COMPOSITE GELATIN DELIVERY SYSTEM FOR BONE REGENERATION

by

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1. ABSTRACT

In this thesis, the chemical/mechanical properties and biocompatibility of gelatin were investigated to produce a gelatin scaffold for the release of bone morphogenetic proteins (BMPs) from composite particles. This delivery system, designed to regenerate bone, holds much promise as an alternative to bone grafts.

The chemical properties of gelatin were examined through zeta potential measurements, swelling studies, optical microscopy, environmental scanning electron microscopy (ESEM), and collagenase degradation. Compressive tests and mercury porosimetry were performed to study the mechanical and structural properties of the scaffold. The biocompatibility of the scaffold was determined through cell optical imaging and DNA quantification studies.

Based on findings of this research, the material choices were made and the synthesis method for the gelatin scaffold was developed. Gelatin A, 300B, derived from bovine collagen, with an isoelectric point of ~ 9, was selected. Crosslinking was accomplished by reacting 10 w/v% glutaraldehyde with 10 w/v% gelatin solution. The most effective crosslinking condition was found to be 5 hours at room temperature. Glycine rinses were conducted to cap any non-reacted (toxic) aldehyde groups, and the necessary length of time was found to be at least 48 hours at 37°C. Finally, based on pore size distribution and mechanical stability, an optimal lyophilization method was developed with initial freezing at –20°C for 1 day, followed by lyophilization of the scaffold for 1-2 days. In terms of mechanical properties of the gelatin and amount of protein delivered, the most effective loading of poly(lactic-*co*-glycolic acid)/apatite/protein composite particles was found to be 10% of the mass of the gelatin.

2. INTRODUCTION

Much research is currently being conducted in the fields of drug delivery and bone tissue engineering.[1] In the field of drug delivery, the challenge is to achieve tunable zero-order release over extended periods of time, and to control the amount of drug released at different time intervals. In the area of bone tissue engineering, the time factor again plays a critical role since bone needs many weeks to regenerate. Bone substitutes or scaffolds also need to provide similar mechanical properties as natural bone.[2]

Proteins are known to facilitate bone generation, but they are not widely used for bone regeneration or for tackling osteoporosis.[3] A current clinical procedure makes use of bone morphogenetic proteins (BMPs) for spinal fusion.[4] To increase the number of clinical applications of BMPs, it is crucial for a delivery system to be developed that would provide for slow release, since the bone-inducing proteins must be present at the defect site for a sufficient time for bone to grow.

2.1. BACKGROUND

Autologous and allogenic bone grafts are currently the best available options for treating bone loss, but they suffer from limited supply and risks of disease transfer (see Figure 2.1).[2,5] They differ in that the latter often involves non-viable cells in order to control immunogenecity.[5,6] Permanent synthetic grafts constructed of metals and ceramics are also used, but their mechanical incompatibility with bone tissue can lead to implant failure. Due to these concerns, there has been increasing interest in alternative methods of regenerating bone to fill defect sites.[7]

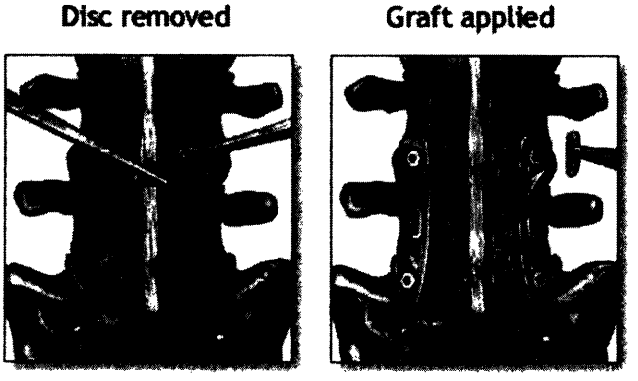


Figure 2.1. Bone graft for spinal surgery.[5]

2.2. BONE GROWTH INDUCERS

BMP delivery systems present a promising option for regenerating bone.[1] BMPs have been found to be potent inducers of bone growth.[8] They are manufactured by bone cells (osteoblasts) and are retained in the bone matrix.[9] When bone fractures, BMPs induce chemotaxis of mesenchymal stem cells and osteoblasts to the fracture site, and promote the differentiation of mesenchymal stem cells into osteoblasts.[8, 10] The design of this work takes advantage of the osteoinductive capacity of BMPs to induce ectopic bone formation subcutaneously in rat models as a measure of the potential of our delivery systems. In this study, preliminary experiments were conducted with the use of model proteins.

Currently ~ 14 forms of BMPs have been recognized; BMP-2 and BMP-7 are the most commonly used in biomedical research.[11] The BMP used in our experiments was recombinant human BMP-2 (rhBMP-2) harvested from Chinese Hamster Ovarian (CHO) cells (R & D Systems). The goal of this research was to optimize the delivery of BMPs to induce bone formation.

2.3. APATITE-POLYMER COMPOSITES

Over the past few years, our laboratory has developed a method for the synthesis of apatite-polymer composite particles with tunable controlled release properties.[12] These particles allowed us to harness the benefits of bioresorbability, osteoconductivity and protein affinity of apatite, and the bioresorbability, compositional flexibility and controlled release properties of biodegradable polymers.[13, 14] By varying parameters such as apatite particle size and polymer molecular weight, it was possible to control the protein release from these particles.

2.4. METHOD OF DELIVERY

The scaffold from which the BMPs will be released should deliver the proteins at the desired rate. It must also support cell attachment, migration and growth so as to promote bone regeneration.[1] For our application, BMP-loaded composite particles would be dispersed in a scaffold. This would allow different composite particles to be used in one scaffold, hence providing a wider range of physical, chemical and release properties.[15]

2.5 GELATIN SCAFFOLD

The focus of this thesis was on developing the gelatin scaffold on which BMP-loaded composite particles would be dispersed. Gelatin has been chosen because of its non-toxicity, its efficacy in delivery applications, and because it is a natural material derived from collagen, the proteinaceous component of bone.[16] It was purchased in a powder form, and needed to be dissolved in water and crosslinked to form a polymer network.

In designing the gelatin system, both chemical and mechanical properties were considered. The goal was to achieve sustained, zero-order release of BMPs from apatite-polymer composite particles with minimal interference from gelatin. In addition, the gelatin scaffold should provide a favorable environment for cell infiltration and growth.

2.6. CHARACTERISTICS OF GELATIN

Collagen derivatives are a logical choice for a delivery system because much of the body, and especially bone, is comprised of collagen. The organic matter in mammals consists of 30% collagen.[17] Collagen contains significant amounts of, glycine, proline, alanine and hydroxyproline (see Table 2.1), and its composition may vary.[17] It also has a slightly basic isoionic pH.[17]

Table 2.1. Components of collagen.[17]

Amino Acid (or Other Component)	Residues (per 100 Total Residues)
Glycine	33.65
Proline	12.90
Alanine	10.66
Hydroxyproline	9.41

Gelatins are proteinaceous materials that are typically derived from the degradation of collagen fibers. By this process, gelatin is made water-soluble with a much lower internal order than collagen.

Swelling is frequently used as a way of comparing different types of gelatin.[18-20] Since swelling is related to degradation, it is a pertinent parameter for drug release. Swelling is also

important because it is a measure of the extent of network crosslinking.[19] Swelling degree is inversely proportional to the number of crosslinks, which is proportional to the number of "restraints" applied to the gelatin structure.[19]

Gelatin samples are defined by their production method and Bloom value.[21] Bloom value is proportional to molecular weight.[21] The Bloom value is determined by pressing a plunger into the gelatin sample and reading the value of the force at a particular deflection. Thus the Bloom number is a measure of rigidity and is proportional to molecular weight as well as the grade and price of the gelatin.[21]

Scaffolds will bind proteins of the opposite charge, so it is necessary to tune the isoelectric point (IEP) of the scaffold to ensure that BMP can be released. The IEP of gelatin differs depending on the processing. Type A gelatin is processed with acids with a typical IEP of 7-9 (Figure 2.2).[16] Type B gelatin is processed with bases with a typical IEP of 3-5.[16] Since BMPs are slightly basic, with an IEP ~ 9, type A gelatin was hypothesized to be more suitable.

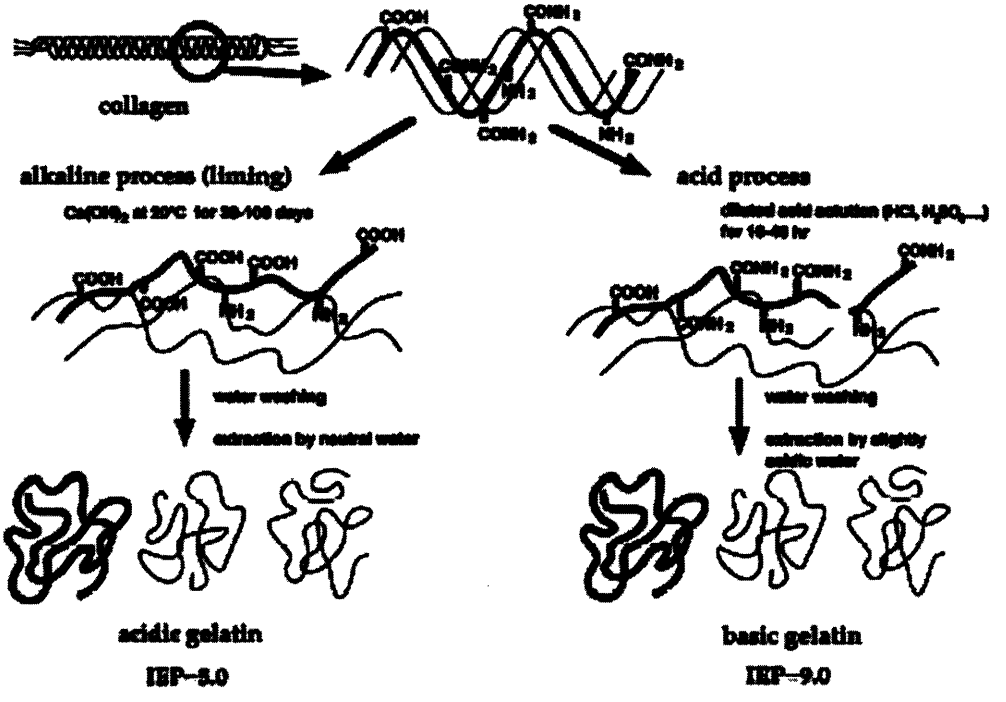
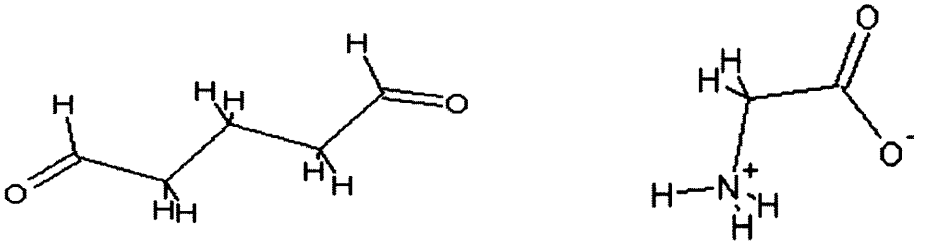


Figure 2.2. Preparation of gelatin and its different IEPs.[16]

2.7. DETOXIFICATION OF GELATIN SCAFFOLD

To form a network gelatin scaffold, the gelatin must be crosslinked. The crosslinking agent used for our research was glutaraldehyde (Figure 2.3a), which is toxic if unreacted.[22] To detoxify the gelatin scaffold, the gelatin was reacted with glycine (Figure 2.3b).[23] In this process, the aldehyde and amino groups reacted to form an imine group.



(a) (b)

Figure 2.3. The chemical structures of (a) glutaraldehyde and (b) glycine.

3. APPROACH AND GOALS

The approach of this project is to develop a gelatin scaffold by crosslinking a gelatin solution in which apatite-polymer composite particles have been dispersed. From this scaffold, rhBMP-2 encapsulated in the composite microparticles would be released.

The goals of this project are as follows: (1) understand the properties of the materials involved, (2) investigate the variables in the gelatin scaffold synthesis, and (3) evaluate the physical, mechanical, biological and release properties of the scaffolds. The desirable characteristics for the scaffold are described in Table 3.1.

Table 3.1. Desirable characteristics of scaffold.

Category	Desirable Characteristics
Pores	High porosity and large pores ($d \geq 100$ μm) to facilitate cell infiltration and vascularization.
Degradation Rate	~ 1-2 months. Too rapid a rate will frustrate complete healing, whereas too slow a rate will impede bone growth.
Strength	Scaffold must not buckle or allow soft tissue encroachment.
Biocompatibility	Scaffold must be non-toxic and support cell growth.
Protein Release	Low burst, tunable release.

4. SCAFFOLD SYNTHESIS AND CHARACTERIZATION

4.1. SYNTHESIS

The general synthesis method (Figure 4.1) consisted of dissolving gelatin powder (Sigma Aldrich) in deionized water by heating the vial containing the gelatin solution in a water bath at 37°C.

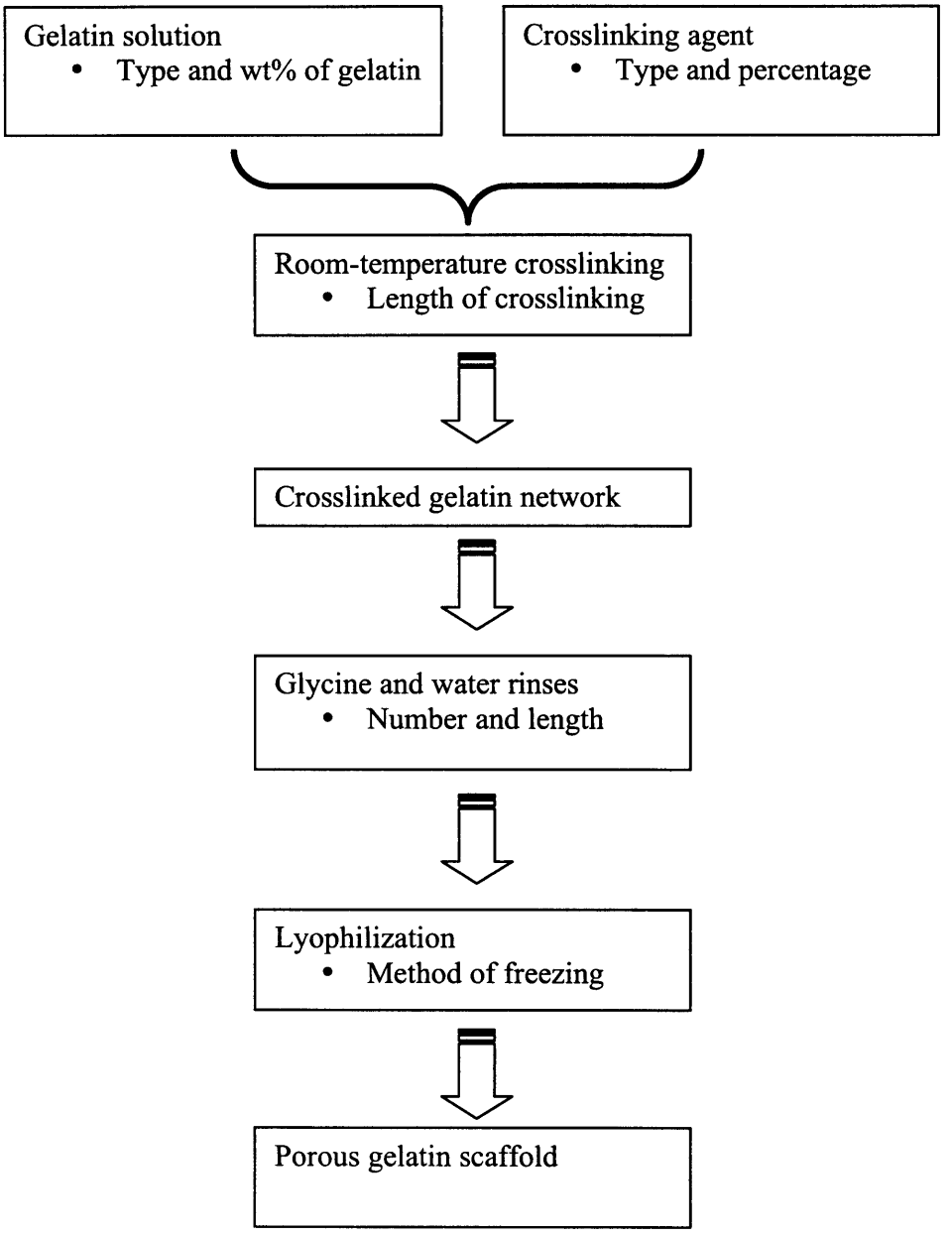


Figure 4.1. Synthesis scheme of gelatin scaffold.

The gelatin solution was then removed from the water bath, and glutaraldehyde was quickly added. The solution was poured into a peel-away mold (Polysciences) or a petri dish, covered and left to crosslink at room temperature. The resulting gel was removed from the mold or petri dish. In the latter case, a metal punch (10 mm dia.) was used to punch out circular gel disks.

The gels were placed in a 5 w/v% solution of glycine to cap any unreacted glutaraldehyde groups. After ~ 1 hour, the glycine solution was removed and replaced with deionized water. Two additional rinses with deionized water were performed. All rinses took at least 1 hour. The gels were then lyophilized for 1-2 days.

4.1.1. Crosslinking Method

Gelatin can be converted into a gelatin network by using a chemical crosslinking agent, UV radiation, or thermal treatment. The method we chose involved using a stock solution of 25 w/v% glutaraldehyde in water (Sigma Aldrich) to chemically crosslink the gelatin chains.

4.1.2. Type and Amount of Gelatin

We purchased samples of Gelatin 75, 175, 225 and 300 Bloom (B) from Sigma Aldrich. Gelatin 175 B and 300 B samples were of type A, and Gelatin 75 and 225 B samples were of type B. In our experiments, 10 w/v% solutions of gelatin in deionized water were employed. For peel-away molds and petri dishes, 6 ml and 20 ml of solutions were used, respectively.

4.1.3. Amount of Crosslinker

Drug-loaded hydrogels release their therapeutic contents as they swell. The release may be controlled by swelling and by the temperature or pH of the environment. Since swelling rate and degree are functions of network density, the concentration of crosslinker is an important factor. Glutaraldehyde solutions of various concentrations (1, 5, 10, 15 and 20 w/w%) were prepared with respect to the gelatin weight. For example, when gelatin was crosslinked in petri dishes, 20 ml of deionized water was combined with 2 g of gelatin and 800 μL of the glutaraldehyde stock solution (25 w/v% glutaraldehyde in water).

4.1.4. Crosslinking Time

Another way to vary the network density is to alter the crosslinking time. In our studies, the crosslinking time was varied between 1, 3, 5 and 7 hours.

4.1.5. Apatite-Polymer Composite Particle Loading

Apatite-polymer composite particles were loaded into the gelatin scaffolds to determine the optimal loading. The particles were added to the gelatin solutions immediately after removing them from the water bath. The solutions were then centrifuged to ensure a homogeneous distribution of particles. Lyophilization, as described in Section 4.1.5, was then conducted.

4.1.6. Lyophilization Method and Post-Lyophilization Sample Preparation

The gelatin scaffolds were subjected to either freezing at –20°C in a conventional freezer or freezing in liquid nitrogen. The size of ice crystals formed is proportional to freezing time, and affects the porosity and pore size of the scaffolds. Several methods of lyophilization were used, involving keeping the gels at different temperatures prior to sublimation. In the first method, termed "fast-freezing", gels were placed in freeze-drying vials after the glycine/water rinses were complete. The vials were immersed in liquid nitrogen for freezing and connected to a vacuum system for water sublimation. The gels were freeze-dried for 1-2 days, or until all water was removed.

In the second method, termed "slow-freezing", gels were not immediately lyophilized, but were first left in a freezer at –20°C overnight. The frozen materials were then lyophilized for 1-2 days.

After lyophilization, low molecular weight gelatin would appear as white fluff. This fluffy material was removed since it might detach during the swelling experiments. Gels were then weighed for further analysis.

4.2. CHARACTERIZATION OF CHEMICAL AND MECHANICAL PROPERTIES

4.2.1. Isoelectric Point

The ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments) was used to measure the zeta potential and the mean mobility. Dilute solutions (0.1 w/v%) of Gelatin A (300 B) and Gelatin B (225 B) were prepared. The pH of each solution was varied using 0.1 N solutions of NaOH and HCl.

4.2.2. Recovered Masses

The amount of gelatin that can be recovered is important since gels that lose a significant portion of their mass may become less mechanically stable. Also, a lower yield reduces the amount of gelatin without significantly affecting the amount of crosslinker, so the overall crosslinker concentration will increase, affecting the *in vivo* degradation rate. In this experiment, two Gelatin B samples (75 B and 225 B) were examined. The gels had the same initial weight of 0.500 g, and the ratio of masses before and after lyophilization was recorded. It was expected that the Bloom number would affect the amount of gelatin recovered since it is related to the quality of a gelatin sample.

4.2.3. Swelling Studies

Swelling studies were performed by immersing lyophilized gels in water and measuring the water content by weight change at different time points. Swelling experiments were also conducted in phosphate buffered saline (PBS) solutions of pH 3, 5, 7, 9 and 11. It was important to vary the pH as this can affect the net charge on gelatin, and consequently, how strongly any released BMPs are held to the scaffold.

4.2.4. Optical and ESEM Imaging

Gel morphology and pore structure were observed with optical microscopy (Leica optical microscope) and environmental scanning electron microscopy (ESEM) (Philips/FEI XL30 FEG-ESEM).

4.2.5. Mechanical Testing

During gel synthesis, the apatite-polymer composite particles provided by the Ying laboratory were mixed into gelatin at loadings of 5, 10 and 20 w/w% gelatin. Crosslinking concentrations of 5, 10 and 15 w/w% were examined. All gel samples were made in peel-away molds. Gels were lyophilized, cut, and swelled again in water prior to testing with a Zwick Roell Stand Alone Universal Machine, series Z010.

4.2.6. Mercury Porosimetry

Pore size distributions were obtained by mercury porosimetry with a Quantachrome 4 Pore Master 33. Gels were synthesized as described in Section 4.2.5 to examine the effects of the lyophilization method and the composite particle loading.

4.2.7. Collagenase Degradation

Collagenase is a protein that catalyzes the cleavage of collagen, the parent protein of gelatin, and can be used to enzymatically degrade collagen or gelatin hydrogels. By measuring the rate of gel dissolution in the presence of collagenase, we could estimate the effects of crosslinker concentration and autoclaving.

In the degradation experiments, solutions of 1 w/v% collagenase (from Clostridium histolyticum) in PBS buffer solutions were prepared. Gel A (300 B) and glutaraldehyde concentrations of 5, 10 and 20 w/w% in gelatin were used. Another set of gels was prepared with autoclaved gelatin solution and 10 w/w% glutaraldehyde in gelatin. This was done because autoclaving might denature part of the gelatin or otherwise alter the degradation rates. The various gels were all crosslinked for 5 hours at room temperature.

Freeze-dried gels that had been presoaked in deionized water were immersed in the collagenase PBS solution. Each gel piece weighed 13 mg. The time for complete dissolution of each sample was recorded.

4.3. RESULTS

4.3.1. Determination of Isoelectric Point

The IEP values of Gelatin A and Gelatin B were determined to be 9.2 and 4.9, respectively (Figures 4.2 and 4.3). These results confirmed the IEP values provided by Sigma-Aldrich and the literature.

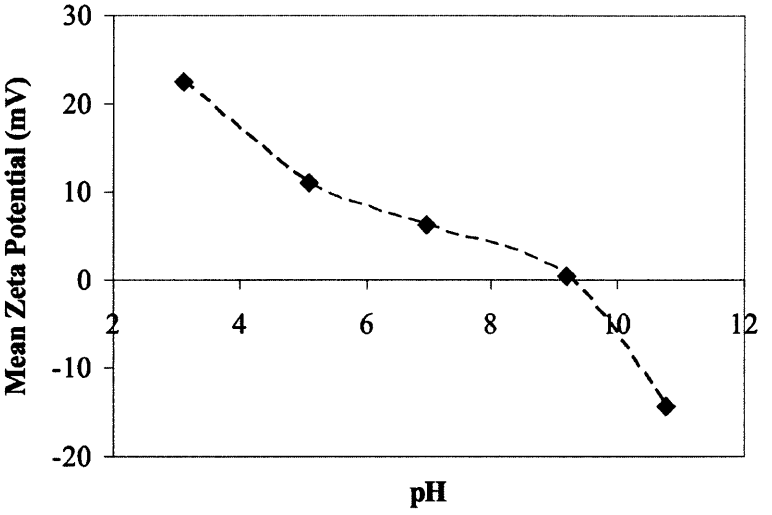


Figure 4.2. Zeta potential vs. pH for Gel A (300 B).

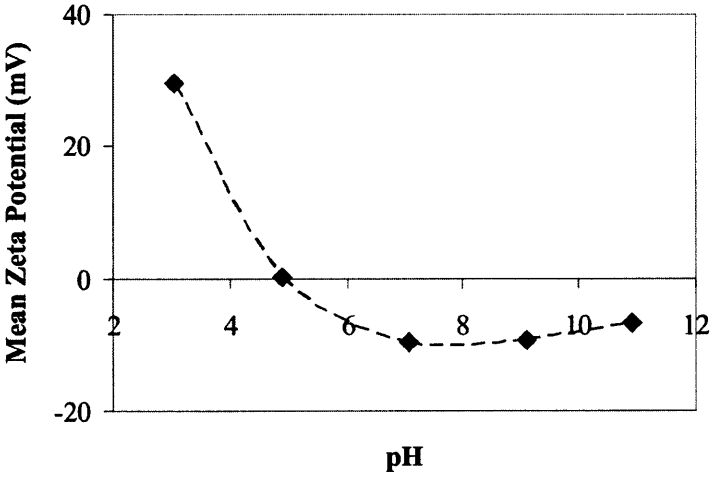


Figure 4.3. Zeta potential vs. pH for Gel B (225 B).

Since their IEP is ~ 9, BMPs will be positively charged at physiological pH (pH 7.4). This experiment verified that Gel A was positively charged at physiological pH, and would

likely repel BMPs, facilitating their release into the surrounding medium. Thus, Gel A would be more suitable for use in our application.

4.3.2. Effect of Gelatin Type

The type of gelatin is important because it affects the mechanical properties and the degradation characteristics of the scaffold. In examining the effects of gelatin type, we measured the amount of gelatin that could be recovered after lyophilization. Figure 4.4 shows that the gelatin with a lower Bloom number (75 B) lost more mass at all glutaraldehyde concentrations examined. These results suggested that gelatins with higher Bloom numbers would provide better mechanical properties.

The effect of gelatin on swelling was examined for two Gel B samples. Less swelling is desirable since it is associated with slower *in vivo* degradation.[16] Swelling also reflects the porosity of gelatin, which determines the diffusion of molecules. Figure 4.5 shows that Gel B (225 B) seemed to reach equilibrium in swelling after 72 hours. The increase in water content past the first 100 hours was probably related to gelatin degradation instead of true swelling.

Swelling data for Gel B (75 B) were taken up to 72 hours. As negligible uptake of water was noted between 48 hour and 72 hours, the data at 48 hours were presented in Figure 4.6 as the equilibrium points. Since the Gel B (75 B) samples swelled more quickly than Gel B (225B) samples, they would be expected to release BMPs at a much faster rate. For our applications, BMPs needed to be delivered over a long period of time. Thus, Gel B sample with the larger Bloom number would be more suitable.

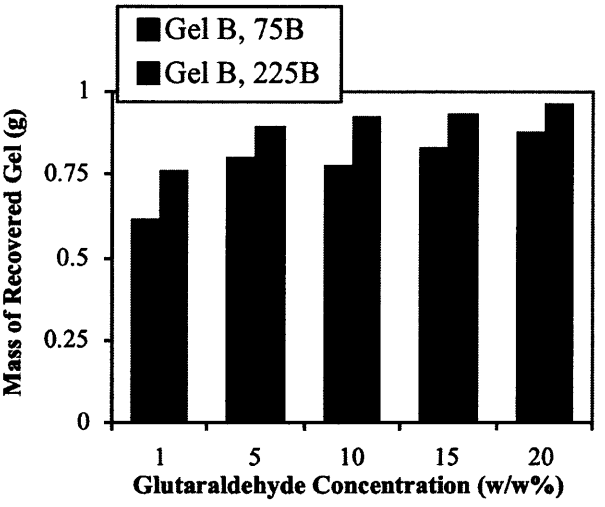


Figure 4.4. Mass recovered for two different types of gelatin after lyophilization.

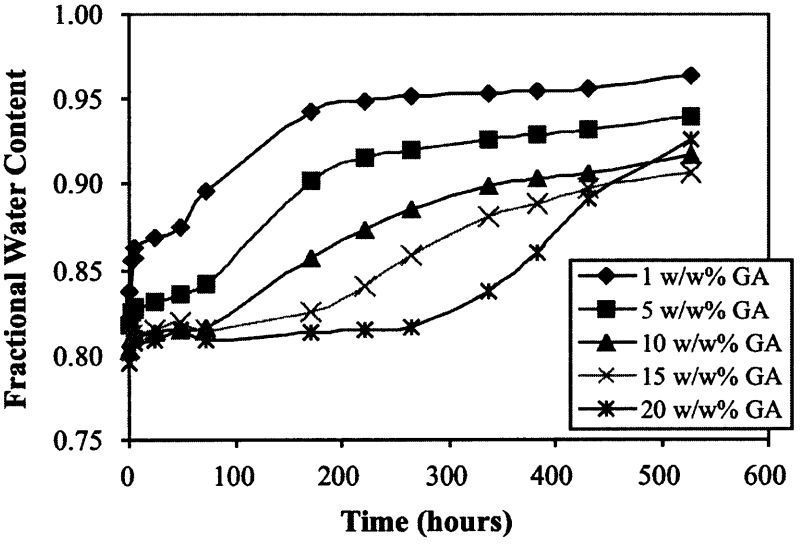


Figure 4.5. Swelling of Gel B (225 B) with different glutaraldehyde concentrations.

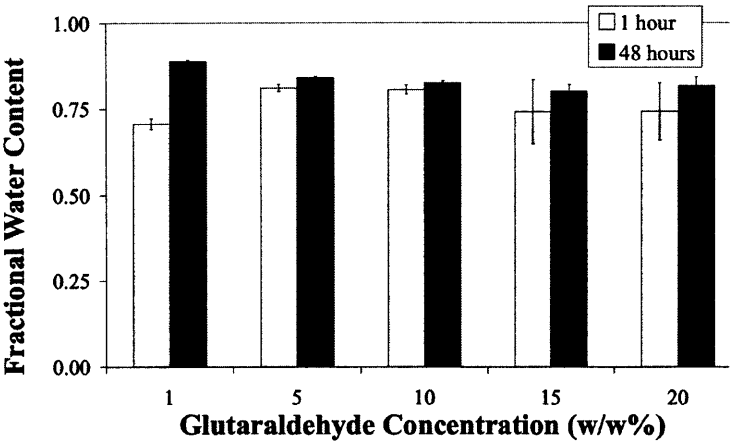


Figure 4.6. Swelling of Gel B (75 B) at 1 and 48 hours (n = 4).

4.3.3. Effect of Glutaraldehyde Concentration

Figures 4.5, 4.6, 4.7 and 4.8 illustrate the effect of glutaraldehyde concentrations on the swelling of Gel B (225 B), Gel B (75 B), Gel A (175 B) and Gel A (300 B), respectively. The degree of swelling was expected to be inversely proportional to the glutaraldehyde content. This trend was observed in general, except in the case of 20 w/w% glutaraldehyde for Gel B (75 B). The high glutaraldehyde concentration might have caused this gel with low Bloom number to be over-crosslinked, becoming too brittle and thus displaying different mechanical characteristics.

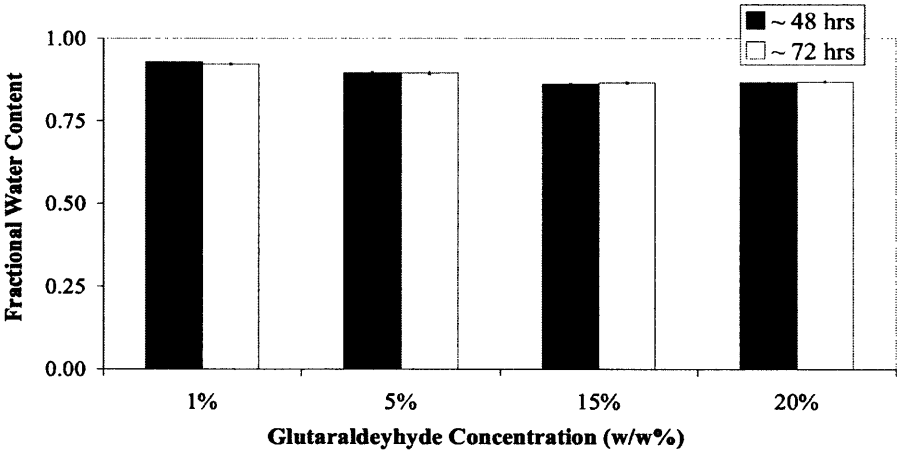


Figure 4.7. Swelling of Gel A (175 B) at 48 and 72 hours (n = 4).

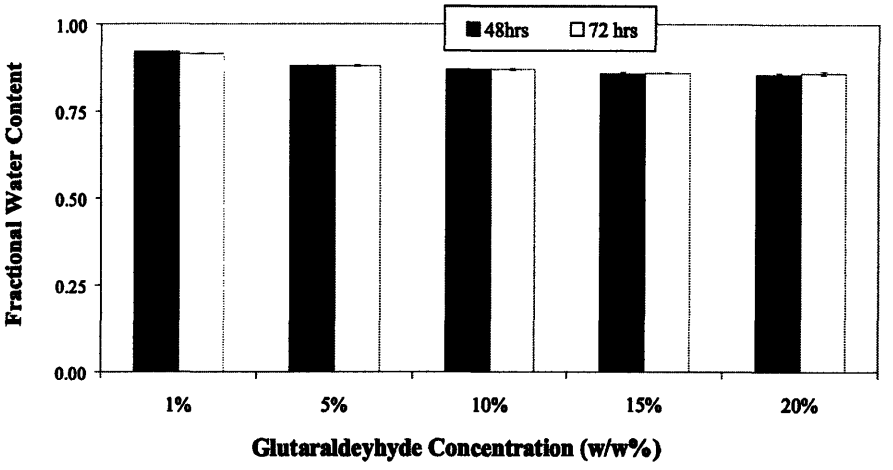


Figure 4.8. Swelling of Gel A (300 B) at 48 and 72 hours (n = 4).

The effect of glutaraldehyde was also investigated via collagenase degradation. Degradation time was found to increase with increasing glutaraldehyde concentration (Figure 4.9). The autoclaved and non-autoclaved gelatin samples prepared with the same glutaraldehyde concentration did not differ statistically in degradation time. This finding was important since preliminary tests were conducted on non-autoclaved gelatin, but the use of these gelatinous scaffolds in *in vivo* studies required that the synthesis be conducted under aseptic conditions, whereby starting materials, such as gelatin, would be sterilized.

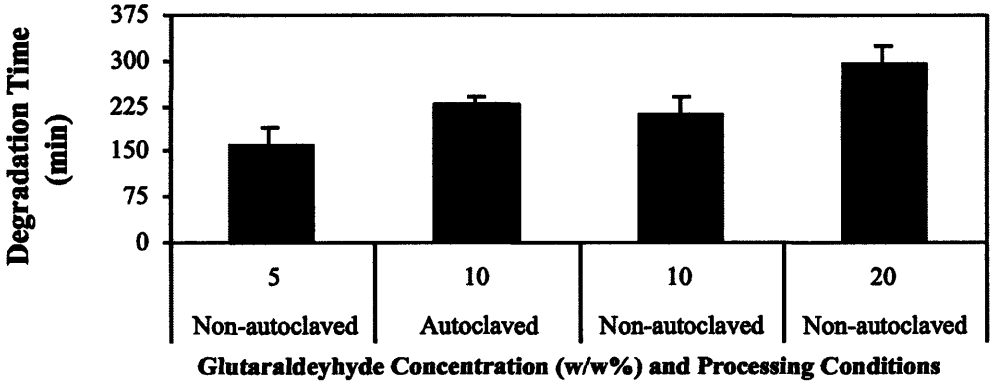


Figure 4.9. Time required for complete gel degradation by collagenase (n = 3).

For cell ingrowth, scaffold porosity is crucial since cells must be able to move into the scaffold, and nutrients and waste products must be able to move in and out of the scaffold, This

suggests that a lower glutaraldehyde concentration is desired, since less crosslinked networks have higher porosities. An excessive amount of glutaraldehyde would also be problematic since it would lead to toxicity and over-crosslinking. On the other hand, inadequate crosslinking would lead to rapid gel swelling and degradation, giving insufficient time for healing and bridging of defects. Considering these various factors, glutaraldehyde concentrations of 10 and 15 w/w% were selected for further studies.

4.3.4. Effect of Crosslinking Time

The effect of crosslinking time was examined with Gel B (225 B), Gel A (175 B) and Gel A (300 B) prepared with 15 w/w% glutaraldehyde. Figures 4.10 and 4.11 show that crosslinking time did not produce any discernable trends in water uptake by the three types of gels after either 48 or 72 hours of swelling.

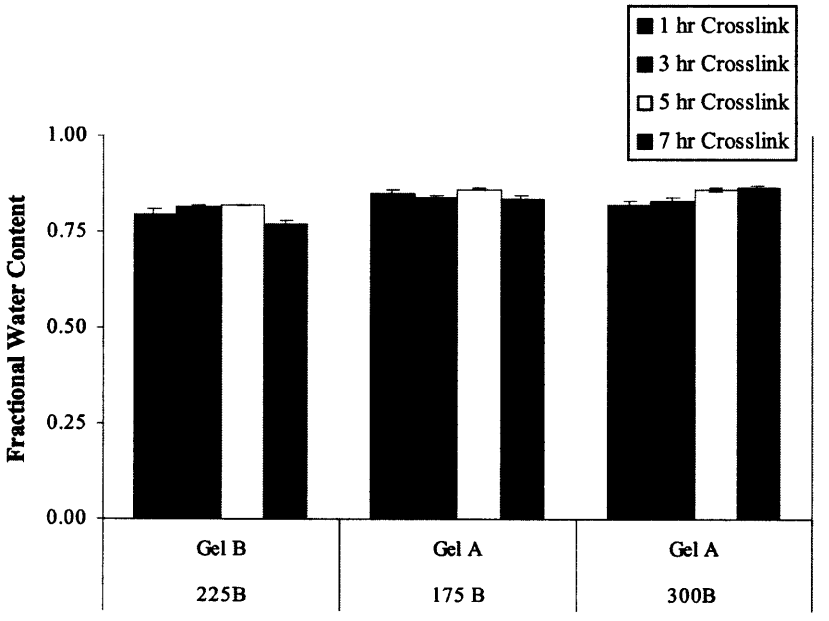


Figure 4.10. Effect of crosslinking time on gel water content after 48 hours of swelling (n = 4).

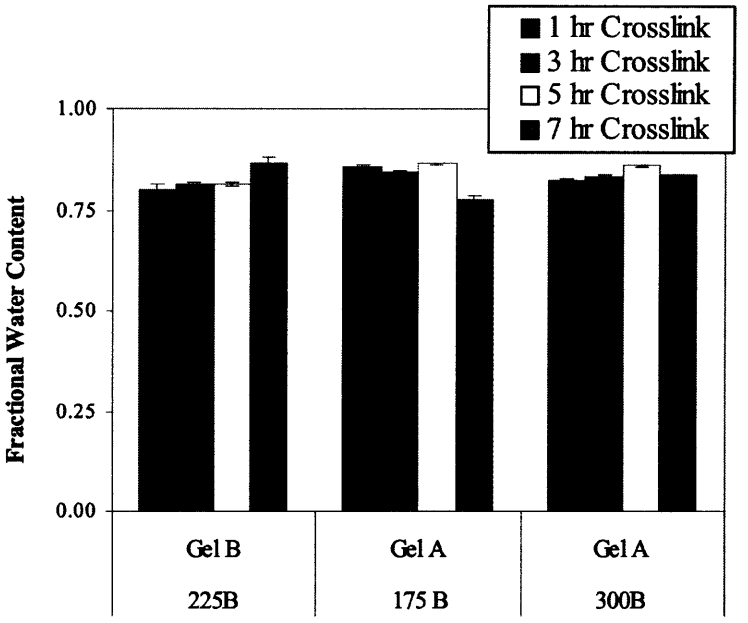


Figure 4.11. Effect of crosslinking time on gel water content after 72 hours of swelling (n = 4).

4.3.5. Effect of Lyophilization Method

Table 4.1 illustrates that disks of Gel A (300 B) prepared with 10 w/w% glutaraldehyde experienced shrinkage and lower water content when subjected to fast-freezing. Water contents and dimensions were measured at 48 hours. Normalized dimensions were determined by dividing the diameter of the gelatin disk at 48 hours by the original diameter. The sample subjected to slow-freezing showed higher water content and increased dimensions.

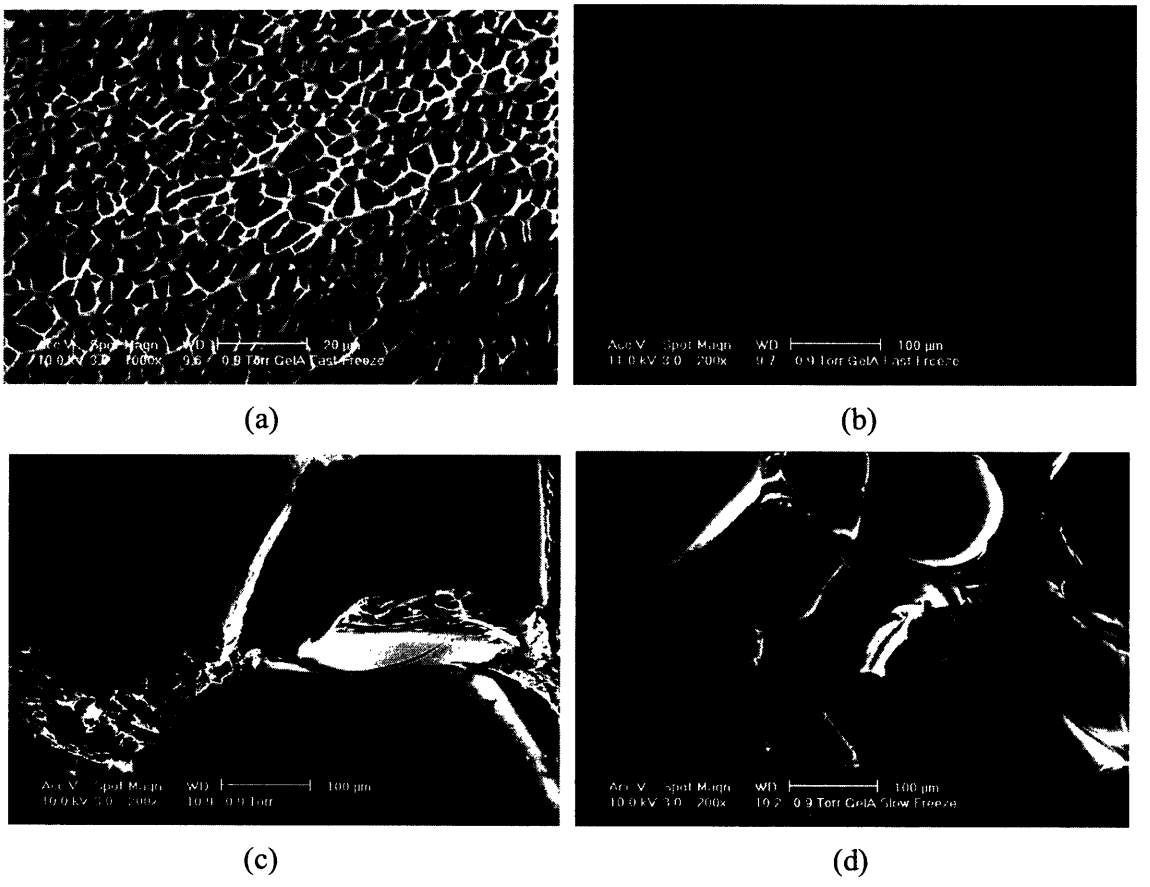
Table 4.1. Effect of lyophilization methods on the swelling of Gel A (300 B) (n = 3 for slow-freezing, n = 4 for fast-freezing).

	Average Fractional Water Content	Normalized Dimensions
Slow-Freezing	0.884	1.957
Fast-Freezing	0.860	-6.139

Figure 4.12 illustrates that gelatin has a bimodal pore size distribution, with pores in the ranges of 5-10 μm and 100-200 μm. The latter meets the criteria for cell infiltration (pore size >

100–500 μm). The ESEM images also indicated that the gels prepared by fast-freezing have smaller pores than those prepared by slow-freezing.

Mercury porosimetry confirmed the presence of broad pore size distributions in the gels without particles (Figures 4.13 and 4.14). The pore size distribution was altered when the gel prepared by slow-freezing was loaded with particles (Figure 4.15). It was difficult to compare the mercury porosimetry results with the ESEM findings since the former would not be able to characterize pores larger than 200 microns.



(a) (b)

(c) (d)

Figure 4.12. ESEM images of gels produced by (a,b) fast-freezing and (c,d) slow-freezing.

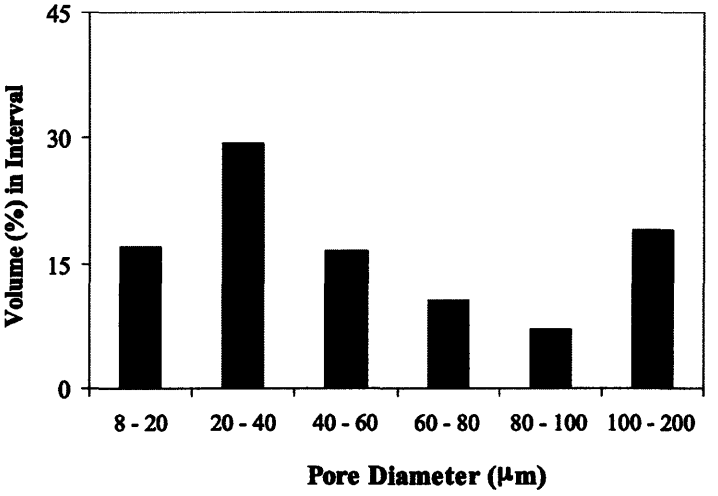


Figure 4.13. Mercury porosimetry of particle-free gel prepared by fast-freezing.

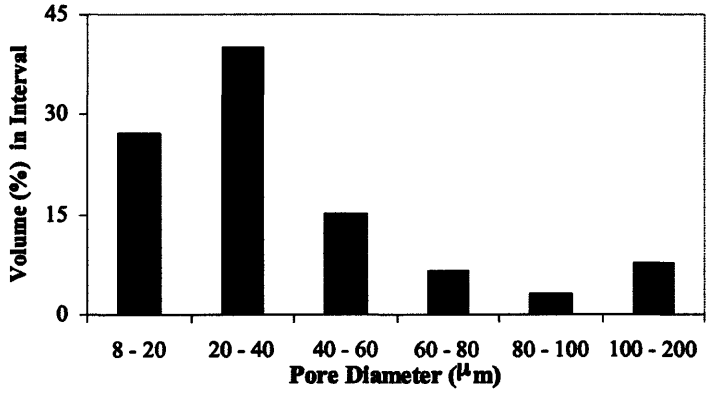


Figure 4.14. Mercury porosimetry of particle-free gel prepared by slow-freezing.

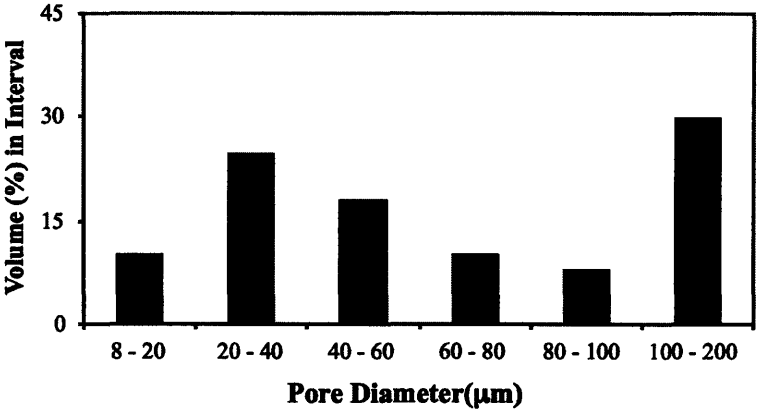


Figure 4.15. Mercury porosimetry of particle-containing gel prepared by slow-freezing.

The effect of lyophilization method on gelatin's mechanical properties was also examined. Compressional tests were performed on wet, swollen gels. Figure 4.16 shows a sample stress-strain curve for Gel A (300 B). The Young's modulus was determined by taking the slope of the initial linear portion of the curve (indicated by the arrow marked E). Figure 4.17 shows that the gel subjected to fast-freezing has a Young's modulus similar to non-lyophilized gel, and that autoclaving did not affect the mechanical properties of the former. A significantly higher Young's modulus was attained by the gel subjected to slow-freezing. To determine whether these results were consistent with the morphology observed by SEM, it would be necessary to examine the total porosity in the different types of gelatin. The total porosity could then be correlated with Young's modulus to illustrate the expected trend of higher porosity corresponding with a lower Young's modulus. However, only the pore size distribution, not the total porosity, was measured in this work, so a comparison of results from mechanical and microscopy studies could not be undertaken till further research is performed.

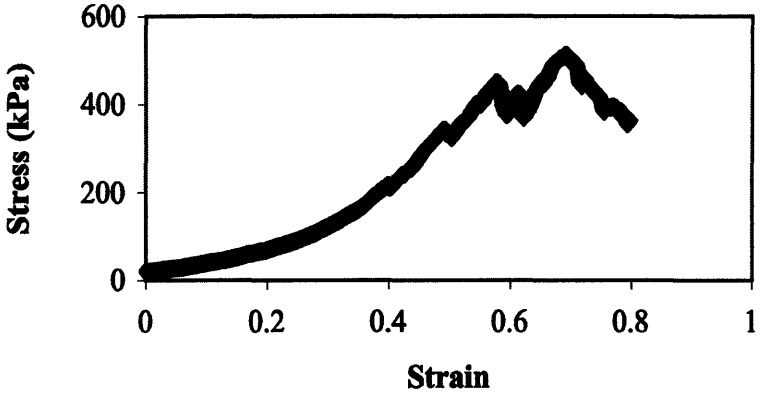


Fig. 4.16. Characteristic stress-strain curve for a gelatin scaffold without particles.

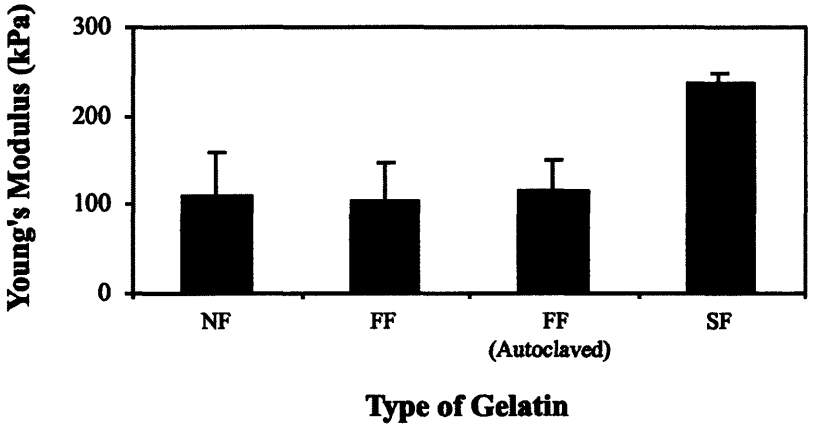


Figure 4.17. Young's moduli of gelatins subjected to no freezing (NF, n = 3), fast-freezing (FF, n = 3), fast-freezing and autoclaving (n = 3) and SF (slow-freezing, n = 6).

4.3.6. Effect of pH

Figure 4.18 shows the effect of pH on the swelling of Gel A (300 B), which has an IEP of ~ 9. As expected, the phosphate buffer with a pH of 3 resulted in the most significant changes in swelling. Buffers of other pH values (5, 9 and 11) did not significantly alter the equilibrium swelling value and swelling rate from those at the physiological pH of 7.4, suggesting that the delivery system should perform reliably over the range of pH values expected in the body.

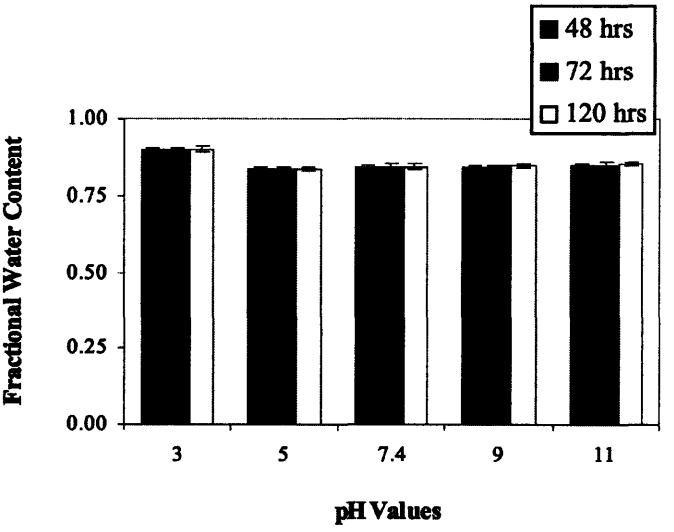


Figure 4.18. Effect of pH on the swelling of Gel A (300 B) (n = 6).

5. BIOCOMPATIBILITY STUDIES

To realize biocompatibility, a literature search was performed to ensure that all materials used were naturally non-toxic or chemically reacted with solutions for detoxification. Aseptic synthesis methods were then developed for all materials, which involved sterilizing all raw materials, and crosslinking and processing the gels under sterile conditions. The gelatin scaffolds derived were tested for biocompatibility in cultures of C3H10T1/2, a pluripotent murine embryonic fibroblast, using optical microscopy and cell proliferation through DNA quantification.

5.1. SYNTHESIS

5.1.1. Aseptic Treatment of Starting Materials

For aseptic preparation of gelatin solutions, the first method involved warming up 10 w/v% gelatin solution in a water bath, and then filtering it with a low protein binding membrane. However, there was a problem in filtering the solution due to the fast solidification rate. In the second method, a proper amount of sterile H_2O was added to gelatin powder (Sigma-Aldrich) in a laminar flow hood. In the third method, gelatin was autoclaved; this was the method of choice.

To aseptically construct gelatin scaffolds, the gelatin solution was autoclaved, and kept in a sterile environment. When it was needed for use, it was warmed in a water bath at 37°C and the stock glutaraldehyde solution (25 w/v% in water) was added to it in a laminar flow hood. Sterile-filtered solutions of 5 w/v% glycine and water were then used as rinses. The glycine rinse was conducted in an incubator at 37°C since these conditions were more favorable for the capping of aldehyde groups by glycine.

5.1.2. Synthesis of Nanocrystalline Apatite Particles

Syntheses of nanocrystalline hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ or HAP) and carbonated apatite (CAP) were developed in our laboratory over the past few years.[12] CAP better matched the organic, mineral component of bone, and was used in the bone regeneration experiments.

HAP particles were synthesized by first preparing a solution of $(NH_4)_2HPO_4$ with a pH of 10.6 combined with Tween 80 (Sigma-Aldrich). A $Ca(NO_3)_2$ solution was then added to it using a peristaltic pump. To produce CAP particles, a solution of $(NH_4)HCO_3$ and $(NH_4)_2HPO_4$ was

first prepared, and then added to $Ca(NO_3)_2$ with a peristaltic pump. The precipitated HAP and CAP particles were subsequently subjected to aging, washing and drying.

5.1.3. Production of Protein-loaded Composite Particles

The production of apatite-polymer nanocomposite particles by solid-in-oil-in-water technique is illustrated in Figure 5.1. The apatite particles produced in Section 5.1.2 were combined with a model protein (e.g. bovine serum albumin (BSA)) or a therapeutic protein (e.g. BMP). This complex was then combined with poly(lactic-co-glycolic acid) (PLGA) dissolved in an organic solvent to form a solid-in-oil suspension. The suspension was dispersed in an aqueous surfactant solution by homogenization to create a solid-in-oil-in-water suspension. The organic solvent was then evaporated to yield protein-encapsulated composite microparticles.

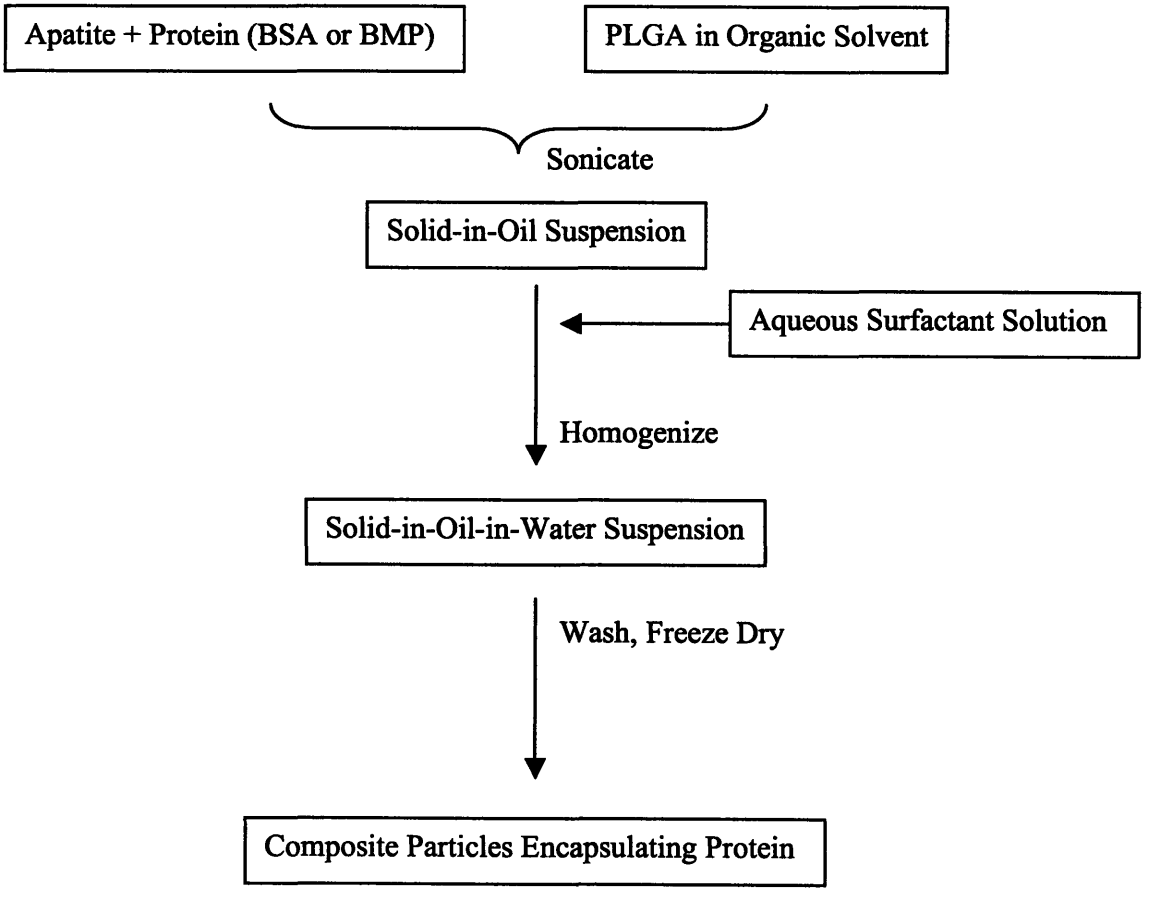


Figure 5.1. Synthesis of protein-loaded apatite-polymer nanocomposite particles.

In the proposed delivery system, these composite particles would be dispersed throughout the gelatin scaffold. After implantation in the fracture site, swelling of the gel would lead to hydrolytic degradation of the particles, and the protein would be released.

5.1.4. *In Vitro* Studies

In vitro studies were conducted with pluripotent embryonic mouse fibroblasts (C3H10T1/2) that could be induced to differentiate into osteoblasts by rhBMP-2. 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (antibiotic) were added to the medium, Basal Medium Eagle (BME) (Sigma-Aldrich).

5.2. CHARACTERIZATION

5.2.1. Cell Viability

Glycine rinses were used to detoxify the uncapped glutaraldehyde groups. In preliminary experiments, gels were exposed to glycine for ~ 1 day. To determine if a longer rinse was necessary, additional studies on cell viability were performed.

To test cell viability and cell proliferation, CyQUANT DNA-binding dye (Molecular Probes) was used to quantify the amount of DNA in cell lysates. Gel A (300 B) samples crosslinked with 10 w/w% glutaraldehyde were placed in a transwell insert with cells plated in the well below at a density of 6,000 cells/cm^2. After 7 days, the cells in the plates were lifted with trypsin and spun into a pellet. The pellet was washed with PBS and then frozen. To lyse the cells, the pellet was freeze-thawed in cell lysis buffer. The RNA was then digested with RNAse since only a count of the DNA was desired in this experiment. Fluorescent dye that would bind to DNA was then added. Since the DNA amount was 6 pg/cell consistently, it was possible to translate the total amount of DNA into the total number of cells.

Cell viability was also determined optically with thin disks of Gel A (300 B). The samples were subjected to 24 and 48 hours of glycine rinses or no glycine rinse. They were then seeded with cells, which were allowed to grow for 3 days before imaging.

5.2.2. *In Vitro* Release

Mechanical testing was conducted to examine the effects of particle loading on the mechanical properties of the gelatin scaffold.

5.2.3. Release Studies

BSA was employed as a model protein to simulate BMP release. Fluorescein isothiocyanate (FITC)-labeled BSA was added to apatite during apatite synthesis. The release of BSA from the composite particles was then measured using a fluorescence reader.

5.3. RESULTS

5.3.1. Effect of Glycine Rinse Period on Cytotoxicity

To examine the effectiveness of glycine rinses, cell viability was investigated through DNA quantification by CyQUANT. Gel A (300 B) scaffolds were exposed to 5 w/v% glycine solution for 4, 6 and 24 hours to detoxify the unreacted glutaraldehyde groups. Control cells were not exposed to the gelatin scaffolds. Figure 5.2 indicated that increasing glycine rinse period significantly decreased the toxicity of the scaffolds. A glycine rinse period of 24 hours was shown to lead to a similar cell viability as the control.

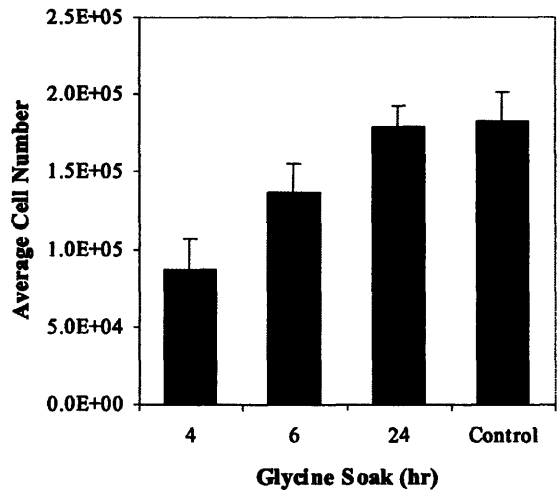


Figure 5.2. Effect of glycine rinse period on the cell viability (n = 5 for control and for samples soaked for 4 hours and 24 hours; n = 2 for sample soaked for 6 hours).

Optical imaging was used to examine the viability of cells seeded on gelatin samples that had been rinsed in glycine for 0, 24 and 48 hours. The cells on the control (with no glycine rinse) did not appear well-adhered. For the samples subjected to 24 and 48 hours of glycine rinse, the cells seemed more well-adhered (see Figure 5.3). The rounded appearance of the cells on the control also suggested that these cells were rather unhealthy, while the cells on the samples subjected to 24 and 48 hours of glycine rinse looked viable and had started to spread out. These results confirmed that a glycine rinse period of at least 24 hours was necessary.

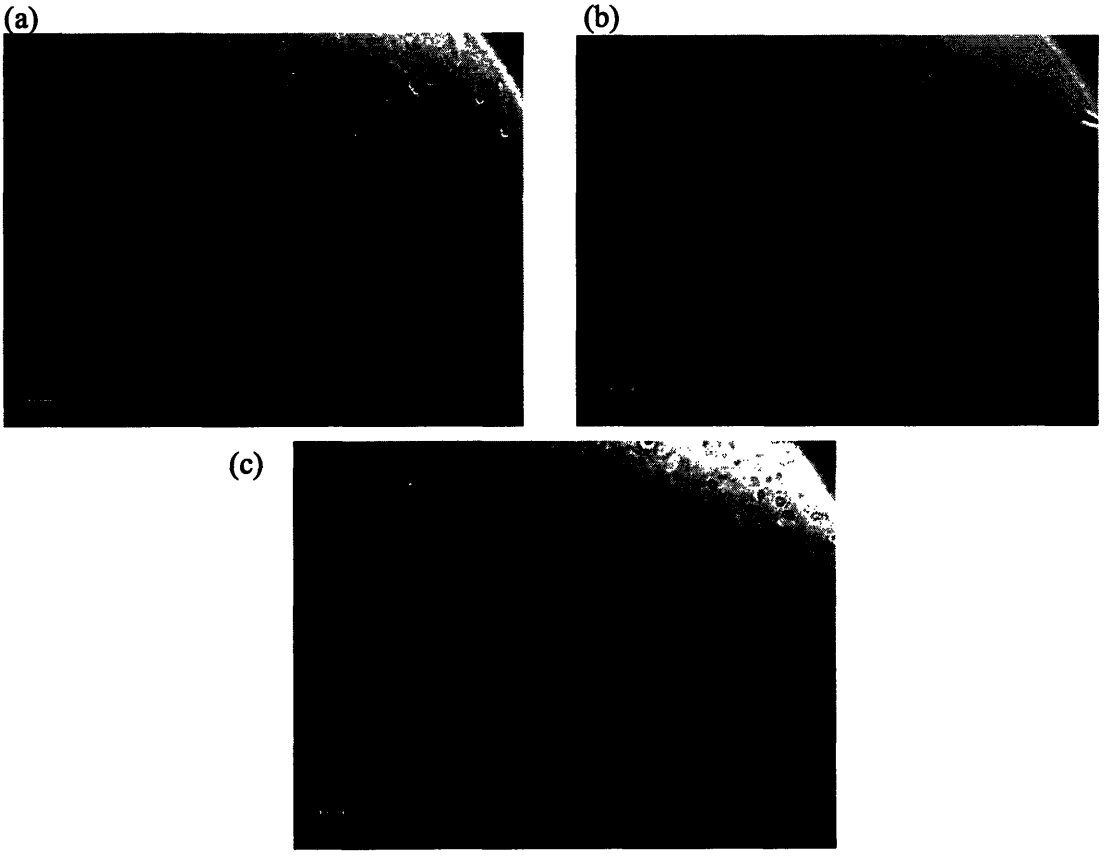


Figure 5.3. Optical imaging of cells seeded onto gelatin samples subjected to (a) 24 hours and (b) 48 hours of glycine rinse, and (c) no glycine rinse.

5.3.2. Effect of Particle Loading on Mechanical Properties

Figure 5.4 illustrates that the Young's modulus of a slow-freezed gelatin scaffold without particles was significantly different from those of particle-loaded scaffolds. The amount of

particle loading only gave rise to minor variation in the mechanical properties of the resulting scaffolds.

Although the PLGA/CAP particles have a higher Young's modulus than the gelatin scaffold, their introduction actually lowered the Young's modulus of the resulting scaffold. We hypothesized that this was because the particles disrupted the gelatin network. There was no chemical bonding to enhance the integration of and interactions between the two components, so only a physical mixture was obtained. The particle-loaded scaffold could not resist deformation to the same degree as the particle-free scaffold due to the decreased connectivity of the gelatin network, thus resulting in a reduced Young's modulus. We note that despite their lower Young's moduli, the particle-loaded scaffolds were easier to handle.

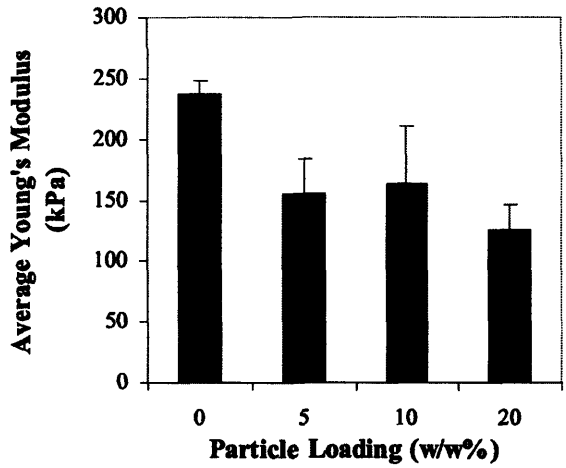


Figure 5.4. Effect of PLGA/CAP particle loading on the Young's modulus of slow-freezed gelatin (n=6 for 0 w/w%, n=4 for 5 w/w%, n=5 for 10 w/w%, and n=3 for 20 w/w%).

5.3.3. Protein Release from a Gelatin Scaffold

FITC-BSA was loaded onto a set of composite particles of CAP and PLGA of low molecular weights (2:1 mixture 6 kD and 24 kD). Figure 5.5 shows the release profile of BSA from these particles, which were designed to have a fast release profile. An initial burst in BSA release was observed.

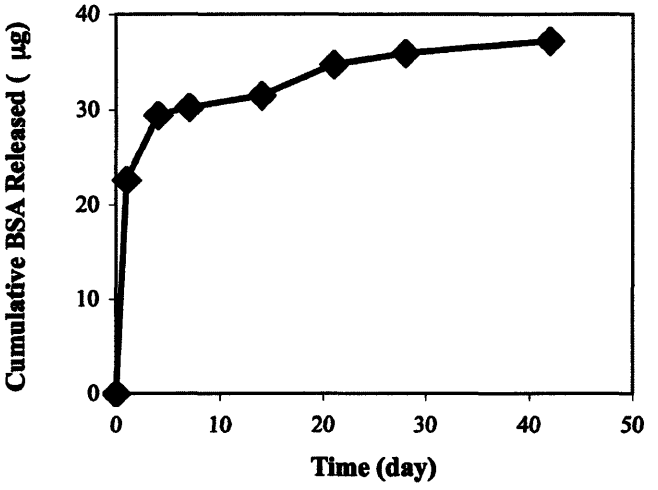


Figure 5.5. BSA release profile from PLGA/CAP composite particles.

BSA release from a gelatin scaffold loaded with PLGA/CAP composite particles is shown in Figure 5.6. Gel B completely degraded by 8 weeks, while Gel A was still intact at 12 weeks. Initial bursts in BSA release were noted with these particle-containing gelatin scaffolds, but were less significant compared to that shown by composite particles alone in Figure 5.5. Thus, a more sustained BSA release was achieved with composite particles incorporated in the gelatin scaffolds.

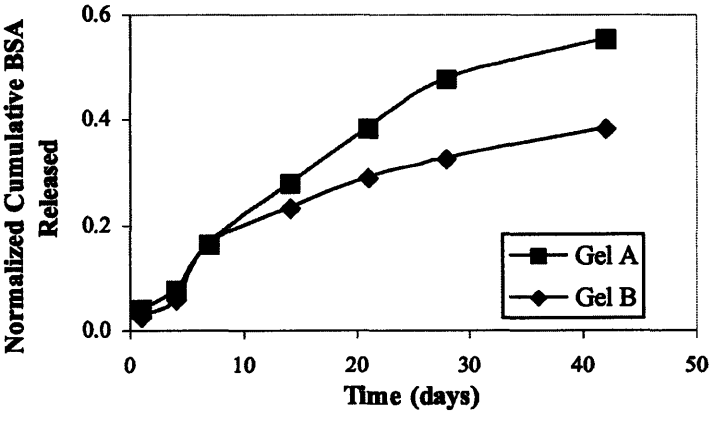


Figure 5.6. BSA release profiles from composite particles in gelatin scaffolds.

6. CONCLUSIONS AND FUTURE WORK

The goals of this project were to understand the properties of the materials involved, to investigate the variables involved in the synthesis of gelatin scaffolds, and to develop a suitable process for producing biocompatible and bioactive composite gelatin scaffolds. Table 6.1 summarizes the recommended materials and synthesis parameters.

Table 6.1. Recommended materials and synthesis parameters for the BMP delivery system.

Type of Gelatin	Gelatin A (300 B) derived from bovine collagen with an IEP of 9.
Crosslinking Method	Chemical crosslinking with 10 w/v% glutaraldehyde for 5 hours at room temperature.
Detoxifying Agent	Single or multiple glycine rinses for $\geq$ 24 hours at 37°C.
Lyophilization Method	Initial freezing at –20°C for 1-2 days, followed by lyophilization for 1-2 days.
Particle Loading	10 w/w% PLGA-CAP composite particles in gelatin scaffolds.

We have successfully achieved the following desirable characteristics for the scaffolds: (1) large pore size, (2) adequate Young's modulus, and (3) biocompatibility. Although the degradation of the gelatin scaffolds was slow, more work would be needed to achieve degradation rates that would sustain BMP concentration for 1-2 months. Currently, gelatin degradation became significant after ~ 1-2 weeks, depending on humidity and temperature (see Section 4.3.2). Since gelatin would degrade much faster in the body due to enzymes, future work should relate *in vitro* degradation to *in vivo* degradation, and determine methods for delaying gelatin degradation.

Additional work should be devoted to investigating the release rates of BMP and their effects on promoting bone growth. This would involve incorporating BMPs (instead of the model protein, BSA) in the composite particles, and loading these particles into the gelatin scaffolds for studying the release and bioactivity of BMPs.

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