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Phase separation, pore structure, and properties of nanofibrous gelatin scaffolds

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Abstract

The development of three-dimensional (3D) biomimetic scaffolds which provide an optimal environment for cells adhesion, proliferation and differentiation, and guide new tissue formation has been one of the major goals in tissue engineering. In this work, a processing technique has been developed to create 3D nanofibrous gelatin (NF-gelatin) scaffolds, which mimic both the physical architecture and the chemical composition of natural collagen. Gelatin matrices with nanofibrous architecture were first created by using a thermally induced phase separation (TIPS) technique. Macroporous NF-gelatin scaffolds were fabricated by combining the TIPS technique with a porogen-leaching process. The processing parameters were systematically investigated in relation to the fiber diameter, fiber length, surface area, porosity, pore size, interpore connectivity, pore wall architecture, and mechanical properties of the NF-gelatin scaffolds. The resulting NF-gelatin scaffolds possess high surface areas ($>32 \text{ m}^2/\text{g}$), high porosities ($>96\%$), well-connected macropores, and nanofibrous pore wall structures. The technique advantageously controls macropore shape and size by paraffin spheres, interpore connectivity by assembly conditions (time and temperature of heat treatment), pore wall morphology by phase separation and post-treatment parameters, and mechanical properties by polymer concentration and crosslinking density. Compared to commercial gelatin foam (Gelfoam[®]), the NF-gelatin scaffold showed much better dimensional stability in a tissue culture environment. The NF-gelatin scaffolds, therefore, are excellent scaffolds for tissue engineering.

Introduction

Tissue engineering offers a new promising approach to tissue and organ repair [1]. In one approach, cells are seeded onto a three-dimensional (3D) biodegradable scaffold followed by *in vivo* implantation to repair tissue or organ defects [2]. Since the scaffold acts as an artificial extracellular matrix (ECM), it should mimic certain critical features (including chemical composition and physical architecture) of natural ECM to facilitate cells adhesion, proliferation, differentiation and new tissue formation [2]. Collagen is the main component of natural ECM of many tissues in the body, such as bone, skin, and tendon. Collagen forms fiber bundles which vary in diameter from 50–500 nm [3]. The nanofibrous architecture of collagen is known to be important for cell attachment and growth [4–6]. Natural collagen has been used as a scaffold for tissue engineering [7–9]. However, immunogenicity and pathogen

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transmission associated with collagen has always been a concern. Therefore, considerable efforts have been made to fabricate collagen-like nanofibrous scaffolds [10–13].

Gelatin is derived from collagen by acidic or basic hydrolysis and its chemical composition is very similar to that of collagen. Therefore, gelatin is a good candidate to mimic the chemical composition of natural collagen. Since gelatin is a denatured protein, the denaturing hydrolysis process eliminates the potential pathogens. As a natural biopolymer, gelatin has been used in tissue engineering [14–16]. Gelfoam®, a commercial gelatin foam, has been shown to be supportive of chondrogenic matrix production *in vitro* [17]. It has also been demonstrated that gelatin foam seeded with bone marrow stromal cells is capable of repairing craniofacial defects *in vivo* [18]. While the gelatin foam has essentially the same chemical composition of collagen and has shown potential for guiding tissue regeneration, it lacks the nanofibrous architecture which has been shown to modulate cell adhesion and function [19–21]. Recently, nanofibrous gelatin (NF-gelatin) mats were reported by using an electrospinning technique [22,23]. However, the electrospinning technique typically forms two-dimensional gelatin sheets, and is incapable of fabricating 3D NF-gelatin scaffolds with well-defined pore size and pore geometry. In the tissue engineering strategy, the 3D porous structure is critical to the development of biological functions of tissues [24,25]. To successfully engineer functional tissues and organs, the scaffolds have to be designed to facilitate cell distribution and guide tissue regeneration in three dimensions.

In this article, we report a comprehensive study of a novel and effective method to fabricate 3D NF-gelatin scaffolds with well-defined pore structure. Three-dimensional NF-gelatin scaffolds with well-defined macropore network were prepared by combining a new thermally induced phase separation (TIPS) technique and a porogen-leaching technique. The processing, structure, mechanical properties and their relationships of the NF-gelatin scaffolds were systematically examined.

Materials and Methods

Materials

Gelatin (type B, from bovine skin, approx 225 Bloom) was purchased from Sigma Chemical Company (St. Louis MO). Gelfoam® was purchased from Pharmacia & Upjohn Company (Kalamazoo, MI). N-hydroxy-succinimide (97%) (NHS) and (2-(N-morpholino) ethanesulfonic acid) hydrate (MES) were purchased from Aldrich Chemical (Milwaukee, WI). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) was purchased from Pierce Biotechnology (Rockford, IL). Ethanol, hexane, cyclohexane and 1,4-dioxane were purchased from Fisher Scientific (Fair Lawn, NJ).

Preparation of NF-gelatin matrices

Gelatin was dissolved in ethanol/water (or methanol/water) solvent mixture at 50°C to make a gelatin solution of 5.0–10.0% (wt/v). The ethanol/water (or methanol/water) ratio ranged from 20/80 (v/v) to 50/50 (v/v). Gelatin solution (1.0 mL) was added into a Teflon vial and was phase separated at –76°C for 4 h or longer. After the phase separation, the vial containing the gel was first immersed into cold ethanol (–18°C) for 24h. The gel was then taken out from the vial and was immersed into 1,4-dioxane to exchange solvent for 24 h. The gel was removed from 1,4-dioxane, blotted with a piece of filter paper, and was frozen at –18°C for 4h. The frozen gel was lyophilized for 1 week. The dried porous gelatin matrices were then stored in a desiccator until characterization or use.

Preparation of 3D NF-gelatin scaffolds

Three-dimensional NF-gelatin scaffolds were fabricated by combining a TIPS technique and a porogen leaching technique. Paraffin spheres were prepared as the porogen [26]. Paraffin spheres (0.40 g) of selected size (diameter range: 150–250 μm , 250–420 μm , or 420–600 μm) were added to Teflon molds (cylinders with a diameter of about 17 mm), and the top surface was leveled. The molds were then preheated at 37°C for 20 min (or 50, 200, 400 min) to ensure that the paraffin spheres were interconnected. Gelatin (1.0g) was dissolved in water (10 mL) and ethanol (10 mL) solvent mixture at 45°C and this solution (0.35 mL) was cast onto the paraffin sphere assemblies. The gelatin solution in the paraffin assembly was then phase-separated at –76°C for at least 4 h.

The gelatin/paraffin composite was then immersed in cold ethanol (–18°C) for 24 h, then transferred into 50 mL 1,4-dioxane for 24 h for solvent exchange (fresh 1,4-dioxane was replaced at every 8 h). The composite was then kept in a freezer at –18°C for 12 h until completely frozen. The frozen composite was freeze-dried in a salt-ice bath for 4 days and then vacuum dried at room temperature for another 3 days.

The gelatin/paraffin composite was cut into samples with a thickness of 2.0 mm. The composite was soaked in 50 mL hexane to leach out paraffin spheres. Hexane was changed every 12 h for 6 times to ensure paraffin removal from the scaffold. To accelerate the dissolution of paraffin spheres, this was done in an oven at 37°C. Cyclohexane was then used to exchange hexane in the scaffold. The gelatin scaffold was frozen at –18°C for 12 h and freeze-dried at between –10°C and –5°C in a salt-ice bath for 4 days and then vacuum dried at room temperature for another 3 days.

Solid-walled gelatin (SW-gelatin) scaffolds were fabricated for comparison with NF-gelatin scaffolds. A similar procedure was used to prepare the SW-gelatin scaffolds except that there was no TIPS process. After the gelatin solution was cast onto the paraffin spheres assembly, the gelatin/paraffin composite was air-dried for 1 week. Hexane was then used to leach out paraffin in the gelatin/paraffin composite and SW-gelatin scaffolds were obtained by direct air-drying.

Chemical crosslinking of 3D NF-gelatin scaffolds

Chemical crosslinking of 3D gelatin scaffolds with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) and N-hydroxy-succinimide (NHS) was carried out in (2-(N-morpholino)ethanesulfonic acid) hydrate (MES) buffer (pH 5.3, 0.05 M) at 4°C for 24 h. To maintain the microstructure and prevent the swelling of gelatin matrices in water, dioxane/water (or acetone/water) solvent mixtures were chosen instead of pure water. The scaffolds were washed with distilled water at 37°C for 3 times. They were then frozen at –18°C for at least 12 h and were freeze-dried for 4 days and vacuum dried at room temperature for another 3 days. The dried gelatin foam was then stored in a desiccator for later use.

Characterization of 3D NF-gelatin scaffolds

Surface morphology examination—The surface morphology of the scaffolds was examined using scanning electron microscopy with an accelerating voltage of 10 kV (SEM, Philips XL30 FEG). The scaffolds were coated with gold using a sputter coater (DeskII, Denton vacuum Inc). During the process of gold coating, the gas pressure was kept at 50 mtorr, and the current was 40 mA. The coating time was 200 s.

Surface area measurement—The surface area was measured by N₂ adsorption experiments at liquid nitrogen temperature on a Belsorp-Mini adsorption apparatus (Bel Japan Inc., Japan), after evacuating samples at 25°C for 10 h ($<7 \times 10^{-3}$ Torr) [27]. Surface area of

scaffolds was calculated from Brunauer-Emmett-Teller (BET) plot of adsorption/desorption isotherm using adsorption points in the P/P_0 range of 0.1–0.3 (BELSORP-mini analysis software).

Porosity—The porosity ε was calculated as: $\varepsilon = 1 - D_p/D_0$ [10]. Where D_p is the skeletal density of gelatin foam, and D_0 is the density of gelatin. D_p was determined by: $D_p = 4m/(\pi d^2 h)$, Where m was the mass, d was the diameter, and h was the thickness of the foam. For gelatin type B (from calf skin, approx. 225 Bloom), $D_0 = 1.35 \text{ g/cm}^3$.

Fiber diameter and length—The average fiber diameter and length between two conjunctions (unit length) was determined using SEM images, where at least 100 measurements of fibers between noticeable conjunctions were selected throughout the matrix [28]. Their averages and standard deviations were reported.

Swelling volume ratio measurement—The swelling volume ratio of scaffolds was measured after the scaffolds were immersed in water for 24 h and was considered to reach the equilibrium of water uptake. The scaffold swelling volume ratio was quantified as V/V_0 , where V_0 is the original volume before crosslinking, and V is the scaffold volume after crosslinking and immersing in water until equilibrium.

Mechanical test—Compressive modulus of scaffolds was measured using an MTS Synergie 200 mechanical tester (MTS Systems Corporation, Eden Prairie, MN) [13]. All samples were circular discs (16 mm in diameter and 2 mm in thickness). Six specimens were tested for each sample. The averages and standard deviations were reported.

Cell seeding on scaffolds—The thawed MC3T3-E1 osteoblasts (clone 26) were cultured in ascorbic acid-free α -MEM supplemented with 10% fetal calf serum (FBS), 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin in a humidified incubator at 37°C with 5% CO_2 . The medium was changed every other day. The scaffolds were sterilized with ethylene oxide for 24 h. The scaffolds were soaked in PBS for 1 h under vacuum. Afterwards, the scaffolds were washed with a complete medium (α -MEM, 10% FBS, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin) twice for 2 h each time on an orbital shaker (3520, Lab-Line Instruments, INC), and each scaffold was seeded with 5×10^5 cells. The medium was changed every 12 h while in the Teflon seeding trays. After 48 h, the cell-scaffold constructs were moved from the Teflon seeding trays and transferred into 6-well tissue culture plates containing 3 mL of complete medium. The constructs were then cultured on the orbital shaker at 100 rpm in the humidified incubator at 37 °C with 5% CO_2 and the medium was changed every other day.

Statistical analysis—All data were presented as means $\pm$ standard deviations (SD). In order to test the significance of observed differences between the study groups, an unpaired Student's t-test was applied. A value of $p < 0.05$ was considered to be statistically significant.

Results and Discussions

Gelatin solution is in a sol state at temperatures above about 37°C. When gelatin concentration is high enough (around 1%, depending on the gelatin composition) and the temperature is decreased to below about 37°C, the gelatin solution becomes a gel. Porous gelatin foam could be obtained by directly freeze-drying an aqueous gelatin solution (Figure 1a). The gelatin foam was composed of closed pores with an average pore size of about 100 μm . The pore wall surface of the gelatin foam was smooth and no nanofibrous architecture was observed by using the conventional freeze-drying method (Figure 1b). In this work, we developed a thermally induced phase separation (TIPS) technique to prepare nanofibrous gelatin matrix. A typical NF-gelatin matrix was shown in Figure 1c and Figure 1d. The architecture of the gelatin matrices was a

3D continuous fibrous network. The nano-fiber diameter of the gelatin matrices ranged from tens to hundreds of nanometers, which was the same range as that of natural collagen matrices.

Generally, the TIPS technique involves several steps, including dissolving a polymer in a proper solvent, phase separation, solvent exchange and freeze-drying. Among them, the selection of proper solvent is one of the most important steps of nanofibrous structure formation. By choosing the ethanol/water and methanol/water solvent systems, nanofibrous architecture of gelatin was created (Figure 1c, Figure 1d). Ethanol and methanol are non-solvents of gelatin. The addition of ethanol and/or methanol to the aqueous gelatin solution was to adjust the interactions between the gelatin molecules and the solvent molecules, thus altering the phase separation conditions when the gelatin solution underwent gelation. A few other solvent mixture systems, such as acetone/water, dioxane/water and THF/water, were also used to investigate the possibility of NF-gelatin matrix formation. However, no typical NF-gelatin matrices were created from these solvent mixtures. These results indicate that controlling the interactions between gelatin and solvent molecules is critical to creating NF-gelatin matrices, although more studies are needed to thoroughly understand the mechanism of the NF-gelatin matrix formation.

After the gelatin solution was phase-separated and became a gelatin gel, it underwent the solvent exchange, which was another important step in maintaining the nanofibrous structure of gelatin. When the gelatin gel was directly freeze-dried without solvent exchange after phase separation, the obtained gelatin foam had a considerable amount of volume shrinkage and formed a smooth pore wall surface, similar to that prepared by conventional freeze-drying method. In our study, cold ethanol (-18°C) and 1,4-dioxane were added to exchange the solvent mixtures in the gelatin gel, and the nanofibrous architecture of the gelatin gel was well retained after freeze-drying (Figure 1c, Figure 1d).

The gelatin foam microstructure was affected by the solvent mixture composition. Gelatin micro-beads were created when the ethanol/water ratio in the gelatin solvent mixture was 10/90 (v/v) (Figure 2a). At an ethanol/water ratio of 20/80(v/v), typical gelatin nano-fibers were observed (Figure 2b). The gelatin nano-fibers were still retained as the ethanol/water ratio was increased to 50/50 (v/v). When the ethanol/water ratio was increased to 60/40 (v/v), the gelatin could only be dissolved at higher temperatures ($>70^{\circ}\text{C}$), and gelatin micro-spheres of various sizes were formed (Figure 2c). Further increasing the ethanol/water ratio led to the insolubility of gelatin and no gelatin gel.

In order to build predesigned interconnected macroporous architecture in the NF-gelatin matrices, a porogen-leaching technique was introduced during the scaffolding fabrication. Paraffin spheres of various sizes (150–250 μm , 250–420 μm , or 420–600 μm) were prepared and used as the porogen of gelatin scaffolds. Because the gelatin solution made of ethanol/water solvent mixture was easy to penetrate inside the paraffin sphere assembly, the ethanol/water solvent mixture system was used to fabricate 3D gelatin scaffolds in this study. Gelatin scaffolds with smooth pore-wall structure (SW-gelatin) were created when the gelatin/paraffin composite was directly air-dried skipping the phase-separation step (Figure 3a, 3b). NF-gelatin scaffolds were created by combining the TIPS and paraffin-leaching technique (Figure 3c–3e). The pore walls consisted of gelatin nano fibers with a diameter ranging from 50 to 500 nm, which was the same diameter range of natural collagen fibers (Figure 3e). The structural parameters of NF-gelatin matrices are summarized in Table 1. When the gelatin concentration was 5.0% (wt/v), the average diameter of gelatin fiber was 177 ± 62 nm. Although the average diameter of gelatin fibers decreased slightly with increasing gelatin concentration, the changes were not statistically significant. The average fiber length (fiber length between two conjunctions) decreased with gelatin concentration. When the gelatin concentration was 5.0% (wt/v), the average fiber length was 1181 ± 413 nm. As the gelatin concentration increased to

10.0% (wt/v), the average fiber length decreased to 497 ± 62 nm, which was less than 50% of the gelatin fiber length fabricated with 5.0% (wt/v) gelatin solution. The NF-gelatin scaffolds exhibited very high porosity. For example, the scaffold porosity was $97.51 \pm 0.03\%$ when the gelatin concentration was 7.5%. The scaffold porosity could be further increased by decreasing the gelatin concentration. NF-gelatin scaffolds possessed extremely high surface areas. For example, the NF-gelatin scaffold prepared with a gelatin concentration of 7.5% had a surface area of $34.76 \text{ m}^2/\text{g}$. In contrast, the SW-gelatin scaffold with the same gelatin concentration and porosity had a surface area of less than $0.1 \text{ m}^2/\text{g}$. The high surface area of a scaffold has been demonstrated to enhance protein adsorption, thus promoting cell adhesion [19].

Both the pore size and the interconnectivity between pores are important parameters of the scaffolds. The fabrication technique in this study could easily control the scaffold pore size and the interconnectivity between pores of the scaffold. The pore size of the scaffold was controlled by the size of paraffin spheres, that is, larger paraffin spheres led to larger scaffold pore size (Figure 4). The interconnectivity between pores of the scaffold could be conveniently tailored by the heat treatment time of the paraffin sphere assembly (Figure 5). The scaffold had low interconnectivity as the paraffin sphere assembly was heat-treated at 37°C for 20 min, while the gelatin sphere assembly heat-treated at 37°C for 50 min resulted in a gelatin scaffold with a moderate pore opening size. Heat treatment at 37°C for 200 min generated a scaffold with high interconnectivity between pores. Further increasing heat treatment time to 400 min led to even higher interconnectivity between pores.

Since the gelation temperature of gelatin is very close to cell culture temperature (37°C), the NF-gelatin scaffold must be crosslinked to improve its thermal and mechanical stability prior to its tissue engineering application. Several physical and chemical crosslinking methods for gelatin have been reported [29–33]. Glutaraldehyde is a widely used crosslinker for gelatin [32,33]. However, the toxicity has always been a serious concern, because the glutaraldehyde is built into the gelatin molecules after crosslinking. The water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) has been considered to be non-toxic and biocompatible because it introduces crosslink without incorporating foreign structures into the biomaterial network. EDC does not remain as part of the linkage after crosslink formation [15]. Therefore, EDC was selected to crosslink NF-gelatin scaffolds in this study.

It was found that the volume of the NF-gelatin scaffold crosslinked with EDC in aqueous solution had a considerable increase ($>50\%$) compared to its original volume before chemical crosslinking. Additionally, the pore wall of the crosslinked gelatin scaffold was smooth and the nanofibrous feature disappeared (Figure 6b). To prevent the volume swelling as well as to retain nanofibrous architecture after crosslinking, a novel crosslinking system has to be developed for the NF-gelatin scaffolds.

We developed two solvent mixture systems (acetone/water and dioxane/water) to crosslink the NF-gelatin scaffolds. It was found that even a small amount of acetone or dioxane was added in the crosslinking solution, the swelling volume ratio (V/V_0) of the crosslinked NF-gelatin scaffolds was dramatically decreased (Figure 7). For example, with the acetone/water solvent mixture composition of 10/90 (v/v), the V/V_0 was $84.9 \pm 1.1\%$, which was about one half of the V/V_0 ($156.3 \pm 4.7\%$) of the scaffold crosslinked in aqueous solution. The V/V_0 decreased as the ratio of acetone/water increased. Interestingly, the V/V_0 was the lowest when the acetone/water ratio reached 60/40 (v/v). Further increasing the acetone/water ratio led to an increase of V/V_0 . As acetone/water ratio increased to 90/10 (v/v), the volume of the crosslinked NF-gelatin scaffold was almost the same as that of the original scaffold before crosslinking. Most importantly, the scaffold crosslinked under this condition retained the nanofibrous morphology (Figure 6c). Similar result was also observed from the dioxane/water solvent mixture system (Figure 7).

It has been known that EDC easily loses its activity in aqueous solution [34,35], and the addition of organic solvents has been reported to prevent the deactivation of EDC [36]. Since acetone and dioxane are both non-solvents of gelatin, the addition of acetone and/or dioxane in solvent mixtures would prevent both EDC deactivation and gelatin scaffold swelling during the crosslinking process, thus leading to higher crosslinking density. Therefore, the equilibrium swelling volume ratio decreased with increasing the amount of non-solvents (acetone and dioxane) in crosslinking reaction systems. On the other hand, both acetone and dioxane also are non-solvents of EDC. As the amount of acetone/dioxane in the crosslinking solution further increased (e.g. acetone/water > 60/40 (v/v)), EDC could not be completely dissolved in the solvent mixture. The amount of EDC that actually participated in the crosslinking reaction decreased and the crosslinking density decreased. Therefore, the swelling volume ratio increased. Our study has shown that the optimal solvent mixture (acetone/water and dioxane/water) ratio is 90/10 (v/v), because the NF-gelatin scaffolds can retain both their original size and nanofibrous structure under this crosslinking condition.

The mechanical properties of the NF-gelatin scaffolds, SW-gelatin scaffolds, and Gelfoam[®] are examined (Figure 8a). While all three scaffolds had high porosities (>97.0%) and similar macropore sizes, the NF-gelatin and the SW-gelatin scaffolds had much higher compressive modulus than Gelfoam[®]. For example, the compressive modulus of the NF-gelatin scaffold was 10 times higher than that of Gelfoam[®]. The compressive modulus of the NF-gelatin scaffold was also higher than that of the SW-gelatin scaffold, although it is not statistically significant ($p = 0.159$). The mechanical strength of the NF-gelatin scaffolds could be easily increased by using higher concentration of gelatin solution (Figure 8b). The compressive modulus of the NF-gelatin scaffolds did not change significantly with macropore size (150–250 μm , 250–420 μm , 420–600 μm) (Figure 8c). With macropore sizes of 150–250 μm and 250–420 μm , NF-gelatin scaffold had a higher compressive modulus than the SW-gelatin scaffold, although it was not statistically significant ($p = 0.145$ and 0.159 respectively). With a macropore size of 420–600 μm , the compressive modulus of NF-gelatin scaffold became significantly higher than that of SW-gelatin scaffold ($p < 0.05$) (Figure 8c).

The crosslinked NF-gelatin scaffolds showed a significantly greater dimensional stability to support tissue regeneration. As shown in Figure 9, the cell/NF-gelatin construct maintained its size after two and four weeks of MC3T3-E1 pre-osteoblast cell culture. In contrast, the diameter of cell/Gelfoam[®] construct shrunk to about 50% and 40% of its original size after two and four weeks of MC3T3-E1 pre-osteoblast cell culture, respectively. In tissue engineering, a critical requirement for a scaffold is that the scaffold should have adequate mechanical strength to maintain the spaces required for cell in-growth and matrix production until later neo-tissue formation. This result demonstrates that the NF-gelatin scaffold is a significantly better scaffold than Gelfoam[®] to maintain 3D geometry for tissue regeneration.

Conclusions

Macroporous and nanofibrous gelatin scaffolds have been fabricated in this work by a phase separation and paraffin leaching technique. This technique has shown great control over porosity, macropore size, interpore connectivity, mechanical properties as well as pore wall morphologies in 3D scaffolds. The NF-gelatin scaffolds possess high surface areas and porosities, well-connected macropores, good mechanical properties, and nanofibrous pore wall morphology. The crosslinked NF-gelatin scaffolds showed great dimensional stability to support tissue regeneration. Since the NF-gelatin scaffolds mimic both the physical architecture and chemical composition of collagen (the main ECM component of many tissues), the biomimetic NF-gelatin scaffolds may provide better environments for a variety of tissue engineering applications.

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Figure 1.

SEM micrographs of gelatin matrices fabricated from different solvent mixtures and processing conditions: (a) water, lyophilized; (b) high magnification of (a); (c) ethanol/water = 50/50, phase separated at -76°C , solvent exchanged, and freeze-dried; (d) methanol/water = 30/70, phase separated at -76°C , solvent exchanged, and freeze-dried. The gelatin concentration was 5.0% (wt/v) for all samples.

Figure 2. SEM micrographs of gelatin matrices fabricated using ethanol/water solvent mixtures. (a) ethanol/water = 10/90 (v/v); (b) ethanol/water = 20/80 (v/v); (c) ethanol/water = 60/40 (v/v). The gelatin concentration was 5.0% (wt/v) for all samples.

Figure 3.

SEM micrographs of SW-gelatin scaffolds and NF-gelatin scaffolds. (a) SW-gelatin scaffold, $\times 50$; (b) pore wall morphology of SW-gelatin scaffold, $\times 1000$; (c) NF-gelatin scaffold, $\times 50$; (d) pore wall morphology of NF-gelatin scaffold, $\times 1000$; (e) high magnification of (d), $\times 10000$. Scaffolds were prepared from a 7.5% (wt/v) gelatin solution in ethanol/water mixture (ethanol/water = 50/50) and the paraffin spheres were 250–420 μm in diameter. Paraffin spheres were heat treated at 37°C for 40 min.

Figure 4.

SEM micrographs of 3D NF-gelatin scaffolds with varying macropore sizes: (a) 150–250 μm ; (b) 250–420 μm ; (c) 420–600 μm . Scaffolds were prepared from a 7.5% (wt/v) gelatin solution in ethanol/water mixture (ethanol/water = 50/50) and the paraffin spheres were heat treated at 37°C for 30 min.

Figure 5.

SEM micrographs of 3D NF-gelatin scaffolds with varying inter pore connectivities under different heat treatment conditions: (a) 37°C for 20 min; (b) 37°C for 50 min; (c) 37°C for 200 min; (d) 37°C for 400 min. Scaffolds were prepared from a 7.5% (wt/v) gelatin solution in ethanol/water mixture (ethanol/water = 50/50) and the paraffin spheres were 250–420 μm in diameter.

Figure 6. SEM micrographs of pore wall morphology of gelatin scaffolds before and after crosslinking in different solvent systems. (a) before crosslinking; (b) after crosslinking in aqueous EDC solution; (c) after crosslinking in EDC/Acetone/water solution (acetone/water = 90/10, v/v). Scaffolds were prepared from a 10.0% (wt/v) gelatin solution in ethanol/water mixture (ethanol/water = 50/50) and the paraffin spheres were 250–420 μm in diameter.

Figure 7. Swelling volume ratios in water after chemical crosslinking in acetone/water and dioxane/water systems. Scaffolds were prepared using 7.5% (wt/v) gelatin solution; the pore size was 250–420 μm . EDC concentration was 5 mmol/L, EDC/gelatin = 5/1 (mg/mg).

Figure 8.

(a) The compressive modulus comparison of Gelfoam[®], NF-gelatin and SW-gelatin scaffolds. The NF-gelatin and SW-gelatin scaffolds were prepared from a 7.5% (wt/v) gelatin solution in an ethanol/water mixture (ethanol/water = 50/50), had a macropore size of 250–420 μm and a porosity of about 97.5%. (b) The effects of gelatin concentration on the compressive modulus of NF-gelatin scaffolds. The NF-gelatin scaffolds had a macropore size ranging of 250–420 μm. (c) The effect of macropore size on compressive modulus of both NF-gelatin scaffolds and SW-gelatin scaffolds. The NF-gelatin and SW-gelatin scaffolds were prepared from a 7.5% (wt/v) gelatin solution in an ethanol/water mixture (ethanol/water = 50/50).

Figure 9.

The size variation of scaffolds (NF-gelatin and Gelfoam[®]) after culturing MC3T3-E1 osteoblasts for 2 weeks and 4 weeks. NF-gelatin scaffolds were prepared from a 7.5% (wt/v) gelatin solution in an ethanol/water mixture (ethanol/water = 50/50) with a macropore size of 250–420 μm .

Table 1

Structural parameters of 3D NF-gelatin scaffolds prepared with different gelatin concentrations.

Gelatin concentration (%)	Fibers diameter (nm)	Fiber length (nm)	Porosity (%)	Surface area (m ² /g)
5.0	177±62	1181±413	98.06±0.07	33.63
7.5	161±45	666±156	97.51±0.03	34.76
10.0	157±49	497±62	96.45±0.19	36.92