CHAPTER III

LITERATURE REVIEWS

In this chapter, the literature reviews are summarized into 3 parts as follows:

1. Influence of scaffolds on dermal wound healing.
2. Processing parameters for scaffold fabrication.
3. Characterization of collagen, collagen/chitosan, and chitosan scaffolds.

1. Influence of scaffolds on dermal wound healing.

In 1996, Pachence [41] reviewed the reasons behind the use of collagen for soft tissue repair, particularly in devices used for wound care. Collagen-based medical devices have a major impact in the field of soft tissue repair. Collagen has become the biomaterial of choice for a number of important medical applications because of several reasons as follows:

- Know-how for obtaining large quantities of medical grade collagen is well developed.
- There are a number of established collagen products, some of which are well known.
- Collagen has a good safety profile as a biomaterial.
- Collagen can be produced in forms that are easily used in minimally invasive procedures.
- The understanding of its role in wound healing, metabolism, and catabolism, and the interaction between cells and collagen has been greatly improved.

The collagen implants are populated with a number of cell types, including macrophages and an occasional giant cell, but primarily with fibroblasts. It has been

found that new collagen production by fibroblasts is increased when the cells are bound to an extracellular matrix, such as implanted collagen. The implanted collagen sponge remains for 3-6 weeks on average (depending on the degree of crosslinking), until it is degraded into peptide and amino acid components by the fibroblast-produced collagenase. The implant is then remodeled and replaced by fibroblast-derived native type I collagen. But more importantly, wound contraction at the collagen-treated wound site is significantly reduced compared with the control or the dressing treated sites. Collagen in the form of fibers, sponges, and fleeces has a long established clinical history as a hemostatic agent. Collagen sponges present an additional benefit for soft tissue repair because their wet strength allows for suturing the material to soft tissue, providing a template for new tissue in growth. It has been well documented that biodegradation of collagen followed a course that was identical to normal wound healing, i.e. implanted collagen is degraded through native enzymatic pathways that lead to peptide fragments and amino acid residues. Animal and clinical testing of collagen dressings used for full thickness or partial thickness wounds have shown them to be safe and effective. More importantly, collagen and collagen peptide fragments are a normal part of the wound healing mechanism, which is a significant advantage over materials used in other commercially available products (gauze, hydrocolloids, alginates, etc.). Therefore, collagen-based wound dressings should experience widespread uses in the near future.

2. Processing parameters for scaffold fabrication.

In 2004, O' Brien and coworkers [3] studied the influence of freezing rate on pore structure in freeze-dried collagen-glycosaminoglycan (CG) scaffolds. The conventional freeze drying process for fabricating CG scaffolds creates variable cooling rates throughout the scaffold during freezing, producing a heterogeneous matrix pore structure with a large variation in average pore diameter at different locations. Scaffold heterogeneity has been shown to lead to variable cell adhesion and to affect the ability of the cell to produce a uniform distribution of extracellular matrix proteins. The scaffold synthesis process was modified to produce more homogeneous freezing by controlling the rate of freezing during fabrication. The rapid and

uncontrolled quench freezing process was typically used in fabricating porous scaffolds via freeze drying results in space- and time-variable heat transfer through the suspension, leading to non-uniform nucleation and growth of ice crystals and, ultimately, scaffold heterogeneity. The CG scaffolds were fabricated from a collagen-glycosaminoglycan suspension contained 0.5% (w/w) collagen and 0.05% (w/w) chondroitin-6-sulfate using a freeze drying method. The CG suspension was frozen at three distinct cooling rates, where the freeze dryer shelf temperature was ramped from 20°C to the final temperature of -40°C in 65, 90 and 115 min. The frozen samples were sublimated under vacuum (<100mTorr) at 0°C for a period of 24 h. The constant cooling rate technique with a freezing time of 65 min which was corresponded to cooling rate of 0.9°C/min displayed significantly improved freezing homogeneity compared to the quenching technique. When the scaffolds were compared, it was apparent that using the new constant cooling rate technique, the pores were more uniform in size and shape, and no obvious variation in mean pore size, pore structure, or alignment was shown. The pore structure of scaffolds had a small difference in pore size between the longitudinal (96.8 ± 11.1 μm) and transverse (94.5 ± 13.9 μm) planes.

In 2005, O' Brien and coworkers [2] studied the effect of pore size on cell adhesion in collagen-glycosaminoglycan (CG) scaffolds. To study the relationship between cell attachment and viability in scaffolds and the scaffold structure, CG scaffolds with four different pore sizes corresponding to four levels of specific surface area (at fixed composition and solid volume fraction (0.005)) were manufactured using a lyophilization technique. The CG suspension was produced by combining microfibrillar, type I collagen (0.5% (w/w)) isolated from bovine tendon and chondroitin-6-sulfate (0.05% (w/w)) isolated from shark cartilage in a solution of 0.05 M acetic acid (pH 3.2). The CG suspension was frozen using the same constant cooling rate, but difference in the final temperatures of freezing (-10°C, -20°C, -30°C and -40°C) were used to produce porous scaffolds with four different mean pore sizes. All CG scaffolds were then crosslinked via a dehydrothermal process at a temperature of 105°C under a vacuum of 50 mTorr for 24 h. Scaffolds fabricated at a final temperature of freezing of -40°C, -30°C, -20°C and -10°C were determined to have a

mean pore size of 95.9, 109.5, 121.0 and 150.5 μm, respectively. For scaffolds produced at freezing temperatures of -40°C, -30°C, -20°C, no significant difference was found between the mean pore sizes in the transverse and longitudinal planes. A significant difference in mean pore sizes of the longitudinal (163.9 ± 31.6 μm) and transverse (130.4 ± 20.6 μm) planes was observed for CG scaffolds produced at a final freezing temperature of -10°C due to a predominant direction of heat transfer during the freezing process. The mean pore size of the scaffolds had a significant effect on cell attachment after 24 and 48 h. In the scaffolds with the smallest mean pore size (T_f = -40°C) over 40% of cells seeded remained attached to the scaffolds at 24 and 48 h compared to approximately 20% of cells that remained attached to the scaffolds with the largest mean pore size (T_f = -10°C). No significant difference in cell attachment was found between the intermediate scaffolds (T_f = -20°C, -30°C) with approximately 30% of cells remaining attached after both 24 and 48 h groups. As all scaffolds were fabricated from the same stock CG suspension, the density of ligands available for binding on each scaffold was assumed to be proportional to the scaffold specific surface; the specific surface area was calculated to be inversely proportional to the mean pore size. The increasing cell viability with decreasing pore size would not be expected to continue as pore size would eventually drop to the point where cells could no longer fit into the pores.

3. Characterization of collagen, collagen/chitosan and chitosan scaffolds.

In 1989, Yannas and coworkers [42] studied the synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Highly porous and crosslinked collagen-glycosaminoglycan (CG) copolymers have induced morphogenesis of skin when seeded with dermal and epidermal cells and then grafted on standard, full-thickness wounds in an adult guinea pig. These chemical analogs of extracellular matrices were morphogenetically active providing the average pore diameter ranged between 20 and 125 μm. The resistance to degradation by collagenase exceeded a critical limit, and the density of analogous dermal and epidermal cells inoculated was more than 5×10^5 cells per cm^2 of wound area. Unseeded copolymers with physical structures that were within these limits

delayed the onset of wound contraction by about 10 days. Seeded copolymers not only delayed contraction but eventually arrested and reversed it while new skin was being regenerated. Morphogenetic activity was shown that both CG copolymers and seeded CG copolymers possessed values of the mean pore diameter and enzymatic degradation rate. When grafted with CG copolymers that was seeded with at least 5×10^5 cells per cm^2 of graft area, the wound initially displayed contraction kinetics that were roughly similar to those of cell-free. The contraction rate then reached a maximum and eventually slowed down until the wound perimeter showed almost no change between days 35 and 50. Thereafter, the wound perimeter expanded at a rate significantly in excess of the normal growth rate of the animal until it asymptotically reached $72 \pm 5\%$ of the original wound area. Furthermore, the control of mean pore diameter by appropriate control of freeze drying conditions was a very effective procedure for delaying the onset of wound contraction. The degradation rate could also be manipulated by glutaraldehyde crosslinking step affected the kinetics of contraction independently from the mean pore diameter.

In 1996, Geesin and coworkers [43] has compared skin model system (SMS) and living skin equivalent (LSE) in vitro matrices for their ability to use as a replacement for a dermis in a culture system using human epidermal cells. Skin model system or SMS was denoted for the matrix containing keratinocytes and living skin equivalent or LSE was manufactured by mixing living human fibroblasts with soluble collagen under conditions where the collagen forms a gel. Three-dimensional collagenous matrices were prepared from lyophilization of 3% (w/w) collagen dispersed in 0.001 N HCl and then crosslinked by a dehydrothermal treatment. The matrices were sealed and sterilized with gamma irradiation using 2.5 Mrad of ^{60}Co and stored in a dry state. Dermal equivalents were prepared by adding 1 ml of cell suspension in cell culture medium containing $1.0\text{-}1.4 \times 10^5$ cells and 2 ml of the same medium into 1 ml of rat tail type I collagen at a concentration of 2.5 mg/ml in 10 mM acetic acid. The cell suspension-collagen solution was poured into a 35 mm petri dish and incubated in a 37°C tissue culture incubator in 5% CO_2 for 5 days to allow contraction to occur. The matrices that were crosslinked by the dehydrothermal technique, achieved cell numbers of approximately 70% of those achieved on plastic

after 7 days. Human keratinocytes were cultured on the collagen matrices produced and on LSEs. In human neonatal foreskin, a dermal layer consisting of connective tissue and fibroblasts was apparent over a stratified epidermal layer containing keratinocytes of varying degrees of differentiation. When keratinocytes were cultured on dermal equivalents for 10 days, stratified layers similar to those seen in foreskin were evident. When collagen sponges with fibroblasts were first used as a support for keratinocytes without considering the importance of the surface pore size, no distinct layers were formed. Epidermal cells grew across the surface of the matrix and within open spaces exposed to the surface. The pore size of SMS developed was designed to be 50-300 μm in which the smaller pore size could be found on the container exposed side of the matrix. For this reason, the epidermal cells were then seeded and grown on the container exposed side of the matrix. Under these conditions, the epidermal component of the model formed stratified layers similar to skin with the epidermal cells differentiating as they approached the surface. As a substrate for epidermal development, the epidermal layer produced in the skin model system was almost the same as that seen in the LSE cultures (thickness and stratified layers). The ability of crosslinked collagen matrix was demonstrated to serve as a substrate for the assembly of an in vitro skin model system that compares favorably to living skin equivalent and normal skin.

In 1996, Taravel and Domard [44] investigated some biological and mechanical properties of collagen and chitosan. Bovine atelocollagen and high molecular weight fully deacetylated chitosan, depending on the conditions, formed complexes whether by means of purely electrostatic interactions or by hydrogen bonding. In the purely electrostatic interactions, the maximum proportion of chitosan in the complex was relatively low (approximately 10%) and it was difficult to conclude whether chitosan prevented collagen digestion by collagenase or not. On the contrary, in the hydrogen bonding complex, chitosan induced a strong protection toward the specific enzyme. This kind of interaction certainly contrasts with the recognition of the collagen structure, in particular by a very important steric hindrance related to the great proportion of chitosan in the surrounding collagen chains. It was

suggested that chitosan brought softening rather than hardening to the system and the complex behaved like some polymer blends interacted with ionic bonding.

In 1999, Sundararajan and coworkers [6] reviewed wide array of tissue engineering applications exacerbates the need for biodegradable materials with broad potential. Chitosan, the partially deacetylated derivative of chitin, may be one such material. In this study, they examined the use of chitosan for formation of porous scaffolds of controlled microstructure in several tissue-relevant geometries. Porous chitosan materials were prepared by controlled freezing and lyophilization of chitosan solutions and gels. The materials were characterized via light and scanning electron microscopy as well as tensile testing. The scaffolds formed included porous membranes, blocks, tubes and beads. Mean pore diameters could be controlled within the range 1-250 μm, by varying the final freezing temperatures. Freshly lyophilized chitosan scaffolds could be treated with glycosaminoglycans to form ionic complex materials which retained the original pore structure. Chitosan scaffolds could be rehydrated via an ethanol series to avoid the stiffening caused by rehydration in basic solutions. Hydrated porous chitosan membranes were at least twice as extensible as non-porous chitosan membranes, but their elastic moduli and tensile strengths were about tenfold lower than non-porous controls. The methods and structures described provided a starting point for the design and fabrication of a family of polysaccharide based scaffold materials with potentially broad applicability.

In 2001, Howling and coworkers [8] investigated the effect of chitin and chitosans with various deacetylation levels and polymer lengths on human dermal fibroblasts and keratinocyte proliferation in vitro using both an immortalised human keratinocyte cell line (HaCaT), and primary human keratinocytes. The chitin/chitosan samples were dissolved in 17 mM acetic acid, filter sterilised though a 0.2 μm polycarbonate filter and then diluted for use with fibroblast growth medium (0, 2.5, 5, 50, 500 μg/ml). The effect of chitin and chitosan on keratinocyte proliferation was initially studied using HaCaT cells. When cultured with chitosan at initial concentrations of 2.5, 5 and 50 μg/ml, these cells showed a reduced proliferation rate compared to controls, whilst chitin appeared to have no significant effect on HaCaT

cell proliferation. The screening of the biopolymers for their effects on human dermal fibroblast proliferation showed that chitosan with the shorter chain length had the greatest mitogenic activity at all concentrations tested. The deacetylation level of chitosan seems to be a key factor in the mitogenic activity on fibroblasts with the molecular mass of the biopolymer being somewhat less important. The requirement of serum for chitosan stimulated fibroblast proliferation may be due to an interaction with components in the serum such as heparin or growth factors. Chitosan might bind these components and in the process stabilize and activate them, thus stimulating proliferation. The mitogenic stimulation by low molecular weight chitosan in responsive fibroblasts was dependent on the presence of serum, implying that chitosan might interact with serum components or act as a progression factor. However, the mechanism by which chitosan may interfere with HaCaT cell proliferation is unclear. It might interact with growth factors in the serum or metal ions (e.g. calcium), reducing the availability of these to cells. These results indicated that highly deacetylated chitosan samples were more biologically active than chitin and less deacetylated chitosans and therefore possibly had more potential as wound healing agents or dressing materials.

In 2001, Shanmugasundaram and coworkers [7] used collagen and chitosan (natural polymers) to develop scaffolds in the form of interpenetrating polymeric network (IPN) using glutaraldehyde as a crosslinking agent. This scaffold was then characterized by FT-IR, DSC. Swelling study was carried out using phosphate buffer (pH 7.4). In vitro culture studies were performed using human epidermoid carcinoma cells (HEp-2, Cincinnati) and the growth characteristics of the epidermoid cells over the scaffold were investigated. The FT-IR spectra of various scaffolds (30/70, 40/60, 50/50, 60/40 and 70/30 (collagen/chitosan)) suggested that the collagen and chitosan were certainly crosslinked through glutaraldehyde without any significant changes in the chemical property. The observation from DSC curve of collagen/chitosan scaffolds developed at the ratio of 30/70 and 40/60 showed no transition band due to the decomposition of core polymeric molecules, which was otherwise presented in the DSC curves of individual polymer molecules. Reasons for this might be due to crosslinking of polymers and also disappearance of further thermal decomposition till

400°C proved the thermal stability of the scaffolds. The DSC curves of scaffolds developed in the ratio of 50/50, 60/40 and 70/30 proportionate mixtures of collagen and chitosan also showed similar characteristic bands except the fact that the decomposition peak of chitosan disappeared at temperature around 290°C, in contrast to the results shown by individual molecules. This showed that as proportion of chitosan increased the stability also increased. For swelling properties of scaffolds, the scaffold developed from 60 parts of collagen and 40 parts of chitosan showed maximum swelling of 70.9% at the fourth hour in phosphate buffer and reached the equilibrium stage there after. These scaffolds were considered to possess the optimum characteristics to perform culture studies. Human epidermoid carcinoma cells (HEp-2 cells) were cultured over these scaffolds. The cells maintained their growth and multiplication by supplementing the sample with extra 10% FCS after every 7 days. The cells proved to have the capability of maintaining growth characteristics. The results of various characteristic studies proved that the scaffold developed using chitosan and collagen, can be used to culture HEp-2 cells.

In 2003, Ma and coworkers [1] studied the collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. Collagen and chitosan was dissolved separately in 0.5 M acetic acid solution to prepare a 0.5% (w/v) solution. The chitosan solution was mixed into collagen suspension at the ratio of 9:1 (collagen:chitosan). The collagen/chitosan blend was frozen at -20°C for 1 h and then lyophilized for 24 h to obtain a porous collagen/chitosan scaffold. Glutaraldehyde (GA) with different concentrations (0.05 – 0.25%) was used to crosslink the scaffolds at 4°C for 24 h to improve their biostability. The GA concentrations had a slight effect on the cross-section morphology and the swelling ratios of the crosslinked scaffolds. The swelling property of the uncrosslinked scaffold was doubled than that of the GA treated scaffolds because of the decrease of the hydrophilic groups. However, the crosslinked scaffolds did not show obvious difference regardless of the GA concentration. The collagenase digestion test proved that the presence of chitosan could obviously improve the biostability of the collagen/chitosan scaffold under the GA treatment, where chitosan might function as a crosslinking bridge. A detailed investigation found that a steady increase of the biostability of the collagen/chitosan

scaffold was achieved when GA concentration was lower than 0.1%, then was less influenced at a still higher GA concentration up to 0.25%. After crosslinked with 0.25% GA, the biostability of the pure collagen scaffold (collagen-GA) was greatly enhanced, where only 12.8% was degraded in 12 h. In vitro culture of human dermal fibroblasts proved that the GA-treated scaffold could retain the original good cytocompatibility of collagen to effectively accelerate cell infiltration and proliferation. In vivo animal tests further revealed that the scaffold could sufficiently support and accelerate the fibroblasts infiltration from the surrounding tissue.

In 2003, Wang and coworkers [5] developed the crosslinked collagen/chitosan matrix (CCM) for artificial lever applications. In this study, 1% type I collagen and 1% chitosan with degree of deacetylation of 80% and M_w of 5.3×10^4 in 1% acetic acid were mixed at ratio of 1:1 by volume. Solution were cast on a Teflon plate and air-dried at room temperature. Half of the films were used as controls while the other half were crosslinked by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in N-hydroxysuccinimide (NHS) and a 2-morpholinoethane sulfonic acid (MES) buffer system. The chemical characteristics were evaluated by Fourier-transformed infrared (FT-IR) spectroscopy. FT-IR spectrum of the EDC crosslinked collagen/chitosan suggested that carboxyl groups of collagen reacted with amino groups of chitosan and with hydroxyl groups of chitosan resulting in the formation of amide and ester linkages. The addition of chitosan to the matrix provided many more amino groups than required for crosslinking which led to a decrease in the relative crosslinking degree and the tensile strength. The platelet deposition showed that CCM had excellent blood compatibility. The reason was that crosslinking of collagen and chitosan could decrease platelet deposition due to a decrease in the number of free carboxylic acid groups of aspartic and glutamic acid residues of collagen and in the amino groups of chitosan. For hepatocyte culture, the number of hepatocytes on the CCM made great progression in the following 2 days and remained significantly high until the end of the experiment (28 days). The effects of the chitosan on the cell behavior might be explained as follows. Collagen, when crosslinked by EDC, the specific cell binding amino acids which interacted with cell surface integrin adhesion receptors might be consumed. The addition of chitosan might provide much more

amino groups for cell adhesion. The composition of CCM was closer to native tissue and had been shown to be more effective than that of collagen alone. There were not as many hepatocytes on the pure chitosan matrix as found on the CCM throughout the experiment. The growth and division of the hepatocytes may be inhibited by the extremely high affinity of hepatocytes with chitosan. The results suggested that the CCM was a promising candidate matrix for implantable bioartificial livers.

In 2004, Sionkowska and coworkers [45] used a variety of techniques to characterize collagen and chitosan (CC) interactions after irradiated by light with a wavelength of 254 nm. Collagen and chitosan complexes were characterized by viscometry, wide angle X-ray scattering and Fourier transform infrared (FT-IR) spectroscopy. The viscosity of blends was shown to be greater than that of either collagen or chitosan alone. This indicated a specific interaction between CC that could not be accounted by a simple biphasic mixture. Their hypothesis was that a gelatin-like component was responsible for the enchanced viscosity of blends with an optimum at around 50% collagen and 50% chitosan (by weight). Since gelatin had a higher intrinsic viscosity than collagen, it pointed to a more disordered form of collagen in the complex. Their FT-IR evidence also pointed to the presence of a novel hydrogen bonding in the CC interactions and they concluded that the collagen in the complexes was denatured. From the X-ray diffraction data, it was attractive to speculate that miscibility induced a deviation of the collagen helix structure to a more disordered phase, similar to gelatin. Production of such a third phase in the blend would be consistent with increased viscosity and a loss of helical diffraction peaks. The conclusion was that collagen and chitosan interactions could alter the collagen helical character and therefore the overall physical parameters of the blend. The explanation of the changes in viscosity was through a triphasic system where the CC blend contained a third gelatin-like phase. The presence of a collagen substrate without collagen helical characteristics might be beneficial for biomaterial design.

In 2004, Yannas and coworkers [46] studied the changes in the microstructure and compressive modulus of a highly porous, type I collagen–GAG scaffold during degradation. Type-I bovine tendon collagen was dissolved in 0.05 M acetic acid and

glycosaminoglycan (GAG) (chondroitin-6-sulfate) was added at the ratio of 9:1 (collagen:GAG). The solution was then frozen at an approximately constant cooling rate from 20°C to -40°C over a period of 65 min. The frozen solution was sublimated for 17 h (100 mTorr, 0°C) to produce the porous collagen–GAG structure. The matrix was then crosslinked in four different ways, as follows. Control matrix freeze dried without additional crosslinking (Nx). Physical crosslinked matrices were performed by exposing to 105°C under a vacuum of <50 Torr for 24 h (Dx). Chemical crosslinked matrices were carried out by EDAC/NHS treatment involved immersion of the matrix in EDAC/NHS in EDAC/NHS crosslinking solution (Ex). Physical and chemical crosslinked matrices were done by 24 h DHT followed by 2 h EDAC/NHS (DEx). The enzymes bacterial collagenase and chondroitinase were used to degrade the collagen and GAG (chondroitin-6-sulfate) components, respectively. The intact scaffold micro-structure was made up of a foam-like interconnected network of thin struts and films. The inverse of the swelling ratio increased for the various crosslinking treatments in the following order: Nx (control with no additional crosslinking), Dx (24 h crosslinked by dehydrothermal process, DHT), Ex (2 h EDAC/NHS crosslinked) and DEx (24 h DHT + 2 h EDAC/NHS crosslinked). Regression analysis indicated that the compressive modulus, E; of the intact matrix was linearly related to the inverse swelling ratio. The Nx samples were almost completely solubilized after only 90 min while Dx samples were almost completely solubilized after 120 min. Samples crosslinked with 2 h EDAC/NHS (Ex) or 24 h DHT + 2 h EDAC/NHS (DEx) appeared completely intact, and there was no detectable difference between micrographs taken before and after degradation even after 120 min collagenase exposure. After 1 week of exposure to collagenase, the Ex samples showed slight pitting while the DEx samples exhibited very little evidence of degradation. For matrices degraded using collagenase, a decrease in compressive modulus with degradation time was observed in the Nx and Dx samples. The compressive modulus did not appear to change for samples treated with Ex or DEx even after 1 week exposure to collagenase. At low levels of crosslinking, the mechanical behavior is dominated by viscous sliding of collagen matrices and fibrils, and thus depends on collagen fibril length and axial ratio. At higher levels of crosslinking, mechanical behavior is dominated by elastic stretching of non-helical

ends, crosslinks, and collagen triple helix. For samples degraded with chondroitinase, the compressive modulus of all sample types did not change with degradation time. The finding that highly crosslinked samples were more resistant to chondroitinase, not just to collagenase, strongly supported the theory that crosslinks were located both between collagen chains and between collagen–GAG chains.