. 45, pp. 5361-5368, 2004.
- [164] C. Tonin, A. Aluigi, A. Varesano, and C. Vineis, "Keratin-based Nanofibres," in *Nanofibers*, A. Kumar, Ed., ed. Croatia: INTECH, 2010, pp. 139-158.
- [165] M. Spei and R. Holzem, "Thermoanalytical investigations of extended and annealed keratins," *Colloid & Polymer Science*, vol. 265, pp. 965-970, 1987.
- [166] K. Morihara and H. Tsuzuki, "Specificity of proteinase K from *Tritirachium album Limber* for synthetic peptides," *Agricultural and Biological Chemistry*, vol. 39, pp. 1489-1492, 1975.
- [167] W. Appel, "Chymotrypsin: molecular and catalytic properties," *Clinical biochemistry*, vol. 19, pp. 317-322, 1986.
- [168] P. X. Ma and R. Zhang, "Synthetic nano-scale fibrous extracellular matrix," 1999.
- [169] H. N. Kim, A. Jiao, N. S. Hwang, M. S. Kim, D.-H. Kim, and K.-Y. Suh, "Nanotopography-guided tissue engineering and regenerative medicine," *Advanced drug delivery reviews*, vol. 65, pp. 536-558, 2013.
- [170] H. N. Kim, Y. Hong, M. S. Kim, S. M. Kim, and K.-Y. Suh, "Effect of orientation and density of nanotopography in dermal wound healing," *Biomaterials*, vol. 33, pp. 8782-8792, 2012.
- [171] G. E. Piérard and C. M. Lapière, "Microanatomy of the dermis in relation to relaxed skin tension lines and Langer's lines," *The American journal of dermatopathology*, vol. 9, pp. 219-224, 1987.

-
- [172] S. Shang, F. Yang, X. Cheng, X. F. Walboomers, and J. A. Jansen, "The effect of electrospun fibre alignment on the behaviour of rat periodontal ligament cells," *Eur Cell Mater*, vol. 19, pp. 180-192, 2010.
- [173] B. M. Baker and R. L. Mauck, "The effect of nanofiber alignment on the maturation of engineered meniscus constructs," *Biomaterials*, vol. 28, pp. 1967-1977, 2007.
- [174] H. Lu, Z. Feng, Z. Gu, and C. Liu, "Growth of outgrowth endothelial cells on aligned PLLA nanofibrous scaffolds," *Journal of Materials Science: Materials in Medicine*, vol. 20, pp. 1937-1944, 2009.
- [175] S. Patel, K. Kurpinski, R. Quigley, H. Gao, B. S. Hsiao, M. M. Poo, *et al.*, "Bioactive nanofibers: synergistic effects of nanotopography and chemical signaling on cell guidance," *Nano letters*, vol. 7, pp. 2122-2128, 2007.
- [176] J. Ma, X. He, and E. Jabbari, "Osteogenic differentiation of marrow stromal cells on random and aligned electrospun poly (L-lactide) nanofibers," *Annals of biomedical engineering*, vol. 39, pp. 14-25, 2011.
- [177] J. Meng, Z. Han, H. Kong, X. Qi, C. Wang, S. Xie, *et al.*, "Electrospun aligned nanofibrous composite of MWCNT/polyurethane to enhance vascular endothelium cells proliferation and function," *Journal of Biomedical Materials Research Part A*, vol. 95, pp. 312-320, 2010.
- [178] C. H. Lee, H. J. Shin, I. H. Cho, Y. M. Kang, I. Kim, K. D. Park, *et al.*, "Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast," *Biomaterials*, vol. 26, pp. 1261-1270, 2005.
- [179] S. Rahmani, M. Rafizadeh, and F. Afshar Taromi, "Statistical analysis of nanofibers alignment in magnetic-field-assisted electrospinning including an alignment percentage formula," *Journal of Applied Polymer Science*, vol. 131, 2014.
- [180] V. Milleret, B. Simona, P. Neuenschwander, and H. Hall, "Tuning electrospinning parameters for production of 3D-fiber-fleeces with increased porosity for soft tissue engineering applications," *Eur Cell Mater*, vol. 21, pp. 286-303, 2011.
- [181] F. H. Epstein, A. J. Singer, and R. A. F. Clark, "Cutaneous wound healing," *New England Journal of Medicine*, vol. 341, pp. 738-746, 1999.
- [182] I. Aukhil, "Biology of wound healing," *Periodontology 2000*, vol. 22, pp. 44-50, 2000.
- [183] F. H. Epstein, A. J. Singer, and R. A. Clark, "Cutaneous wound healing," *New England journal of medicine*, vol. 341, pp. 738-746, 1999.
- [184] W.-S. Kim, B.-S. Park, J.-H. Sung, J.-M. Yang, S.-B. Park, S.-J. Kwak, *et al.*, "Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts," *Journal of dermatological science*, vol. 48, pp. 15-24, 2007.
- [185] T. Hurme, H. Kalimo, M. Sandberg, M. Lehto, and E. Vuorio, "Localization of type I and III collagen and fibronectin production in injured gastrocnemius muscle," *Laboratory investigation; a journal of technical methods and pathology*, vol. 64, pp. 76-84, 1991.

-
- [186] G. Lawrie, I. Keen, B. Drew, A. Chandler-Temple, L. Rintoul, P. Fredericks, *et al.*, "Interactions between alginate and chitosan biopolymers characterized using FTIR and XPS," *Biomacromolecules*, vol. 8, pp. 2533-2541, 2007.
- [187] Z. X. Xu, T. Li, Z. M. Zhong, D. S. Zha, S. H. Wu, F. Q. Liu, *et al.*, "Amide-linkage formed between ammonia plasma treated poly (D, L-lactide acid) scaffolds and bio-peptides: Enhancement of cell adhesion and osteogenic differentiation in vitro," *Biopolymers*, vol. 95, pp. 682-694, 2011.
- [188] R. Makarem and M. Humphries, "LDV: a novel cell adhesion motif recognized by the integrin alpha 4 beta 1," *Biochemical Society Transactions*, vol. 19, pp. 380S-380S, 1991.
- [189] T. A. Petrie and A. J. García, "Extracellular Matrix-derived Ligand for Selective Integrin Binding to Control Cell Function," in *Biological Interactions on Materials Surfaces*, ed: Springer, 2009, pp. 133-156.
- [190] J. Gailit, M. Pierschbacher, and R. A. Clark, "Expression of Functional α 4 β 1 Integrin by Human Dermal Fibroblasts," *Journal of investigative dermatology*, vol. 100, pp. 323-328, 1993.
- [191] P. Ayala, J. I. Lopez, and T. A. Desai, "Microtopographical cues in 3D attenuate fibrotic phenotype and extracellular matrix deposition: implications for tissue regeneration," *Tissue Engineering Part A*, vol. 16, pp. 2519-2527, 2010.
- [192] E. Lamers, R. v. Horssen, J. t. Riet, F. v. Delft, R. Luttge, X. Walboomers, *et al.*, "The influence of nanoscale topographical cues on initial osteoblast morphology and migration," 2010.
- [193] T. Zako, M. Sakono, N. Hashimoto, M. Ihara, and M. Maeda, "Bovine insulin filaments induced by reducing disulfide bonds show a different morphology, secondary structure, and cell toxicity from intact insulin amyloid fibrils," *Biophysical journal*, vol. 96, pp. 3331-3340, 2009.
- [194] K. S. Midwood, Y. Mao, H. C. Hsia, L. V. Valenick, and J. E. Schwarzbauer, "Modulation of cell-fibronectin matrix interactions during tissue repair," in *Journal of Investigative Dermatology Symposium Proceedings*, 2006, pp. 73-78.
- [195] J. M. Tour, L. Jones, D. L. Pearson, J. J. Lamba, T. P. Burgin, G. M. Whitesides, *et al.*, "Self-assembled monolayers and multilayers of conjugated thiols, α , ω -dithiols, and thioacetyl-containing adsorbates. Understanding attachments between potential molecular wires and gold surfaces," *Journal of the American Chemical Society*, vol. 117, pp. 9529-9534, 1995.
- [196] G. E. Davis, K. J. Bayless, M. J. Davis, and G. A. Meininger, "Regulation of tissue injury responses by the exposure of matricryptic sites within extracellular matrix molecules," *The American journal of pathology*, vol. 156, pp. 1489-1498, 2000.
- [197] E. Ruoslahti, "RGD and other recognition sequences for integrins," *Annual review of cell and developmental biology*, vol. 12, pp. 697-715, 1996.
- [198] S. Soliman, S. Pagliari, A. Rinaldi, G. Forte, R. Fiaccavento, F. Pagliari, *et al.*, "Multiscale three-dimensional scaffolds for soft tissue engineering via multimodal electrospinning," *Acta biomaterialia*, vol. 6, pp. 1227-1237, 2010.

-
- [199] H. Yoshimoto, Y. Shin, H. Terai, and J. Vacanti, "A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering," *Biomaterials*, vol. 24, pp. 2077-2082, 2003.
- [200] M. I. Santos, K. Tuzlakoglu, S. Fuchs, M. E. Gomes, K. Peters, R. E. Unger, *et al.*, "Endothelial cell colonization and angiogenic potential of combined nano-and micro-fibrous scaffolds for bone tissue engineering," *Biomaterials*, vol. 29, pp. 4306-4313, 2008.
- [201] W.-J. Li, R. Tuli, X. Huang, P. Laquerriere, and R. S. Tuan, "Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold," *Biomaterials*, vol. 26, pp. 5158-5166, 2005.
- [202] G. T. Christopherson, H. Song, and H.-Q. Mao, "The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation," *Biomaterials*, vol. 30, pp. 556-564, 2009.
- [203] L. Smith and P. Ma, "Nano-fibrous scaffolds for tissue engineering," *Colloids and surfaces B: biointerfaces*, vol. 39, pp. 125-131, 2004.
- [204] K. M. Woo, V. J. Chen, and P. X. Ma, "Nano-fibrous scaffolding architecture selectively enhances protein adsorption contributing to cell attachment," *Journal of biomedical materials research Part A*, vol. 67, pp. 531-537, 2003.
- [205] A. S. Badami, M. R. Kreke, M. S. Thompson, J. S. Riffle, and A. S. Goldstein, "Effect of fiber diameter on spreading, proliferation, and differentiation of osteoblastic cells on electrospun poly (lactic acid) substrates," *Biomaterials*, vol. 27, pp. 596-606, 2006.
- [206] M. Chen, P. K. Patra, S. B. Warner, and S. Bhowmick, "Role of fiber diameter in adhesion and proliferation of NIH 3T3 fibroblast on electrospun polycaprolactone scaffolds," *Tissue Engineering*, vol. 13, pp. 579-587, 2007.
- [207] A. Balguid, A. Mol, M. H. van Marion, R. A. Bank, C. V. Bouten, and F. P. Baaijens, "Tailoring fiber diameter in electrospun poly (ϵ -caprolactone) scaffolds for optimal cellular infiltration in cardiovascular tissue engineering," *Tissue Engineering Part A*, vol. 15, pp. 437-444, 2008.
- [208] W. W. Sampson, *Modelling Stochastic Fibrous Materials with Mathematica®*: Springer Science & Business Media, 2008.
- [209] S. J. Eichhorn and W. W. Sampson, "Statistical geometry of pores and statistics of porous nanofibrous assemblies," *Journal of the royal society Interface*, vol. 2, pp. 309-318, 2005.
- [210] Q. P. Pham, U. Sharma, and A. G. Mikos, "Electrospun poly (ϵ -caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration," *Biomacromolecules*, vol. 7, pp. 2796-2805, 2006.
- [211] S. Yang, K.-F. Leong, Z. Du, and C.-K. Chua, "The design of scaffolds for use in tissue engineering. Part I. Traditional factors," *Tissue engineering*, vol. 7, pp. 679-689, 2001.
- [212] M. Van Lieshout, C. Vaz, M. Rutten, G. Peters, and F. Baaijens, "Electrospinning versus knitting: two scaffolds for tissue engineering of the aortic valve," *Journal of Biomaterials Science, Polymer Edition*, vol. 17, pp. 77-89, 2006.
- [213] K. Tuzlakoglu, N. Bolgen, A. Salgado, M. E. Gomes, E. Piskin, and R. Reis, "Nano-and micro-fiber combined scaffolds: a new architecture for bone tissue

- engineering," *Journal of Materials Science: Materials in Medicine*, vol. 16, pp. 1099-1104, 2005.
- [214] K. Sivamani, M. S. Garcia, and R. R. Isseroff, "Wound re-epithelialization: modulating keratinocyte migration in wound healing," *Frontiers in bioscience: a journal and virtual library*, vol. 12, pp. 2849-2868, 2006.
- [215] 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," *The Journal of cell biology*, vol. 132, pp. 381-397, 1996.
- [216] R. O. Hynes and K. M. Yamada, "Fibronectins: multifunctional modular glycoproteins," *The Journal of cell biology*, vol. 95, pp. 369-377, 1982.
- [217] R. Clark, *The molecular and cellular biology of wound repair*: Springer Science & Business Media, 2013.
- [218] W. S. To and K. S. Midwood, "Plasma and cellular fibronectin: distinct and independent functions during tissue repair," *Fibrogenesis Tissue Repair*, vol. 4, p. 21, 2011.
- [219] B. Larivière, M. Rouleau, S. Picard, and A. D. Beaulieu, "Human plasma fibronectin potentiates the mitogenic activity of platelet-derived growth factor and complements its wound healing effects," *Wound repair and regeneration*, vol. 11, pp. 79-89, 2003.
- [220] R. M. Kaake, X. Wang, and L. Huang, "Profiling of protein interaction networks of protein complexes using affinity purification and quantitative mass spectrometry," *Molecular & Cellular Proteomics*, vol. 9, pp. 1650-1665, 2010.

Publications

Sow WT, Lui YS, Ng KW. Electrospun Human Keratin Matrices as Templates for Tissue Regeneration. *Nanomedicine* 8(4):531-41. 2013

Bhardwaj N, Sow WT, Devi D, Ng KW, Mandal BB, Cho NJ. Potential of silk fibroin-keratin based 3D scaffolds as dermal substitute for skin repair and regeneration. *Integrative Biology (Camb)* 7(1):53-63. 2014

Zhao X, Lui YS, Choo CKC, Sow WT, Huang CL, Ng KW, Tan LP, Loo JSC. Calcium phosphate coated Keratin-PCL scaffolds for potential bone tissue regeneration. *Materials Science and Engineering C: Materials for Biological Applications* 49:746-53. 2015

Conferences

Sow WT, Ng KW, *Electrospun Keratin Fibrous Matrices*
Tissue Engineering & Regenerative Medicine International Society, Asia Pacific Chapter Annual Meeting. Singapore. 3-5 August 2011.

Sow WT, Ng KW, *Characterization of Electrospun Fibrous Scaffolds Derived from Keratin*
Materials Research Society, International Conference of Young Researchers Advanced Materials. Singapore. 1-6 July 2012

Sow WT, Ng KW, *Behavior of Fibroblasts on Human Hair Keratin Fibrous Matrices*
Tissue Engineering & Regenerative Medicine International Society, Asia Pacific Chapter Annual Meeting. Shanghai, China. 23-26 October 2013

Sow WT, Ng KW, *Electrospun Human Hair Keratin Matrices Affect Human Fibroblast Behaviour Through Topographical Cues*
European Society for Biomaterials, 26th Annual Conference. Liverpool, United Kingdom. 1-3 September 2014

Sow WT, Tan LP, Ng KW, *An in-vitro Examination of Human Hair Keratin*
European Materials Research Society, 2015 Spring Meeting. Lille, France. 11-15 May
2015