Human hair keratin hydrogel : fabrication, characterization and application

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NANYANG
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**HUMAN HAIR KERATIN HYDROGEL:
FABRICATION, CHARACTERIZATION
AND APPLICATION**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2014

HUMAN HAIR KERATIN HYDROGEL: FABRICATION, CHARACTERIZATION AND APPLICATION

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Abstract

Human hair keratins are readily available, easy to extract and eco-friendly materials with promising bioactivities. These properties make them the ideal building blocks for a scaffold. This research was aimed at developing keratin-based materials with an emphasis on keratin hydrogels. We started with keratin extraction from human hair. The optimized extraction method yields $24 \pm 1\%$ soluble proteins from human hair. The major components were keratins and keratin associated proteins. The most abundant amino acids were cysteine (>10 mol %), Glutamine/ Glutamic acid (13.6 mol %) and Proline (11 mol %). The first application was using CaCl_2 induced keratin hydrogels as 2D cell culture substrates. The CaCl_2 induced keratin hydrogels were first tested for physical and mechanical properties. In the *in vitro* experiment, L929 cells seeded on keratin hydrogels had an average proliferation rate 76% compared to cells on collagen hydrogels. They were also less elongated and formed localized proliferative cell colonies. The second application was using citrate buffer induced keratin hydrogels as 3D cell culture scaffolds. A new keratin gelation method using citrate buffer was developed. Live cell encapsulation was facilitated by controlling factors such as concentration, pH and temperature. This 3D cell culture system was able to support cell viability and proliferation. Further *in vitro/ in vivo* preliminary studies of keratin-based material demonstrated promising biocompatibility. Thus this novel keratin hydrogel technique has the potential to be used as either 2D or 3D soft tissue templates.

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List of Abbreviations

2D	Two Dimensional
3D	Three Dimensional
BME	Beta Mercapto Ethanol
BMP-2	Bone Morphogenetic Protein-2
CD	Circular Dichroism
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded DNA
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
GSH	Glutathione
H&E	Haematoxylin And Eosin
HDF	Human Dermal Fibroblast
KAP	Keratin Associated Protein
LDV	Leu Asp Val

MT	Masson's Trichrome
PAA	Poly(Acrylic Acid)
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PDDA	Poly (DiallylDimethylAmmonium chloride)
PEO	Poly(Ethylene Oxide)
PLA	Poly(Lactic Acid)
PLGA	Poly(Lactic-Co-Glycolic Acid)
PLLA	Poly(L-Lactic Acid)
Redox	Reduction-oxidation
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
TCPS	Tissue Culture Poly Styrene
TGA	Thermogravimetric Analysis
TRX	Thioredoxin

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Patents

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Awards

- Silver award for oral presentation on Fibroblasts Migration Suppression On Calcium-Keratin Hydrogel. Graduate student category, Singapore Biomedical Engineering Society 5th Scientific Meeting, Singapore, 2011.

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Chapter 1: Introduction

1.1 Background

2D cell culture systems have their limitations. In this over simplified model, cells are grown on flat surface of tissue culture plates, where cell arrangement, cell-material interactions and cell-cell interactions are completely different from natural tissues (8). To overcome those limitations, many 3D cell culture models were developed using synthetic or natural materials.

3D cell culture models based on synthetic polymers have controllable and reproducible material properties. However, the disadvantages of synthetic polymers include lack of bioactivity, lack of degradability and risk of unknown degradation products (9-12).

Protein-based materials on the other hand, are composed of amino acids that are also found in natural tissues. Thus, they can be degraded in host through enzymatic or hydrolytic process. After that, degradation products can be cleared through physiological processes (13, 14). In addition, protein-based materials can inherit unique bioactivities. One of the most important bioactivities for biomaterials are promoting cell attachment and proliferation. For example, ECM component proteins including elastin, collagen, hyaluronic acid and fibrin contain cell binding sequences that promote cell attachment and proliferation (15-17). They are prominent for biomedical applications (17, 18). Some proteins also have the potential to bind specifically or none specifically to growth factors. Proteins such as silk have bioactivities that can protect cells from oxidative stress. They

However, limited availability of those proteins hindered their use for clinical applications. The most established protein production technologies are organic chemistry peptide synthesis and recombinant protein production. Neither method could fulfill the demands for protein-based materials. The first method could not effectively produce large functional proteins (>50 amino acids), while the later method is too expensive to be practiced at industrial scale (19, 20). Some proteins can be extracted from various animals. However, issues such as immune intolerance response as well as cross-species disease transmissions restrict their clinical applications (21, 22). Therefore it is anticipated that there will be strong demands for proteins of human origin for biomedical applications.

1.2 Keratin-based materials

Human hair keratins have emerged as potential materials to address some of these challenges. Besides advantages such as being largely available, bioactive and biocompatible, human hair keratins also possess realistic potential as autologous materials since patient's own hair can be easily collected as starting materials for transplant fabrication (23, 24). A number of soluble human hair keratins extraction methods were used in previous studies (25, 26). The resulting soluble keratin solutions were used for fabrications of keratin films (27-29), sponges (30, 31), fibers (32-34) and as the emphasis of this study hydrogels (5, 35).

Keratins were intermediate filaments proteins that made up intracellular cytoskeletons (36). Despite not being ECM component proteins, 11 out of 17

keratin subtypes contain cell binding sequence LDV (leu-aspartic-val), suggesting cell attachment on keratin materials through LDV recognition by $\alpha_4\beta_1$ integrin (37). Thus, keratin films and coatings have been used as cell culture substrate and demonstrated ability to support cell attachment and growth(38).

1.3 Hypothesis

The overarching hypothesis of this study is that human hair keratins have bioactive and physical properties which can be restored in the form of hydrogels that are cell compliant and therefore have potential to be used for biomedical applications.

1.4 Objectives

1. Optimize keratin extraction from human hair to give high yields without compromising protein quality.
2. Examine the physical, mechanical and biochemical properties of CaCl_2 induced keratin hydrogels and test them as 2D cell culture scaffolds.
3. Develop a keratin hydrogel system that allows living cell encapsulation and 3D cell culture.
4. Confirm keratin hydrogels' biocompatibility by *in vitro* and *in vivo* studies.

1.5 Scope

The scope of this study includes:

1. Comparing and optimizing different human hair keratin extraction methods.
2. Characterizing extracted soluble keratins using different techniques.
3. Developing methods to induce keratin hydrogel formation.
4. Characterizing physical, mechanical and chemical properties of keratin hydrogels.
5. Characterizing cell and tissue compliance of keratin hydrogels using *in vitro* cell culture studies and *in vivo* subcutaneous implantations.

1.6 Organization of this thesis

This thesis contains 8 chapters.

Chapter 1 gives a brief background to the development of materials as cell culture substrates, a brief introduction to keratin-based materials, hypothesis, objectives, scope and thesis organization.

Chapter 2 presents a thorough literature review of relevant topics.

Chapter 3 records the materials and methods used in this study.

Chapter 4-7 report all experimental results generated for this thesis.

Chapter 8 summarizes the significant conclusions from the experimental data and provides future recommendations to further this research.

Chapter 2: Literature review

2.1 Hair structure

Every human hair is constituted by a hair follicle that is embedded in the skin and a hair shaft that protrudes out of the skin. The hair shaft is the portion that is used for keratin extraction. It typically consists of the cuticle cells and the cortex. In certain regions, there is also a medulla, which is the central core of the shaft that is made of loosely packed cells and hollow spaces. The cuticle is the outer most layer of the hair shaft. It consists 0.3-0.5 μm thick overlapping scale-like cells (39).

Figure 2.1 Hair structure. Figure adapted from(1).

2.2 Keratin subgroups

The human hair keratin family consists of 17 members (Table 2.1). They can be divided into two groups: acidic (or type I) group and basic (or type II) group. The acidic keratin group contains 11 members. Their molecular weights are in the range of 45-56kDa and their isoelectric points are between 4.8-5.1. The basic keratin group contains 6 members, with molecular weights 53-65kDa and isoelectric points 6.4-7.4 (40, 41).

	Name	Molecular Weight (Da)	Length	Isoelectric Point	LDV position
1	K31	47236.9	416	4.5342	343-345
2	K32	50319.8	448	4.4894	383-385
3	K33a	45940.2	404	4.4782	343-345
4	K33b	46213.5	404	4.4995	343-345
5	K34	49423.5	436	4.7215	385-387
6	K35	50360.5	455	4.5515	384-386
7	K36	52246.9	467	4.6012	380-382
8	K37	49747	449	4.6188	391-393
9	K38	50479.8	456	4.4933	391-393

10	K39	55622.9	491	4.8649	383-385
11	K40	27479	431	4.5977	376-378
12	K81	54971.2	505	5.2382	NA
13	K82	56652.5	513	6.7169	283-285
14	K83	54168.2	493	5.1429	NA
15	K84	64842.2	600	7.6124	NA
16	K85	55802	507	6.4906	NA
17	K86	53500.5	486	5.3537	NA

Table 2.1 The human hair keratin family. Table information extracted from the Human Intermediate Filament Database

[\[http://www.interfil.org/proteinsTypeInII.php\]](http://www.interfil.org/proteinsTypeInII.php)

2.3 Keratin filament assembly

All 17 human hair keratins share a common basic molecular structure. As Figure 2.2 shows, each keratin molecule is composed of α -helical central rod domain and non-helical head and tail domains. Compatible acidic and basic keratin peptides align in parallel forming coiled-coil heterodimers. They then undergo multiple-step antiparallel assembly into 10-nm intermediate filaments (microfibrils) (42, 43).

Microfibrils are embedded by matrix proteins and aggregate into macrofibrils, which are the major components of hair cortex (44).

Figure 2.2 Hierarchy of keratin filament assembly. Figure adapted from (2).

2.4 Keratin subgroup distribution within hair

Different keratin subgroups are distributed at different locations of hair. As shown in Figure 2.3, six keratins (4 Type I and 2 Type II) are cuticle-restricted, beginning with co-expression of K32, K35 and K85 followed by K82 before K39 and K40. (3, 45). K38 exhibits a heterogeneous expression in distinct, randomly scattered cortex cells and is possibly involved in the determination of hair fiber curvature (45). Keratins K37 and K84 are unidentifiable in terminal scalp hair follicles; K37 was

found only in the cortex of the small, non-pigmented vellus hair that rarely persists into adulthood while K84 was identified in the dorsal tongue (46, 47).

Figure 2.3 Each keratin subgroup distribution within hair fiber. Figure adapted from (3).

2.5 Keratin extraction methods

Many keratin extraction methods have been reported in the literature (7, 28, 48-50). But there has been no systematic studies done to compare them. The main differences between each extraction method are temperature, duration and chemicals used. However, the overall procedure of each extraction methods is similar. They all involve pretreatment of hair, incubation with chemical solutions, filtration and dialysis. The pre-treatment of hair includes washing and delipidizing. Washing is done with water and detergent to remove dirt. Many hair shampoos contain polymers that attach to hair surface after washing, which should thus be avoided. After washing, hair was subjected to delipidization, although in some papers this step was skipped (4, 51, 52). Several delipidization methods are listed in Table 2.2. Delipidized hair is typically air dried and cut into short fragments followed by chemical extractions. Table 2.3 lists different extraction solution recipes. Among them, the first method which was named “shindai method” is the mostly widely used (28, 29, 48, 53-55). Keratins extracted with oxidative chemicals were named keratose (5, 52, 56) while keratins extracted with reductive chemicals were named kerateines (57-60). Keratins extracted with different methods may have different activities and should be differentiated. For example, kerateines were found to be hemostatic while keratoses were not (5, 35, 61). After chemical incubation, remaining undissolved residues are separated by filtering. Unwanted chemicals can then be removed by dialysis. Keratin solutions can be used directly for downstream processes or lyophilized into keratin powder for long term storage.

Delipidization chemicals	Incubation time	References
n-hexane	12 hour	(28)
Petroleum ether	NA	(33, 34)
chloroform/methanol (2 : 1, v/v)	24 hour	(7, 29, 48, 49)

Table 2.2 Human hair delipidization methods.

	Extraction method	References
1	Tris pH8.5 (25mM), thiourea (2.6M), urea (5M), 2-mercaptoethanol (5 v/v %), 50°C, 72 hour	(28, 48) (29, 53-55)
2	Na ₂ S (0.125M), 40°C, 4 hour	(7, 49)
3	Urea (8M), m-bisulfite (0.5M), SDS (0.05M), pH 6.5, 65°C, 2-2.5 hour	(32-34)
4	Urea (8M), m-bisulfite (0.5M), pH 6.5, 65°C, 2 hour	(62)
5	Urea (8M), SDS (0.2M), Na ₂ S ₂ O ₅ , (0.5-0.6M), 100°C, 0.5 hour	(63) (27, 64)
6	Dimethylsulfoxide (DMSO), 115°C, 24 hour	(65)

7	NaOH (5 w/v %), until all hair dissolved	(66)
8	Dithiothreitol (DTT, 0.03M), guanidine hydrochloride (6M), nitrogen atmosphere, 50°C, 15 hour	(50)
9	Peracetic acid (2 w/v %), 37°C, 10 hour / overnight	(5, 52, 56)
10	Thioglycolic acid (0.5M, pH 11), 15 hour	(57, 58)
11	Thioglycolic acid (1M, pH 10.2), 12 hour	(59, 60)

Table 2.3 Keratin extraction methods.

2.6 Keratin Template fabrication methods

Attempts to fabricate keratin solution into functional materials for biomedical applications have been underway since two decades ago. Highlighted below are the most prevalent forms of keratin materials: film, fiber, sponge and gel.

2.6.1 Keratin film

Studies on keratin films had focused on enhancing film mechanical properties and testing for applications such as cell culture substrates, skin patches, nail plate model and ocular surface reconstruction.

Different approaches, such as compression, combinations with other synthetic or natural polymers and adjusting film casting ionic conditions, were tested to enhance keratin film mechanical properties. Keratin films made by compression molding at 120°C have enhanced ultimate strength and Young's modulus (27). Synthetic polymers, such as poly (diallyldimethylammonium chloride) (PDDA) or PDDA and poly(acrylic acid) (PAA), were mixed with keratins and fabricated into thick films using layer by layer self-assembly method (50). Natural polymers such as chitosan were mixed with keratin for film casting as well. It was found that the resulting keratin/ chitosan composite films exhibited in general weaker mechanical properties compared to pure chitosan films. Moreover, their mechanical properties could be greatly affected by UV irradiation (67). Another important factor that could result in changes to keratin film physical properties is ionic condition. Exposing keratin films to post-casting solutions with or without divalent ions could result in changes in flexibility, transparency and surface structure (55).

Keratin coatings or keratin films were used as cell culture substrates (27-29). As table 2.4 shows, at least 14 primary cell types or cell lines were tested, demonstrating compatibility in terms of cell adherence and cell growth (28). Keratin films that were reinforced with cotton gauze were tested as skin patches. During the period of five days, no significant adverse reactions were observed, revealing the possibility of keratin film as wound dressing materials (55). As keratins are the major proteins in nails, mechanical stable and water resistant keratin films were used as a model for nail plate drug permeation studies (53). Notably, recent study of replacing human amniotic membrane with keratin films in

the ocular surface reconstruction showed improved light transmission, enhanced mechanical strength and comparable corneal epithelial cell growth. (54)

	Keratin origin	Scaffold	Tissue origin	Cell types
1	Human hair	Coating	Human colon adeno-carcinoma	Caco-2 (28)
2	Human hair	Coating	Human skin epidermis	HaCaT (28)
3	Human hair	Coating	Rabbit cornea epithelium	SIRC (28)
4	Human hair	Coating	Human cornea epithelium	CEPI (28)
5	Human hair	Coating	Human cornea endothelium	HENC (28)
6	Human hair	Coating	Human cornea stroma	HCK (28)
7	Human hair	Coating	Human cornea stroma	HUFIB (28)
8	Human hair	Coating	Human cornea epithelium	HCE-T (28)
9	Human hair	Coating	Human skin keratinocytes	PHK (28)
10	Human hair	Coating	Lung adeno-carcinoma	Calu-3 (28)
11	Human hair	Coating	Nasal septum squamous cell carcinoma	RPMI 2650 (28)
12	Human hair	Film	Human cornea epithelium	HCE-T (54)

13	Wool	Film	Mouse subcutaneous areolar and adipose tissue	L929 (27)
14	Human hair	Coating and Sponge	Mouse embryonic fibroblast	NIH3T3 (29)

Table 2.4 List of cells tested on keratin films/coatings.

2.6.2 Keratin fiber

Keratin fibers can be formed by poly ionic complexation or electrospinning. Poly ionic complexation is driven by polyanionic and polycationic interactions at the interface of two solutions. Water insoluble complexes were formed and can be drawn into fibers with mico-meter range diameters (68-70). For example, in the keratin-gellan-chitosan ternary complex, keratins formed particles on the fiber surfaces. Fiber diameter ranged from 50 μ m to 100 μ m with increasing keratin concentration from 0 to 35 wt %. This hybrid fiber showed good mechanical strength and biodegradability (71). Electrospinning is driven by electrostatic forces. Each fiber is stretched as it accelerates towards a grounded collector in an electrific field and solidifies as the solvent evaporates (72-75). Electrospinning can produce fibers in the nanometer range. Keratin has been blended with poly(lactic acid) (PLA) (76), poly(L-lactic acid) (PLLA) (66), poly (lactic-co-glycolic acid) (PLGA) (77), poly(ethylene oxide) (PEO) (32-34) or silk fibroin (62) to produce electrospun fibers with diameters ranging from around 0.1 μ m to 1.5 μ m.

2.6.3 Keratin sponge

Porous keratin sponges can be obtained by lyophilization (31). Highly flexible and porous keratin sponges can be made by lyophilization with calcium alginate beads embedding (30). Keratin sponges with controlled pore size and porosity can be made from compression-molding followed by particulate-leaching (64).

Keratin sponges have been used as 3D cell culture scaffolds and drug carriers. L929 mouse fibroblast-like cells could infiltrate into porous keratin sponges and proliferated within (30, 31). Keratin sponges have been tested as lysozyme and bone morphogenetic protein (BMP)-2 carriers. In the lysozyme delivery model, lysozyme was covalently immobilized to keratin sponge through disulfide or thio-ether bonds. Disulfide bonded lysozyme was gradually released during 20days in *in vitro* experiments, while thio-ether bonded lysozyme remained stable (78). In the BMP-2 releasing studies, keratin sponges were made from either chemically modified keratines or keratose. Chemically modified keratine sponges were made to present large amounts of negatively charged carboxyl groups. Thus, (BMP)-2 molecules with positive charges could be absorbed through electro static attractions. Preosteoblasts seeded in these sponges were found differentiated while cells grown outside of the scaffold remained undifferentiated (79). Keratose sponges were evaluated in a critical-size rat femoral defect model. Results demonstrated that using keratose sponges used as BMP-2 carriers could promote critical-size segmental long bone defect regeneration. Interestingly, keratose

sponge alone without BMP-2 showed more bone outgrowth and reduction of adipose tissue (51). These findings suggested the feasibility of using keratin sponges as delivery systems for bone regeneration.

2.6.4 Keratin gel

Hydrogels are 3D networks that can hold water. They draw many researchers' interest as potential tissue engineering scaffolds due to their high water content that resembles natural tissue environment. The study of keratin hydrogels are still at early stage. Based on fabrication methods, 2 types of keratin hydrogels have been discussed in previous studies (4)(5)(6).

2.6.4.1 Divalent ions induced keratin hydrogel

As shown in figure 2.4, the keratin hydrogel induced by $MgCl_2$ was opaque. Mechanical properties of the hydrogel was not tested, however, authors estimate that the stiffness would be similar to brain tissues(4). LG3 domain of laminin was incorporated into the hydrogel to improve attachment of neural progenitor cells on the surface.

Figure 2.4 Image of keratin hydrogel induced by MgCl_2 . Image adapted from (4).

2.6.4.2 Keratin hydrogel fabricated through disulphide bonds reformation

As shown in figure 2.5 keratin hydrogels fabricated through the reformation of disulphide bonds were brownish and translucent. They were tested as neuron conducting materials for peripheral nerve regeneration (5, 35). Keratin hydrogel was injected into the lumen of collagen nerve guide until all empty space was filled (Figure 2.5 A and B). This keratin filled conduit was tested in a rabbit peripheral nerve regeneration model. The result demonstrated keratin hydrogel filler could stimulate the neuron regeneration compared to empty conduit control.

The hemostatic property of keratin hydrogels were tested in rabbit lethal liver injury model (21) and porcine lethal femoral artery injury model (6). In rabbit model, keratin hydrogels were directly applied on the liver injury surface. The 24 hours survival rate was increased from 0% (negative control) to 75% (with keratin hydrogel treatment) (21). In the porcine model, keratin hydrogel was first spread on gauze before applied to the wound site to prevent being washed off by blood.

The survival rate after 180 minutes was improved from around 28% (empty gauze) to around 60% (keratin hydrogel treatment group) (6).

Figure 2.5 Keratin hydrogel fabricated through disulphide bonds reformation. Keratin hydrogel was injected into collagen guide (A) until the middle lumen was completely filled (B). Keratin hydrogel on gauze as hemostats (C). Image A and B were adapted from (5), image C was adapted from (6).

2.6.4.3 Gaps in current keratin hydro gel research

Current applications of keratin hydrogel as 3D cell culture scaffolds were only limited to seeding on the surface, which led to problems such as inhomogeneous distribution of cells. However, existing keratin hydrogel fabrication methods could not support direct live cell encapsulation. The excess divalent ions used in the first keratin hydrogel fabrication method were toxic to cells. On the other hand, the sol-gel transition process in the second method was

too slow to support cells in 3D after encapsulation. In this project, these gaps were filled by developing a new keratin hydrogel fabrication method as well as a simplified live cell encapsulation procedure.

Chapter 3: Materials and methods

3.1 Keratin extraction and characterization

Extraction of human hair proteins

Raw human hair samples were collected from local hair salons. There was no distinction in terms of age, gender or ethnic group. Both age and ethnic group could affect hair pigmentation. Mammalian melanocytes produce two kinds of pigments: eumelanin and pheomelanin. Typical black or brown hair is eumelanin preponderant, while typical red hair is pheomelanin preponderant. White hair occurs due to decreased amounts of pigments (80, 81). It is still unclear how gender affects hair composition. Male hair generally has higher cysteine content than female hair.

Prior to extraction, hair was washed with detergent to remove dirt and then soaked in 95% ethanol for 6 hours with the purpose of sterilization. Then, samples were air dried and delipidized by soaking in 2:1 (v/v) chloroform/ methanol mixture for 24 hours. The delipidizing process removed lipids from the surface of hair, making the hair more permeable to the extraction cocktail. Then the treated hair was air dried and cut into roughly one centimeter long fragments using a pair of scissors. During the extraction step, 5-50g of hair fragments was immersed in 1 liter extracting solution that consisted of 0.125M sodium sulphide (Sigma Aldrich) solution in deionized water. Incubation was carried out at 40 °C for 2 hours. The resulting mixture was then filtered to separate the insoluble residues. The filtrate was dialysed against deionized water using cellulose dialysis tubings (Sigma Aldrich MWCO=12 kDa).

Protein concentration measurement

Soluble human hair protein concentrations were measured using the 660nm protein assay kit (Thermo Fisher Scientific). A serial dilution of 2000µg/ml BSA (Thermo Fisher Scientific) solution was used as standard. 10µl of standard or protein solution was added in transparent 96-well flat bottom plate. Then 150µl protein assay reagent was added in each well. The plate was covered and protected from light for 1 minute incubation at room temperature. Absorbance was subsequently measured at 660nm using a microplate reader (INFINITE F200, TECAN). Samples were run in either duplicates or triplicates.

Coomassie blue staining

About 10µl of 1mg/ml keratin sample was mixed with 5µl LDS loading buffer (Invitrogen) and 2µl sample reducing agent (Invitrogen) and topped up to total volume of 22µl with deionized water. Samples were heated at 75°C for 15min for protein denaturing. 20ul of sample was loaded into each well of NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen). Electrophoresis was run at 120V for 90 min. After electrophoresis, the gel was stained with coomassie blue (SimplyBlue SafeStain solution, Thermo Fisher Scientific) for 60 min and subsequently washed overnight in deionized water.

Scanning Electron Microscopy

Human hair samples before or after extraction were washed with water and air-dried. Keratin hydrogel samples were frozen at -20°C for more than 8 hours and lyophilized. All samples were gold-sputtered at 18mA for 10 sec. Images were taken with a scanning electron microscope (JSM-6360, JEOL, USA) at an accelerating voltage of 5kV/10kV under high vacuum.

Thermogravimetric analysis (TGA)

Thermal stability of freeze-dried human hair proteins was analyzed by thermogravimetric analysis (TGA; TGA2950, TA Instruments). Tests were carried out from room temperature up to 800°C at the heating rate of $10^{\circ}\text{C}/\text{min}$ under constant air flow, using 2.3mg of samples.

Western blotting

Electrophoresis of keratin protein was done as described above except total protein amount loaded in each well was about $1\mu\text{g}$. After electrophoresis, separated protein bands were transferred onto nitrocellulose membranes using iBlot Dry Blotting System (Invitrogen) at condition of 20V and 6min. Membranes were blocked with blocking buffer (5% non-fat milk dissolved in PBS containing 0.05% Tween-20 (PBST)) for 30 min under constant shaking. Blocked membrane was incubated with primary antibody against total human hair keratin (Abcam) (1:1000

diluted in blocking buffer) for 60min at room temperature. Unbounded primary antibody was washed off with PBST and blots were incubated with secondary HRP-conjugated antibody (1:4000 in blocking buffer) for 30 min. Chemiluminescence was developed by applying Super Signal West Pico (Thermo Fisher Scientific) chemiluminescent substrate on the surface of membrane.

Circular dichroism (CD) spectroscopy

Far-UV light (180nm to 260nm) CD spectra of soluble human hair proteins at various temperatures were measured with a chirscan spectropolarimeter (Applied Photophysics). CD spectra of human hair proteins extract at 2mg/ml concentration in PBS buffer were recorded under temperatures of either 37°C, 50°C, 70°C or 90°C.

3.2 Fabrication of hydrogels

Fabrication of CaCl₂ keratin hydrogel

To prevent microorganism contamination, soluble human hair keratin solution was sterilized by filtering through 0.2µm cellulose filter. Initial protein solution concentration was adjusted to 10mg/ml. Keratin gelation was induced by mixing keratin solution with 1M CaCl₂ at the ratio of 50:1 (v/v) followed by incubation at 37°C overnight. Prior to cell seeding, keratin hydrogels were very gently rinsed with sterile ultra pure water and cell culture medium to remove excess CaCl₂.

Fabrication of collagen hydrogel

According to manufacturer's instruction, calculated volume of rat tail collagen I (BD biosciences) was neutralized with NaOH and PBS buffer to achieve final concentration of 1mg/ml. The mixture was kept at 37°C for 30 mins to allow complete gelation.

Oscillatory rheology

Rheology studies were done using a rheometer (Anton Paar, Germany). Keratin or collagen hydrogels were cast in petri dishes with diameters of either 25mm or 50 mm. An empty petri dish was used for zeroing the gap height. Petri dishes containing hydrogels were attached to the platform surface with double sided tape to prevent slippage. To start the measurement, 50 or 25 mm diameter parallel plates were lowered until they merely touched the hydrogel surface. The linear viscosity ranges were determined to be below 5% strain by strain sweeps. During frequency sweep, both storage modulus (G') and loss modulus (G'') were monitored as a function of angular frequency.

Light microscopy imaging of keratin flocs

Effect of concentrations on the formation of keratin flocs was observed using phase contrast light microscopy. Flocculation was triggered by mixing keratin solutions with 1ml pH 3 PBS/citrate buffer (made from 1ml PBS with 0.1ml 0.1M pH3

citrate buffer). Keratin solutions were prepared over a serial concentration range from 0.2 mg/ml to 4mg/ml. One drop of each flocculated keratin solution was placed on a glass slide and observed using an upright phase contrast light microscope (CKX41, Olympus, Japan).

UV-Vis spectrometry

The keratin flocculation process was associated with an increase in solution turbidity that was measured by the absorbance profiles at a wavelength of 400nm using a UV-Vis spectrophotometer (UV-1700, Shimadzu, Japan). To investigate the influence of pH on the flocculation process, PBS and 0.1M, pH 3 citrate buffers were mixed at the volume ratios of 10:0, 10:0.3, 10:0.5, 10:1 and 10:2. Accordingly, the resulting PBS/citrate buffer pH values were 7.2, 4.5, 4, 3.5 and 3.3. 3ml of each PBS/citrate buffer was mixed with 3 ml of 15mg/ml keratin solution. 2 ml of each mixture was measured in a quartz cell with 10 mm path length using the UV-Vis spectrophotometer. Sample buffers were used as reference. About 4 ml remaining samples were poured into 25mm Petri dishes for photo imaging.

In the temperature-dependent keratin flocculation stabilizing experiment, keratin flocculation was triggered by mixing 30ml of 15mg/ml keratin solution with 30 ml of pH3.5 PBS/citrate buffer that was prepared as mentioned above. 2ml aliquots of the mixture were made for stabilizing treatment with respect to incubation time and temperature. There were three incubation treatments: “PBS”, “Water” and “PBS, 4°C”. For “PBS, 4°C” group, incubation was carried out at 4°C. For the other

groups, incubation was carried out at 37°C. Each group was monitored over 9 time points from 0 hour to 8 hours. At each time point, flocculated keratin was spun down at 1000g for 10min. The supernatant was discarded and the keratin floc pellets were re-suspended in either 2ml water (“Water” group) or 2ml PBS (“PBS” and “PBS, 4°C” groups). Subsequently, absorbance of the resulting suspensions was measured immediately using UV-Vis spectrophotometer as described above.

Computational porosity analysis of SEM images

Keratin hydrogel porosity was estimated from SEM images using image processing techniques, in collaboration with the School of Computer Engineering, Nanyang Technological University. Based on the local brightness contrast, the pore areas were segmented against the surface of the material. First, each SEM image was divided into overlapping small blocks. Then, each block was converted into binary image in which pore areas and surface areas were separated. Lastly, all binary image blocks were fused together to have a binary image of the original. To remove background noise, an image filter was applied and statistical analysis of pore area/ number was done based on the filtered binary image. This image processing was programmed and run using MATLAB 8.3 software.

3.3 Cell culture

2D culture of L929 cells on keratin/collagen hydrogels/films

L929 murine fibroblast-like cells were maintained in 75 cm² tissue culture polystyrene (TCPS) flasks (Corning) with DMEM (Gibco, Invitrogen) medium

supplemented with 10% FBS (Hyclone, Invitrogen), 1nM non-essential amino acids (Gibco, Invitrogen), 1mM sodium pyruvate (Gibco, Invitrogen), 0.2mM L-glutamine (Gibco, Invitrogen), 100Units/ml penicillin (Gibco, Invitrogen) and 100ug/ml streptomycin (Gibco, Invitrogen). Cells were split 1 to 4 every 2 days using 0.25% trypsin (Gibco, Invitrogen). Prior to seeding, keratin/collagen hydrogels/films were rinsed with complete cell culture medium. L929 cells were harvested by trypsinization, followed by counting using hemocytometer. For 2D culture, cell suspensions were gently dropped onto the surfaces of hydrogels/films at the density of 10,000 cells/cm².

Histological examination

In vitro cell populated scaffolds and *in vivo* subcutaneously implanted scaffolds together with surrounding tissue were harvested and fixed in 4% paraformaldehyde (PFA) (Sigma Aldrich) for 48 hours at room temperature. They were processed with standard histological protocols for paraffin embedding. 5 µm serial sections were made, mounted on glass slides and stained either with haematoxylin and eosin (H&E) or Masson's trichrome (MT). Brightfield images of stained sections were obtained using an upright light microscope (CKX41, Olympus, Japan).

3D cell encapsulation in citrate buffer induced keratin hydrogel

Sterile keratin solution, 0.1M pH3 citrate buffer and cell culture medium (DMEM) were needed for live cell encapsulation. PBS/citrate buffer was prepared by mixing PBS with citrate buffer at the ratio of 10:1, and mixed with equal volumes of

keratin solution. The resulting mixture was kept in 37°C for 12 hours. After keratin flocs were stabilized, they were washed with cell culture medium (DMEM). After 2-3 repeats of washing, keratin flocs were centrifuged at 1000 g for 10 min. About 3% of total proteins were found to be lost in the supernatant during the washing process (data not shown). However, for consistency, a starting protein concentration of 25mg/ml was used across the experiments. The keratin flocs were mixed with live cells in suspension and calculated volumes of complete cell culture medium to adjust the final protein concentration to 25mg/ml. Then, the cell loaded keratin mixture was cast into cell vessels and left undisturbed for several hours to allow 3D hydrogel formation. This gelation process was likely due to the reformation of disulfide bonds between cysteine groups in hair keratins.

Live/Dead staining

Cell viability, morphology and distribution were visualized by Live/Dead staining (Invitrogen). Live cell marker calcein AM could penetrate through cell membrane where it was converted into fluorescent molecule calcein by intracellular esterase, and is retained within the cytoplasm. In contrast, EthD-1 could enter necrotic cells (but not apoptotic cells whose plasma membranes were still intact) through damaged plasma membrane to give strong fluorescence when bound to nucleic acids. Thus, necrotic cell nuclei were marked with red fluorescence while live cell cytoplasm will fluoresce green. Cell culture medium was removed followed by rinsing with PBS. Live/Dead reagent was diluted in PBS at the ratio of 1:1000 for both calcein AM and EthD-1. The resulting staining reagent was added to wash monolayer cell cultures or immerse 3D cell loaded hydrogels. After 30 min

incubation at 37°C and 5% CO₂ in an incubator, excessive staining reagent was removed. Samples were then rinsed once with PBS, before imaging under a florescent microscope (CKX41, Olympus, Japan).

2D Cell proliferation rate measurement

Cell proliferation of monolayer cultures was monitored by quantifying double-stranded DNA (dsDNA) level, using PicoGreen assay (Invitrogen). At each cell culture time point, cells were rinsed gently with PBS, followed by 30 min incubation with cell lysis buffer (10 mM Tris-HCl pH8 buffer containing 0.01 % SDS) at room temperature. After that, samples and DNA standards diluted in the same cell lysis buffer were mixed with working PicoGreen reagent in a 96-well plate. Florescent readings were measured at excitation and emission wavelengths of 480 nm and 520 nm, using a microplate reader (INFINITE F200, TECAN). Calibration curves of dsDNA standards were generated to determine the relative dsDNA amount in each sample, and directly correlated to cell proliferation.

3D Cell proliferation rate measurement

Unlike for monolayer cultures, cells encapsulated in 3D cell culture systems had to be released in order for efficient cell lysis and access to dsDNA (82). Here, both mechanical force and proteinase K digestion were used to break down the keratin and collagen hydrogels. Cell culture medium was removed before each sample was incubated with digesting buffer (10 mM Tris-HCl pH8 buffer containing 0.5 % SDS and 0.3 µg/ml proteinase K). Digestion was carried out at 37 °C overnight

under constant shaking. 0.3 µg/ml of proteinase K was replenished every 12 hours until no visible fragments were left.

Digested samples were further purified using phenol. First, digested samples were volume adjusted from around 2 ml to 5 ml using deionized water. 1ml of each digested sample was then mixed with 1 ml tris-saturated phenol. The mixtures were inverted several times until emulsions were formed. These were then centrifuged at 20,000 g for 3 minutes to create two clearly visible separated phases. Polar DNA molecules were collected in the top aqueous phase, while denatured protein molecules were separated to the bottom organic phase. Without disrupting the organic phase, the top aqueous phase was carefully transferred into a new tube. 1 ml of chloroform was added to the separated aqueous phase, followed by repeating the same phase separation procedure. The second phase separation cycle was included to remove possible traces of phenol in the aqueous phase, which could interfere with the PicoGreen assay that was used to quantify dsDNA, as described above.

3.4 Preliminary *in vivo* studies

All *in vivo* studies were carried out with ethical approvals from the institutional authorities. Short-term *in vivo* studies were carried out in collaboration with the School of Biological Sciences, Nanyang Technological University, using female C57Bl/6 mice. Surgery sites were shaved before two 3mm incisions were made on the dorsal skin of each mouse using surgical scissors. A subcutaneous pocket was

made through the incision using a spatula. One keratin hydrogel disk (40mg/ml, 3mm in diameter and 2mm thick) was inserted into each pocket. The incision was closed with adhesive skin closure tape. The mice were sacrificed at day 7 (n=2) and the implants were retrieved and fixed with 4% paraformaldehyde. Long-term *in vivo* studies were carried out in collaboration with the Shanghai Key Laboratory of Tissue Engineering, using Sprague Dawley rats. Operational procedures were the same as described above, except that 1ml keratin hydrogel was injected into the subcutaneous compartments using a syringe through 30mm incisions and closed with surgical sutures. Implants were retrieved at after 90 days, as described above. All harvested samples were sectioned and processed for H&E and Masson's Trichrome staining using standard procedures (83, 84).

Chapter 4: Keratin extraction and characterization

This is the first result chapter. In this chapter, the optimized keratin extraction procedure would be described, followed by extracted keratin characterization results. Objectives and experimental design flowchart are shown below. Detailed experimental procedures can be found in chapter 3.

4.1 Objectives:

1. Compare two widely used keratin extraction methods (BME method and Na₂S method), in terms of extraction yield and keratin solubility.
2. Optimize extraction procedures and extraction solution recipes.
3. Purify protein solution to remove unwanted chemicals.
4. Identify and characterize human hair protein extracts.

4.2 Experimental design:

Figure4.1 Chapter 4 experimental design illustration.

4.3 Comparison of human hair protein extraction methods

All the documented keratin extraction methods listed in table 2.3 shared similar extraction procedures. As illustrated in figure 4.2 they consisted of pre-extraction hair treatment (washing, delipidization and fragmentation), extraction and purification (filtering and dialysis). The major difference among those extraction methods was in extraction recipes, especially the reducing agents used. Here, two reducing agents: sodium sulphide (Na_2S) and beta mercaptoethanol (BME) were tested.

Figure 4.2 Graphical illustration of human hair protein extraction procedures.

Protein yield of each extraction method has been used as one of the selection criteria. As figure 4.3 shows, both methods produced protein yields of 15%-22% of original hair mass. The highest yield using Na_2S was around 22%, while the highest yield using BME was around 19%.

Figure 4.3 Comparison of protein yields with various Na_2S (A) or BME (B) concentrations.

Another selection criterion was protein solubility. As figure 4.4 shows, after removing chemicals by dialysis, proteins extracted by Na_2S method were soluble. The solutions were brownish in color and transparent. In contrast, proteins extracted by BME tend to precipitate. They appeared milky and yellowish. The

distinctive difference in protein solubility was due to different chemicals used during extraction process. BME method contained concentrated urea and thiourea. Both are strong protein denaturant. During extraction, urea and thiourea denatured hair proteins and exposed the hydrophobic peptide chains. Once urea and thiourea were removed during dialysis, denatured keratins were not able to refold properly. Thus denatured proteins aggregated to form precipitates. In contrast, Na_2S method does not contain urea or thiourea. Hydrophobic protein peptides were not exposed, and thus the proteins remain soluble even after dialysis

As protein solubility was critical for downstream applications, Na_2S method was decided as the primary extraction method.

Figure 4.4 Photographs of soluble human hair protein solutions extracted by Na_2S method (top) and BME method (bottom) after dialysis

4.4 Na₂S extraction method optimization

Na₂S extraction method was optimized by varying Na₂S concentrations as well as incubation time (Figure 4.5). By increasing Na₂S concentration from 0.0625M to 0.25 M and incubation time from 1 hour to 3 hours, protein yields increased from minimum 10% to a maximum of 35%. However, high Na₂S concentration and elongated incubation time caused protein degradation as shown in SDS PAGE (figure 4.6).

Figure 4.5 Comparison of protein yields with various Na₂S concentrations and incubation time. Data were presented as average presented as means $\pm$ standard deviation. n=3, *p<0.05 **p<0.01 (Student's t-test).

Figure 4.6 SDS PAGE of human hair proteins extracted with various Na_2S concentrations and incubation time.

4.5 SEM image of human hair before/after extraction

We also investigated which part of human hair was more prone to solubilisation by Na_2S . Before extraction, SEM images of human hair cross section showed a fully-filled central cortex (Figure 4.7 A). Human hair surfaces were covered with defined cuticle scales which were smooth (figure 4.7 B). After extraction, the central hair cortex appeared hollow (figure 4.7 C), while the hair cuticle remained relatively intact (figure 4.7 D).

Figure 4.7 SEM images of natural human hair before extraction: cross section (A), lateral surface (B); and after extraction: cross section (C), lateral surface (D).

4.6 Human hair protein extracts identification and characterization

Human hair protein extracts were subjected to purification processed including filtration and dialysis with the purpose of removing insoluble residues and unwanted chemicals. The efficiency of chemical removal was tested using TGA and FTIR.

4.6.1 TGA analysis

In TGA, freeze-dried keratin powder samples had two major weight loss stages (Figure 4.8). The first stage with 5.5% weight loss below 100 °C was due to moisture evaporation. Protein degradation started around 220 °C and peaked around 315 °C. This is consistent with typical protein sample TGA studies.

Figure 4.8 TGA analysis of the freeze-dried human hair proteins at a heating rate of 10 °C/min.

4.6.2 FTIR analysis

As shown in figure 4.9, without dialysis 2 strong peaks were observed in FTIR spectrum: one broad peak at 1100 cm^{-1} and the other sharp peak at 980 cm^{-1} . They

were assigned to remaining chemical SO₄ and S₂O₃ groups, which were produced during the extraction process by the following reactions:

According to literature, SO₄ groups have only one characteristic peak at 1100 cm⁻¹, while S₂O₃ groups possess two characteristic peaks at 995 cm⁻¹ and 1115 cm⁻¹ (85). Therefore, the broad peak at 1100 cm⁻¹ could be the result of overlapping characteristic peaks of SO₄ at 1100 cm⁻¹ and of S₂O₃ at 1115 cm⁻¹. The sharp peak at 980 cm⁻¹ could be assigned solely to S₂O₃ groups. In the FTIR spectrum of protein extracts after dialysis, these two peaks disappeared, indicating the clearance of chemical contaminants. In both spectrums, peptide bond C=O stretching (amide I 1700-1600 cm⁻¹) and N-H bending (amide II close to 1500 cm⁻¹) peaks were observed. These findings are in good agreement with typical protein sample spectrums.

Figure 4.9 FTIR spectra of extracted human hair proteins solution indicate removal of chemical contaminations while retaining proteins. Figure adapted from (7).

4.6.3 Amino acid analysis

Amino acid analysis of freeze-dried extracted hair proteins showed 11.2 mole % cysteine contents (Table 4.1). Other major amino acids (>10 mol %) are Glutamine/ Glutamic acid (13.6 mol %) and Proline (11 mol %). Those findings agree with reported results of hair keratin amino acid analysis (52).

Amino acid analysis

Amino acid	Mol %
Alanine	4.7
Arginine	7.7
Aspartic acid/ Asparagine	6.3
Cysteine	11.2
Glutamic acid/Glutamine	13.6
Glycine	5.8
Histidine	0.9
Isoleucine	3
Leucine	7
Lysine	2.4
Methionine	0.5
Phenylalanine	1.8
Proline	8
Serine	11
Threonine	7.6
Tryptophan	0.5
Tyrosine	2.1
Valine	5.9

Table 4.1 Amino acids composition (mol %) of human hair extract

4.6.4 Keratin identification by SDS PAGE and Western blotting

Coomassie blue stained SDS PAGE gels (Figure 4.10 a) showed the presence of acidic keratin monomers (~45 kDa), basic keratin monomers (~50 kDa), as well as keratin heterodimers (80~100kDa). Multiple keratin subtypes (40, 86) were suggested by two strong bands in the acidic keratin region. Within the keratin dimer region (80~100kDa), multiple bands were observed which suggested different heterodimer combinations. There were faint bands in the region below 20 kDa, which came from keratin associated proteins (KAPs). Besides SDS-PAGE, Western blotting was performed to confirm the identity of

human hair keratins. Polyclonal mouse antibody that could recognize total human hair keratins was used for identifying keratin bands. (Figure 4.10 b). Based on SDS PAGE and Western blotting data, it was concluded that the major components of human hair protein extracts were keratins and keratin associated proteins. Since keratins are the primary components, human hair protein extracts will be referred to as human hair keratin extracts henceforth.

Figure 4.10 Protein SDS PAGE stained with Coomassie blue (a) and Western blot (b) of extracted human hair proteins demonstrate the presence of keratin monomers and heterodimers. Figure adapted from (7).

4.6.5 Human hair keratin secondary structure analysis

Secondary structure of human hair keratins was determined from CD spectra (Figure 4.11). CD spectrum recorded at 37°C reached maximum at 189 nm and minima at 208nm and 222nm. These suggest the presence of α -helical structures. As the temperature elevated from 37°C to 50°C, 70°C and 90 °C, reached maximum at 189 nm and minima at 208nm and 222nm became less significant. These indicate the protein unfolding and were in agreement with the protein denaturation processes at elevated temperature.

Figure 4.11 CD spectra under various temperatures.

4.7 Major findings:

1. Optimized human hair extraction method: Human hair was subjected to washing, delipidizing and cutting. 5-50g of processed hair fragments were immersed in 1 L extracting solution that consisting of 0.125M Na₂S solution. After incubation at 40°C for 2 hours, the resulting mixture was filtered. Soluble protein solution was separated from insoluble residues and dialyzed.
2. The optimized extraction method yields 24 ±1% soluble proteins from human hair.
3. After exhaustive diaysis, chemical contamination was undetectable by either TGA or FTIR.
4. Most abundant amino acids of human hair protein extracts are cysteine (>10 mol %), Glutamine/ Glutamic acid (13.6 mol %) and Proline (11 mol %).
5. Major components of human hair protein extracts are keratins and keratin associated proteins.
6. α -helical structures were present in human hair protein extracts.

Chapter 5: Hydrogel for 2D cell culture

In the previous chapter, keratin extraction methods were optimized. The extracted keratin solution was tested to avoid protein degradation and characterized for properties such as amino acid composition and secondary structure. In this chapter, fabrication of keratin hydrogel induced by CaCl_2 would be reported. Furthermore, this keratin hydrogel's ability to support L929 attachment and proliferation would be demonstrated. Objectives and experimental design flowchart are shown below. Detailed experimental procedures can be found in chapter 3.

5.1 Objectives:

1. Characterize physical and mechanical behaviour of keratin hydrogels induced by CaCl_2
2. Characterize 2D behaviour of L929 mouse fibroblasts-like cells on the surfaces of CaCl_2 induced keratin hydrogel

5.2 Experimental design:

Figure 5.1 Chapter 5 experimental design illustration

5.3 CaCl₂ induced keratin gelation

Keratin gelation was induced by mixing keratin solution with 1M CaCl₂ at the ratio of 50:1 (v/v) followed by incubation at 37°C overnight. Previous to cell seeding, keratin hydrogels were very gently rinsed with sterile ultra pure water and cell culture medium to remove excessive CaCl₂. As shown in figure 5.2 (right), keratin hydrogel was able to retain its shape in ultrapure water despite being too weak to be lifted with forceps without breaking.

Figure 5.2 Photograph showing human hair keratin solution (left) transforming into a hydrogel (right) by adding CaCl₂.

5.4 Keratin hydrogel rheology

The frequency dependence of storage and loss modulus for keratin (10mg/ml) and collagen hydrogel (1mg/ml) at 37°C was shown in figure 5.3. Similar shear thinning behavior was observed for both collagen and keratin hydrogels. They have relatively constant storage modulus (G') of around 5 Pa at lower frequency

followed by rapid decrease at higher frequency (above 10 Hz). Their respective loss modulus (G'') was significantly lower than G' within the frequency region below than 20 Hz, which was indicative of a gel material (87). In general, keratin hydrogels share similar mechanical properties as collagen hydrogel. Its storage modulus was too low to match the storage modulus of dermis (88). To increase mechanical properties, the possibility of incorporating keratin with other hydrogel systems has been discussed as a future recommendation in chapter 7.

Figure 5.3 Plot of storage modulus (G') and loss modulus (G'') as a function of frequency, at 37°C. Figure adapted from (7).

5.5 Keratin hydrogel surface morphology

SEM images of lyophilized keratin hydrogels were presented in figure 5.4. Keratin hydrogels consisted of irregularly shaped leaflets which contained smaller isolated pores (indicated by red arrows). Bigger interconnecting pores (indicated by blue arrows) were present between keratin leaflets branches. In general, keratin hydrogels exhibited highly porous structure. Computational analysis of porosity showed 30 ± 0.5 % total pore area per image. Qualitatively from the SEM images, pore diameter ranged from 6 to 100 μm .

Figure 5.4 SEM image of lyophilized keratin hydrogel. Figure adapted from (7).

5.6 2D L929 cells viability, morphology and distribution

L929 cell line was used here under ASTM international standards for direct contact cell culture evaluation of materials for medical devices (F813-83, reapproved 1988). 10,000/cm² L929 cells were seeded on the surface of tissue culture polystyrene (TCPS control), keratin hydrogel or collagen hydrogel over the course of 6 days. TCPS is the most widely used tissue culture flask plastic. However, TCPS has much higher storage modulus than regular hydrogels. Difference in stiffness may lead to increased cell migration and aberrant proliferation (89). For fair comparison, type I collagen hydrogels which were casted to have similar mechanical properties (shown in our previous rheology results; Figure 5.3) were used as another control group. Type I collagen is the most abundant component of extra cellular matrix in most tissue. They are among the most well-established hydrogels for biomedical researches. They have been used extensively as the gold standards for biomedical applications (90). In this study, cell viability was labeled by live/dead cell staining kit.

In TCPS control group, L929 developed their typical elongated spindle shape from day 2 (Figure 5.5 a) and maintained their morphology through days 4 and 6 (Figure 5.5 d and g). In comparison, L929 in keratin and collagen hydrogel groups still remained rounded at day2 (Figure 5.5 b and c). In collagen hydrogel, a small proportion of L929 started elongating at day 4 (Figure 5.5 f). At day 6, the majority of L929 were elongated (Figure 5.5 i). In the keratin hydrogels, cell morphology development was delayed. They had relatively smaller portion of elongated cells at day 4 and day 6 compared to the collagen hydrogels (Figure 5.5 e and h).

Another observation was that L929 in the keratin hydrogels proliferated in a locally clustered manner. There were still empty spaces in between those cell colonies at day 6 (Figure 5.5 h). In comparison, cells on TCPS and in collagen hydrogels spread evenly over the culture surfaces. These achieved near 100% confluence by day 6 (Figure 5.5 i).

Figure 5.5 Fluorescent microscope images of L929 cells. Cells were seeded on either tissue culture polystyrene (TCPS) (a, d, g), keratin hydrogel (b, e, h) or collagen hydrogel (c, f, i) with initial density of 10,000/cm². At days 2, 4 and 6, live cells were labeled with green fluorescence followed by image taking. Scale bars represent 100µm. Figure adapted from (7).

5.7 2D L929 mouse fibroblast-like cells proliferation

L929 proliferation on TCPS, collagen hydrogel or keratin hydrogel surfaces was measured by the PicoGreen assay. PicoGreen assay quantifies double strand DNA (dsDNA) content of L929 at each time point after seeding. Result was shown in figure 5.6. L929 in all three groups had equal amounts of dsDNA on day 1, suggesting similar cell attachment efficiencies on TCPS, collagen hydrogel and keratin hydrogel surfaces. Beyond day 2, L929 proliferation on collagen and keratin hydrogels was lagged behind that of TCPS control group. This was expected for cells grown on hydrogels, as the stiffnesses of most hydrogels were much lower than TCPS. Between day 2 to day 4, cell number in the control group increased by 10 times, while that in keratin and collagen groups increased by 4 and 7 times. In all three groups, L929 proliferation reached a plateau after 10 days, which was likely to be the result of contact inhibition. In summary, average L929 numbers at each time point in keratin hydrogels were 76% of that in collagen I hydrogels.

Figure 5.6 L929 proliferation rate on TCPS (control), keratin hydrogel and collagen hydrogel. Over 12 days of culture, L929 proliferation was represented by relative double-stranded DNA (dsDNA) levels. Data at each time point were normalized to day 1 control group, presented as means $\pm$ standard deviation. n=4, *p<0.05 (Student's t-test); **p<0.05 (ANOVA, control versus both hydrogel groups) Figure adapted from (7).

5.8 Cell induced keratin hydrogel surface contraction

As the result of forces generated by fibroblast contraction on the surface, keratin hydrogels contracted significantly over the cell culture period (Figure 5.7). The contraction was limited to the surfaces while the rest of the hydrogels remained stable, thus forming a ring of depression on the hydrogel surfaces. The contracted areas made up about 65% of initial surface area on day 4 and 45% on day 6.

Similar cell-induced scaffold contraction phenomena has been described in other *in vitro* systems studies (91, 92) and in wound contraction studies (93). It is speculated that this was a result of fibroblasts differentiating into smooth muscle actin expressing myofibroblasts. In addition, the possibility that the observed phenomena resulted from keratin degradation by fibroblast secreted proteases cannot be ruled out.

Figure 5.7 Keratin hydrogel surface contractions induced by L929. Photographs of keratin hydrogel surfaces and cross sectional illustrations (a) before cell seeding, (b) 4 days and (c) 6 days after cell seeding. Arrows indicate directions of combined cell contracting forces. Scale bar represents 2mm. Figure adapted from (7).

5.9 Cell penetration into keratin/collagen hydrogels

L929 penetration into the keratin/collagen hydrogels was assessed by histology (figure 5.8). At each time point, cell seeded keratin/collagen hydrogels were fixed and sectioned for H&E staining. In both groups, cells were scattered on the hydrogel surfaces on day 2. Collagen hydrogel matrix had fibrous structure, while

keratin hydrogel matrix had globular structure with small branches and appeared denser. At day 4, cells grew into a continuous cell layer. A small proportion of them penetrated into the hydrogel. The penetration depth at this stage was about 500 μ m. In keratin hydrogels, most of the hydrogel surface was covered by a cell monolayer, while some area of collagen hydrogel surface was covered by cell multilayers. On day 6, more cell penetration was evident. The penetration depth increased to about 1mm. In agreement with the florescent microscope images in figure 5.8, more L929 cells in collagen hydrogels developed the elongated spindle shape compared to that in keratin hydrogels. At the same time, keratin hydrogel matrix was pulled towards the cell populated areas, suggesting a similar matrix remodeling and contraction process that has been seen in granulation tissue wound (93, 94).

Figure 5.8 H&E staining of cell seeded hydrogel cross sections. Histology slides of L929 seeded (A1-3) keratin and (B1-3) collagen hydrogels were taken at day (A1, B1) 2, (A2, B2) 4 and (A3, B3) 6 of culture. Scale bar represents 100 μ m. Figure adapted from (7).

5.10 Major findings:

1. Keratin hydrogels supported high cell viability.
2. L929 cells seeded on keratin hydrogels have an average proliferation rate 76% compared to cells on collagen hydrogels.
3. Different distribution and morphology were observed in cells grown on keratin hydrogels compared to collagen hydrogel. Keratin hydrogels gave rise to localized proliferative cell colonies.
4. L929 cells were capable of penetrating and remodeling the keratin matrices.

Chapter 6: Keratin hydrogel for 3D cell culture

In the last chapter, it was shown that keratin hydrogel can be induced by calcium ions. However, excessive calcium ions have to be washed off before those keratin hydrogels were able to support 2D cell culture. In this chapter, the development of a pH induced keratin gelation method which allows living cell encapsulation under physiological condition would be reported. Furthermore, the potential of using cell-encapsulated keratin hydrogel as a novel 3D cell culture would be demonstrated. Objectives and experimental design flowchart are shown below. Detailed experimental procedures can be found in chapter 3.

6.1 Objectives:

1. Develop novel way of inducing keratin gelation while maintaining physiological conditions for cell encapsulation
2. Characterize physical and mechanical behaviour of keratin hydrogels induced by pH3 citrate buffer
3. Demonstrate feasibility of cell encapsulation in keratin hydrogel using L929 mouse fibroblast-like cells

6.2 Experimental design:

Figure 6.1 Chapter 6 experimental design illustration

6.3 Factors to control keratin gelation: concentration

It is critical to adjust the keratin gelation process in order to fulfill the requirement of live cell encapsulation. We managed to control the keratin gelation process by 3 factors. They were concentration, pH and temperature.

There were two stages in keratin gelation process: initial keratin flocculation and gelation with stabilized keratin flocs. It is first noticed that the first stage could be triggered by lowering the pH to 3.5 and this process was affected by keratin concentration. As figure 6.2A shows, keratin flocs appeared as interconnecting networks at higher keratin concentration. The number of keratin flocs increased as keratin concentration increased from 0.1mg/ml to 0.5mg/ml. clustering of keratin flocs started from 0.75 mg/ml and formed interconnected networks at 2 mg/ml.

6.4 Factors to control keratin gelation: pH

Then range of pH at which keratin flocculation could be triggered was established using techniques such as visual inspection and UV-Vis spectrophotometer. Many proteins have the tendency to undergo spontaneous association which was driven mainly by hydrophobic interactions. However, at neutral pH, keratin molecules carried net negative charges ($pI < 7$). Electrostatic repulsions kept the keratin molecules apart from each other. Thus at neutral pH, keratin was dissolved and keratin solution appeared brownish semi-transparent (figure 6.2B). By gradually lowering pH, the negative net charges of keratin molecules were reduced. Hydrophobic interactions became the dominant force and keratin flocculation started. Clustering keratin flocs scattered light which resulted in increased turbidity.

The semi-transparent solution turned into opaque gels. By monitoring solution turbidity changes when solution pH was adjusted from 7.2 to 3.3. It is concluded that keratin flocculation was triggered between pH4 to 4.5.

6.5 Factors to control keratin gelation: Temperature

Keratin flocculation was initialized at pH3.5 but can be resolubilized at neutral pH. However, since acidic pH was not ideal for cell culture, the keratin flocs have to be stabilized with incubation at 37°C until they can be maintained at neutral pH. Further, this stabilizing process was found to be temperature-dependant. As shown in figure 6.2C, re-suspending keratin flocs in water (water group) resulted in unchanged high absorbance. That was because water has low neutralizing capacity. There was no re-solubilization as the pH had not been neutralized. Instead of water, if PBS was used to re-suspend keratin flocs (PBS group), the absorbance dropped to low background level at time 0 (no stabilizing time). Keratin flocculation was reversible at that time point without the stabilizing. By increasing the incubation time from 1 hour to 4 hours, absorbance reading increased as well, which was a sign that keratin flocs became more stable. After 5 hours' incubation at 37°C, absorbance readings reached constantly high plateau. After that time point, keratin flocs were stable at neutral pH.

Lower temperature slowed down this stabilization process. If the incubation temperature was kept at 4°C, keratin flocs were still unstable and completely re-soluble even after 8 hours incubation. Stabilization of keratin flocs could also be

achieved at room temperature (25°C) with doubling incubation time (data not shown).

Figure 6.2 Establishing controllable gelling conditions. 3 crucial factors that affect keratin gelation are concentration, pH and temperature. (A) Phase contrast light

microspe images of keratin solutions at pH3.5 with concentrations ranging 0.1-2 mg/ml. Increased keratin concentration resulted higher floc density and size. Scale bar represents 100µm. (B) Turbidity variations as the result of pH triggered keratin gelation. During keratin gelation process, formation of keratin floc cause light scattering that affect tubidity. Turbidity changes were observed by visual inspection.and quantified by UV-Vis spectrophotometer. (C) Correlation of temperature and keratin flocs stability. Stability of keratin flocs was represented by the visible light absorbance. It was established that 5 hours incubation at 37°C is needed for stable keratin gelation at neutral pH (PBS).

6.6 Keratin hydrogel fabrication

Based on the key factors that has been identified; a protocol that allows live cell encapsulation and cultivation was formulated. The resulting hydrogels were very fragile. They could not be taken out of normal plates without breaking. Cylindrical molds were made, in order to facilitate easy transportation for downstream measurement such as microscopy and histology (Fig. 6.4). Detailed descriptions of those procedures were available in chapter 3.

Figure 6.3 Live cell encapsulation into 3D keratin hydrogel. Keratin gelation was triggered at pH3.5 by mixing keratin solution with PBS/citrate buffer. Resulting keratin flocs were stabilized at 37°C for 12 hours and then washed with PBS or DMEM. Living cells were mechanically suspended into washed keratin flocs followed by casting into culture vessels.

Figure 6.4 Assembling of custom-made molds. Custom-made molds were used to provide convenient transportation of cell-seeded keratin hydrogels during microscopy and histology processing. As shown, polyethylene rings were adhered onto glass cover slips using PDMS. They can be peeled off later without breaking keratin hydrogel.

6.7 Keratin hydrogels surface morphology

SEM images of lyophilized keratin hydrogels were presented in figure 6.5. Keratin hydrogels exhibited highly porous structure. Based on visual inspection of the SEM images, increasing keratin concentration from 15mg/ ml to 50 mg/ml reduced porosity. This observation was supported by quantitative image analysis (figure 6.6). Porosity of high concentration (40mg/ml, 50mg/ml) keratin hydrogels was significantly lower than low concentration (15mg/ml, 25mg/ml) keratin hydrogels. There was no significant difference between 15mg/ml or 25mg/ml citrate buffer induced keratin hydrogel and 10mg/ml CaCl_2 induced keratin hydrogel.

Figure 6.5 SEM images of lyophilized keratin hydrogel with variant keratin concentrations.

Figure 6.6 Computational analyses of keratin hydrogel relative porosity and pore numbers based on SEM images.

6.8 Keratin hydrogels mechanical properties

The frequency dependence of storage modulus of hydrogels with different concentrations was shown in figure 6.7. By increasing keratin concentration from 15mg/ml to 50mg/ml, the storage modulus increased from a few Pa to a few hundred Pa. This range covers the storage modulus of very soft tissues such as brain (95), but could not match the storage modulus of dermis (88).

Figure 6.7 Plot of storage modulus (G') as a function of frequency which suggest higher keratin concentration increased keratin hydrogel stiffness.

6.9 L929 cells viability in 3D keratin hydrogels

50,000/cm³ L929 cells were encapsulated inside keratin or collagen hydrogels. Cell morphology and distribution was captured using fluorescence microscope every 2 days until day 16 (figure 6.8). Randomly distributed L929 in both keratin and collagen hydrogels remained rounded at day2. Cell doublets were observed since day 4, which indicated cell proliferation. From day 6 to day 12, growing cell clusters were observed in both groups. Until day 12, very few dead cells were found. Therefore, it is concluded that L929 viability was preserved during the encapsulation process and was maintained at this stage. Cell culture was continued until Day 16. Cells grew almost confluent at day 14 and day 16. More dead cells were observed since day 14 as the result of contact inhibition.

Figure 6.8 Fluorescent microscope images of L929. Cells were encapsulated in either keratin hydrogel or collagen hydrogel with initial density of $50,000/\text{cm}^3$. Every 2 days from day 2 until day 16, live cells were stained with live/dead cell kit followed by image taking. In both groups, cell doublets were observed since day 4. From day 6 to day 12, growing cell clusters were observed in both groups. Until

day 12, very few dead cells were found. Cells grew almost confluent at day 14 and day 16. More dead cells were observed as the result of contact inhibition.

6.10 L929 proliferation in 3D keratin hydrogel

Proliferation of L929 encapsulated in collagen or keratin hydrogels was determined by measuring dsDNA level using PicoGreen assay. Previous to PicoGreen assay, collagen or keratin hydrogels were first digested with proteinase K. Then the DNA content of cell lysate was separated from DNA content using phenol extraction method. Result was shown in figure 6.9A. L929 in both keratin/ collagen hydrogel groups had equal amount of dsDNA on day 1. Between day 2 and day 10, cells in keratin groups proliferated slightly faster than in collagen groups. In keratin group, L929 number reached maximum plateau at day 12, while collagen group caught up keratin group after day 12 and reached maximum at day 14. Cell numbers in both groups dropped at day 16. During day 14 and 16, cells in both groups were becoming locally confluent, nutrient transport and space became the dominant limiting factors. Nevertheless, keratin hydrogels were able to support encapsulated L929 proliferation at the same level of collagen hydrogel.

6.11 Cell-induced keratin /collagen hydrogel contraction

Despite similar cell numbers in both hydrogel groups through cell culture period, collagen hydrogel contraction was significantly more than keratin hydrogel (figure 6.9B). Both keratin and collagen hydrogels were maximally contracted at day 8

before cell culture was terminated at day 16. Once they reached the most contracted stage, collagen hydrogels diameter shortened from 12mm to around 6mm, while keratin hydrogels contracted only slightly from 12 mm to 10mm.

Figure 6.9 Cell proliferation and cell-induced hydrogel contraction (A) Proliferation rate of encapsulated L929 in keratin hydrogel and collagen hydrogel.

Over 16 days of culture, L929 proliferation was represented by relative double-stranded DNA (dsDNA) levels. Data at each time point were normalized to day 1 collagen group, presented as means $\pm$ standard deviation. n=4, *p<0.05 (Student's t-test). (B) L929 induced keratin and collagen hydrogels contraction. Over culture period, keratin and collagen hydrogels contracted by 2mm (17%) and 6mm (50%) respectively.

6.12 Cell distribution in 3D keratin hydrogels

As florescent microscopy was mainly for 2D cell visualization, it can only capture cells within a limited depth. Therefore L929 distribution in 3D keratin hydrogels was assessed by histology (figure 6.10). At day 2, day 6 and day 12, cell seeded keratin hydrogels were fixed and sectioned for H&E staining. At early stage of cell culture (day 2 and day 6), cells were found randomly distributed throughout the depth of the hydrogels, which indicated homogenous encapsulation and keratin hydrogel's ability to hold cells suspended. However at the late stage of cell culture (day 12), more cells were found near to the hydrogel surface where nutrient transportation was more efficient.

Figure 6.10 H&E staining of cell seeded scaffolds cross sections. Whole cross section of L929 seeded keratin hydrogels were taken at day 2 (A), 6 (B) and 12 (C) of culture. The scale bar represents 500 μm . A1, B1 and C1 represent enlarged regions of day 2, 6 and 12. Scale bar represents 20 μm . Cells were distributed randomly across the entire depth at day 2 and day 6. More cells were found near to the periphery at day 12.

6.13 Major findings:

1. Acidic pH induced keratin gelation process was studied. 3 factors (concentration, pH, temperature) were identified to be able to control the gelation process.
2. Procedures of encapsulating live cells into 3D keratin hydrogels were developed.
3. Cell viability was supported in 3D keratin hydrogel.
4. A new method which combined enzymatic digestion with DNA phenol extraction for quantitative analysis of cell proliferation in 3D cell culture was developed.
5. Cell proliferation rate in 3D keratin hydrogel was comparable to collagen I hydrogel.

Chapter 7: *in vitro* / *in vivo* biocompatibility study of keratin-based material

As reported in chapters 4 and 5, keratin hydrogels were suitable 2D/3D cell culture material for L929. In this chapter, a broader spectrum of keratin biocompatibility was studied. Additional cell types (L929, human dermal fibroblasts and keratinocyte) were tested for their behaviour and interaction with different format of hair keratins (solution, film/coating and hydrogel). Moreover, preliminary *in vivo* studies were done to show that subcutaneously implanted keratin hydrogels/sponges did not cause acute hosts tissue response. Objectives and experimental design flowchart are shown below. Detailed experimental procedures can be found in chapter 3.

7.1 Objectives:

1. Examine the effects of soluble hair keratins on keratinocyte proliferation.
2. Exam keratin coating/film bioactivity in supporting L929 and HDF cell culture.
3. Study keratin hydrogel / sponge immunogenicity by *in vivo* subcutaneous transplantation in immunocompetent mice.

7.2 Experimental design:

Figure 7.1 Chapter 7 experimental design illustration

7.3 Effect of soluble keratin on keratinocyte proliferation

Proliferation of keratinocyte was a critical event during wound healing. It directly affected re-epithelialisation process. Keratin-based materials were generally quite stable as demonstrated by the *in vivo* studies. However, soluble keratins and keratin fragments were released into surrounding tissues during degradation. Thus, as potential wound dressing material, it is important to determine how soluble keratin affected keratinocyte proliferation. $10^4/\text{cm}^2$ keratinocytes were seeded on TCPS. At day1, cell culture medium was supplemented with soluble keratin solution with final concentration ranged from 0 (control group) to 1000 $\mu\text{g}/\text{ml}$ and cell culture was continued until day 5. Results are shown in figure 7.2. At lower concentration (below 30 $\mu\text{g}/\text{ml}$) there was no significant difference with or without soluble keratin supplement. As keratin concentration increased to 100, 300 and 1000 $\mu\text{g}/\text{ml}$, keratinocyte proliferation rate dropped to around 80%, 45% and 20% respectively of control group. However, the release of soluble keratins or keratin fragments was expected to be slow and gradual. Soluble keratin levels should be within low concentration range.

Figure 7.2 Keratinocyte proliferations in soluble keratin supplemented cell culture medium. At day 5 after cell seeding, keratinocyte proliferation was represented by relative double-stranded DNA (dsDNA) levels. Data were normalized to control group (no keratin supplement), presented as means $\pm$ standard deviation. n=4, *p<0.05 (Student's t-test).

7.4 *In vitro* study of keratin coating/film

L929 and human dermal fibroblast (HDF) proliferation on keratin films made from soluble keratins or flocculated keratins were tested. Two types of keratin films have different microstructures. As suggested in previous studies, soluble keratins films have tightly packed smooth surface, while flocculated keratin films have

sponge-like network surface(28). Collagen films and TCPS were used as control groups. The result of L929 proliferation was shown in figure 7.3A. L929 grown on collagen film seemed to have a longer lag phase. However, there was no significant difference between the 2 keratin films. On the contrary, HDF had a slower proliferation rate on flocculated keratin film than on soluble keratin film (figure 7.3B). Similar observation was made by a previous study (28). This could be due to changed accessibility of cell binding motif as the result of keratin flocculation. Using K31 as an example, an analysis of its amino acid sequence showed that the LDV motif is located in the hydrophobic region of the protein (figure 7.4). Similar prediction was made for the other 10 type I keratins that contained the known cell binding sequence LDV. During the flocculation process, these LDV motifs could have been partially buried. It was clear that different cells responded differently to keratin materials that have different micro-structures.

Figure 7.3 L929 (A) and Human dermal fibroblast (HDF) (B) proliferation rate on TCPS control, flocculated keratin film, keratin film and collagen film. Over 5 days of culture, L929 and HDF proliferation was represented by relative double-

stranded DNA (dsDNA) levels. Data at each time point were normalized to day 1 collagen film group, presented as means $\pm$ standard deviation. n=4, *p<0.05 (Student's t-test)

Figure 7.4 Protein hydrophobicity plot of Keratin K31. Scales were determined by Kyte-Doolittle method. Regions that have values above 0 were characterized as hydrophobic residues. LDV was located at 343-345, arrow indicate amino acid position 344.

7.5 *In vivo* subcutaneous implantation

Keratin hydrogels were studied for immune response after subcutaneous implantation. Both short- and long-term results of pH3 induced keratin hydrogel were shown in figure 7.5. Keratin implants were found intact after 7 days. They integrated into surrounding host tissue with only mild fibrotic response (Figure 7.5 A, B). Both fibroblasts (elongated spindle shape) and neutrophils (round shape with dense nuclei) were found around the implants and infiltrating into the implants. Together with cell infiltration, certain degree of matrix remodeling was also evident (Figure 7.5 C, D). 90 days post implantation, only 30% of original keratin hydrogel remained visible. Implants were fragmented and surrounded by a thin layer of encapsulating cells. There was no obvious vascularization inside keratin hydrogels (Figure 7.5 E, F).

Only short-term study was done with CaCl₂ induced keratin hydrogel. Results were shown in figure 7.7. Similarly, the boundary of keratin implants remained relatively intact 7 days post transplantation. No signs of adverse immune response were found.

Figure 7.5 Subcutaneous pH3 keratin gel implantation. MT staining (A) and H&E staining (B, C and D) at day 7 post-implantation showed intact implants, cell infiltration and collagen deposition. Position of keratin implants were indicated

with dashed oval (A and B). Examples of fibroblasts (elongated spindle shape) were indicated with red arrows; while neutrophils (round shape with dense nuclei) were indicated with green arrows. Hydrogel boundary was indicated by dashed lines with an asterisk. Appearance of cells within hydrogel boundary indicates cell migration from surrounding tissue (D).

Figure 7.6 H&E staining of subcutaneous pH3 keratin gel implantation at day 90 showed pieces of remaining implants and signs of vascularisation around the implants.

Figure 7.7 H&E and MT staining of subcutaneous implanted CaCl_2 gel. At day 7 post-implantation, relatively intact cell capsule and new collagen deposition were found around implants, cells infiltration was evident. Position of keratin implants were indicated with dashed line (A and B). Hydrogel boundary was indicated by dashed lines with asterisk. Cells were found migrating into the hydrogel (C and D).

7.6 Major findings:

1. No sign of hindering keratinocyte proliferation was observed until 100µg/ml.
2. L929 had similar proliferation rate on keratin films made from soluble keratins or flocculated keratins, while HDF proliferated slower on the later.
3. Keratin hydrogels were low-immunogenic in subcutaneous transplantation model.

Chapter 8: Conclusions and future recommendations

8.1 Conclusions

Human hair keratins are readily available, easy to extract and eco-friendly materials with promising bioactivities. In this PhD study, keratin extraction from hair was optimized; fabricated keratin hydrogel using both CaCl_2 as well as pH induction were tested as 2D or 3D cell culture scaffolds. Collectively, the following conclusions were drawn from experimental results generated:

1. Soluble keratins can be obtained from human hair in high yield and without protein degradation.
2. Keratin gelation can be triggered by increasing salt concentration using CaCl_2 or by reducing pH to about 4 using citrate buffer.
3. CaCl_2 induced keratin hydrogels have suitable physical, mechanical and biochemical properties that support 2D cell growth.
4. Live cell encapsulation in citrate buffer induced keratin hydrogels can be facilitated by controlling the three key factors: keratin concentration, pH and temperature.
5. Citrate buffer induced keratin hydrogels could maintain the viability of encapsulated cells and support cell proliferation in 3D environment.
6. Preliminary *in vitro* and *in vivo* keratin-based studies demonstrated cell and tissue compatibility.

This thesis demonstrated that keratins extracted from human hairs are able to form hydrogels which have bioactive and physical properties that meet the needs of either 2D or 3D cell culture requirements and therefore have potential to be used for biomedical applications.

8.2 Future recommendations

8.2.1 Cell binding mechanism

Human hair keratin materials can support cell adhesion and proliferation. However, the cell attachment mechanism has yet to be discovered. It has been suggested that LDV (Leu-Asp-Val) was involved in cell adhesion. Similar to fibronectin and laminin, human hair keratins contain the LDV (Leu-Asp-Val) cell adhesion motif, which is recognized by the integrin receptor $\alpha_4\beta_1$ (28). Among the 17 known human hair keratins, all 11 type I keratins contain the LDV motif, while only K82 out of the 6 type II keratins contain LDV (Table 2.1). Since in hair, keratins filaments were assembly as a heterodimers of type I and type II keratins in the ratio 1:1, so more than half of the extracted keratins contain LDV. However, contradictory results have been obtained from different cell types. Platelets grown on keratin films had better adhesion and spreading morphology. Those adhesion was demonstrated to be β_1 and β_3 integrin dependent which suggest the involvement of LDV (60). However, a study on hepatocyte attachment on keratin material showed that β_1 and β_3 integrins were not mediating the adhesion. This study suggested that instead of LDV, hepatic asialoglycoprotein receptor (ASGPR)

was more likely to be involved in cell adhesion (58). Those conflicting results suggest multiple cell binding pathways exist for cell binding mechanisms on keratins. It is also important to consider the accessibility of the LDV motif on keratins extracted with different methods and subsequent processes.

8.2.2 Keratin filament assembly

Human hair mechanical properties are influenced by factors such as temperature (96), humidity (97, 98) and racial groups (99, 100). Despite those variances, it is still one of the strongest natural fibers. The elastic modulus of human hair is in the range of 1-3.5 GPa. The ultimate tensile strength of human hair is in the range of 100 to 300 MPa (39, 101-103), which is comparable to aluminum but weighs only half as much. Human hair's strength and lightness makes it attractive to various high performance applications. However, none of current keratin-based materials has achieved or has even come close to the same mechanical strength. One of the missing keys here is the reassembly of keratin. Keratins have the intrinsic potential to self-assemble into keratin intermediate filaments, *In vitro* human hair keratin assembly has been achieved for years, although the exact assembling mechanism has not been completely understood (104-108). However, those re-assembling processes have not been integrated into keratin-based material studies. One common mistake is assuming that undamaged keratin monomers are obtained through the extraction processes and that they are able to spontaneously self-assemble into keratin filaments once the extraction chemicals are removed. There

is no study of how current extraction methods affect keratin refolding. There is no experimental evidence of keratin intermediate filament formation in any keratin material studies. We believe this to be the area that more attention should be devoted to eventually revolutionize applications using keratin-based templates.

8.2.3 Keratin antioxidant activity

There is no proven mechanism that could explain the bioactivity of keratin-based material. As commonly suggested in other biomaterial studies, cell binding motifs found in keratin sequences was the most prevalent theory. The influence of a high cysteine content, which is the most distinct characteristics of keratins, has not yet been brought up for discussion. Given the fact that free-thiol groups are well known scavengers of Reactive oxygen species (ROS), here we propose another possible mechanism: the antioxidant effect of keratin-based materials.

ROS are produced primarily by mitochondria during normal cell metabolism. They are also produced by phagocytes to combat micro-organisms, destroy neoplastic cells and mediate inflammatory response. However, it is well documented that excess ROS can cause damage to cellular proteins, DNA, lipids and carbohydrates, inhibiting normal cell function. Reduction-oxidation (redox) state imbalance has been linked with various human diseases including diabetes mellitus, inflammatory diseases, neurodegenerative disorders, coronary artery disease, cancer and ageing. (109-114) Thus, it is crucial to maintain the redox homeostasis. Several mechanisms are involved in redox balance. Molecules involved in those

mechanisms includes vitamins (C and E), enzymes (catalase, glutathione peroxidase and superoxide dismutase) and most importantly the small sulfhydryl containing molecules (such as glutathione (GSH) and thioredoxin (TRX)) that can be reversibly reduced or oxidized depending on the cellular environment (115-118).

Amino acid analysis of extracted human hair proteins revealed 9-10% cysteine content(59, 63). Using reductive extraction method, cysteines are reduced to their free thiol (S-H) state. Future studies should be done on quantifying free-thiol groups in keratin-based materials after different methods of extraction, purification and fabrication. After that, correlations can be tested between observed effects and the amount of free-thiol groups.

8.2.4 Keratin hydrogel mechanical strength enhancement

The storage modulus of keratin hydrogels that were fabricated through either CaCl_2 induced or citrate buffer induced methods ranged between a few Pa to a few hundred Pa. This range covers the storage modulus of very soft tissues such as brain (95), but was too weak to match the storage modulus of dermis (88). To overcome this problem, several approaches could be used to enhance the gel's mechanical properties.

It is suspected that limited number of linkages among keratin flocs accounted for the gel weakness. Adding thiol-containing crosslinkers to form disulfide bonds with keratin's thiol groups could potentially bridge those flocs. Crosslinkers could be either synthetic polymers such as thiolated poly acrylic acid (PAA), polyethylene (glycol) diacrylate (PEGDA) or thiol-rich proteins such as KAPs.

Another approach could be to incorporate keratin hydrogel with other hydrogel systems such as chitosan or gellan gum. Change in gel strength and porosity can be measured with rheometer and SEM.

8.2.5 Safety and toxicity of human hair keratin

Using human hair keratins as biomaterials is relatively new. Although it is speculated that using one's own hair would lower risk of toxicity and immunogenicity, the physiological influence of this material, including potential toxicity, has not been systematically established. Future studies should be done in 3 aspects: human hair heavy metal contents, *in vitro* enzymatic degradation and *in vitro/in vivo* immunogenicity studies.

Heavy metals such as mercury and lead are able to accumulate in human hair by binding to cysteine-rich keratin proteins (119, 120). Most of the cysteine-bounded metals could be released during reductive extraction procedure. Further experiments should be done to monitor if there is any heavy metal contamination. From literature, keratins extracted from human hair or wool can be degraded by the serine protease trypsin, chymotrypsin and proteinase K (63, 121-123). However, it has not been studied how keratin degradability can be influenced by keratin extracting methods, scaffold fabrication methods, cross-linking density, scaffold porosity, fiber diameter and keratin concentrations. A more systemic *in vitro* enzymatic degradation study needs to be done to understand keratin enzymatic degradation process. Despite promising result from preliminary keratin subcutaneous implantation studies, very little is known regarding keratin *in vivo* immunogenicity. Next step could be *in vitro/ in vivo* study on macrophages

response to keratin-based material. Macrophages play important role in immune response, inflammation and foreign body reactions. It is important to determine if keratin-based material can activate macrophages and if keratin-based material can absorb proteins such as stress proteins (heat shock proteins), plasma fibronectin, and fibrin from surrounding tissues that activate macrophages.

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