The development of crosslinked keratin-alginate sponges as novel biomaterials

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**THE DEVELOPMENT OF CROSSLINKED KERATIN-
ALGINATE SPONGES AS NOVEL BIOMATERIALS**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2016

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**SCHOOL OF MATERIALS SCIENCE AND
ENGINEERING**

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degree of Doctor of Philosophy

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Abstract

As one of the prospective alternative biomaterials that has recently been explored, keratin offered several advantages over other natural protein-based biomaterials such as its natural abundance, biocompatibility, presence of cell binding motifs that could improve cellular attachment and possibility to obtain autologous material that could minimize immunoresponse. Nonetheless, their further application in the biomedical field has been limited by their fragile and brittle characteristics as well as their poor mechanical properties.

In this study, keratin extracted from human hair was crosslinked with alginate, a relatively bioinert material, using 1-Ethyl-3-dimethylaminopropyl Carbodiimide (EDC) as a crosslinking agent in order to improve its mechanical properties. Variations of crosslinking agent concentration and keratin-alginate ratio were studied in order to determine the effects of these differences on the physical and mechanical properties, as well as cell compliance.

Successful crosslinking was confirmed with free amine groups determination as well as analysis of IR spectra, where increasing crosslinking degree (up to 83%) was achieved with higher crosslinking agent concentration. Higher crosslinking degree and higher alginate content were confirmed to increase the strength and modulus of the resulting material (from tensile, compression and flexural tests). Additionally, increasing the alginate content would increase the water uptake capacity by up to 6 times its original weight while increasing the crosslinking degree would initially increase the water uptake capacity to a certain point before reducing it. The crosslinked sponges were also shown to exhibit lower water vapor transmission rate, a characteristic that is desirable for wound dressing applications, compared to commercially available wound dressing Kaltostat®. Moreover, sponges with the highest keratin content were also revealed to be degraded by proteinase K by up to 75% of their original weight.

Our results also revealed that matrices with higher keratin content enhanced the proliferation of both L929 murine fibroblasts and human dermal fibroblasts

(HDF) in both 2D and 3D environment compared to matrices with higher alginate content. The resulting matrices with higher keratin content were also shown to support cell viability as well as extracellular matrix proteins, cytokines, and growth factor production with even distribution of cells inside the matrices.

Matrices with higher keratin content were also revealed to upregulate production of tissue factor by HDF, suggesting it would be beneficial for hemostasis application. Based on these findings, crosslinked keratin-alginate matrices were shown to be a promising tunable materials for cell carriers or wound dressing purposes.

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Table of Contents

Abstract	i
Acknowledgements	iii
Table of Contents	vii
Table Captions.....	xiii
Figure Captions	xv
Abbreviations.....	xxi
Chapter 1 Introduction.....	1
1.1 Background	2
1.1.1 Keratin as a Prospective Biomaterials	2
1.1.2 Keratin-containing Hybrid Materials for Biomedical Applications	3
1.1.3 Alginate as a Crosslinking Partner for Keratin	4
1.2 Hypotheses	5
1.2.1 Hypothesis 1.....	5
1.2.2 Hypothesis 2.....	5
1.3 Objectives and Scopes	5
1.3.1 Objectives	5
1.3.2 Scopes.....	5
1.3.2.1 Scope 1.....	5
1.3.2.2 Scope 2.....	6
1.4 Dissertation Overview.....	6
1.5 Findings and Originality.....	7

References	8
Chapter 2 Literature Review	11
2.1 Human Hair Keratin and Its Potential as Prospective Biomaterials	12
2.1.1 Properties of Human Hair Keratin	12
2.1.2 The Use of Keratin for Biomedical Application	13
2.1.2.1 Keratin Films	13
2.1.2.2 Keratin Sponges	15
2.1.2.3 Keratin Fibers	16
2.1.2.4 Keratin Hydrogels	18
2.2 Alginate as Biomedical Materials	20
2.2.1 Properties of Alginate	20
2.2.2 The Use of Alginate for Biomedical Application	21
2.3 Desired Properties of Materials for Wound Healing Purposes	22
2.4 Keratin-containing Hybrid Materials	23
2.5 Crosslinking of Keratin and Alginate	24
References	25
Chapter 3 Experimental Methodology	31
3.1 Rationale for Selection	32
3.2 Materials	33
3.3 Methods	33
3.3.1 Extraction of Keratin from Human Hair	33
3.3.1.1 Delipidization of Human Hair	33
3.3.1.2 Keratin Extraction	34
3.3.1.3 Preparation of Lyophilized Keratin Powder	34
3.3.2 Characterization of extracted keratin	34

3.3.2.1 Fourier Transform Infra-Red Spectroscopy	34
3.3.1.2 Coomassie Blue Staining	38
3.3.3 Preparation of Crosslinked Keratin-Alginate Mixture	39
3.3.4 Fabrication of Keratin Alginate Sponges	40
3.3.5 Characterization of Crosslinked Keratin Alginate Sponges	40
3.3.5.1 Scanning Electron Microscopy	40
3.3.5.2 Determination of Crosslinking Degree Based on the Calculation of the Remaining Free Amine Groups	42
3.3.5.3 Measurement of Infrared (IR) Spectra of Crosslinked Sponges	42
3.3.5.4 Determination of Secondary Structure by Curve-Fitting of Amide I Band Profile	42
3.3.5.5 Compression Test	43
3.3.5.6 Tensile Test	43
3.3.5.7 Three-point Bend Test	44
3.3.5.8 Water Uptake Study	44
3.3.5.9 Water Vapor Transmission Study	45
3.3.5.10 Degradation	45
3.3.6 Cell Culture	45
3.3.6.1 Culture of L929 on Crosslinked Keratin-Alginate Coated-surfaces	45
3.3.6.2 Culture of L929 in Crosslinked Keratin-Alginate Sponges	46
3.3.6.3 Culture of Primary Human Dermal Fibroblasts on Crosslinked Keratin-Alginate-Coated-Surfaces	47
3.3.6.4 Culture of Primary Human Dermal Fibroblasts in Crosslinked Keratin-Alginate sponges	48
3.3.6.5 Histology of Cross-sectional Sponges	48
3.3.6.6 Evaluation of ECM Production by Immunohistochemistry	49
3.3.6.7 Evaluation of Growth Factor Expression	50

3.3.7 Statistical Analysis	50
References	52

Chapter 4 Fabrication and Characterization of Crosslinked Keratin-Alginate Sponges

4.1 Extraction of Human Hair Keratin.....	56
4.2 Fabrication of Crosslinked Keratin Alginate Sponges	58
4.3 Compression Study	62
4.4 Tensile Study	64
4.5 Three-Point Bend Study	67
4.6 Water Uptake Capacity	69
4.7 Water Vapor Transmission Study	71
4.8 Degradation	73
References	78

Chapter 5 *In Vitro* Cell Culture Studies of Crosslinked Keratin-Alginate Sponges

5.1 Culture of L929 and HDF on 2D-coated Surfaces and 3D Sponges	82
5.2 Matrices Contraction	93
5.3 Histology	95
5.4 Evaluation of Extracellular Matrices' Production by Immunohistochemistry	97
5.5 Growth Factor Expression	101
5.5.1 Tissue Factor	101
5.5.2 CD26	106
5.5.3 Pentraxin 3	106
5.5.4 Interleukin-8	107
5.5.5 MMP-9	107
5.5.6 MCP-1/CCL2	108

5.5.7	Angiopoietin-1, Angiopoietin-2	109
5.5.8	Urokinase Plasminogen Activator (uPA)	109
5.5.9	PAI-1	110
5.5.10	Tissue inhibitor of metalloproteinases (TIMP)	110
5.5.11	Insulin-like growth factor binding proteins (IGFBP)	110
5.5.12	TSP-1	111
5.5.13	Endostatin	112
5.5.14	Endothelin-1	112
5.5.15	Summary of Growth Factor Expression Evaluation	113
References		114

Chapter 6 General Discussions, Conclusions and Future Recommendations

.....		123
6.1	General Discussions	124
6.2	Conclusions	127
6.3	Future Recommendations.....	130
6.3.1	Culture of Keratinocytes	130
6.3.2	Understanding Cell-Material Interaction through Mechanism of Cell Attachment	130
6.3.3	Incorporation of Active Agents for Various Biomedical Applications	131
6.3.4	Material Characterization during Hydrated State	131
6.3.5	Degradation Study	131
6.3.6	<i>In vivo</i> Study	132
References		133

List of Publications, Patents and Conferences.....	134
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Table Captions

Table 3.1 Fabrication of crosslinked keratin-alginate by varying EDC concentrations and varying keratin-alginate composition.

Table 3.2 Regions of each components of proteins' secondary structures (amide I band)

Table 5.1 List of coordinates and analytes on the multiarray membranes.

List of Figures

Figure 2.1 Keratin films.

Figure 2.2 Keratin sponges

Figure 2.3 Scanning Electron Microscope images of Keratin/PEO (9:1)

Figure 2.4 Keratin hydrogel

Figure 2.5 EDC-mediated crosslinking reaction between carboxylic acid and amine groups

Figure 4.1 Freeze dried keratin powder

Figure 4.2 Coomassie blue-stained SDS PAGE gel of keratin

Figure 4.3 IR Spectra of reconstituted freeze-dried keratin powder and, freshly extracted soluble keratin solution and alginate

Figure 4.4 Crosslinked keratin alginate sponges

Figure 4.5 SEM image of crosslinked keratin alginate sponges with different EDC concentration

Figure 4.6 Average pore area (μm^2) of crosslinked keratin alginate sponges with different EDC concentrations (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

Figure 4.7 Crosslinking degree of crosslinked keratin-alginate sponges with varying EDC concentration ($p<0.05$, ANOVA, Tukey's test vs 1 mM, n=3)

Figure 4.8 IR spectra of crosslinked keratin-alginate sponges with varying EDC concentration

Figure 4.9 Reconstructed image of curve-fitting analysis on amide I band profile obtained from FTIR spectroscopy for KA11-100mM

Figure 4.10 Percentage of amide I band secondary structure composition based on relative AUC

Figure 4.11 Compression modulus of crosslinked keratin-alginate sponges with different EDC concentration (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

Figure 4.12 Compression modulus of crosslinked keratin-alginate sponges with different keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-1mM)

Figure 4.13 Tensile modulus of crosslinked keratin-alginate sponges with varying EDC concentration (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

Figure 4.14 Ultimate tensile strength of crosslinked keratin-alginate sponges with varying EDC concentration (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

Figure 4.15 Tensile modulus of crosslinked keratin-alginate sponges with varying keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-1mM)

Figure 4.16 Ultimate tensile strength of crosslinked keratin-alginate sponges with varying keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-1mM)

Figure 4.17 Flexular modulus of crosslinked keratin-alginate sponges with

varying EDC concentration (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

Figure 4.18 Flexular modulus of crosslinked keratin-alginate sponges with varying keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-1mM)

Figure 4.19 Water uptake capacity of keratin-alginate sponges with varying EDC concentration (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

Figure 4.20 Water uptake capacity of keratin alginate sponges with varying keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-1mM)

Figure 4.21 Water vapor transmission rate of crosslinked keratin alginate sponges with varying EDC concentration (n=3, $p<0.05$, ANOVA, Tukey's test vs Kaltostat®)

Figure 4.22 Water vapor transmission of crosslinked keratin alginate sponges with varying keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs Kaltostat®)

Figure 4.23 Remaining weight percentages of crosslinked keratin-alginate sponges with varying EDC concentration vs time of treatment with α -chymotrypsin, proteinase K and tris buffer (control), (mean $\pm$ SD, n=3)

Figure 4.24 Remaining Weight percentages of Keratin Sponges, Alginate Sponges, Crosslinked keratin-alginate Sponges with Varying Keratin-Alginate Composition vs Time of treatment with α -Chymotrypsin, Proteinase K and Tris buffer (control), (mean $\pm$ SD, n=3)

Figure 5.1 Relative amount of dsDNA of L929 murine fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture with varying crosslinking degree (mean $\pm$ SD, n=4),

* $p < 0.05$ vs day 7 keratin, ANOVA, Tukey's test). Data normalized against control day 1.

Figure 5.2 Relative amount of dsDNA of L929 murine fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture with varying keratin and alginate ratio (mean $\pm$ SD, $n=4$), * $p < 0.05$ vs day 7 keratin, ANOVA, Tukey's test). Data normalized against control day 1

Figure 5.3 Live-Dead staining images of L929 murine fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture (day 7 post-seeding)

Figure 5.4 Relative amount of dsDNA of L929 murine fibroblasts cultured on collagen, keratin, alginate and crosslinked keratin alginate sponges. data were normalized against alginate on day 1. (mean $\pm$ SD, $n=4$), *, #, + $p < 0.05$ vs keratin, ANOVA, Tukey's test) where * day 7, # day 14 and + day 21

Figure 5.5 Live-Dead staining images of L929 murine fibroblasts cultured on collagen, keratin, alginate and crosslinked keratin alginate sponges (day 7 and 14 post-seeding). Images taken using confocal microscope

Figure 5.6 Relative amount of dsDNA of human dermal fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture with varying crosslinking degree (mean $\pm$ SD, $n=4$), * $p < 0.05$ vs day 7 keratin, ANOVA, Tukey's test). Data normalized against control day 1

Figure 5.7 Relative amount of dsDNA of human dermal fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture with varying keratin and alginate ratio (mean $\pm$ SD, $n=4$), * $p < 0.05$ vs day 7 keratin, ANOVA, Tukey's test). Data normalized against control day 1

Figure 5.8 Live-Dead staining images of human dermal fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture (day 7)

Figure 5.9 Relative amount of dsDNA of human dermal fibroblasts cultured on collagen, keratin, alginate and crosslinked keratin alginate sponges with varying keratin and alginate ratio. Data were normalized against alginate on day 1. (mean $\pm$ SD, n=4), *, #, + $p < 0.05$ vs keratin, ANOVA, Tukey's test) where * day 7, # day 14 and + day 21

Figure 5.10 Live-Dead staining images of human dermal fibroblasts cultured on collagen, keratin, alginate and crosslinked keratin alginate sponges. Images were taken using confocal microscope

Figure 5.11 Percentage of matrices' diameter after 14 days of culture from initial diameter. (n=3, $p < 0.05$, ANOVA, Tukey's test vs collagen)

Figure 5.12 Matrices' contraction of collagen, keratin, alginate and crosslinked keratin-alginate sponges after 14 days of culture. Upper arrows represent sponges' diameter on day 0 of culture and lower arrows represent sponges' diameter on day 14 of culture.

Figure 5.13 Hematoxylin and eosin-stained crosssection of collagen, keratin, alginate and crosslinked keratin-Alginate sponges after 14 days of culture.

Figure 5.14 Immunohistochemical staining of collagen III on collagen, keratin, alginate and crosslinked keratin-alginate sponges crosssection on day 14 post seeding. Cell nuclei were stained with DAPI (blue)

Figure 5.15 Immunohistochemical staining of fibronectin on collagen, keratin, alginate and crosslinked keratin-alginate sponges crosssection on day 14 post seeding. Cell nuclei were stained with DAPI (blue)

Figure 5.16 Growth factor and cytokines expressions from HDFs after 14

day of culture on various 3D sponges. Detected by multiarray membranes. Images taken using the image analyzer. Names and coordinates of analytes are listed on Table 5.1

Figure 5.17 Wound healing scheme.

Figure 5.18 Relative expression of growth factor, cytokines and enzymes produced by HDFs after 14 days of culture on collagen, keratin, alginate and crosslinked keratin alginate matrices.

Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
ANG	Angiopoeitin
BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle Medium
EDC	1-Ethyl-3-dimethylaminopropyl Carbodiimide
EDGE	Ethylene glycol diglycil ether
ET	Endothelin
FGF	Fibroblasts Growth Factor
FTIR	Fourier Transform Infrared Spectroscopy
H&E	Hematoxylin and Eosin
HDF	Human Dermal Fibroblasts
IF	Intermediate Filaments
IGFBP	Insulin-like growth factor binding proteins
IL	Interleukin
KIF	Keratin Intermediate Filaments
MCP-1	Monocyte chemoattractant protein
MMP	Matrix Metalloproteinase
MOPS	3-(N-morpholino) propane sulfonic acid
MW	Molecular Weight
MWCO	Molecular Weight Cut-off
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PCL	Poly Caprolactone
PEO	Polyethylene Oxide
PLGA	poly-lactic-co-glycolic acid
PLLA	Poly-lactic acid
PTX3	Pentraxin 3
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy

SMA	Smooth Muscle Actin
TCPS	Tissue Culture Polystyrene Surface
TEWL	Trans-Epidermal Water Loss
TF	Tissue Factor
TIMP	Tissue Inhibitor of Metalloproteinases
TSP	Thrombospondin
uPA	Urokinase Plasminogen Activator
WVTR	Water Vapor Transmission Rate

Chapter 1

Introduction

The limited availability of autologous, biocompatible and naturally-derived protein-based biomaterials led to the search of alternative materials for biomedical purposes. The potential of keratin, a biocompatible material that can support cell attachment and proliferation, as a candidate for these purposes has been hindered by its poor mechanical properties. Crosslinking keratin with alginate was anticipated to provide a solution to these issues by improving its mechanical properties while maintaining its cell-compliance. This chapter explains the background, hypotheses, objectives and scopes of fabricating crosslinked keratin-alginate sponges as novel biomaterials.

1.1. Background

1.1.1. Keratin as a Prospective Biomaterial

The exploration of naturally-derived protein-based biomaterials has recently gained increased interest. Proteins such as collagen, albumin, gelatin, fibrin and keratin have been extensively studied for various biomedical applications [1-6]. Nonetheless, these proteins are mostly obtained from animal origin or synthetically produced, thus presenting an issue of biocompatibility. Fibrin, which is one of the most reliable sources of human-derived protein for biomedical purposes [7], also possesses some limitations due to the limited availability of human blood for fibrin extraction [8]. Among all of these proteins, keratin-based biomaterials have recently showed their potential for biomedical applications due to their biocompatibility, biodegradability, natural abundance, ability to self-assemble and ability to promote cell attachment [1, 9-11].

Keratin is known to be present in various sources such as feathers, nails, hair and skin from many different species such as human, chicken, sheep and others. Among different sources available, human-hair-derived keratin is shown to be the most promising candidate for clinical application since it is highly abundant and enables the possibility to obtain autologous materials [1, 12]. By obtaining autologous materials, it is expected that the chances of receiving excessive immunological reaction that may occur from the usage of allogenic human materials or those of animal origins could be minimized.

Regardless of its advantages, recent studies have shown that extracted human-hair keratin possessed unfavorable mechanical properties. These studies demonstrated that human-hair keratin exhibited hard, fragile and brittle properties, thus limiting its usage in biomedical applications [1, 13]. Therefore, in order to improve its mechanical properties, modifying hair keratins with another material was proposed in this study.

1.1.2. Keratin-containing Hybrid Materials for Biomedical Applications

Previous investigations have studied mixtures of keratin with either natural or synthetic polymers and they were shown to display improvement in their mechanical properties,

specifically in terms of strength and flexibility. A study on physical blends of wool keratin with chitosan [14], wool keratin with hydroxyapatite, [15] wool keratin with polyethylene oxide [16, 17] and horn keratin with collagen [18] showed improvement in final matrices' properties such as increased strength and modulus. However, these studies also reported that even though they were able to improve the mechanical properties of the keratin mixture in the dried state, they failed to maintain the mixture's structure in the hydrated state. Keratin-polymer mixtures tend to collapse and partially dissolve in the presence of water. This property was disadvantageous for biomedical application since it is expected that the resulting matrix will come in contact with a relatively significant amount of body fluid [14-19]. Since all of the previous studies mentioned above solely work on physical blends of keratin with a partnering material, chemically crosslinking of keratin with a partnering material might resolve this issue.

Another study using diepoxy crosslinkers as crosslinking agents for wool keratin managed to show that the resulting films were able to have improved mechanical properties, in which the films produced from the crosslinked wool keratin became flexible and tenacious enough to handle. Unfortunately, the resulting crosslinked keratin films displayed waterproof characteristics and inability to uptake water, thus making it disadvantageous if it were to be utilized for biomedical applications [20].

Several approaches can be pursued in order to chemically crosslink keratin with partnering polymers. Ionic and covalent crosslinking are one of the possible options in forming crosslinking between the two materials. In this study, covalent crosslinking was preferred since it would result in a stronger and more stable chemical bond, thus making the resulting product less susceptible to loss of mechanical strength due to the loss of ionic interaction [21].

1.1.3. Alginate as a Crosslinking Partner for Keratin

Alginate was chosen as the partnering material since it is known to be abundant, has the ability to form hydrogels, low-cost and easy to handle. Alginate also has been commercially used for numerous biomedical purposes and proven to be biocompatible. Although it is a well-established material for biomedical purposes such as wound

dressings, it is lacking cell-binding motifs thus making alginate biologically inactive [22]. Therefore, by combining keratin with alginate and performing crosslinking on the materials, it is anticipated that there will be improvement in mechanical properties and biological activities of the resulting material.

Alginate possesses free carboxylic acid groups which could be modified or activated so that they could chemically interact with the amino acid's side chain of keratin. One of the possible chemical linkages that is able to be formed is amide linkages between carboxylic acid groups of alginate and amine groups from keratin's N-terminal and amino acids' side chain. Amide bond linkages were aimed and preferred in this study since it is expected to be biodegradable by enzymatic degradation and it is also less prone to hydrolysis compared to ester bond. Beside amide bond formation between keratin and alginate, crosslinking may happen between the different hair keratin subtypes and associated proteins by the formation of amide bonds between the amine and carboxylic groups present in the keratin mixture [21]. This research therefore targeted to ascertain if effective crosslinking between hair keratins and alginate, through amide bond formation, could take place using established crosslinking chemistry.

By carrying out different variations of crosslinking conditions, it is expected that different properties of the final crosslinked materials will be acquired, since previous works have shown that performing crosslinking would increase the stiffness and moduli of the final materials [20, 23]. In this context, this study was carried out to correlate the degree of crosslinking as well as the variation of keratin and alginate content in the resulting mixture with the changes of mechanical properties. In addition, the extent of crosslinking of the keratin-alginate matrices alongside the varying keratin and alginate composition were also further associated with their ability to support fibroblasts' viability and proliferation on matrices over a time course of culture. By having a better understanding regarding these matters, achieving tunable properties of the material will be made possible, in relation to their potential application as matrices for tissue regeneration and wound healing.

1.2. Hypotheses

1.2.1. Hypothesis 1

The formation of amide linkages can be successfully generated between hair keratin and alginate in a heterogeneous keratin-alginate mixture with the facilitation of carbodiimide in order to produce a microporous sponge with tunable physical and mechanical properties depending on the variation of crosslinking degree and keratin-alginate composition.

1.2.2. Hypothesis 2

Crosslinked keratin-alginate sponges with higher keratin content can enhance fibroblast proliferation and viability compared to sponges with higher alginate content.

1.3. Objectives and Scopes

1.3.1. Objectives

The objectives of this research are:

- to produce novel microporous keratin-alginate sponges with tunable physical and mechanical properties as well as,
- to understand the properties of these sponges as potential mammalian cell carriers or wound dressings.

In line with these objectives, the corresponding scope of work was identified.

1.3.2. Scope

1.3.2.1. Scope 1

The first scope of work included:

- extracting keratin from human hair,
- characterizing the efficacy and specificity of crosslinking by measuring the functionalization of carboxylic acids, quantifying the amount of free amine groups in the mixture, as well as evaluating and analyzing the infrared (IR) spectra,
- evaluating changes in the tensile, compressive and flexural properties of crosslinked sponges and correlate them with varying concentrations of keratin/alginate and varying types and concentrations of crosslinking agents,
- evaluating changes in water uptake capacity and water vapor transmission rate of the crosslinked sponges and correlate the changes with varying concentrations of keratin/alginate and varying types and concentrations of crosslinking agents,
- evaluating the degradation profile and correlate them with varying concentrations of keratin/alginate and varying types and concentrations of crosslinking agents.

1.3.2.2. Scope 2

The second scope of work included carrying out cell culture studies using L929 mouse fibroblasts and primary human dermal fibroblasts (HDFs) on 2D keratin-alginate substrates and 3D keratin-alginate sponges as well as performing the following characterization, such as:

- evaluating cell viability and cell proliferation over a 7-21 day culture period,
- evaluating cell distribution over a 14 day period of culture,
- measuring extracellular matrix production and growth factor expression over a 14 day period of culture,
- measuring contraction of 3D keratin-alginate sponges over 14 day period of culture.

1.4. Dissertation Overview

This thesis addresses the fabrication and characterization of crosslinked keratin-alginate sponges as well as their capability in supporting fibroblasts proliferation and viability in relation to their intended application as a novel alternative material for cell

carriers or wound dressing

Chapter 1 explains the limited availability of autologous, biocompatible and naturally-derived protein-based biomaterials which led to the search of alternative materials for biomedical purposes. The potential of keratin, a biocompatible material that can support cell attachment and proliferation, as a candidate for these purposes has been hindered by its poor mechanical properties. Crosslinking keratin with alginate was anticipated to provide a solution to these issues by improving its mechanical properties while maintaining its cell-compliance. This chapter explains the background, hypotheses, objectives and scopes of fabricating crosslinked keratin-alginate sponges as novel biomaterials.

Chapter 2 elaborates that despite keratin's advantages over other naturally-derived materials, keratin's potential as an alternative biomaterial faced challenges for further clinical use due to its brittle, fragile and poor mechanical characteristics. Therefore, this study explains the possibility of overcoming the limitation addressed on previous studies by performing crosslinking between human-hair keratin and alginate through formation of amide bond by carbodiimide-mediated reaction. This chapter elaborates the properties of human-hair keratin and alginate as well as their potential and limitation as materials for biomedical applications based on past reviews and studies. This chapter also explains the chemistry of crosslinking between keratin and alginate with the facilitation of carbodiimide.

Chapter 3 explains in detail the methodology used in the extraction of keratin by using sodium sulfide as reducing agent as well as the characterization of the extracted keratin protein bands by gel electrophoresis and keratin's chemical composition and structure by Fourier-Transform Infrared (FTIR) spectroscopy. The method of fabrication of crosslinked keratin-alginate sponges with the facilitation of carbodiimide is further explained alongside the characterization of the crosslinking degree (by means of ninhydrine reaction), mechanical properties (by means of compression, tensile and flexural tests), physical properties (by means of evaluation of morphology, water uptake capacity and water vapour transmission test) and degradation profile (with proteinase K and chymotrypsin) of the crosslinked sponges. Methods of cell culture on 2D surfaces and 3D sponges using L929 murine fibroblasts and human dermal fibroblasts are

elaborated in details. In addition, the methodology of the evaluation of cell viability (Live/Dead[®] assay), cell proliferation (dsDNA measurement by picogreen[®]), cell distribution (Hematoxylin and Eosin staining of histology section), extracellular matrices production (by immunohistochemistry) and growth factor production (by using multiarray membrane) were further explained.

Chapter 4 discusses the result of the fabrication and characterization of crosslinked keratin-alginate sponges. Keratin was successfully extracted from human hair and the extracted keratin was successfully crosslinked with alginate as confirmed with free amine groups determination as well as analysis of IR spectra. Porous and flexible crosslinked sponges was obtained through freeze drying and sponges with higher crosslinking degree and higher alginate content were confirmed to increase the strength and modulus of the resulting material (from tensile, compression and flexural tests). Moreover, higher alginate content proven to increase the water uptake capacity by up to 6 times its original weight while higher crosslinking degree was shown to increase the water uptake capacity to a certain point before reducing it. The crosslinked sponges were also shown to exhibit lower water vapor transmission rate, a characteristic that is desirable for wound dressing applications, compared to commercially available wound dressing Kaltostat[®]. Additionally, sponges with the highest keratin content were shown to be degraded by proteinase K by up to 75% of their original weight. Therefore, these results confirmed the potential of producing keratin-hybrid materials with tunable physical and mechanical properties for biomedical purposes (such as cell carriers and wound dressing) by means of varying crosslinking extent or the composition of keratin and partnering material (alginate).

Chapter 5 discusses about the result of L929 and HDF culture on 2D and 3D environment as well as evaluation of ECM, growth factor and cytokines production of HDF cultured on crosslinked keratin-alginate matrices. The result of this study revealed that matrices with higher keratin content enhanced the proliferation of both L929 murine fibroblasts and human dermal fibroblasts in both 2D and 3D environment compared to matrices with higher alginate content. Matrices with higher keratin composition were also shown to support cell viability as well as extracellular matrices proteins, cytokines, and growth factor production with even distribution of cells inside the matrices. Interestingly, higher keratin content were also revealed to upregulate

production of tissue factor by HDF, suggesting it would be beneficial for hemostasis application. Based on these results, crosslinked keratin-alginate matrices were shown to be a promising alternative for cell carriers or wound dressing purposes.

Chapter 6 summarizes the conclusion obtained from this study as well as elaborate the proposed future works to be carried out following this study. It is revealed from this study that the fabrication of crosslinked sponges made of human-hair keratin and alginate with tunable physical and mechanical properties by carrying out chemical crosslinking with the facilitation of carbodiimide derivative (EDC) or varying the keratin-alginate composition was made possible. Additionally, this study also displayed that crosslinked keratin-alginate sponges with dominant keratin content were able to improve fibroblast (both L929 and HDF) proliferation and viability compared to sponges with higher alginate content. These results confirmed that crosslinking human hair keratin with alginate offers the alternative of fabricating hybrid biomaterials with tunable physical and mechanical properties which could be utilized as 3D cell carriers or wound dressing.

1.5. Findings and Originality

This research revealed several original findings as follows:

1. It is possible to fabricate crosslinked sponges made of human-hair keratin and alginate with tunable physical and mechanical properties by carrying out chemical crosslinking with the facilitation of carbodiimide derivative (EDC),
2. Crosslinked keratin-alginate sponges with dominant keratin content improve fibroblast (both L929 and HDF) proliferation and viability compared to pure alginate sponges.

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Chapter 2

Literature Review

Despite keratin's advantages over other naturally-derived biomaterials, keratin's potential as an alternative biomaterial faced challenges for further clinical use due to its brittle, fragile and poor mechanical characteristics. Therefore, this study explains the possibility of overcoming the limitation addressed on previous studies by performing crosslinking between human-hair keratin and alginate through formation of amide bond by carbodiimide-mediated reaction. This chapter elaborates the properties of human-hair keratin and alginate as well as their potential and limitation as materials for biomedical applications based on past reviews and studies. This chapter also explains the chemistry of crosslinking between keratin and alginate with the facilitation of carbodiimide.

2.1. Human Hair Keratin and Its Potential as Prospective Biomaterials

2.1.1. Properties of Human Hair Keratin

Keratins are a part of the fibrous proteins' family which are associated as the main structural protein in epithelial cells that construct cytoplasmic network consisting of intermediate filaments (IFs) [1, 2]. These proteins can be divided either as “hard” and “soft” keratin or type I (acidic) and type II (neutral-basic) keratin. Soft keratins usually assemble into cytoplasmic IFs bundles that are loosely-packed as well as give mechanical flexibility to epithelial cells. On the other hand, hard keratins usually are assembled into ordered arrays of IFs deposited in a matrix of proteins with high content of cysteine that crosslink with each other, thus resulting in a tough structure. Another classification are type I keratin, which consists of K9-K23 and the hair keratins Ha1-Ha8, and type II keratin which consists of K1-K8 and the hair keratins Hb1-Hb6. Other than the previously mentioned classification, keratin could also be divided as alpha keratin and beta keratin according to their secondary structure [1-6].

Human hair keratins are keratins extracted from human hair fibers. Hair fibers are defined as structures that consisted of hard keratins which are already keratinized and highly crosslinked. The fibers are composed of and divided into three main parts, namely the cuticles, cortexes, and medullas. The cuticles are the outer surface of the hair fiber. They are thin, consisting of layers that are scaly and tubular, and composed of flattened cells that overlap each other. The hair cuticles mostly contains beta-keratins while the main part of the hair fiber, known as the cortexes, mainly consists of keratin-filament-containing spindle-shaped cells. Moreover, the medullas, which consist of loosely-connected keratinized cells, would be located at the center of the fiber and occasionally occur [7].

Two major groups of proteins are present in the cortexes of hair fibers. These proteins are called the low-sulfur, “alpha” keratins (with a MW ranging from 40–60 kDa) and the high-sulfur, matrix proteins (with a MW ranging from 10–25 kDa). The hair fibers mainly comprises of alpha keratins (around 50-60%) and matrix proteins (around 20-30%) [7]. The alpha keratins would assemble into microfibrillar structures named as the keratin intermediate filaments (KIFs) which are responsible for providing toughness to

the hair fiber. Meanwhile, the matrix proteins are mainly responsible as a disulfide crosslinker which hold the structure of the hair together. They are also known as the keratin associated proteins or KAPs. Recently, there are over 17 human hair keratin genes (11 type I; 6 type II) as well as 85 KAP genes that has been discovered and were suspected to potentially comprised the structure of the human hair [3].

Human hair keratins were reported to possess cell binding motifs within their sequence. The cell binding residues present in human hair keratin sequence are known to be LDV (leucine-aspartic acid-valine) and EDS (glutamic acid-aspartic acid-serine). These cell binding motifs will likely support cell attachment which is favorable for tissue engineering application [8-10].

2.1.2. The Use of Keratin for Biomedical Application

Recently, the study of keratin utilization as materials used for biomedical purposes has received increased interest. Several reasons for its exploitation as biomaterials candidate are its ability to self-assemble which enables the formation of a three-dimensional structure matrix and its capability to support cell attachment by the existence of cell-binding motifs (LDV and EDS) [8, 9]. LDV is known to bind to $\alpha_4\beta_1$ integrin, which is known to be expressed in cells such as fibroblasts and endothelial cells [10, 11].

Some examples of commercially available keratin-based wound care include KeragelTM, KeramatrixTM and KerasorbTM from Keraplast Technologies, Inc. These products use ReplicineTM, a technology based on wool keratin as the pharmacologically active substance. Previous study reported that ReplicineTM acted as the active ingredients which gave physiological effect to the process of wound healing by improving epithelization rate. It was hypothesized that the improved epithelization rate was the result of increased proliferation and migration of keratinocytes cells as well as the increased level of collagen IV and VII[12, 13]. It was also previously reported by several clinical studies involving patients with chronic and acute wounds treated using ReplicineTM that the dressings were able to reduce scarring, improve wound size reduction and accelerate wound healing [14-17]. Until recently, KerastatTM from Keranetics LLC is the only biomedical product in development as a wound dressing .

KerastatTM is a hydrogel product made of human-hair keratin. Based on several clinical trials, KerastatTM showed the capability to successfully treat partial-thickness wound and burns caused by radiation and thermal insult as well as hemorrhaging [18]. Unfortunately, there are limited reports available regarding the mechanism of its action on wound. Hence, explanation regarding the biological or molecular mechanism of keratin in a physiological environment is limited. Additionally, aside from wound dressing and cosmetic usage, there is no commercially human-hair keratin product available in the market for other biomedical purposes, leaving very little information regarding human hair keratins' behavior in a physiological and biological setting.

Besides commercially-available products, previous studies have also shown the possibility of keratin to be fabricated into several different forms such as films, hydrogels, sponges and fiber form. Detailed description as follows:

2.1.2.1. Keratin Films

Keratin has been successfully fabricated into films according to several studies. Keratin films produced by casting method have shown fibroblast proliferation and adherence despite its fragile, brittle, poor strength and poor flexibility without the addition of plasticizer [19-21]. Moreover, keratin films with decent flexibility were successfully fabricated with the addition of glycerol as a plasticizer. However, the water uptake was relatively low. The glycerol as the plasticizer would also possibly dissolve with the presence of water, thus impairing its physical structure and mechanical properties [22]. Besides fibroblasts, keratin films have also shown its ability to support proliferation, migration, adhesion and differentiation of other types of cells such as human epithelial corneal cell line, making it a promising alternative for ocular surface reconstruction [23, 24]. Films made from keratin and silk fibroin blend generated biodegradable matrices, therefore showing their potential as tissue engineering scaffolds [25].

Modified keratin has also been produced into films and studied for its biological and mechanical properties. One study utilized the cysteine-residues possessed by keratin, which make the modification with RGDS possible, thus enhancing the cell adhesion on the resulting modified keratin [26]. A compression-molded S-sulfo keratin films made from extracted wool keratin were also revealed to support mammalian cells'

proliferation. Nevertheless, the obtained films were insoluble and showed limited swelling in water, unless a reducing agent was introduced to break the disulfide linkages [27]. Similar phenomenon was also observed on crosslinked keratin films obtained with the facilitation of diepoxy crosslinkers. It was reported that the crosslinked films possessed improved mechanical properties such as good strength and good flexibility.

However, the crosslinking resulted in a hydrophobic material with almost no swelling capability, making this crosslinked films unsuitable for biomedical applications [28]. A study by Cui et al also discovered that transglutaminase modified wool-keratin films exhibited slight increase in tensile strength and water stability compared to non-modified films. These modified films were also found to have adequate biocompatibility, thus making it a possible candidate for tissue engineering applications [29].

Figure 2.1 Keratin films. Image reproduced with permission from [22].

2.1.2.2. Keratin Sponges

It was discovered that keratin produced into sponge forms by freeze-drying method has shown good cell compatibility and supported cell attachment and proliferation [8]. Another study by Verma et al regarding lyophilized sponges made from human hair keratins revealed that porous sponges which could support NIH3T3 cells' proliferation were able to be produced. However, the resulting sponges displayed limited swelling capabilities (less than 50%). No results were reported regarding the mechanical properties [30].

The cysteine residue presents in keratins also allows them to undergo further modification as shown in previous researches which dealt with the fabrication of chemically modified keratin sponges using iodoacetic acid, iodoacetamide, and 2-bromoethylamine to produce carboxy-, amido-, and amino-sponges, respectively. These studies showed that the modified keratin sponges were able to support osteoblasts differentiation [31, 32]. S-sulfo keratin extracted from wool was also successfully fabricated into porous sponges by a compression molding/particulate-leaching method, where the resulting sponges showed more stable structure and water-resistant properties [33].

Porous sponges could also be produced by blending keratin with other polymers followed by particulate-leaching method. Study by Li et al revealed that a blend of polylactic acid (PLLA) and keratin with paraffin microspheres as porogens were able to successfully produce sponges with porous structure [34]. Similar study by Hamasaki et al also demonstrated that porous keratin sponges with good flexibility could be obtained by the assistance of calcium alginate beads as porogens [35].

Figure 2.2. Keratin sponges. Image reproduced with permission from [30, 35]

2.1.2.3. Keratin Fibers

It is also reported that keratin has been successfully fabricated into fibers by electrospinning method. However, due to poor mechanical characteristics of keratin, it is unlikely to produce electrospun fibers using pure keratin alone. By mixing keratin with other polymer (e.g Polyethylene oxide/PEO, silk fibroin) the fabrication of fibrous mat was made possible [36-38]. .

Various keratin/PEO blends' formulation and their effect on the mechanical properties of the resulting fibers have been extensively studied [36, 37, 39, 40]. The keratin/PEO fibers were shown to be able to support L929 fibroblasts proliferation [41]. Crosslinked keratin/PEO fibers were also successfully produced by using ethylene glycol diglycid ether (EDGE) as a crosslinking agent. The resulting crosslinked keratin/PEO fibers were shown to have improved water-resistance [42]. Another study involving crosslinking reported that by modifying keratin with iodoacetic acid prior to crosslinking with glutaraldehyde would result in higher viability of NIH3T3 cells. Additionally, the crosslinked keratin fibers were easily degraded in trypsin, where within 2 h the fibers has lost their fibrous structures and degraded in 12 h [43].

Other than keratin/PEO blend, electrospun keratin and PLLA have also been studied for biomedical applications. Keratin/PLLA fibers were shown to be biodegradable and also improved osteoblasts proliferation compared to PLLA only membranes. Nonetheless, over half of keratin was removed in the first few hours of degradation, suggesting that the system is not resistant to aqueous environment [44]. It was also reported that by blending keratin for electrospinning with poly-lactic-co-glycolic acid (PLGA), the resulting fibers exhibited increased tensile strength by 8.2 % and improved bone mesenchymal stem cells proliferation compared to pure PLGA fibers [45]. Besides PLLA and PLGA, electrospun keratin/Polycaprolactone (PCL) mixture reportedly supported 3T3 viability alongside possessing adequate mechanical properties and uniform fibrous structures, providing another alternative for tissue engineering purposes [46].

Electrospun keratin with silk fibroin has also shown promising result as tissue engineering matrices [47]. The keratin/silk fibroin fibers were also shown to be biodegradable by trypsin [48]. However, despite the attempts of increasing the processability of keratin by combining pure keratin with other materials, the resulting fibers obtained were reportedly still possessing water instability and poor mechanical strength thus limiting them to be used for biomedical applications [36-38].

Figure 2.3. Scanning Electron Microscope images of keratin/PEO (9:1) nanofibres image reproduced with permission from [37]

2.1.2.4. Keratin Hydrogels

Keratin hydrogels have been extensively studied for tissue engineering and regenerative medicine purposes recently [49]. Several studies reported the use of keratin hydrogels as a conduit filler to serve as a neuroinductive provisional matrix that can facilitate nerve regeneration and promote neuromuscular recovery [50]. Keratin biomaterial hydrogel has proven to show a more immediate migration of Schwann cells compared to other control groups in a sciatic nerve injury rat model [51]. Keratin hydrogels were also displaying larger nerve area and higher myofiber density compared to collagen conduit filler in a median nerve injury model in non-human primates [52]. Similar result was also observed in rodent sciatic nerve injury model [53].

Keratin hydrogels were also investigated for treatment of skin injury or wound healing. Soluble keratin was shown to sustain viability and improve proliferation of thermally stressed fibroblast. In the same study, keratin was able to retard enlargement of wound area and facilitate wound closure [54]. Clinically, keragel[®], a commercially available keratin hydrogels wound dressing, has shown improved healing of blisters in patients with recessive dystrophic epidermolysis bullosa [13].

Other studies also demonstrated the utilization of keratin hydrogels as a hemostatic agent by stimulating the formation of thrombus and creating physical barricade on the site of the wound, acting as a scaffold which enable infiltration of cells and formation

of granulo tissue [55]. A following study revealed that keratin-hydrogel-coated surfaces has comparable platelet adhesion level to collagen-coated surfaces. In this study, it was demonstrated that β_1 and β_3 integrin play a role in the integrin-mediated platelet adhesion since the unblocked adhesion was significantly higher than those that were blocked by β_1 or β_3 antibodies. Additionally, talin staining on platelet cultured on keratin showed a halo shape which suggested activation and localization, unlike those cultured on albumin [56]. Another study explained the hemostatic properties of keratin hydrogels by their possible activation of the clotting cascades. The study revealed that keratin has a role in hemostasis by decreasing plasma clotting time. The presence of keratin was also confirmed to increase the assembly of fibrin network [57].

Besides nerve regeneration, wound healing and hemostatic purposes, keratin hydrogels were also known to support the adhesion and proliferation of fibroblasts [58, 59] and human umbilical vein endothelial cells [60], providing an option for keratin hydrogels to be utilized as a three-dimensional scaffold for tissue engineering applications.

Figure 2.4. Keratin hydrogel. Image is reproduced with permission from [58]

2.2. Alginate as Biomedical Materials

2.2.1. Properties of Alginate

Alginate is an anionic polymer which is typically obtained from brown algae or produced via bacterial biosynthesis by either *Azetobacter* or *Pseudomonas genera*. It is a linear copolymer which consists of α -D-Mannuronate (M) and β -L-Guluronate (G) as its monomers. These monomers were covalently linked via 1,4-glycosidic bond in different sequences or blocks. The blocks or sequences might occur as homopolymer blocks of repeating G residues (GGGGGG), repeating M residues (MMMMMM), and alternating M and G residues (GMGMGM) [61].

Alginate has the ability to form hydrogels by various cross-linking methods whether it is ionic or covalent-crosslinking. The most common method of gelation is ionic cross-linking by introducing divalent cations (e.g Ca^{2+}) which will bind to the Guluronate blocks of the alginate chains. The resulting hydrogel's physical properties are affected by the M or G ratio, the molecular weight and the pH of the gelation [62].

Alginate was known to be a biocompatible material since previous research showed that there is little or no immunoresponse given from alginate implants. Other research also confirmed this biocompatibility by reporting that there is no inflammatory response observed from subcutaneously injected alginate hydrogel [63, 64]. Despite its biocompatibility, alginate biodegradability has been debatable. Since mammals lack the enzyme alginase which could cleave the polymer chains, its degradation will depend on its solubility and partial oxidation of alginate chains. Even if the gel dissolves, the complete removal of alginate will depend on the molecular weight of the polymer in order for it to pass the renal clearance threshold. Meanwhile, partial oxidation with periodate might lead to the cleavage of carbon-carbon bond between the *cis*-diol group in the uronate residue. These will change the chair conformation and enable the degradation of the alginate backbone which leads to the reduction of the molecular weight [65, 66].

2.2.2. The Use of Alginate for Biomedical Application

Alginate has been extensively used for various biomedical applications due to its biocompatibility and relatively low cost. It has been commercially used as wound dressings (e.g AlgicellTM (Derma Sciences), AlgiSite MTM (Smith & Nephew), Comfeel PlusTM (Coloplast), KaltostatTM (ConvaTec), SorbsanTM (UDL Laboratories), and TegagenTM (3M Healthcare)) since it provide a moist environment that could aid wound healing. Alginate dressings are mainly commercially available in freeze-dried porous sheet or fibrous non-woven form. The dry form of the dressing will enable the absorption of the wound exudate hereby providing a physiological moist environment while concurrently preventing infection at the wound site [61].

Alginate has also been widely researched in pharmaceutical application, whether in combination with other materials or alginate alone, as a matrix for drug delivery. The possibility to modify alginate properties in order to regulate the kinetics of the drug release has been the main interest in some researches. The interaction of alginate with the respective drugs and also the physical properties that affect the manner in which the drug is released were investigated [61].

Besides pharmaceutical applications and wound dressings, alginate has been researched as a model system for mammalian cell culture for biomedical study purposes. However due to the lack of cell-binding sequence in alginate, RGD-modified alginate are primarily utilized for cell-culture substrate purposes nowadays. By varying the amount and type of the RGD-containing-peptide incorporated in the alginate matrix, control of the intended cell growth in various cell types will be made possible [67].

The use of alginate for tissue regeneration purposes has also gained growing interest. Numerous researches have been done in the utilization of alginate gels in the delivery of cells and growth factor to promote blood cell formation, bone regeneration, restore damaged cartilage, and mediate the regeneration of various tissues and organs such as liver, pancreas, nerve and muscle [61].

2.3. Desired Properties of Matrices for Wound Healing Purposes

The approach of a biomaterial design for wound dressing purposes would depend on the properties of the type of wound and tissue (such as whether the wound is an open, closed, acute or chronic wounds) as well as the treatment goal. For example, a dressing could be designed to absorb exudates from the wound, to remove foreign object from the wound site hence debriding the wound, to degrade and release a bioactive substance to aid in the wound healing process, or to simply provide a barrier between the wound and external environment thus avoiding infection [68]. For example, materials intended to be used for internal wounds should have the ability to block and damage microbes and other infectious agents. Additionally, wound dressings that are porous and hydrophilic, which allow absorption of exuded liquids or adequate swelling capacity, would be able to fill voids in damage tissue and provide a moist wound environment. A biocompatible material that is degradable on a time scale fitting to wound healing process would provide minimal pain during dressing change [68-70].

The goal of the treatment would also affect the design of the biomaterial for dressing purposes. A dressing could be hydrophilic or hydrophobic depending on the desired control rate of the fluid passage to or from the wound as well as the preferred swelling capacity. Porosity would affect the rate of diffusion and release of encapsulated drug into the wound as well as the swelling capacity, which would affect the amount of wound exudates absorbed and the moisture content in the wound. The degradation rate of the material would affect the release rate of the pharmacologically active agents encapsulated or included in the materials to assist in wound healing and tissue regeneration. Dressings for acute wounds might require greater hydrophilicity and swelling capacity to absorb exudates, antimicrobial properties to prevent infection, as well as hemostatic properties to stop bleeding [68]. Meanwhile, in chronic wounds, regeneration of damaged tissue is needed thus designing a matrix that would aid tissue regeneration is a main concern. Dressings which provide ECM structures such as collagen and hyaluronan has been used to allow cellular adhesion, aid ECM remodeling and therefore accelerate the healing process . Moreover, active substances that trigger remodeling can be incorporated into the matrices. Proteins and growth factors could be included in a dressing to allow granulation, improve epithelization, induce ECM synthesis, or promote cellular migration and proliferation[71, 72]. Aside from these,

anti-bacterial substances or anti-inflammatory agents could be added in order to prevent infection and excessive inflammatory responses, respectively [68]. In conclusion, by tuning the physical, chemical and biological properties of the materials, a novel wound-healing biomaterial could be designed to achieve their respected optimal properties depending on the intended usage .

2.4. Keratin-containing Hybrid Materials

Mixtures of keratin with either synthetic or natural polymers in order to increase its processability and mechanical properties have been previously studied. Blends of keratin with PEO [36], PLLA [44], PLGA [45] or PCL [46] have been carried out to improve their processability for electrospinning . However, most of these hybrid materials were only physical blends of keratin with other materials, thus once the hybrid materials come into contact with water, the issue of erosion and dissolving would occur, making these hybrid materials disadvantageous for biomedical applications.

In order to improve their water-resistance, crosslinking keratin with other materials had been carried out where EDGE was used as a crosslinking agent to improve its hydrophobicity [42]. However, the resulting material became too hydrophobic, which would be an issue in clinical settings since if the materials were to be applied for tissue engineering, cell carrier or wound dressing purposes, they would need to have the capability to uptake fluid.

In this study, by crosslinking keratin with alginate using EDC as a crosslinking agent, the mechanical strength of the resulting material was expected to be improved. Furthermore, performing variation of crosslinking agent concentration as well as the ratio of keratin and alginate, the water uptake and the biocompatibility were also expected to still be acceptable for biomedical use. Additionally, it was also expected that the covalent crosslinking which happened as a result of amide bond formation with the assistance of EDC would still be cleavable by proteases, making the resulting material biodegradable.

2.4. Crosslinking of Keratin and Alginate

There are various choices of crosslinking agent and crosslinking method available in order to form crosslinking between the designated protein and the chosen crosslinking partner. Crosslinking between protein and the chosen partner could be designed to form ionic or covalent crosslinking. Most of the time crosslinking is directed towards the protein functional groups such as their amino acids' side chains. This crosslinking could be carried out either with or without prior modification of the protein. The crosslinking could also be designed to incorporate a bridge spacer between the linkage [73].

In this study keratin and alginate were crosslinked by the formation of amide bond linkage between the carboxylic groups of alginate and the amine groups available in keratins' amino acid side chain or N-terminal. Amide bond formation is the preferred covalent bond linkage in this study since it is suspected to be cleavable by protease enzymes that are present *in vivo*, thus enabling the crosslinked materials' biodegradation. EDC were chosen as the crosslinking agent due to the properties of its derivative (EDC HCl) which is easily soluble in water hence providing ease in the crosslinking and fabrication process. Carbodiimide derivatives also has the advantage of not requiring prior activation of the carboxylic acid, thus enabling a one-step reaction [73, 74].

Figure 2.5. EDC-mediated crosslinking reaction between carboxylic acid and amine groups

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Chapter 3

Experimental Methodology

This chapter explains in detail the methodology used in the extraction of keratin by using sodium sulfide as reducing agent as well as the characterization of the extracted keratin protein bands by gel electrophoresis and keratin's chemical composition and structure by Fourier-Transform Infrared (FTIR) spectroscopy. The method of fabrication of crosslinked keratin-alginate sponges with the facilitation of carbodiimide is further explained alongside the characterization of the crosslinking degree (by means of ninhydrine reaction), mechanical properties (by means of compression, tensile and flexural tests), physical properties (by means of evaluation of morphology, water uptake capacity and water vapour transmission test) and degradation profile (with proteinase K and chymotrypsin) of the crosslinked sponges. Methods of cell culture on 2D surfaces and 3D sponges using L929 murine fibroblasts and human dermal fibroblasts are elaborated in details. In addition, the methodology of the evaluation of cell viability (Live/Dead[®] assay), cell proliferation (dsDNA measurement by picogreen[®]), cell distribution (Hematoxylin and Eosin staining of histology section), extracellular matrices production (by immunohistochemistry) and growth factor production (by using multiarray membrane) were further explained.

3.1. Rationale for Selection

Extraction of keratin from human hair was carried out using a method that has been previously optimized by utilizing sodium sulfide as a reducing agent that could cleave disulfide bonds between keratin monomers and making these dissolvable in water [1, 2]. In order to determine the quality of extracted human hair keratin, a measurement of the IR spectra (which provide the information to identify the chemical structure) and coomassie blue staining of SDS-PAGE (which provides information of the molecular weight of the protein extract to help identify keratin quality) were carried out.

Crosslinking of keratin and alginate was carried out by the formation of amide bonds, using EDC HCl as a crosslinking agent. EDC HCl was chosen due to their simple one-step reaction in crosslinking between amine and carboxylic acid groups as well as their solubility in water [3]. The crosslinking was confirmed by IR measurement as well as the determination of remaining free amine groups using the ninhydrin reagent.

The obtained crosslinked sponges were then further characterized in terms of their changes in physical and mechanical properties. The morphology was recorded using the scanning electron microscope (SEM) and the average pore area was calculated using ImageJ software. Changes in mechanical properties were evaluated by measuring the moduli and strengths of the obtained sponges by means of compression, three-point bending and tensile tests. Studies on their water uptake capacity were also performed to evaluate the materials' behaviour in a hydrated and aqueous environment. In addition, degradation studies were also carried out using proteinase K and chymotrypsin (enzymes that are well-known to degrade keratin) to evaluate their degradation rate and behaviour.

Cell culture studies were carried out in order to prove that crosslinked keratin-alginate mixtures were able to support proliferation and viability of cells in both 2D and 3D environment. L929 murine fibroblasts were chosen as one of the cell type used since it has already been established as an ASTM standard for cytotoxicity evaluation. Moreover, relating to its application as wound dressing and cell carrier, study by using HDF were also carried out due to fibroblasts well-known role in skin regeneration. The viability was evaluated by using Live/Dead® assay kit since this assay has the capability

to differentiate live cells (which are stained green) and dead cells (which are stained red) during the visualization under fluorescent microscope. Meanwhile, the proliferation was measured by quantifying the dsDNA amount of cultured cells by Picogreen[®] assay with the understanding that the amount of dsDNA correlate to the amount of live cells.

Moreover, histology study was also carried out. Hematoxylin and eosin staining was performed on paraffin-embedded cross section of the cultured matrices in order to visualize the distribution of the cells inside the matrices, since the homogenous distribution of cells was required for effective tissue regeneration.

Additionally, to evaluate the cell behaviour and function inside the matrices, measurement of ECM, cytokines and growth factor production was also carried out. ECM production was evaluated by performing immunohistostaining on the paraffin-embedded crosssection of the cultured matrices to visualize the production of collagen III and fibronectin. Meanwhile, growth factor and cytokines expression were measured using the protein multiarray membranes due to their ease of use and effectiveness.

3.2. Materials

Human hair was obtained from local barbershop. All other chemicals were purchased from Sigma-Aldrich international unless stated otherwise while 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (EDC HCl) was purchased from Chemimpex International Inc., USA.

3.3. Methods

3.3.1. Extraction of Keratin from Human Hair

3.3.1.1. Delipidization of Human Hair

Hair retrieved from local barbershop was washed with soap then rinsed with 95 % ethanol twice. Afterwards, the hair was air-dried in order to remove the remaining ethanol. Subsequently, the dried hair was delipidized by soaking the hair in a mixture

of chloroform-methanol (2:1) for 24 h. The obtained delipidized hair was then cut into 1-2 mm length with scissors and used for extraction.

3.3.1.2. Keratin Extraction

Sodium sulfide solution was used to extract keratin from delipidized human hair. Briefly, 30 g of delipidized human hair was weighed and then dispersed in 1 L of 0.125 M Sodium sulfide solution. Subsequently, the mixture was incubated in 40 °C for 4 hours and then filtered afterwards by using filter paper in order to remove the residual hair debris.

3.3.1.3. Preparation of Lyophilized Keratin Powder

In order to remove the remaining sodium sulfide, the filtered keratin solution was exhaustively dialyzed in dialysis membranes (molecular weight cut off/MWCO : 10000 Dalton) against deionized water (DI water). The resulting dialyzed keratin solution was then transferred into 50 mL centrifuge tube and frozen on -80 °C overnight. The frozen aliquot was then freeze dried for 2 days to obtain dry keratin powder. The dry keratin powder obtained was then stored at -20 °C until further use.

3.3.2. Characterization of extracted keratin

3.3.2.1. Fourier Transform Infra-Red (FTIR) Spectroscopy

Recently, the usage of FTIR for the determination of biomacromolecules structure has been increasing. Even though the chosen method to evaluate the three-dimensional structure of a protein is still through X-Ray crystallography method, this method is not possible for some proteins since this method required the protein molecule to form a well-ordered crystal [4, 5].

Another method that can be used as an alternative is nuclear magnetic resonance spectroscopy. This method enables the determination of protein structure in solution, however it is only suitable for proteins with small-medium molecular weight since the data interpretation for large protein will be complex. Moreover, crosslinked protein

would likely face solubility issues, making this method unsuitable for them. Another method that enables the determination of protein structure is circular dichroism spectroscopy. This method could provide the secondary structure of protein, however, the protein needs to be dissolved in a solution, thus also limiting this method to be used for crosslinked protein.[6, 7]

Fourier transform infrared (FTIR) spectroscopy is a technique that is used to measure and record infrared spectra. The measurement is carried out by guiding infrared light through an interferometer before letting it pass through the sample and vice versa. Inside the infrared spectroscopy apparatus, a beam splitter, a detector, light source, a fixed mirror and a moving mirror existed, where half of the light from the source is reflected, while the other half is transmitted. Following reflection by these two mirrors, each of the two beams (half reflected by fixed mirror the other half by moving mirror) were split at beam splitter and were guided to the source and the detector. The signal that were recorded were called an "interferogram", which represented the light output as a function of mirror position. Fourier transform is a common algorithm used to turn this raw data into the desired result which is the sample's spectrum (or also known as the light output) as a function of infrared wavelength (or also known as wavenumber). As what has been previously described, the sample's spectrum is compared to a reference. Due to this characteristic, IR spectroscopy is often used to identify structures since different functional groups give rise to various characteristic bands both in terms of intensity and position (frequency). The positions, range and intensity of these bands (which are assigned to a particular functional groups) has been well characterized as listed on the table below [8-10].

Table 3.1. Table of IR absorption and band assignment [11]

Bond	Type of bond	Specific type of bond	Absorption peak (cm ⁻¹)	Appearance
C=O	aldehyde/ketone	saturated aliph./cyclic 6-membered	1720	
		α,β -unsaturated	1685	
		aromatic ketones	1685	
		cyclic 5-membered	1750	
		cyclic 4-membered	1775	
		aldehydes	1725	
	carboxylic acids/derivates	saturated carboxylic acids	1710	

Bond	Type of bond	Specific type of bond	Absorption peak (cm ⁻¹)	Appearance
O—H	alcohols, phenols	unsat./aromatic carb. Acids	1680–1690	broad
		esters and lactones	1735	
		anhydrides	1760	
		acyl halides	1820	
		amides	1800	
		carboxylates (salts)	1650	
		amino acid zwitterions	1550–1610	
		low concentration	1550–1610	
		high concentration	3610–3670	
		low concentration	3200–3400	
N—H	carboxylic acids	high concentration	3500–3560	broad
		low concentration	3000	
		primary amines	3400–3500	
		Any	1560–1640	
		secondary amines	>3000	
		Any	2400–3200	
		ammonium ions	1040–1060	
		primary	~1100	
		secondary	1150–1200	
		tertiary	1200	
C—O	phenols	any	1120	two bands (distinct from ketones, which do not possess a C—O bond)
		aliphatic	1220–1260	
		aromatic	1250–1300	
		carboxylic acids	1100–1300	
		any	1020–1220	
		esters	1615–1700	
		any	2250	
		aliphatic amines	2230	
		any	2165–2110	
		any	2140–1990	
C=N	C≡N (nitriles)	unconjugated	1000–1100	similar conjugation effects to C=O
		conjugated	1100–1200	
		any	540–760	
		any	500–600	
		any	500	
		ordinary	1540	
		trifluoromethyl	1380	
		any	1520	
		any	lower if conjugated	
		any	lower if conjugated	
C—X	fluoroalkanes	any	1540	stronger
		any	1380	
		any	1520	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
N—O	chloroalkanes	any	540–760	weaker
		any	500–600	
		any	500	
		any	1540	
		any	1380	
		any	1520	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
N—O	bromoalkanes	any	540–760	stronger
		any	500–600	
		any	500	
		any	1540	
		any	1380	
		any	1520	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
N—O	iodoalkanes	any	540–760	weaker
		any	500–600	
		any	500	
		any	1540	
		any	1380	
		any	1520	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	
		any	lower if conjugated	

In this study, characterization using FTIR spectroscopy was carried out since the obtained spectra could provide fingerprint information of the chemical composition of the protein and/or crosslinked protein as well as the secondary structure by performing

deconvolution of amide band. In addition, this method is suitable for this study since powdered form of keratin or freeze-dried crosslinked protein could be used for the evaluation.

The IR absorbance spectra of keratin powder or crosslinked keratin-alginate sponges were measured by using a Fourier Transform Infra-Red (FTIR) Spectroscope (Perkin Elmer Spectrum GX). The keratin powder or crosslinked sponges was grinded, mixed with dried (KBr) powder and then compressed to form a disk-shape pellet preceding measurement. The measurement of the IR spectrum was performed between a range of 4000 and 400 cm^{-1} , a resolution of 2 cm^{-1} , an interval of 0.5 cm^{-1} with an average of 16 number of scans per sample. The obtained spectra were processed using spectrum software version 5 by Perkin Elmer.

Additionally, it is known that amide I band shape of proteins is characteristic of their secondary structure. This characteristic enables the determination of secondary structure of protein by analysing the amide I band. The analysis is based on the principle and assumption that spectrum of single narrow bands representing each of the component of the secondary structure was broadened in both liquid and solid state. These bands (where each is assigned to a characteristic secondary structure (Table 3.3)) overlap each other and is difficult to distinguish. Therefore, by performing curve fitting procedure, the quantitative estimation could be carried out by comparing the area of each component that represent each type of secondary structure [12-14]. Previous work by Byler and Susi showed the deconvolution of amide I band with a Lorentzian line shape function and a deconvoluted spectrum fitted with the Gaussian band shapes through an iterative curve fitting procedure. The study showed that the result complied with the secondary structure data obtained from the X-Ray crystallography, signifying that this method could be used to analyse protein secondary structure [14].

The secondary structure of the keratin-alginate mixture before and after crosslinking were determined by performing curve-fitting of the amide I band from the obtained FTIR Spectra. A curve fitting treatment was carried out in order to calculate the relative proportion of each type of secondary structure assigned that is represented in the amide I region, namely α -helix, β -sheet and unordered or random coil (Table 2). The curve fitting was calculated and performed using OPUS software and the linear baseline was

set at the wavelength of 1560-1720 cm^{-1} . The band shape was fitted with the Gaussian shape and the algorithm used was Levenberg-Marquardt. Bands' components' position were fixed while the bandwidth were able to be adjusted to perform the profiling of amide I band curve-fitting. The percentage of each secondary structure components was calculated by dividing the area under curve of the representative structure to the total area under curve of all amide band I components.

Table 3.2. Regions of each components of proteins' secondary structures (amide I band)

Structure	Region (cm^{-1})
Beta-turn	1682-1662
Alpha-helix	1662-1645
Beta-sheet	1637-1613 1689-1682
Random coil/unordered	1645-1637

3.3.2.2. Coomassie Blue Staining

In order to confirm the presence of keratin in the freeze dried keratin sample, SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was performed. Ten micrograms of keratin was first diluted in 10 μL of ultrapure water and then followed by mixing of 5 μL of LDS sample buffer and 2 μL of sample reducing agent. The volume of the mixture was then adjusted with ultrapure water until it reached 22 μL . The samples were then heated at 90°C for 20 min. Subsequently, 20 μL of sample was loaded in each lane of the NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen, USA). 3-(N-morpholino) propane sulfonic acid buffer (MOPS) (Invitrogen, USA) was used as running buffer. Separation was performed at a constant voltage of 120V. During the end of the separation, the gels were washed three times using ultrapure water and then stained for 60 minute using SimplyBlue SafeStain® solution (Invitrogen, USA). The gels were then washed with ultrapure water for 2 hours in order to clear the background.

3.3.3. Preparation of Crosslinked Keratin-Alginate Mixture

EDC HCl was used as a crosslinking agent to chemically crosslink keratin and alginate. The crosslinking reaction was carried out by varying the amount of EDC HCl and keratin-alginate composition. Alginate was mixed with the desired amount of EDC HCl in DI water for 1h (under magnetic stirring, at room temperature) in order to activate the carboxylate groups. Subsequently, keratin solution in DI water was added into alginate-EDC mixture until desired concentration is achieved. The obtained final mixture was incubated for 24 h under magnetic stirring at room temperature and then exhaustively dialyzed against DI water in a dialysis membrane (MWCO 10000 Da) in order to remove the remaining crosslinking agent and its byproducts. Keratin solution and reagents were sterile filtered before use and all processes were carried out inside the biosafety cabinet in order to maintain sterility.

Table 3.3. Fabrication of crosslinked keratin-alginate by varying EDC concentrations and varying keratin-alginate composition.

Sample Abbreviation	Keratin-Alginate final concentration (% w/v)	EDC (mM)	Keratin-alginate ratio (w/w)
KA11-0mM	2	0	1:1
KA11-1mM	2	1	1:1
KA11-10mM	2	10	1:1
KA11-100mM	2	100	1:1
KA14-10mM	2	10	1:4
KA12-10mM	2	10	1:2
KA21-10mM	2	10	2:1
KA41-10mM	2	10	4:1

3.3.4. Fabrication of Keratin Alginate Sponges

Keratin-alginate sponges were fabricated by freeze drying the final crosslinked mixture. One milliliter of the final crosslinked mixture was casted on a 24-well plate and then frozen on -80°C for 24 h. The resulting frozen mixture was then freeze-dried for 1 day in order to obtain dry sponges.

3.3.5. Characterization of Crosslinked Keratin Alginate Sponges

3.3.5.1. Evaluation of Sponges' Morphology by Scanning Electron Microscopy

The scanning electron microscopy (SEM) is a technique that uses focused beam of high-energy electrons to scan the specimen surface which will then produce signals at the surface of solid specimens. The interactions between sample and electron would generate signals that reveal the information about the samples such as the external morphology, crystalline structure, chemical composition, texture and materials' orientation. A 2-dimensional image which shows the spatial variations could be generated from data collected over a selected area of the surface of the sample. The conventional techniques of SEM could image areas from approximately 1 cm to 5 microns in scanning mode (enabling high magnification of images) as well as carrying out analyses of selected point locations on the sample. The analysis carried out using energy-dispersive X-Ray spectroscopy (EDS) can determine chemical composition qualitatively or semi-quantitatively while electron backscatter diffraction (EBSD) enables the determination of crystalline structure and crystal orientations [15, 16].

In SEM, accelerated electrons carry significant amounts of kinetic energy which is dissipated as a variety of signals generated by interactions between electron and the sample when the electrons are decelerated in the sample. These signals include secondary electrons which produce SEM images, backscattered electrons which are useful for compositional imaging, diffracted backscattered electrons which are used to determine crystal structures and orientations of mineral, photons which are characteristic of X-rays that are used for elemental analysis and continuum X-rays, visible light (cathodoluminescence–CL), and heat. Generally, secondary electrons and backscattered electrons are used for samples' imaging, where secondary electrons

imaging (SEI) is utilized for displaying morphology and topography on samples while backscattered electrons imaging (BEI) is utilized for illustrating contrasts in composition in multiphase samples (such as for rapid phase discrimination). SEM analysis is considered as a non-destructive method since x-rays generated by electron interactions will not lead to loss of the sample, thus enabling the materials to be analysed repeatedly [15, 16].

SEM is routinely utilized to produce high-resolution pictures of shapes of objects (SEI) as well as to show spatial variations in chemical compositions by acquiring elemental maps or spot chemical analyses using EDS, discrimination of phases based on mean atomic number (commonly related to relative density) by using BEI, and retrieving compositional maps based on differences in trace element "activators" using cathodoluminescence. SEM is also widely used to identify phases based on qualitative chemical analysis and/or crystalline structure. Precise measurement of very small features and objects down to 50 nm in size is also accomplished using the SEM. SEM equipped with diffracted backscattered electron detectors (EBSD) can be used to examine crystallographic orientation in many materials [15, 16].

Samples must meet certain requirements before being subjected to analysis using SEM. Firstly, the samples must be solid and dry. Moreover, their size needs to fit into the microscope chamber. For most instruments, it is required for the sample to be stable in a vacuum. In addition, an electrically conductive coating is needed to be applied to insulate samples electrically for observation using conventional SEM's, especially if the sample has low conductivity (which is the general case for organic materials). SEM requires minimal sample preparation as well as enabling rapid data acquisition, which explains why it is a preferred method for morphology measurement in this study [17].

The microarchitecture of the samples was measured using the secondary electron imaging (SEI) mode of the Scanning Electron Microscope (SEM; JEOL 5410). Preceding imaging with SEM, the samples were dipped in liquid nitrogen (N_2) until frozen and then cut with a thin razor knife in order to expose their clean-cut cross-section. Using a carbon tape, the obtained cross-section of the sample was then mounted on the sample holder. The mounted samples were subjected to gold sputtering at 18 mA for 15 seconds prior to observation under SEM. The image was recorded using SEM

under an accelerating voltage of 5kV and 8 spot size. The obtained images were then further analyzed using ImageJ software to calculate the average pore area of each samples (n=3). Measurements were scaled as 1.47 $\mu\text{m}/\text{pixel}$.

3.3.5.2. Determination of Crosslinking Degree Based on the Calculation of the Remaining Free Amine Groups

The determination of the remaining free amine groups was quantified based on the ninhydrin reaction. One milliliter of ninhydrin working solution (0.2 g of ninhydrin in 50 mL water) was mixed with 10 mg of pulverized sample, and then incubated at 90 °C in a water bath for 20 min. Subsequently, the mixture was cooled down to room temperature preceding the measurement of the optical absorbance of the mixture's supernatant. The optical absorbance at 570 nm was determined using a microplate reader. The degree of crosslinking was calculated by the crosslinking index which was defined as:

$$\text{Crosslinking index (\%)} = (\text{NH}_0 - \text{NH}_t) / \text{NH}_0 \times 100$$

Where NH_0 is the amount of free amine groups in a sample before crosslinking (keratin-alginate sponges without EDC); NH_t is the amount of free amine groups in a sample after crosslinking.

3.3.5.3. Measurement of Infrared (IR) Spectra of Crosslinked Sponges

The IR absorbance spectra of crosslinked keratin-alginate sponges were measured by using a Fourier Transfer Infra-Red (FTIR) Spectroscope (Perkin Elmer Spectrum GX) as previously mentioned on 3.3.2.1.

3.3.5.4. Determination of Secondary Structure by Curve-Fitting of Amide I Band Profile.

The secondary structure of the keratin-alginate mixture before and after crosslinking were determined by performing curve-fitting of the amide I band from the obtained FTIR Spectra as explained in 3.3.2.1.

3.3.5.5. Compression Test

Performing a compression test enables the determination of materials' behavior under crushing loads. The deformation of compressed specimen at various loads is recorded and the compressive stress and strain is plotted as a stress-versus-strain diagram, where compressive stress is the applied compressive force per area and strain is the change in thickness per original thickness. The linear portion of the stress-strain curve is used to measure the compressive modulus of the material. The modulus value represents the stiffness of the material, where the higher the modulus, the stiffer the material is [18].

The compression modulus of the sample was measured using an Instron Mechanical Tester 5567 (Instron co., MA) until it reached 80 % strain. The dimension of the samples used in this study were 4 mm in thickness and 16 mm in diameter. The machine was equipped with a 500 N load cell and the loading speed was set at 0.01 mm/s. Prior to performing the test, the sponges were pre-loaded with 0.05 N. The modulus was determined by calculating the slope of the initial linear phase of the stress-strain curve.

3.3.5.6. Tensile Test

Tensile testing is a test that is carried out in order to determine the materials' behaviour when subjected to controlled tension until it reaches failure. Generally, for most specimens, the relationship between the applied tensile force per area (stress) and the elongation per original length (strain) is linear in the initial part of testing, where it follows the "Hooke's Law" where the ratio of stress to strain is a constant. The slope of the line in this region where stress (σ) is proportional to strain (ϵ) is called the "Modulus of Elasticity" or "Young's Modulus", which represents the stiffness of the material. The higher the value of the modulus is, the higher the stiffness is. Additionally, the ultimate tensile strength (UTS) value, which represents the maximum load the specimen can withstand before it reaches failure, can also be obtained from this tensing. The UTS value is not always the strength at break, depending on whether the material experience plastic deformation [18-20].

The tensile modulus of the sample was measured using Instron Mechanical Tester 5567 (Instron co., MA). The samples used for measurement were casted into a dimension of

4 mm in thickness, 5 mm in width and 40 mm in length. Each end of the sponges was mounted on epoxy resin and clamped to the grip. The machine was equipped with a 500 N load cell and the sponge was then pulled at a constant speed of 0.5 mm/min until failure was reached. The requirement for the results to be accepted was when the observed failure was within the gauge length. The modulus was determined by calculating the slope of the initial linear phase of the stress-strain curve.

3.3.5.7. Three-point Bend Test

The flexure test method enables the measurement of materials' behaviour when they are subjected to simple beam loading. Stress and strain are calculated for each increments of load and the values are plotted in a stress-versus-strain diagram. Flexural modulus could be calculated by obtaining the value of the slope from the linear portion of the stress vs. strain curve. The modulus value represents the stiffness of the material, whereas the higher the value the stiffer the material is [18, 21].

The flexural modulus of the sample was measured using Instron Mechanical Tester 5567 (Instron co., MA). The samples used for measurement were casted into a dimension of 4 mm in thickness, 5 mm in width and 40 mm in length. The machine was equipped with a 500 N load cell and the crosshead speed was set at a constant speed of 0.1 mm/min until 80% strain was reached. The modulus was determined by calculating the slope of the initial linear phase of the stress-strain curve.

3.3.5.8. Water Uptake Study

The sponges' water uptake capacity was evaluated by immersing the dried samples in deionized water at 25°C. The immersed samples were taken out after 1 minute and filter paper was used to remove the excess liquid by gently blotting the samples. The air-dried samples before and after immersion were weighed using a balance. The water uptake capacity was calculated according to the formula:

$$\text{Water uptake (\%)} = (W_t - W_o) / W_o \times 100$$

where W_o is the weight of the dried sample and W_t is the weight of the hydrated sample.

3.3.5.9. Water Vapor Transmission Study

The samples' capability to prevent trans-epidermal water loss (TEWL) was evaluated using the Tewameter[®] TM 300 (Courage + Khazaka Electronic GmbH, Germany). Samples were placed over healthy adult forearm skin and the TEWL across the samples was measured. TEWL measured across healthy adult forearm skin without any material mounted was used as a comparison. The TEWL were recorded as soon as a stable reading was achieved in about 20 s. Kaltostat[®] (alginate) and Aquacel[®] (sodium carboxymethylcellulose microfibre), which are widely available commercial wound dressings, were used as controls.

3.3.5.10. Degradation

The extent of degradation of crosslinked keratin-alginate sponges against proteinase K and chymotrypsin was determined in this study. Briefly, each of the crosslinked sponges (approximately 1 x 1 cm, 4 mg) initial weight was recorded before placing it inside a cell culture insert with a 0.4 μ m pore size. The cell inserts were then mounted in a 24-well plate and 1 mL of the respective enzyme solutions (1 IU/mL in tris buffer, pH 8) was added into each well (350 μ L inside of the insert, 650 μ L outside of the insert). Samples were then kept in an incubator with the temperature maintained at 37 °C and the enzyme solutions were replenished every 3 days. At pre-determined time points (day 3, 7, 14 and 30), the samples were taken out and freeze-dried. The weights of the dried samples were measured and the percentage of weight retained was calculated.

3.3.6. Cell Culture

3.3.6.1. Culture of L929 on Crosslinked Keratin-Alginate Coated-surfaces

Firstly, a 2D L929 culture study was carried out in order to better understand the cellular response of cells to crosslinked keratin-alginate. Briefly, 100 μ L of either 2 % keratin, 2 % alginate, 2% collagen or crosslinked keratin-alginate mixtures was coated on the tissue culture polystyrene surface (TCPS) in 48-well culture plates and was allowed to

air-dry at room temperature before use. All processes were carried out inside the biosafety cabinet to ensure sterility. Separately, L929 murine fibroblasts were cultured on tissue culture polystyrene (TCPS) in DMEM supplemented with 10 % FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate with 100 IU/ml penicillin (Gibco, USA) as an antimicrobial agent. L929 cells were harvested at sub-confluency level with 0.25 % trypsin (Gibco, USA) and the cell numbers were calculated using a haemocytometer before being seeded on top of the coated surfaces at a cell density of 10,000 cells/cm². Cell viability was analyzed by Live/Dead[®] assay and proliferation was evaluated by Picogreen[®] assay on day 1, 3 and 7 following seeding.

The cell viability was measured by means of visualization of L929 cells stained with Live/Dead[®] assay kit (Molecular Probes, USA). The ethidium homodimer of the Live/Dead[®] kit would stain the nuclei of the dead cells red meanwhile the calcein AM would stain the live cells green. These staining could be observed under the fluorescent microscope (Olympus CKX41, Japan). The assay was carried out in accordance to the instructions given by the manufacturer.

On the other hand, cell proliferation was investigated by measuring the amount of double-stranded DNA (dsDNA) with the Picogreen[®] assay (Molecular Probes, USA). The assay was carried out after discarding the medium and repeatedly rinsing the surfaces with PBS. Subsequently, in order to harvest the dsDNA, the cultured L929 cells were subjected to incubation for 4 hours with 100 µL of 1% Triton-X lysis buffer on 37 °C. Following the end of incubation, 20 µL of the sample was mixed with the working solution of Picogreen[®] reagent (1:200 in TE buffer). The fluorescence intensity of the mixture was then measured at 480 and 520 nm (excitation and emission wavelengths) on a 96-well plate by a microplate reader (TECAN INFINITE F200, Switzerland). The amount of dsDNA was calculated using a calibration curve generated from various dsDNA concentrations (250-2000 ng/mL) as standards.

3.3.6.2 Culture of L929 in Crosslinked Keratin-Alginate Sponges

Culture of L929 fibroblasts on three-dimensional sponges was carried out in order to observe fibroblasts' cellular behavior in a 3D setting. Keratin sponges, alginate sponges and collagen sponges were used as controls. Keratin sponges were produced from 2%

keratin solutions induced to gelling by using a pH 3 citric buffer, while collagen sponges were produced from 0.5 mg/mL rat tail collagen (type 1) (BD Biosciences, USA) induced to gelling by addition of NaOH in PBS, at 37 °C for 1 h. Both gels were frozen at -80 °C overnight following freeze-drying to obtain dried sponges. Meanwhile, crosslinked keratin-alginate sponges were fabricated using a method previously described above.

Prior to seeding, a sub-confluent culture of L929 murine fibroblasts were harvested with 0.25 % trypsin, and the number of harvested cells were counted using a haemocytometer. Then, harvested L929 cells were seeded on top of the three-dimensional sponges at a density of 200,000 cells/cm³. The viability and proliferation of L929 were measured on day 1, 3, 7, 14 and 21 post-seeding by Live/Dead[®] assay and Picogreen[®] assay as previously described, respectively.

Prior to dsDNA quantification by Picogreen[®] assay, a series of digestion were performed in order to separate the dsDNA from the matrices. The digestion was carried out by incubating each samples overnight on 37 °C with 1 mL of proteinase K (5 IU/mL) and SDS (1 %) on Tris-HCl buffer to degrade the matrices and harvest the dsDNA. The cycle was repeated for 3 times until no debris from the matrices was visibly observed. The separation of dsDNA from the denatured protein was carried out by performing phase separation using phenol. The collected digested samples were adjusted into a volume of 5 mL using DI water. Subsequently, 1 mL of the digested sample was mixed with 1 mL of phenol to carry out a liquid-liquid extraction. The molecules of the polar dsDNA would be accumulated in the aqueous phase and thus this aqueous part could be used for Picogreen[®] assay as previously described.

3.3.6.3 Culture of Primary Human Dermal Fibroblasts on Crosslinked Keratin-Alginate-Coated-Surfaces

Following the culture of L929 on crosslinked sponges, 2D cell culture study using human dermal fibroblasts cells were carried out. Human dermal fibroblasts (HDFs) are the family of cells that play a crucial role in skin regeneration and wound healing. Therefore, evaluating the cellular behavior of HDFs relating to their interaction with the crosslinked sponges was essential for sponges' biomedical usages such as wound

dressings. Additionally, HDFs were also known to produce $\alpha_4\beta_1$ integrin that could bind with LDV cell-binding motifs of keratin, hence why it was chosen in this study.

Firstly, a 2D cell culture study was carried out to investigate the cellular response of HDFs with the crosslinked keratin-alginate mixture without the influence of topographical cues. Tissue culture polystyrene surface (TCPS) in 48-well culture plates was coated with 100 μ l of either 2 % keratin, 2 % alginate, 2% collagen or crosslinked keratin-alginate mixtures and then were allowed to air-dry at room temperature before use. Human dermal fibroblasts were harvested at sub-confluency using 0.25 % trypsin and cell numbers was calculated using a haemocytometer before being seeded on top of the coated surfaces at a density of 3,000 cells/cm². Cell viability was determined by Live/Dead[®] assay and cell proliferation was determined by Picogreen[®] assay on day 1, 3 and 7 post-seeding as previously described.

3.3.6.4. Culture of Primary Human Dermal Fibroblasts in Crosslinked Keratin-Alginate sponges

Cell culture study on crosslinked keratin-alginate sponges, keratin sponges and collagen sponges were carried out in order to evaluate the HDFs cellular behavior in a 3D environment. The sponges were produced as what previously has been described. Human dermal fibroblasts were harvested from a sub-confluent culture by using 0.25 % trypsin, and the amount of cells was calculated by using a haemocytometer. The top of the three-dimensional sponges was seeded with the harvested HDFs at a density of 50,000 cells/cm³. Cell viability and proliferation of HDFs were determined on days 1, 3, 7, 14 and 21 following seeding by means of Live/Dead[®] assay and Picogreen[®] assay as previously described, respectively.

3.3.6.5. Histology of Cross-sectional Sponges

In order to evaluate the distribution of HDFs on crosslinked sponges, histology sectioning of the crosslinked sponges' cross-sections was carried out for samples cultured with primary HDFs on day 14.

Briefly, in preparation of histology sectioning, the samples were washed with PBS 3 times and fixed in 4% Paraformaldehyde overnight at 4 °C. The samples were then

dehydrated with 70% Ethanol, 80% Ethanol, 90% Ethanol, 100% Ethanol and 100% Xylene (each steps was repeated twice). After the dehydration step was completed, the samples were fixed with paraffin at 65 °C for 2x2 hours inside a tissue cassette. The paraffin-embedded sponges were then sectioned into 5 µm thickness. The histology sections were then deparaffinized and stained with hematoxylin and eosin (Thermo Fisher Scientific, USA) afterwards in order to visualize the distribution of the HDF cells.

3.3.6.6. Evaluation of ECM Production by Immunohistochemistry

Production of extracellular matrix was evaluated by immunofluorescence staining of the cross-sectional histology section of the cultured crosslinked keratin-alginate sponges. Initially, prior to staining process, the sections underwent deparaffinization and antigen retrieval. The sections' slides were rehydrated by heating the slides to 65 °C for 30 min and dipping them in 100% Xylene, 100% Ethanol, 90% Ethanol, 80% Ethanol, 70% Ethanol and water. The rehydrated sections were then subjected to antigen retrieval treatment by immersing the slides on antigen retrieval buffer (sodium citrate, pH 6) for 20 mins in 95 °C. Following the antigen retrieval step, the slides were cooled by dipping in running tap water prior to blocking. The sections were washed with PBS and then blocked with blocking solution (1% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline-0.05% Tween 20) for 1 hour under room temperature. Subsequently, the blocked sections were incubated with the primary antibody, either with 100 µL of rabbit polyclonal anti-collagen III (Abcam AB7778, 1:200 in blocking solution) or rabbit polyclonal anti-fibronectin (Abcam AB2413, 1:250 in blocking solution) for 16-18 hours on 4 °C. After the incubation was completed, the sections were washed with PBS 3 times and incubated with the fluorophore-conjugated secondary antibody, goat anti rabbit alexa fluor® 488 (Invitrogen, USA, 1:200) for 1 h in the dark at room temperature. Following the completion of the second incubation, the sections were washed with PBS 3 times and mounted with Prolong® Gold AntiFade with DAPI mounting medium (Invitrogen, USA) prior to imaging with fluorescence microscope. Deparaffinized slides stained with only the secondary antibody were used as negative control.

3.3.6.7. Evaluation of Growth Factor Expression

Growth factor expression was measured using the Human Angiogenesis Multiarray kit from RnDsystems, USA. Briefly, the culture medium after 7 and 14 days of culture were centrifuged to remove cell debris and then 200 to 700 μL of the supernatant were used for the assay. The array membranes were blocked with the blocking buffer (Array buffer 7) for 1 h under room temperature prior to overnight incubation at 2-8 $^{\circ}\text{C}$ with the sample/antibody mixtures (culture medium added with 0.5 mL of array buffer 4, volume adjusted with array buffer 5 until 1.5 mL and added with 15 μL of detection antibody cocktail). Subsequently, the membranes were washed with the wash buffer 3 times and then incubated with the Streptavidin-HRP working solution (1:200 in Array buffer 5) for 30 mins. After the incubation, the membranes were washed with washing buffer for 3 times and then placed onto individual plastic sheet protector. One milliliter of Chemi reagent mixture (chemiluminescent reagent) was pipetted onto the membranes. The incubation with the mixture was carried out for 1 minute prior to chemiluminescent imaging of the membranes using an image analyzer, ImageqQuant LAS 4000 (GE Life Sciences, USA) . Exposure time was set at 2 mins. Prior to the study, a control using serum-supplemented medium was run to confirm negligible interference from the serum. The resulting image was analyzed for its pixel density using Image Studio software. The pixel density was calculated and normalized with the cell numbers.

3.3.7. Statistical Analysis

All values were represented as means $\pm$ standard deviation. The number of samples, n , equals to 3 to 6, depending on the experiment performed. Statistical analyses were carried out using one-way ANOVA followed by Tukey's test as a post-hoc test where p values less than 0.05 indicated significant differences.

Analysis of variance (ANOVA) is known as a type of a statistical model which is used to analyze whether there are any significant differences existed among groups as well as between groups. By performing ANOVA statistical test, finding out whether the means of several groups are equal or significantly different become possible. The use of ANOVA is appropriate for comparing an experimental setting or treatment with three

or more groups or variables for statistical significance. Performing ANOVA would avoid a statistical type I error that is often encountered when performing multiple two sample t -tests to compare several groups of samples. When there are only two groups of samples, the usage of t -test as a statistical test would be deemed more appropriate. However, ANOVA would only be able to point out whether there is a difference among or between groups. In order to pinpoint which groups show statistical difference to among several groups, a post-hoc test is required. Tukey's test was chosen as a post-hoc test in this study. By comparing the different in means between two groups and divide it with the standard error, q_s value was obtained. The obtained q_s value was then compared with the q value calculated from the studentized range distribution in order to determine whether there was any significant difference among the two groups [22].

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Chapter 4

Fabrication and Characterization of Crosslinked Keratin-Alginate Sponges

This chapter discusses the result of the fabrication and characterization of crosslinked keratin-alginate sponges. Keratin was successfully extracted from human hair and the extracted keratin was successfully crosslinked with alginate as confirmed with free amine groups determination as well as analysis of IR spectra. Porous and flexible crosslinked sponges was obtained through freeze drying and sponges with higher crosslinking degree and higher alginate content were confirmed to increase the strength and modulus of the resulting material (from tensile, compression and flexural tests). Moreover, higher alginate content proven to increase the water uptake capacity by up to 6 times its original weight while higher crosslinking degree was shown to increase the water uptake capacity to a certain point before reducing it. The crosslinked sponges were also shown to exhibit lower water vapor transmission rate, a characteristic that is desirable for wound dressing applications, compared to commercially available wound dressing Kaltostat®. Additionally, sponges with the highest keratin content were shown to be degraded by proteinase K by up to 75% of their original weight. Therefore, these results confirmed the potential of producing keratin-hybrid materials with tunable physical and mechanical properties for biomedical purposes (such as cell carriers and wound dressing) by means of varying crosslinking extent or the composition of keratin and partnering material (alginate).

4.1. Extraction of Human Hair Keratin

Human hair keratin was obtained in the form of freeze dried flaky powders (Figure 4.1.). Result from SDS PAGE displayed that with coomassie blue staining the keratin protein's bands were discovered as monomers within the smaller range of 40-60 kDa. These results confirmed that the reduced form of keratins which are soluble in water were present in the freeze-dried powder form. Bands in the large molecular weight area (>100 kDa) were also identified, representing the presence of keratin dimers, meanwhile weak bands in the smaller molecular weight area (>20 kDa) which represents the existence of matrix proteins were also observed (Figure 4.2.)

Figure 4.1. Freeze dried keratin powder

Figure 4.2. Coomassie blue-stained SDS PAGE gel of keratin

The IR spectrum shown (Figure 4.3) displayed the existence of amide band I, II and III for both freeze-dried and solution forms at 1655 cm^{-1} , 1543 cm^{-1} and 1240 cm^{-1} , respectively. The spectrum also displayed the signature N-H stretching vibration peaks of amides A and B at 3301 cm^{-1} and 2925 cm^{-1} , respectively for both groups. No difference was observed between the keratin's spectrum from the reconstituted freeze-dried keratin powder and the fresh keratin solution, indicating that keratin did not undergo any significant chemical or conformational changes during storage. Meanwhile, in the alginate sample, the identification peaks of mannuronate and guluronate groups were also evident.

Figure 4.3. IR spectra of reconstituted freeze-dried keratin powder and freshly extracted soluble keratin solution and alginate

4.2. Fabrication of Crosslinked Keratin Alginate Sponges

Figure 4.4. Crosslinked keratin alginate sponges

The crosslinked keratin-alginate sponges obtained after freeze-drying were porous and flexible. The resulting sponges were shown to have porous structure with interconnected pores. The average pore area obtained from ImageJ analysis showed that with increasing crosslinking degree the average pore area decreased.

Figure 4.5. SEM image of crosslinked keratin alginate sponges with different EDC concentration

Figure 4.6 Average pore area (μm^2) of crosslinked keratin alginate sponges with different EDC concentrations ($n=3$, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

The results of the crosslinking degree study showed that the higher the crosslinking concentration used, the higher the crosslinking degree would be achieved. Based on the ninhydrin reaction, the amount of free amine bond presents in the crosslinked keratin-alginate mixture would correlate with the intensity of the color on the supernatant and the absorbance on the visible area (570 nm), indicating that lower absorbance level was the result of higher crosslinking degree.

Figure 4.7 Crosslinking degree of crosslinked keratin-alginate sponges with varying EDC concentration ($p<0.05$, ANOVA, Tukey's test vs 1 mM, $n=3$)

Our results revealed that with 10mM of EDC, 70 % of crosslinks were able to be achieved. Further increase of the crosslinking concentration only resulted in a marginal rise of the crosslinking degree, as increasing the EDC concentration by 10-fold (100 mM) only gave 82.1 ± 1.3 % crosslinking (Figure 4.7).

Figure 4.8. IR spectra of crosslinked keratin-alginate sponges with varying EDC concentration

The obtained FTIR spectra of the crosslinked sponges signified the formation of amide linkages by registering increases in the amide II and III peak intensities observed at 1555 cm^{-1} and 1260 cm^{-1} , respectively, as EDC concentration increased (Figure 4.8). Additionally, reduced intensities of the peaks between the regions 1190 to 974 cm^{-1} and 1480 to 1370 cm^{-1} were also observed, indicating that reduction of mannuronate and guluronate groups of alginate, respectively, occurred with the increasing crosslinking degree. Furthermore, the shoulder peaks detected in the amide I band of the non-crosslinked samples and samples with low crosslinking degree (KA11-1mM) were found to be disappearing in samples with high crosslinking degree (KA11-10mM and KA11-100mM), indicating that the COO^- stretching in alginate (1600 cm^{-1}) was masked by C=O stretching within amide groups, further confirming the occurrence of

successful crosslinking. Though less evident, the narrowing amide A peak suggested that the previous N-H stretching band that is masked by the broad O-H stretching bend (usually from 2500-3500) became more apparent indicating less O-H groups (from carboxylic acid of alginate) were detected and further confirming successful COOH conversion into amide linkages .

In addition to the changes in peaks' intensity, shifts in amide I and amide II peaks were also detected in samples with high crosslinking degrees (KA11-10mM and KA11-100mM). The amide I peaks of non-crosslinked and low-crosslinking-degree sponges (KA11-0mM and KA11-1mM) were identified at the 1655 cm^{-1} region, indicating a majority of α -helical structure. Meanwhile, amide I peak of high-crosslinking-degree sponges (KA11-10mM and KA11-100mM) shifted to the 1645 cm^{-1} region, demonstrating a majority of random structures. Moreover, a shift of amide II peak from 1540 cm^{-1} to 1555 cm^{-1} detected on sponges with higher crosslinking extent (KA11-10mM and KA11-100mM) suggested less proportion of α -helical structures upon crosslinking, further confirming the effect of crosslinking on the secondary structure of keratin.

Figure 4.9. Reconstructed image of curve-fitting analysis on amide I band profile obtained from FTIR spectroscopy for KA11-100mM.

Further analysis of the FTIR spectra was carried out by performing curve-fitting on amide I band region in order to estimate the relative proportion of each components of keratin's secondary structure before and after crosslinking (Figure 4.9). Based on the calculation of the relative area under curve, it was revealed that the keratin's structure

transformed into a more unordered structure with the increasing crosslinking degree. Before crosslinking, it was shown that there are similar proportion of α -helix and β -sheet structure. Meanwhile, it was found that increasing the crosslinking degree up to 80% resulted in decreasing the relative proportion of α -helical structure (down to 30% from 50%) and β -sheet structure (down to 20% from 50%), alongside increasing the relative proportion of random coil or unordered structure (up to 50%) (Figure 4.10). This finding from the curve-fitting analysis further support the understanding that performing crosslinking altered the secondary structure of protein.

Figure 4.10. Percentage of amide I band secondary structure composition based on relative AUC

4.3. Compression Study

Sponges fabricated only from keratin (2% w/w) were too fragile and brittle to be undergoing any mechanical testing, thus the result of the mechanical study for this group was not reported. Result for compression tests displayed that compression moduli of crosslinked keratin-alginate sponges increased together with crosslinking degree (Figure 4.11). The average modulus of the sponges with the highest crosslinking degree of 82.1 ± 1.3 % (EDC concentration of 100 mM) was 93.2 ± 5.7 kPa, which was a two-

fold increase compared to the sponges with the lowest crosslinking degree of 15.9 ± 7.8 %.

Figure 4.11. Compression modulus of crosslinked keratin-alginate sponges with different EDC concentration ($n=3$, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

Additionally, the compression tests performed on crosslinked sponges with different keratin-alginate ratios showed that higher ratio of alginate resulted in higher compression moduli, where samples with the highest proportion of alginate incorporated into the keratin-alginate sponges (KA14-10mM) displayed an increase of moduli up to 219 ± 52 kPa, an 8-fold increase compared to sponges with the lowest alginate content (KA41-10mM) (Figure 4.12).

Figure 4.12. Compression modulus of crosslinked keratin-alginate sponges with different keratin-alginate composition (n=3, $p < 0.05$, ANOVA, Tukey's test vs KA11-10mM)

Compression tests' results revealed that performing chemical crosslinking by using EDC to facilitate amide bonds' formation between keratin and alginate was proven to improve the stiffness of the resulting material. The utilization of EDC crosslinking has been reported in other systems such as from the study conducted by Harley et al. [1]. The study reported that EDC-crosslinked collagen-glycosaminoglycan scaffolds were able to improve the compression modulus up to 7.2 kPa, 3.5-fold increase in comparison to those with the lowest crosslinking density. Likewise, another study performed by Calderon et al also revealed that an increase in compression moduli from 1.4 kPa (0 mM EDC) to 8.6 kPa (48 mM EDC, highest crosslinking degree) were observed on EDC-crosslinked collagen-hyaluronan hydrogels [2]. The result of these studies was in agreement with our findings.

4.4. Tensile Study

Previous study on alginate-based wound dressing has measured the tensile strength and tensile modulus of Kaltostat® (commercially-available alginate-based dressing) with values of 0.87 ± 0.12 MPa and 1.30 ± 0.19 MPa, respectively [3]. However, due to different size, format (fibrous sheet) and possible different nature of alginate used in

Kaltostat® (different molar mass, gulluronate and manuronate composition, etc) compared to the alginate used in this study, these reported values should serve as qualitative comparison with the data generated from the keratin-alginate sponges used herein .

Tensile tests' results (Figure 4.13) showed that the tensile moduli of sponges with the highest crosslinking degree (KA11-100mM) were increased by about 4 times, from 36.2 ± 5.5 kPa to 135.4 ± 23.1 kPa, compared to those with the lowest crosslinking degree (KA11-1mM). The results of the tensile tests (Figure 4.14) also exposed that sponges with the highest crosslinking degree (KA11-100 mM) underwent an increase of ultimate tensile strength up to 2 times from 3.7 ± 0.5 kPa to 8.0 ± 0.8 kPa in comparison to less crosslinked sponges (KA11-1mM).

Figure 4.13. Tensile modulus of crosslinked keratin-alginate sponges with varying EDC concentration (n=3, $p < 0.05$, ANOVA, Tukey's test vs KA11-0mM)

Figure 4.14. Ultimate tensile strength of crosslinked keratin-alginate sponges with varying EDC concentration (n=3, $p < 0.05$, ANOVA, Tukey's test vs KA11-0mM)

Additionally, it was also observed that sponges with highest proportion of alginate content displayed an increase in tensile modulus and ultimate tensile strength in comparison to those with lower proportion of alginate (Figure 4.15 and 4.16). Therefore, these findings confirmed that higher alginate content or higher crosslinking degree would improve the overall strength and stiffness of the resulting keratin-alginate sponges, which was similar with the trend observed in the compression tests' results.

Figure 4.15. Tensile modulus of crosslinked keratin-alginate sponges with varying keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-10mM)

Figure 4.16. Ultimate tensile strength of crosslinked keratin-alginate sponges with varying keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs KA11-10mM)

Previous study has shown improvement of strength and stiffness by crosslinking. Study by Naficy et al reported that crosslinked alginate and Polyacrylamide hydrogels (with 1% alginate) [4] also recorded an improvement of ultimate tensile strength and Young's modulus, with an increase detected up to 4 times from about 5 kPa to 20 kPa and from about 7 kPa to 30 kPa, respectively, when compared to non-crosslinked hydrogels. In comparison to this system, the keratin-alginate sponges produced in our study exhibited more superior mechanical properties. Another study with similar observation of mechanical properties' improvement were also reported by Chui et al. The study confirmed that crosslinking alginate with Polyethyleneimine and Ethylenediamine with the facilitation of EDC improved the tensile strength of the resulting sponges up to 13 and 8 times compared to those that contained alginate only, respectively [5]. Similar to our findings, these results suggested that the formation of crosslinking provided a more stable structure of the resulting scaffold, resulting in improved mechanical properties.

4.5. Three-Point Bend Study

The result obtained from three-point bent test revealed that the flexural moduli increased with increasing crosslinking degree. The sponges with the highest crosslinking degree of 82.1 ± 1.3 % (EDC concentration of 100 mM) had an flexural modulus value of 730 ± 141 kPa while those with the lowest crosslinking degree of 15.9 ± 7.8 % (EDC concentration of 1 mM) had an average flexural modulus value of 313 ± 19 kPa, a two-fold increase of the value (Figure 4.17). It was also revealed from the three-point bend test that the flexural modulus result showed similar trend to other mechanical test results, where sponges with higher alginate content displayed higher flexural modulus (Figure 4.18).

Figure 4.17. Flexural modulus of crosslinked keratin-alginate sponges with varying EDC concentration (n=3, $p < 0.05$, ANOVA, Tukey's test vs KA11-0mM)

Figure 4.18 Flexural modulus of crosslinked keratin-alginate sponges with varying keratin-alginate composition ($n=3$, $p<0.05$, ANOVA, Tukey's test vs KA11-10mM)

EDC-crosslinked system has displayed improvement in flexural moduli as shown by previous earlier studies. Demineralized dentin collagen was found to have significantly increased its flexural moduli up to 1.5 times just after 60 s of EDC crosslinking [6]. Covalent crosslinking formed by the formation of amide linkages was proven to provide a more stable mechanical structure compared to the non-crosslinked system.

4.6. Water Uptake Capacity

Figure 4.19. Water uptake capacity of keratin-alginate sponges with varying EDC concentration ($n=3$, $p<0.05$, ANOVA, Tukey's test vs KA11-0mM)

Result from the water uptake study revealed that the crosslinked keratin alginate sponges have the capability to uptake water approximately 7 times of their original weight (Figure 4.19). It was also discovered from the water uptake capacity data (Figure 4.20) that with increasing crosslinking degree the water uptake percentage would increase to a peak of approximately 600 % (with EDC concentration of 10mM), before subsequently dropping to approximately 100 % of its original weight when the crosslinking degree reached the highest (when 100 mM EDC was used).

Moreover, it was also observed from this study that higher alginate ratio in a crosslinked keratin-alginate sponge resulted in higher water uptake capacity, suggesting that increased hydrophilicity and possibly increased swelling capability were accomplished with the increasing alginate weight ratio (Figure 4.19).

Figure 4.20. Water uptake capacity of keratin alginate sponges with varying keratin-alginate composition (n=3, $p < 0.05$, ANOVA, Tukey's test vs KA11-10mM)

In biomedical applications, cell and biomolecule exchanges mainly happens in an aqueous environment. Therefore, with this understanding it is important to consider about water uptake capability when designing 3D matrices [7]. Generally as the degree of crosslinking increases, the hydrophilicity and water uptake capacity of a material is expected to be reduced [8]. In our system, water uptake capacity of the sponges revealed to be increased initially with increasing crosslinking density before peaking when 10 mM EDC was used (70 % crosslinking density). As crosslinking density increased

further and reached a plateau when 100 mM EDC was used (reaching 80 % crosslinking density), the water uptake capacity would drop significantly. This phenomena were observed since when crosslinking density was too low, the sponges were likely too weak to maintain a stable form in aqueous environment, thus limiting their water uptake and retention capacity. Instead, further increasing crosslinking degree would result in a more rigid structure with smaller pore sizes, thus restricting the water permeation as well as the sponge's ability to swell and ultimately resulting in a decrease in water uptake capacity. With these findings, the crosslinked keratin-alginate system offers a tunable system which allows crosslinking degree to be adjusted, providing the possibility to achieve optimal balance between mechanical properties and water uptake capacity.

4.7. Water Vapor Transmission Study

It was discovered from the water vapor transmission rates' (WVTRs) result that keratin alginate sponges were able to retard water vapor transmission when compared to alginate only sponges and Kaltostat®, regardless of the crosslinking degree (Figure 4.21). Furthermore, sponges with the highest crosslinking degree (EDC concentration of 10 mM) and sponges with high keratin content (KA21-10mM and KA41-10mM) were found to exhibit lower water vapor transmission rate to the same extent as Aquacel® ($p>0.05$), a dressing that sustains a moist wound bed by maintaining moisture in the wound.

Figure 4.21. Water vapor transmission rate of crosslinked keratin alginate sponges with varying EDC concentration (n=3, $p<0.05$, ANOVA, Tukey's test vs Kaltostat®)

Separately, WVTR measurement performed on sponges with different keratin-alginate ratios showed that sponges with higher alginate content exhibited higher water vapor transmission compared to sponges with higher keratin content ($p<0.05$). This trend was observed probably as a result of lower hydrophilicity of keratin compared to alginate (Figure 4.22).

Figure 4.22. Water vapor transmission of crosslinked keratin alginate sponges with varying keratin-alginate composition (n=3, $p<0.05$, ANOVA, Tukey's test vs Kaltostat®)

The importance of the measurement of water vapor transmission rate across different sponges and commercial dressing is related to their possible application as wound dressing. In order to provide an environment that is ideal for wound healing, it is necessary to keep the wound bed in an environment with enough moisture. With this reasoning, low water vapor transmission rate is desired when designing a dressing, since the dressings' capability to retain water and prevent loss of moisture would avoid the unwanted wound desiccation [9].

Kaltostat® is a non-woven fibrous sodium calcium alginate dressing that is commercially available. This dressing is known to have the capability to absorb wound exudates and keeping moist state around the wounds as well as having an advantage

over gauze as a wound dressing due to its easy removal from the wound tissue, unlike gauze that have the tendency to adhere to the wound thus hindering wound healing process as well as causing pain during removal of dressing [3]. Similar to Kaltostat[®], Aquacel[®] is a sodium carboxymethylcellulose fibers that also has the ability to provide moisture environment to wound, absorb wound exudates and minimize pain during dressing removal [10].

The capability of keratin-alginate sponges to hinder water vapor transmission was found to be comparable to commercially available dressings such as Aquacel[®] and Kaltostat[®]. This discovery was a significant finding in this study in regards to one of their potential applications as a wound dressing, where low water vapor transmission rate is necessary in order to limit wound desiccation and support moist wound healing [9].

4.8. Degradation

Crosslinked keratin-alginate sponges were subjected to enzymatic degradation by using two different enzymes, namely proteinase K and chymotrypsin, with the purpose of investigating their degradation profile. These two enzymes are commonly used and known to degrade keratin, which allows convenient comparison with other matrices reported in the literature[11]. Proteinase K is a broad-range protease that cleaves the carboxyl terminal group on the hydrophobic amino acids and widely known to have the capability to digest keratin. It is also known to be widely used in removing proteins in nucleic acid preparation (usually with concentration of 0.05-1 mg/mL)[12, 13]. Additionally, chymotrypsin, as a digestive enzyme which exists in the physiological environment that is also known to have the ability to cleave the carboxyl terminal group on the hydrophobic amino acid, was used as a comparison. Additionally, previous studies has used both chymotrypsin and proteinase K for digestion and protein determination purposes such as cartilage digestion and determining type II collagen (working concentration 1 mg/mL)[14, 15].

By carrying out this study, the effect of increased degree of crosslinking to the degradability and rate of degradation of keratin and its crosslinked composite could be confirmed. In addition to those, by learning about the degradation profile of the

matrices, it would be possible to include extrinsic proteases to the matrices in order to tune and control their degradation rate, depending on the intended application and period of usage.

The acquired results showed that sponges with highest keratin ratio (KA41-10mM) lost 74.7 ± 4.5 % of their initial weight while sponges with the highest alginate ratio (KA14-10mM) only lost 17.5 ± 3.7 of their original weight. (Figure 4.23), indicating that keratin underwent enzymatic degradation by proteinase K. Additionally, degradation result also demonstrated that significant difference in weight loss percentage ($p < 0.05$) over the period of four weeks were discovered on sponges with lowest degree of crosslinking (KA11-1mM) compared to sponges with highest crosslinking density (KA11-100mM). Within a period of 30 days, it was recorded that crosslinked sponges also underwent weight losses in the range of 7.9 – 18.7 % of their original weight on the control (Tris buffer) and chymotrypsin treated group. This phenomenon was made possible likely due to the partial dissolution or surface erosion of the sponges. Nevertheless, there was no significant difference in weight loss percentages discovered between different samples within control and chymotrypsin groups, suggesting crosslinked keratin-sponges were not degraded by chymotrypsin, implying that there was insignificant proteolytic activity exhibited by chymotrypsin towards keratin.

On the other hand, sponges with different keratin-alginate ratios exhibited variable degradability by proteinase K. It was discovered that sponges with higher alginate ratio managed to retain at least 76.3 ± 1.9 % of their initial weight intact after 30 days. Meanwhile, sponges with higher keratin ratio were only able to maintain about 25.26 ± 4.5 % of their initial weight over the same period of time, signifying that keratin was more prone to degradation by proteinase K compared to alginate (Figure 4.24). Crosslinked sponges with high keratin-content exhibited higher degradation rate, where only 50% of the initial weight remained after being subjected to Proteinase K degradation for 7 days.

Our study concluded that the resulting sponges were still enzymatically degradable by Proteinase K regardless of being subjected to chemical crosslinking. Previous finding by Yamauchi et al [11] revealed that keratin fibers and films from human hair treated with proteinase K (the concentration of proteinase K used was much higher than those

used in this study) were able to reach up to 60% degradation of their original weight. It was reported by Bressolier et al [16] decades ago that proteinase K demonstrated higher proteolytic activity to keratin in comparison to chymotrypsin even though both of the proteases were known to hydrolyze the carboxyl terminal groups of hydrophobic amino acid [17]. The difference in proteolytic activity could be attributed to differences in either the specificity or accessibility of the enzymes to the substrates, especially in a three-dimensional setting. Our results came in agreement with these previous studies in regards to the finding that proteinase K were able to degrade crosslinked keratin-alginate sponges more efficiently than chymotrypsin (which showed little proteolytic activity towards keratin). This finding provide a possibility that templates based on human keratin, such as the crosslinked keratin-alginate sponge investigated in this study, can be further developed into a degradable and bioactive wound dressings, which could be useful as 3D scaffolds to support wound healing or as dissolvable dressings to minimize pain during dressing change.

Figure 4.23. Remaining weight percentages of crosslinked keratin-alginate sponges with varying EDC concentration vs time of treatment with α -chymotrypsin, proteinase K and tris buffer (control), (mean $\pm$ SD, n=3)

Figure 4.24. Remaining Weight percentages of Keratin Sponges, Alginate Sponges, Crosslinked keratin-alginate Sponges with Varying Keratin-Alginate Composition vs Time of treatment with α -Chymotrypsin, Proteinase K and Tris buffer (control), (mean $\pm$ SD, $n=3$)

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Chapter 5

In Vitro Cell Culture Studies of Crosslinked Keratin-Alginate Sponges

This chapter discusses about the result of L929 and HDF culture on 2D and 3D environment as well as evaluation of ECM, growth factor and cytokines production of HDF cultured on crosslinked keratin-alginate matrices. The result of this study revealed that matrices with higher keratin content enhanced the proliferation of both L929 murine fibroblasts and human dermal fibroblasts in both 2D and 3D environment compared to matrices with higher alginate content. Matrices with higher keratin composition were also shown to support cell viability as well as extracellular matrices proteins, cytokines, and growth factor production with even distribution of cells inside the matrices. Interestingly, higher keratin content were also revealed to upregulate production of tissue factor by HDF, suggesting it would be beneficial for hemostasis application. Based on these results, crosslinked keratin-alginate matrices were shown to be a promising alternative for cell carriers or wound dressing purposes.

5.1. Culture of L929 and HDF on 2D-coated Surfaces and 3D Sponges

From previous mechanical testings' results, it was revealed that increasing crosslinking degree and alginate content of the matrices would result in a significant improvement of the hybrid materials' stiffness and strength. However, since alginate was expected to have poor cell compliance, it was uncertain whether improving the mechanical properties using any of these means would compromise the biocompatibility of the resulting material. Hence, due to this reasoning, a biocompatibility assay was needed to be carried out with the aim of investigating to what extent would altering the mechanical properties (either by increasing alginate content or increasing crosslinking degree) affect biocompatibility.

L929 murine fibroblasts were cultured on the 2D-coated surfaces and the 3D matrices of the crosslinked keratin-alginate sponges in order to determine the biocompatibility and cytotoxicity of the material. L929 murine fibroblasts were chosen as the cell type to test these characteristics since they are known as an established American Society for Testing and Materials' (ASTM) standard for cytotoxicity evaluation of materials for medical use. The cytotoxicity was further determined by measuring the viability (by visualization of live and dead cells using the Live/Dead[®] Kit) and quantifying the proliferation of L929 murine fibroblasts (by calculating the total amount of double-stranded DNA using Picogreen[®] Kit). The assessment was carried out in two different groups, with varying keratin-alginate composition and varying crosslinking degree, with the purpose of examining whether any of these variations would affect the biocompatibility of the resulting product.

Our result demonstrated that the increasing crosslinking degree did not affect the proliferation rate of L929 cultured on surfaces coated with crosslinked keratin-alginate sponges with varying crosslinking degree ($p>0.05$) (Figure 5.1). Meanwhile, different result was observed with L929 cultured on crosslinked keratin-alginate sponges with varying keratin-alginate composition, whereas higher keratin content supported higher cell proliferation over a 7-day period of culture (Figure 5.2).

Figure 5.1. Relative amount of dsDNA of L929 murine fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture with varying crosslinking degree (mean $\pm$ SD, $n=4$), * $p<0.05$ vs day 7 keratin, ANOVA, Tukey's test). Data normalized against control day 1.

Figure 5.2. Relative amount of dsDNA of L929 murine fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture with varying keratin and alginate ratio (mean $\pm$ SD, $n=4$), * $p<0.05$ vs day 7 keratin, ANOVA, Tukey's test). Data normalized against control day 1.

Through staining with calcein-AM dye from the live-dead kit, the live cells were stained green meanwhile the dead cells were stained red, thus enabling us to visualize and differentiate between live cells and dead cells under fluorescent microscope, assessing their overall viability. Additionally, besides the overall viability, the calcein-AM would also allow us to examine the morphology of the cultured L929 cells. The captured images from observation under fluorescent microscope (Figure 5.3) showed that by day 7 of culture, L929 cells seeded on TCPS, collagen-coated and keratin-coated surfaces

managed to reach near 100% confluency. Likewise, L929 fibroblasts cultured on crosslinked mixture with dominant keratin content (KA41-10mMM and KA21-10mM) also exhibited confluent distribution though to a less extent (around 80-90% confluency) than the previous three groups (collagen, keratin and TCPS). L929 fibroblasts on all of these coated surfaces also exhibited a spindle-like shape morphology, which is a usual characteristic of the L929 murine fibroblasts, signifying that cells were viable and growing healthily.

A different finding was observed when L929 cells were cultured on surfaces where keratin-alginate content had equal ratio (KA11-10mM). Over 7 days of culture, no significant differences in terms of cell proliferation were observed among groups with different crosslinking degree (Figure 5.1). Nonetheless, though the morphology of the L929 cells was still normal and spindle-like, the confluency dropped to 50-60%, as confirmed by the lower dsDNA amount ($p < 0.05$) when compared with keratin groups, collagen groups, TCPS groups, as well as groups with dominant keratin content (KA41-10mMM and KA21-10mM).

Moreover, when L929 were cultured on groups with dominant alginate content (KA14-10mM and KA12-10mM), only very low cell numbers of live-cells with rounded morphology were detected. These findings signified that when alginate was added in a proportion equal to or higher than keratin in the crosslinked mixtures, the proliferation and viability would be affected, which was in contrast to those groups with higher keratin content. Groups with dominant keratin content (KA41-10mMM and KA21-10mM) showed no significant difference of dsDNA amount as well as similar confluency level when compared to keratin groups, indicating that the proliferation of the L929 cells was comparable with the keratin groups and not yet compromised with the addition of alginate (Figure 5.2 and 5.3)

Figure 5.3. Live-Dead staining images of L929 murine fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture (day 7 post-seeding).

Following the result of the 2D L929 culture studies, selected formulations were chosen to undergo further 3D culture in order to evaluate and analyze L929 cells behavior that grew on 3D environment of the crosslinked keratin-alginate matrices. Similar observation with the 2D result was recorded on L929 cultured on 3D crosslinked sponges, whereas cells cultured on groups with higher keratin content (KA21-10mM) displayed significantly higher proliferation than groups with higher alginate content after 21 days of culture. Cell proliferation plateaued after 14 days (Figure 5.4).

Figure 5.4. Relative amount of dsDNA of L929 murine fibroblasts cultured on collagen, keratin, alginate and crosslinked keratin alginate sponges. data were normalized against alginate on day 1. (mean $\pm$ SD, $n=4$), *, #, + $p<0.05$ vs keratin, ANOVA, Tukey's test) where * day 7, # day 14 and + day 21

Confocal images of Live/Dead stained cells taken at day 14 of culture showed that L929 seeded on collagen displayed a spread and spindle-like morphology meanwhile on other groups they appeared to be more rounded (Figure 5.5). Additionally, based on the significantly higher number of viable cells represented from dsDNA quantification by Picogreen® assay, collagen matrices were shown to have superior cell compliance compared to other groups. Moreover, conforming to the finding from 2D culture studies, L929 seeded on matrices containing keratin only or higher keratin content (KA21-10mM) displayed comparable ($p>0.05$) dsDNA amount over 21 days of culture. Similarly, matrices containing alginate only and dominant alginate content (KA12-10mM) exhibited lower cell number and proliferation compared to keratin, collagen and KA21-10mM groups, suggesting inferior cell compliance (Figure 5.4). These result confirmed that as a material alginate supported less cell proliferation and possessed poor cell compliance when compared to keratin.

Figure 5.5. Live-Dead staining images of L929 murine fibroblasts cultured on collagen, keratin, alginate and crosslinked keratin alginate sponges (day 7 and 14 post-seeding). Images taken using confocal microscope.

After the completion of cell culture study using L929 murine fibroblasts, culture of HDFs cells was carried out on both 2D and 3D environment. HDFs were chosen due to their critical role in wound healing and skin regeneration. On top of that, HDFs were also known to produce $\alpha_4\beta_1$ integrin, which is known to bind to LDV cell binding motifs which are present in keratin.

HDFs seeded on 2D surfaces and 3D crosslinked keratin alginate sponges showed similar trends of proliferation rate with L929 culture. It was demonstrated that HDF cultured on 2D surfaces of keratin-alginate matrices with varying crosslinking degree showed similar proliferation rate (Figure 5.6) while those cultured on keratin-alginate matrices with varying keratin-alginate ratio displayed that matrices with dominant keratin content showed enhanced proliferation rate compared to matrices with dominant alginate content (Figure 5.7).

Figure 5.6. Relative amount of dsDNA of human dermal fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture with varying crosslinking degree (mean $\pm$ SD, $n=4$), * $p<0.05$ vs day 7 keratin, ANOVA, Tukey's test). Data normalized against control day 1.

Figure 5.7. Relative amount of dsDNA of human dermal fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture with varying keratin and alginate ratio (mean $\pm$ SD, $n=4$), * $p<0.05$ vs day 7 keratin, ANOVA, Tukey's test). Data normalized against control day 1.

The level of confluency observed on 2D surfaces also revealed that within 7 days of culture HDFs seeded on control and collagen coated surfaces reached nearly 100 % confluency while those cultured on coated surfaces with dominant keratin content and equal keratin-alginate content reached 70-80 % and 40-50%, respectively. All of the cells cultured on these surfaces exhibited a spread and branched-shape typical of those

of human dermal fibroblasts (Figure 5.8). Additionally, alike to the L929 result, HDFs cultured on alginate only and high alginate content-coated surfaces displayed very low cell number as well as rounded morphology (Figure 5.8), further confirming poor cell compliance of alginate.

Biomaterials may interact with cells and induce changes such as cell phenotype, cell development, biochemical characteristics and morphologies. When a cell interacts with an external substrate, it generates traction forces that are transmitted through cellular receptors onto the ECM which in turn influences the morphology of the cell during spreading [1]. Initially, as cells adhere to the ECM through integrin receptors present on cell surfaces, extension of pseudopodia from the cell body will occur following the integrin-ligand binding. Subsequently, cells will start flattening against the substrate and rearrange their cytoskeleton, forming actin bundles and focal adhesions as well as resulting in the spreading of the cells [1-3]. In order for a cell to spread, it must exert force against its membrane and substrate and the resultant net force between the extensional and contractile forces will generate the cell morphology. Generally, the size of cellular extensions define the extent of adhesions between cell and ECM molecules since it increases the degree of signaling between the ECM to the cell [1, 4, 5]. In our study, fibroblasts exhibited a more distinguished branched shape on a collagen substrate compared to keratin, suggesting more interaction between the cells and substrate via integrin-mediated binding occurring on collagen substrate, which allowed a more defined shape of fibroblasts.

Corresponding to the 3D result of L929 culture, over 21 days of culture, HDFs cultured on collagen matrices showed higher proliferation rate compared to other groups as well as elongated and spread morphology which is typical of the fibroblast cells (Figure 5.9 and 5.10). In contrast, besides lower cell proliferation compared to collagen groups, HDFs cultured on keratin and high-keratin-content crosslinked sponges (KA21-10mM) displayed a more rounded morphology as well as clustered growth of cells. Very little to no proliferation was detected on alginate or high-alginate-content (KA12-10mM) sponges (Figure 5.9 and 5.10).

Figure 5.8. Live-Dead staining images of human dermal fibroblasts cultured on TCPS, surfaces coated with collagen, keratin, alginate and crosslinked keratin alginate mixture (day 7).

Figure 5.9. Relative amount of dsDNA of human dermal fibroblasts cultured on collagen, keratin, alginate and crosslinked keratin alginate sponges with varying keratin and alginate ratio. Data were normalized against alginate on day 1. (mean $\pm$ SD, $n=4$), *, #, + $p<0.05$ vs keratin, ANOVA, Tukey's test) where * day 7, # day 14 and + day 21

DAY 7

KERATIN

COLLAGEN

ALGINATE

KA21-10mM

KA12-10mM

DAY 14

KERATIN

COLLAGEN

ALGINATE

KA21-10mM

KA12-10mM

Figure 5.10. Live-Dead staining images of human dermal fibroblasts cultured on collagen, keratin, alginate and crosslinked keratin alginate sponges. Images were taken using confocal microscope.

It is generally known that alginate lacks cell binding motifs, thus making it biologically inactive. Common approaches carried out to increase its bioactivity include modifying alginate with cell binding peptide (such as RGD) by crosslinking [6]. In compliance to this, it was anticipated that in our experiment scaffold with higher or dominant alginate content displayed low cell compliance. Several previous studies has also reported findings that were in agreement with our result, where alginate did not support proliferation of several cell types such as fibroblasts [7, 8] or endothelial cells [9].

On the other hand, keratin has been revealed to be able to support the growth and adhesion of several type of cells (such as fibroblasts [10-13], hepatocytes [14], mesenchymal stem cells [15], endothelial cells [9] and schwann cells [16] in both 2D and 3D environment. Nevertheless, conflicting results were reported regarding the mechanism of cell attachment to keratin, specifically regarding whether there was any involvement of the β integrin subunit in cell attachment, thus indirectly answering whether the presence of LDV in keratin affect the increase in cell attachment.. Experiment from Richter et al demonstrated that the blockage of the β_1 and β_2 integrin subunit did not affect the attachment of hepatocyte to human-hair keratins significantly, suggesting that cell attachment was due to non-integrin mediated binding rather than the integrin-mediated one [14]. Contradictorily, another study showed that by blocking the β_1 and β_3 subunit, the adhesion of platelet to keratin was decreased significantly compared to the unblocked ones, indicating the involvement of these integrins in cell attachment to keratin [17]. From these earlier studies, it was suggested that the mechanism of cell attachment to keratin is dependent on cell types and possibly also involve different mechanism that is not related to integrin-mediated binding. Our study revealed that crosslinked sponges with dominant keratin content were able to support attachment and proliferation of fibroblasts by acting as scaffolds that offered mechanical and physical support. Nevertheless, further investigation regarding the mechanism of fibroblast attachment would need to be carried out. By understanding this mechanism, exploring the potential application for crosslinked keratin-alginate matrices in the biomedical field was made possible.

5.2. Matrices' Contraction

It was revealed that keratin-containing matrices displayed less contraction compared to collagen matrices. Collagen matrices experienced 50% contraction of their initial diameter while alginate, keratin and crosslinked keratin-alginate sponges could retain 75-85% of their initial diameter following 14 days of culture (Figure 5.11 and 5.12).

Contraction of the 3D scaffold was possibly caused by the contraction of myofibroblasts. During wound healing, fibroblasts would differentiate into myofibroblasts, cells which are known to possess higher contractile force compared to fibroblasts. Hence, by contracting the edges of the wound, myofibroblasts are able to assist in wound closure [18-21].

Possibly, more fibroblasts have differentiated into myofibroblasts in the collagen matrices in comparison to keratin, alginate and keratin-alginate matrices, resulting in higher contraction of the scaffold. Detection of myofibroblasts' marker such as the alpha-smooth muscle actin (α -SMA) would provide an additional confirmation regarding the matrices' contraction phenomena that occurred. However, the occurrences of α -SMA does not necessarily result in the contraction of the cells (and eventually the matrices). Hence, since the observation was done to measure matrices' contraction, the staining was not performed.

Figure 5.11. Percentage of matrices' diameter after 14 days of culture from initial diameter (mean $\pm$ SD, $n=3$), $p<0.05$ vs collagen, ANOVA, Tukey's test)

Figure 5.12. Matrices' contraction of collagen, keratin, alginate and crosslinked keratin-alginate sponges after 14 days of culture. Upper arrows represent sponges' diameter on day 0 of culture and lower arrows represent sponges' diameter on day 14 of culture.

5.3. Histology

Hematoxylin and Eosin (H&E) staining was performed on the paraffin-embedded cross-section of the crosslinked sponges seeded with HDF after 14 days of culture. The staining of the cross-section would allow us to examine the distribution of HDF inside the 3D sponges, since imaging by either fluorescent microscope or confocal microscope would only permit limited depth of visualization around the surface. Hematoxylin would stain the nuclei of the cells with deep-purple blue while eosin would stain proteins nonspecifically, generating varying degree of pink color for the cytoplasm and extracellular matrix [22]. It is necessary to evaluate the distribution of the cells inside the matrices, since homogenous distribution of the cells on 3D scaffolds is necessary for effective tissue reconstruction.

The images of the H&E-stained sections were recorded using a light microscope. The result revealed that HDFs were fairly well distributed on collagen, keratin and high-keratin content (KA21-10mM) sponges, where HDFs cultured on collagen sponges were well attached to the collagen matrices and displayed a more elongated, spread and branched morphology meanwhile the HDFs cultured on keratin-containing matrices exhibited a more rounded morphology (Figure 5.13). Very few cells were observed on alginate sponges and KA12-10mM sponges, which also evidently proved poor cell proliferation and compliance of matrices with high alginate content. Meanwhile, HDF seeded on matrices with higher keratin content (keratin and KA21-10mM sponges) displayed higher cell numbers, confirming keratin is a better material to support cell attachment and proliferation than alginate. Similar to what has been previously discussed on section 5.1, fibroblasts seeded on a collagen substrate exhibited a more distinguished branched and spread morphology compared to a keratin-containing substrate which induced a more rounded morphology. This phenomena suggested that there were more integrin-mediated binding interactions between the cells and collagen compared to keratin. Binding of integrin-receptor on the cells' surfaces to the materials would allow cells to reorganize their cytoskeletal body and undergo actin polymerization, resulting in formation of focal adhesion and cell spreading [1-3]

Figure 5. 13. Hematoxylin and eosin-stained cross section of collagen, keratin, alginate and crosslinked keratin-Alginate sponges after 14 days of culture.

5.4. Evaluation of Extracellular Matrices' Production by Immunohistochemistry

Fibroblasts are known to play important roles in wound healing and skin regeneration. During the wound healing process, fibroblasts would produce growth factor, cytokines and extracellular matrices in order to repair and reconstruct the wounded tissue. Therefore, besides evaluating the proliferation and distribution of the cells inside the crosslinked keratin-alginate matrices, it is also important to analyze the production and distribution of extracellular matrices produced by fibroblasts cultured on these matrices.

In the initial stage of wound healing, collagen III and fibronectin are actively produced by fibroblasts, prior to collagen III being replaced gradually by the tougher and more robust collagen I on the later stages of healing in order to remodel and restore the normal dermal function after wounding. The fibrin and plasma fibronectin that were crosslinked to clot blood would later be degraded by enzymes that are produced by fibroblasts (such as the matrix metalloproteinases and plasmin). Following the degradation, fibrin and plasma fibronectin would be replaced with the cellular fibronectin which then assembled into the insoluble extracellular matrix that would support the tissue. The collagen fibers which were initially in a disordered manner would eventually be crosslinked, aligned and arranged in the maturation phase until the scar tissue would regain up to 80% of its initial tensile strength [23-25].

Immunofluorescence staining of collagen III and fibronectin on the histology sections revealed that HDFs seeded on all sponges produced both collagen III (Figure 5.14) and fibronectin (Figure 5.15.). It was found that collagen III produced by HDFs cultured on keratin-containing matrices and alginate were localized and accumulated around the cytoplasm and surface of the cells. Similar observation was observed for fibronectin staining, whereas the fibronectin was also found to be produced and localized on the cytoplasm and cells' surfaces.

Comparable observation was reported by Pu et al, where HDFs cultured on PLLA-collagen 3D scaffolds displayed localized expression of collagen I and collagen III [26]. Study by Chawla et al also observed similar findings where the HDFs cultured on collagen gels produced extracellular matrices (collagen and elastin) only on the

cytoplasm and surface of the cells. They found that the fibroblasts produced lesser amount of lysyl oxidase, an enzyme that is responsible for crosslinking collagen into a more mature and stable fibrillar network, thus explaining the possible reason of the extracellular matrices' accumulation on the surface and cytoplasm of the cells [27].

Additionally, Dubin-Thaher et al reported that fibroblasts has the tendency to spread anisotropically and that the extent of spreading increased with the fibronectin density [28]. In this study, it was suggested that fibronectin was well deposited in the collagen scaffold, allowing adhesion through integrin-ECM binding and more distinctive spreading of fibroblasts. In contrast, it was observed that fibronectin production was mostly localized in the cytoplasm and surface of the cells seeded on keratin-containing matrices, suggesting a lack of fibronectin deposition on the matrices which possibly resulted in compromised spreading of the cells.

Based on our findings, it was revealed that fibroblasts cultured on crosslinked keratin sponges with high keratin content (KA21-10mM) were able to proliferate and maintain their viability as well as producing extracellular matrices' components comparable with those of keratin sponges, suggesting that the crosslinked keratin-sponges were able to support fibroblasts' growth as well as maintaining their function. This result supported our hypothesis that crosslinked keratin-alginate sponges provide a possible alternative as materials for biomedical application as cell carriers or wound dressing. However it was also noted that keratin did not perform as well as collagen.

Figure 5.14. Immunohistochemical staining of collagen III on collagen, keratin, alginate and crosslinked keratin-alginate sponges cross section on day 14 post seeding. Cell nuclei were stained with DAPI (blue)

Figure 5.15. Immunohistochemical staining of fibronectin on collagen, keratin, alginate and crosslinked keratin-alginate sponges crosssection on day 14 post seeding. Cell nuclei were stained with DAPI (blue)

5.5. Growth Factor Expression

During the process of tissue regeneration or wound healing, fibroblasts would produce extracellular matrices, cytokines and growth factor concurrently. The level of growth factor and cytokines production would influence the outcome of wound healing, angiogenesis or tissue regeneration. Therefore, we determined the growth factor and cytokines expression on HDFs cultured on various sponges in order to evaluate the changes in cellular behavior related to cell and materials interaction.

5.5.1. Tissue factor

Result showed that keratin-containing matrices (Keratin and KA12-10mM groups) induced expression of Tissue Factor (TF or also known as coagulation factor III/thromboplastin) as compared to collagen and alginate matrices where TF expression was not present or too low to be detected. It was also revealed that higher keratin portion generate higher expression level of TF, where HDF cultured on keratin matrices expressed up to 3 times TF level compared to HDF cultured on KA12-10mM sponges (Figure 5.16 and 5.17).

Tissue Factor is an integral membrane glycoprotein that binds to Factor VII/VIIa (FVII) that eventually activates Factor X (FX) and leads to thrombin generation, platelet activation and formation of fibrin clot. Expressed in many type of cells such as fibroblasts and keratinocytes, complex of TF-FVII holds a central role in coagulation and hemostasis, as well as other cell signaling pathways unrelated to clotting such as angiogenesis, tumor metastasis and inflammation. Its activation were known to be triggered by injury and inflammation [29-35].

Our result suggested that keratin-containing matrices offer a potential to be developed as hemostatic agent in comparison to collagen and alginate matrices. This was also supported by previous study from Aboushwareb et al [36] where keratin gel was shown to exhibit comparable if not better performance in comparison to the commercial hemostat available (QuickClot[®] and HemClon[®]). Keratin gel was shown to decrease blood loss in liver injury better than Hemclon[®] and comparable to Quickclot[®], as well as displaying hepatocytes' contact and cell infiltration to the matrices from histology

results, indicating tissue regeneration. Interestingly, their early experiment also revealed that contact of keratin preparations with fresh whole blood initiated excessive red blood cells (RBC) aggregation and rapid clotting. Therefore, one of the explanation for these phenomenon was suggested from our result in which keratin induces TF expression that facilitates blood clotting, making it a promising material for wound dressing for hemostatic purposes.

Further explanation of the interaction between keratin and cell where TF was activated could be hypothesized from recent studies that explored the role of Cys186 and Cys209 on TF to its coagulant activity [37]. It was revealed that by ablating the disulfide bond by mutating cysteine to serine or alanine, TF coagulant activity would be impaired [38, 39]. Meanwhile, treating cells with HgCl_2 as an oxidizing agent that transform dithiols into disulfides was revealed to increase the activity of TF [37, 40]. Other study performed by Usha et al also showed that exposure of oxidizing agent results in increased coagulant activity of TF, although they indicated that the increase arisen from increased anionic phospholipid formation rather than increased disulfide bond formation [41]. Keratin is a cysteine rich protein with higher amount of thiol groups compared to other protein. Hence, it is suggested that this characteristic of keratin might play a role in the induction of TF expression on fibroblasts cells.

Figure 5. 16. Growth factor and cytokines expressions from HDFs after 14 day of culture on various 3D sponges. Detected by multiarray membranes. Images taken using the image analyzer. Names and coordinates of analytes are listed on Table 5.1.

Table 5.1. List of coordinates and analytes on the multiarray membranes.

Coordinates	Analytes	Coordinates	Analytes	Coordinates	Analytes
A1	Control	C1	GDNF	E1	Serpin B5
A2	-	C2	GM-CSF	E2	Serpin E1
A3	Activin A	C3	HB-EGF	E3	Serpin F1
A4	ADAMTS	C4	HGF	E4	TIMP-1
A5	Angiogenin	C5	IGFBP-1	E5	TIMP-4
A6	Angiopoetin-1	C6	IGFBP-2	E6	TSP-1
A7	Angiopoetin-2	C7	IGFBP-3	E7	TSP-2
A8	Angiostatin	C8	IL-1 β	E8	uPA
A9	Amphiregulin	C9	IL-8	E9	Vasohibin
A10	Artemin	C10	TGF- β 1	E10	VEGF
A11	-	C11	Leptin	E11	VEGF-C
A12	Control	C12	MCP-1	E12	-
B1	Tissue Factor	D1	MIP-1a	F1	Control
B2	CXCL16	D2	MMP-8		
B3	DPPIV/CD26	D3	MMP-9		
B4	EGF	D4	NRG1- β 1		
B5	EG-VEGF	D5	Pentraxin 3		
B6	Endoglin	D6	PD-ECGF		
B7	Endostatin	D7	PDGF-AA		
B8	Endothelin-1	D8	PDGF-AB		
B9	FGF-1	D9	Persephin		
B10	FGF-2	D10	Platelet Factor -4		
B11	FGF-4	D11	PIGF		
B12	FGF-7	D12	Prolactin

Figure 5.17. Wound Healing Scheme

Figure 5.18. Relative expression of growth factor, cytokines and enzymes produced by HDFs after 14 days of culture on collagen, keratin, alginate and crosslinked keratin alginate matrices.

5.5.2. CD26

Expression of CD26 was found to increase on HDF cultured on collagen and keratin-containing matrices (Figure 5.16 and 5.17).

CD26 is a protein that is distributed widely in tissues and expressed by fibroblasts, endothelial cells, epithelial cells and also lymphocytes. CD26 participates in immunologic and inflammatory reaction and is known to interact with adenosine deaminase (ADA) to support T cell proliferation. During injury or inflammation, the expression of CD26 was up-regulated during the activation of T cell [42-47].

Our result demonstrated the expression of CD26 on HDF cultured on collagen and keratin-containing matrices, while no expression of CD26 was detected on HDF cultured on alginate matrices. This finding suggested that collagen and keratin-containing matrices triggered inflammation reaction compared to alginate matrices that is known to be bioinert.

5.5.3. Pentraxin 3

Pentraxin 3 (PTX3) is a glycoprotein produced by fibroblasts, endothelial cells, macrophages, myeloid cells and dendritic cells. Its synthesis is stimulated by primary inflammatory signals involved in the inflammation process such as IL-1 β and TNF- α . Some of its roles are modulation of inflammation, modification of angiogenesis and participation in extracellular matrix formation [48-51]. Several studies have demonstrated the utilization of PTX3 as promising marker for inflammation in various diseases such as inflammatory cardiovascular diseases [49]. Additionally, an interesting study by Napoleone et al demonstrated that PTX3 could upregulate TF expression on endothelial cells and monocytes, thus suggesting its role in coagulation and wound healing [52, 53].

Our result revealed that PTX3 was expressed by HDF cultured on keratin-containing matrices (KA12-10mM and keratin groups) on day 14 and collagen matrices. Meanwhile, the expression of PTX3 was not observed in HDF cultured on alginate matrices. As previously mentioned, PTX3 expression indicates inflammatory responses, suggesting that immune reaction was evident on HDF cultured on collagen and keratin-containing matrices (Figure 5.16 and 5.17).

5.5.4. Interleukin-8

Interleukin-8 (IL-8) is a chemokine produced by fibroblasts, keratinocytes, epithelial cells, smooth muscle cells, monocytes, lymphocytes and endothelial cells. It acts as a major bioactive chemoattractant during inflammation and wound healing. It is known to play a role in increasing keratinocyte migration and proliferation, possibly by inducing expression of matrix metalloproteases (MMPs) thus stimulating tissue remodeling. IL-8 expression was found to be increased in acute wounds and high expression of IL-8 was also found to decrease collagen-lattice contraction by fibroblasts. [54-60] Study by Cox et al revealed that secretion of IL-8 was detected on a higher level at formulations that support robust dermal fibroblast proliferation and migration [61]

Our study found that high expression of IL-8 was observed on HDF cultured on collagen and keratin-containing matrices, whereas expression of IL-8 on HDF cultured on alginate matrices was not detected (Figure 5.16 and 5.17). Since IL-8 expression signifies inflammation, this result suggested that HDF cultured on these matrices exhibit higher level of inflammation compared to HDF cultured on alginate matrices.

5.5.5. MMP-9

Matrix metalloproteinase-9 (MMP-9) is a member of the matrix metalloproteinase family which is a class of enzyme involved in the degradation of extracellular matrix components such as several types of collagens during various physiological processes (e.g angiogenesis, cell migration and wound healing) or pathological processes (e.g tumour metastasis). Its involvement in the breakdown of ECM will result in collagen contraction that will aid the closure of wounds. Other than tissue remodelling, MMP-9 is also known to play a role in the inflammatory response by modulating cytokines and chemokines activities [62-66].

Our study showed that MMP-9 was not detected on alginate matrices while cells cultured on collagen matrices showed higher level of MMP-9 expression by two-fold compared to keratin matrices (Figure 5.16 and 5.17). This result might explain the reason why keratin matrices exhibited lower matrix contraction compared to collagen

matrices. The explanation of the MMP-9 downregulation on HDF cultured on keratin-containing matrices could be conjectured from the study done by Galis et al. which revealed that treating macrophages with N-Acetyl cysteine lowered MMP-9 expression [67]. Another study involving cysteine-containing nutrients also demonstrated the decrease in MMP-9 expression [68]. This phenomena could be the result of cysteine-switch involved in activating MMP-9 [69]. Keratin as a cysteine-rich protein might play a role in inhibiting the activation of MMP-9 and thus as a result diminished the MMP-9 expression.

5.5.6. MCP-1/CCL2

Monocyte chemoattractant protein (MCP-1/CCL2) is a chemokine that attracts monocytes, T cells and Natural Killer (NK) cells. This chemokine is produced by many cell types such as epithelial, endothelial, fibroblast, smooth muscle and monocytic cells. MCP-1 plays a role in immune or inflammatory response by modulating the migration and infiltration of monocytes, T cells and NK cells, thus making its involvement in several diseases expected [70]. Studies showed that MCP-1 is involved in the inflammatory and healing process of skin and diabetic wounds, cardiovascular diseases and cancers [70-73].

Our result showed that MCP-1 was expressed by HDF cultured on collagen matrices while no or very low expression was identified on keratin-containing and alginate matrices (Figure 5.16 and 5.17). The study by Adhami et al could offer an explanation to this occurrence. Adhami et al. demonstrated that treatment of N-acetyl-S-farnecyl-L-cysteine (AFC) suppressed MCP-1 production by human dermal microvascular endothelial cells (HMEC-1). The mechanism was not fully understood. However it was hypothesized that cysteine analogs inhibits G-proteins coupling receptor signaling, thus inhibiting MCP-1 expression induced by ATP [74]. Based on this, the suppression of MCP-1 expression on HDF cultured on keratin containing matrices could be related to keratin's characteristic as a cysteine-rich protein.

5.5.7. Angiopoietin-1, Angiopoietin-2

Ang-1 and -2 are known as ligands for the Tie-2 receptor. Ang-1 is an agonist for Tie-2 which induces the tubule formation, migration and survival of endothelial cells. Ang-1 has a role in angiogenesis by promoting the structural integrity of blood vessels in vivo. On the other hand, Ang-2 is a Tie-2 antagonist and thus it interferes with Ang-1-induced vascular stabilization which eventually increases their sensitivity to other proangiogenic factors. It was known from previous study that expression of Ang-1, Ang-2 in fibroblasts/myofibroblasts significantly increased in early scars and then decreased in older scars. The study concluded that the level of expression was also depending on the scar's age [75-77].

Result from our study displayed that these proteins were detected only on keratin and collagen matrices, where Ang-1 expression on day 14 was 5 times higher on HDF cultured on collagen matrices than those cultured on keratin matrices while Ang-2 expression was 3 times higher on HDF cultured on collagen matrices compared to those cultured on keratin matrices (Figure 5.16 and 5.17). Based on this result, it was suggested that collagen matrices support angiogenesis or blood vessel formation better compared to keratin matrices.

5.5.8. Urokinase Plasminogen Activator (uPA)

Urokinase Plasminogen Activator is a serine protease which plays a role in growth factor activation, extracellular matrix degradation and cell signaling. uPA activates plasminogen into plasmin, which is able to degrade fibrin and extracellular matrix (ECM). uPA could also activate other matrix-degrading proteases such as the MMPs. It is also known to co-express together with its inhibitor, plasminogen activator inhibitor (PAI-1). Due to its role in fibrinolysis and ECM degradation, the importance of uPA in wound healing and tissue remodeling process is widely documented [78-81].

Expression of uPA was found to be upregulated on HDF cultured on keratin-containing matrices (keratin and KA12-10mM groups) on day 14. Based on this result, keratin was expected to exhibit higher ECM degradation that could lead to matrices' contraction, as well as an increase in MMPs production. However, in our study it was also revealed

that MMPs production was suppressed and matrices contraction was lower than those of collagen. Therefore, the activation of MMPs as well as matrices contraction were not only dependent on uPA level, but also other pathways and mechanisms involved.

5.5.9. Plasminogen Activator Inhibitor-1 (PAI-1)

Plasminogen Activator Inhibitor-1 (PAI-1) is a serine protease inhibitor that inhibits uPA and tissue plasminogen activator. Due to its effect in inhibiting the plasminogen activator, PAI-1 plays a major role in the regulation of fibrin and ECM degradation during cell migration, wound healing and tissue remodeling. Several studies revealed that the inhibition of PAI-1 function might attenuate wound closure and accelerate wound healing [82-85].

Our result displayed that PAI-1 expression was detected on all groups on day 14 on a comparable level (Figure 5.16 and 5.17). This result suggested that the expression of PAI-1 is unaffected by the matrices that the HDF is cultured on.

5.5.10. Tissue inhibitor of metalloproteinases (TIMP)

Tissue inhibitor of metalloproteinases (TIMP) is an inhibitor of metalloproteinases enzymes. Its inhibition effect on the MMPs giving TIMPs role in the tissue remodeling and wound healing process. TIMPs are usually co-express together with MMPs and the balance of their expression were correlated to the success of wound healing [86-89].

In our study, the expression of TIMP-1 was identified on HDF cultured on all matrices (Figure 5.16 and 5.17). The level of expression was found to be comparable on all groups, suggesting that the matrices that the HDF was cultured on did not affect the expression of TIMP-1.

5.5.11. Insulin-like growth factor binding proteins (IGFBP)

Insulin-like growth factor binding proteins (IGFBPs) are proteins that can modulate the actions of Insulin-like Growth Factor (IGF). It is reported that IGF has a role in cell migration and proliferation [90, 91]. IGFBP-2 is known to bind to IGF-2 and thus inhibits cell proliferation, however studies also found that IGFBP-2 could stimulate cell

proliferation and that this action is independent of IGFs [92-94]. Meanwhile, study by Valentinis et al reported that IGFBP-3 inhibited growth of fibroblast cells through a signaling system not related to IGF-IGF's receptor system [95, 96].

IGFBP-2 was detected on HDF cultured on all matrices, with expression level of those cultured on keratin and collagen matrices almost 2-4 higher than those cultured on keratin-alginate matrices (KA12-10mM). Meanwhile, expression of IGFBP-2 on HDF cultured on alginate matrices was observed to be very low and almost undetected (Figure 5.16 and 5.17). This finding is not surprising since the proliferation of HDF cultured on these matrices are very low. The level of IGFBP-2 expressed proportionally correlate and comply to cell proliferation results (based on the measurement of dsDNA), where matrices containing high alginate content showed lower cell proliferation compared to collagen and keratin matrices. This result support the general understanding that alginate is a biologically inactive material thus it did not support proliferation of human dermal fibroblasts.

Meanwhile, the expression of IGFBP-3 was comparable in HDF cultured on all matrices except collagen. HDF cultured on collagen matrices gave higher expression of IGFBP-3 compared to other matrices. This result was in agreement with our proliferation study where collagen supported higher proliferation compared to other matrices.

5.5.12. Thrombospondin-1 (TSP-1)

Thrombospondin-1 is a glycoprotein which has a function of modulating cell attachment, proliferation, migration and differentiation. It is known to be produced by different types of cells such as fibroblasts, keratinocytes, endothelial cells and macrophages. One of its crucial function is regulating focal adhesion's turnover by driving cell to its intermediate adhesive state which is required for fibroblast cell migration, a critical state in early step of wound healing and tissue remodeling. It was reported that TSP-1 is an essential component for wound healing as inhibition of TSP-1 caused a delay in wound repair. [97-100]

The result in our study revealed that TSP-1 expression was detected on HDF cultured on all matrices (Figure 5.16 and 5.17). Comparable expression level of TSP-1 in all groups regardless of the matrices indicates that its expression was not influenced by the materials.

5.5. 13. Endostatin

Endostatin is the proteolytic fragment of collagen type XVIII and was produced and released by several cells such as endothelial cells and fibroblasts. This peptide was known for its role in inhibiting endothelial cell proliferation, migration and vessel formation by modulating cell-matrix interactions and pericellular proteolysis, thus making it a potent inhibitor for angiogenesis [101-103]. Previous study by Bloch et al revealed that treatment with endostatin decreased the number of functional blood vessels and matrix density in granulation tissue, though the study claimed that it did not significantly affect the overall wound healing process [104].

In this study, endostatin was detected on HDF cultured on keratin-containing matrices and collagen matrices (Figure 5.16 and 5.17). HDF cultured on keratin matrices expressed higher level of endostatin compared to collagen matrices by 2.5 fold. Endostatin expression was not detected or detected at very low levels in HDF cultured on keratin-alginate matrices. This result implied that collagen matrices were more capable of supporting angiogenesis in comparison to keratin.

5.5.14. Endothelin-1

Endothelin-1 (ET-1) is a peptide known as a potent vasoconstrictive agent. ET-1 plays a role in tissue remodelling and wound repair since it has been reported that it could stimulate fibroblasts' collagen synthesis (type I and type III), inhibits MMPs production and promotes collagen gel matrix contraction in vitro [105-107]. A study by Falanga et al revealed that ET-1 did not affect collagen synthesis, conclude that any role of ET-1 in tissue repair is probably due to its hemodynamic effects and not as a growth factor [108].

In our study, the expression of ET-1 was evident on HDF cultured on keratin matrices and collagen matrices. The level of expression was found to be comparable for both days.

5.5.15. Summary of Growth Factor Expression Evaluation

Based on our experiment, we found that keratin upregulated the expression of Tissue Factor and several factors related to inflammation such as CD26 and PTX3. This result indicated that keratin might be more suitable as wound dressing compared to collagen since the increase in tissue factor would assist in haemostasis. However, collagen matrices were found to be more superior for angiogenesis since it upregulate the expression of several factors related to angiogenesis such as Ang-1 and Ang-2. Interestingly, keratin seemed to downregulate MMP-9; although it also upregulated uPA, which both were responsible for ECM degradation. Meanwhile, alginate matrices displayed that they were not able to stimulate comparable expression of growth factors, enzymes and cytokines, indicating that the material is bioinert.

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Chapter 6

General Discussions, Conclusions and Future Recommendations

This chapter summarizes the discussion and conclusion obtained from this study as well as elaborates the proposed future works to be carried out following this study. It is revealed from this study that the fabrication of crosslinked sponges made of human-hair keratin and alginate with tunable physical and mechanical properties by carrying out chemical crosslinking with the facilitation of carbodiimide derivative (EDC) or varying the keratin-alginate composition was made possible. Additionally, this study also displayed that crosslinked keratin-alginate sponges with dominant keratin content were able to improve fibroblast (both L929 and HDF) proliferation and viability compared to sponges with higher alginate content. These results confirmed that crosslinking human hair keratin with alginate offers the alternative of fabricating hybrid biomaterials with tunable physical and mechanical properties which could be utilized as 3D cell carriers or wound dressing.

6.1. General Discussion

Human hair keratins offer new and promising possibilities to the field of biomaterials because they represent abundant and affordable source of human material that can be potentially autologous. Nevertheless, similar to most proteinous materials, poor mechanical properties of keratin limit its application in a clinical setting. Hence, in this study the feasibility of covalently crosslinking keratin with a proven biomaterial, alginate, to produce a new generation of porous 3D sponges with improved mechanical properties compared to each material on its own was evaluated. Indeed, chemical crosslinking by using EDC to form amide bonds between keratin and alginate was revealed to improve the strength and stiffness of the resulting material. The sponges recorded maximum tensile and compression moduli of 135.4 ± 23.1 kPa and 93.2 ± 5.7 kPa respectively. EDC crosslinking has been reported in other hybrid systems as well. [1, 2] where they showed an increase in strength and stiffness of crosslinked composites when compared to non-crosslinked materials

In general, water uptake is an essential consideration for 3D matrices to be used in biomedical applications due to the need for cell and biomolecule exchanges in an aqueous environment [3]. With crosslinking, the hydrophilicity and water uptake capacity of a material is expected to decrease [4]. In our case, water uptake capacity of crosslinked sponges first increased with increasing crosslinking density and peaked when 10 mM EDC was used (70 % crosslinking density). As crosslinking density increased further and plateaued when 100 mM EDC was used (80 % crosslinking density), water uptake capacity dropped significantly. When crosslinking density was low, the sponges were likely too weak to maintain their form in aqueous environment thus limiting their water uptake and retention capacity. On the other hand, increasing crosslinking will result in a more rigid structure which limited the sponge's ability to swell, thus also reducing water uptake capacity. A tuneable system allowing crosslinking degree to be adjusted is thus advantageous in allowing an optimal balance between mechanical properties and water uptake capacity to be achieved, as demonstrated in the keratin-alginate sponges.

The ability of the keratin-alginate sponges to hinder water vapor transmission was a significant finding in this study. Applications such as wound dressings require low

water vapor transmission in order to prevent wound desiccation and offer moist wound healing environment [5]. Keratin-alginate sponges' ability to emulate Aquacel®'s performance in retarding water vapour transmission indicates their potential use as wound dressings.

Despite undergoing chemical crosslinking, the resulting sponges were still enzymatically degradable by proteinase K. Previous study [6] showed that keratin fibers and films from human hair were subjected to proteinase K degradation up to 60% of their original weight and that proteinase K possessed higher proteolytic activity to keratin compared to chymotrypsin [7], despite both enzymes being known to hydrolyze the carboxyl terminal groups of hydrophobic amino acid [8], possibly due to differences in either the specificity or accessibility of the enzymes to the substrates. Our results are in agreement with these reports in that proteinase K degraded the keratin-alginate sponges more efficiently than chymotrypsin.

Unmodified alginate lacks cell binding motifs. It is thus an established approach to crosslink alginate with cell adhesion peptides such as RGD to improve cell compliance [9]. Not surprisingly, alginate did not support the proliferation of fibroblasts [10, 11] or endothelial cells [12] in both 2D and 3D environment. Consequently, we observed in the current study that increasing the proportion of alginate in the keratin-alginate sponges resulted in decreased fibroblast proliferation.

Meanwhile, keratin in various formats has been shown to support the attachment and proliferation of several cell types including fibroblasts [13-16], hepatocytes [17] and Schwann cells. It was speculated that this behavior might be due to the presence of the cell binding motif, LDV, which is known to be recognized by $\alpha_4\beta_1$ integrin [18, 19]. However, previous study [17] suggested that non-integrin cell membrane receptors might be involved in hepatocyte attachment to hair keratins, since blockage of the β_1 and β_2 subunit did not significantly affect cell attachment. In contrast, another study revealed that blocking of β_1 and β_3 subunit resulted in significant decrease of platelet adhesion to keratin, suggesting that there was integrin-mediated mechanism involved in cell attachment to keratin [20]. These earlier studies indicated cell attachment to keratin and the mechanism behind it might be dependent on cell types or mechanisms not related to integrin-mediated binding possibly play a role.

Additionally, our study revealed that despite keratin's ability to support attachment and proliferation of fibroblasts compared to alginate, the morphology of the attached cells were shown to be more rounded and not spread and branched like those cultured on collagen materials (as shown in H&E and live-dead staining). When cells adhere to the ECM via integrin-mediated binding, cells would extend and start flattening against the substrate, rearranging cytoskeleton and forming actin bundles and focal adhesion. These phenomenon lead to the spreading of the cells and define the cell morphology, as a result of the net force between the extensional force and contractile force exerted by the cells [21-23]. In general, the extent of adhesion between cell and ECM is proportionate to the size of cellular extension due to the increase in signaling between cells and ECM [21, 24, 25], suggesting higher interaction between cell and substrate via integrin-mediated binding on collagen materials compared to keratin. In addition, immunohistochemical staining of collagen III and fibronectin also revealed that the production of these ECM were locally expressed only on the cytoplasm and surfaces of the cells as opposed to those cultured on collagen materials. Previous study [26] concluded that the extent of spreading would increase with the fibronectin density on the substrate, suggesting that there was lack of fibronectin deposition on keratin materials compared to collagen, resulting in compromised spreading of the cells.

It was also revealed that cell interaction with keratin resulted changes in growth factor production. The existence of cysteine residues was suggested to play a role in these changes as it was reported from previous study how the production of some growth factors were influenced by the presence of cysteine groups [27-29]. Moreover, evaluation on growth factor production performed in this study further confirmed that alginate is bioinert.

Finally, future studies into cell-specific mechanisms of interaction with hair keratins and the resulting cellular responses will be important in order to exploit the full benefits of using this material for clinically relevant applications.

6.2. Conclusions

From previous studies, keratin was found to be a promising candidate as an alternative material for biomedical applications. As a biomaterial, keratin possesses several advantages such as its natural abundance, ability to obtain autologous material, presence of cell binding motifs that could support cell attachment as well as its biocompatibility. However, their poor, brittle and fragile mechanical characteristics have limited their further translations into clinical use. Many approaches have been carried out in order to improve their mechanical strength. Physical blending of keratin with other partnering material were proven to eliminate the fragility and brittleness thus providing ease in handling in the dried state. However, these properties are not maintained in the hydrated state and the matrices collapsed in an aqueous environment. Crosslinking keratin with diepoxy crosslinking agent was shown to improve its water-resistance, however the resulting matrices become too hydrophobic thus making them unsuitable for biomedical use such as cell carriers. Alginate on the other hand, has been widely used for various biomedical application despite its lack of bioactivity. Hence, in this study, we chemically crosslinked keratin with alginate in order to improve the mechanical properties while maintaining the ability of the resulting hybrid materials to support cell viability and proliferation.

Until now, there hasn't been any work done in performing chemical crosslinking of keratin with partnering material through a carbodiimide-mediated reaction. The effects of these crosslinking method, the extent of crosslinking, and the ratio of keratin and partnering material (alginate) to the resulting materials' physical and mechanical properties remain unknown. Moreover, regarding keratin's potential use for tissue engineering application, there was not many information regarding the cellular behavior of dermal fibroblast in keratin matrices or the keratin-fibroblast cellular-material interaction. More specifically, there was no provided information on how cells would behave in a crosslinked keratin-partnering material (alginate) system. In addition to that, most cell studies performed on keratin system only evaluate the proliferation and viability of the cells without further evaluating the production of extracellular matrices, growth factor and cytokines, which could provide additional information regarding how keratin affect cellular behavior, which was carried out in this study.

We hypothesized that by varying the crosslinking degree or the keratin-alginate ratio of the mixtures, the mechanical and physical properties of the resulting hybrid materials would also vary, thus providing the possibility to tune their properties depending on the desired application. Based on our experimental results, we found that extracted human-hair keratin was successfully fabricated into crosslinked keratin-alginate sponges which were porous and flexible with varying mechanical properties dependent on the crosslinking degree and keratin-alginate content. It was revealed that with increasing crosslinking degree and alginate content, the stiffness and strength would increase, proven by the increasing tensile, compression and flexural modulus as well as the ultimate tensile strength. Additionally, increasing alginate content would increase the water uptake capacity while increasing the crosslinking degree would initially increase the water uptake capacity before decreasing it due to a more stable and rigid structure.

On top of improved mechanical properties, we also hypothesized that the crosslinked keratin-alginate sponges would better support the proliferation and viability of fibroblasts cells compared to matrices with higher component of the non-bioactive alginate. Our result has verified that sponges with higher and dominant keratin content support higher proliferation and viability of both L929 and human dermal fibroblasts in comparison to alginate only sponges or spongers with high alginate content, confirming keratin's bioactivity. Moreover, fibroblasts cultured on crosslinked keratin-alginate sponges were also able to produce extracellular matrices (collagen III and fibronectin), cytokines and growth factors. We discovered in this study that keratin matrices upregulated the expression of CD26 and PTX3, factors that are related to inflammation. Additionally, they also upregulated the expression of tissue factor, factor that plays a role in coagulation, indicating that keratin matrices are more suitable for hemostasis purposes compared to other materials in this study (alginate and collagen). Therefore, these findings provide additional background for further exploration of keratin matrices in relation to their prospective use as wound dressing.

Based on our results, by varying keratin and alginate ratio, it was discovered that tuning the mechanical properties as well as the biological activity of the resulting hybrid materials has been made possible. These findings demonstrated the potential to use human-hair keratin as a realistic source of human biomaterial which may find suitable applications. For example, when the need for mechanical properties outweighs

bioactivity, lower keratin content could be incorporated. On the other hand, when cell compatibility is required (such as for cell carriers, dermal substitutes, 3D cell template for *in vitro* cell cultivation) more keratin could be added into the system. Additionally, degradability of this material makes it possible for exploration as a drug or cell delivery vehicle. Degradation could be controlled by varying the crosslinking degree, keratin-alginate ratio or incorporating extrinsic proteases that could digest the composite matrices. In our study, it was shown that the formulation of KA21-10mM provided the most favorable mechanical properties that allowed ease in handling without compromising the bioactivity (comparable proliferation and viability of cells to keratin matrices).

In conclusion, this study proved that covalent crosslinking of human hair keratin with alginate, via amide bond formation, can be achieved. This strategy offers the potential of fabricating an alternative hybrid biomaterial that is based on an abundant source of human protein. Based on experimental results, these crosslinked matrices could be further developed for *in vitro* applications such as 3D cell culture matrices or *in vivo* applications such as wound dressings.

6.3. Future Recommendations

6.3.1. Culture of Keratinocytes

Since in this study keratin-alginate matrices were proven to support fibroblast growth, cultures of other types of cells could be carried out. Besides fibroblasts, keratinocytes also play an important role in the process of skin regeneration and remodeling. Understanding how keratinocytes behave within the crosslinked sponges would provide an additional insight of how effective crosslinked keratin-alginate mixture would be as either cell carriers or wound dressing.

6.3.2. Understanding Cell-Material Interaction through Mechanism of Cell Attachment and further evaluation of ECM and growth factor production

As previously explained, the mechanism of cells' attachment to keratin is still unclear. Conflicting result where hepatocyte cells did not attach to keratin through integrin-mediated binding mechanism while integrin-mediated binding were involved in platelets attachment to keratin indicated that the interaction of keratin with cells was also dependent on the cell type. Additionally, it was also suggested that other mechanism might play a role in cell attachment besides integrin-mediated binding. Hence, confirming the mechanism involved on fibroblast and keratinocytes attachment to keratin materials would offer more in-depth understanding regarding their cell-material interaction. Since it was suggested by previous studies that the integrin involved for attachment is the $\alpha_4\beta_1$ integrin, a study comparing the extent of cell attachment between keratin blocked with $\alpha_4\beta_1$ antibody and non-blocked keratin could be carried out to confirm this.

Additionally, a more thorough evaluation on ECM production and growth factor could be performed in order to further explain the behavior of cell when interacting with keratin. The growth factor evaluation conducted in this study was just an overview screening of the change in growth factor production when cells were grown on keratin matrices. A more quantitative means (for example by using ELISA) would be able to further confirm the effect of cell-material interaction to growth factor production. Similarly, the evaluation of ECM production was only observed in a semi-quantitative

manner to confirm if there was any major differences of ECM production in between different materials tested. The observation of strut building in the material could be further evaluated, giving additional information regarding the movement of the cells and its effect on migration. Further assessment of other such as talin and actin staining, various types of collagen could be carried out depending on the intended usage.

6.3.3. Incorporation of Active Agents for Various Biomedical Applications

Since crosslinked keratin-alginate sponges has been proven to support cell growth as well as possessing biocompatibility and improved mechanical characteristics, implementing them for further studies regarding their applications in the biomedical field would be made possible. Therefore, we propose to incorporate additional compound to the crosslinked keratin-alginate matrices according to the desired intended applications. For example, incorporating other compounds into the matrices such as growth factor might increase their effectivity for tissue engineering or cell carrier purposes. Furthermore, incorporating antibacterial compounds such as silver particles would also improve their performance as a wound dressing. Additionally, since it was revealed that the degradation of the compound could be manipulated by varying the keratin content, we could also tune the degradation of the matrices to the desired period of time by incorporating varying amount of proteases (such as proteinase K) in order to control their degradation rate.

6.3.4. Material Characterization during Hydrated State

Since the characterization of the mechanical properties performed in this study was carried out on the dried state, measuring the modulus of the crosslinked materials on the rehydrated state would provide additional information about the crosslinked materials' properties. In addition, since during application the sponges would likely be used in aqueous environment, understanding the mechanical characteristics of the materials in their hydrated state would be beneficial in evaluating the feasibility of the materials to be utilized in various biomedical applications. This may include the mechanical characterization following cell culture study.

6.3.5. Degradation Study

Following the preliminary degradation study carried out in this work, further degradation study involving various enzymes that naturally exist in physiological environment, specifically wound bed) such as matrix metalloproteinases, plasmin, and esterase could be performed. By understanding the degradation behavior in a simulated wound environment, it would be possible to predict the fate of the matrices in a physiological setting.

6.3.6. *In vivo* Study

With the completion of the *in vitro* study by performing *in vitro* cell culture with fibroblasts, it was confirmed that the crosslinked keratin-alginate matrices with high keratin content were proven to be biocompatible. Hence, assessing their further interaction with living tissues would be a beneficial additional information in order to exploit their potential in clinical settings.

Implantation of the crosslinked keratin-alginate sponges would be able to let us observe the infiltration of cells inside the scaffold as well as the degradation and remodeling of the scaffolds. Moreover, to evaluate their effectiveness as a wound dressing, an *in vivo* study involving wounded animals could be carried out. By comparing the inflammation as well as the rate of wound closure between the standard of dressing (such as gauze) and crosslinked keratin-alginate sponges, we could analyze their potential for wound healing application.

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List of Publications, Patents and Conferences

Patents

- Ng KW, **Hatrianti P**. Cross-linked Keratin Sponges for Biomedical Applications. PCT/SG2014/000016; 15 Jan 2014.

Conferences

- **Hartrianti P**, Tang BYM, Ng KW “Cross-linked Keratin-Alginate Sponges as Novel Matrices for Tissue Engineering” Bone-Tec conference 2013, Singapore, Dec 16-19, 2013
- **Hartrianti P**, Johanes J, Ng KW “Crosslinked keratin-alginate as alternative 3D matrices for biomedical applications” Termis-AP 2014, South Korea, Sept 24-27, 2014

Publications

- **Hartrianti P**, Ling L, Goh LMM, Ow KSA, Samsonraj RM, Sow WT, et al. Modulating Mesenchymal Stem Cell Behavior Using Human Hair Keratin-Coated Surfaces. Stem Cells International, 2015.
- **Hartrianti P**, Nguyen LTH, Johanes J, Chou SM, Tan NS, Zhu PC, Tang BYM, Ng KW, Preparation and Characterization of a Novel Crosslinked Keratin-Alginate Sponge, Journal of Tissue Engineering and Regenerative Medicine, 2016. → accepted