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Chitosan/gelatin porous bone scaffolds made by crosslinking treatment and freeze-drying technology: Effects of crosslinking durations on the porous structure, compressive strength, and *in vitro* cytotoxicity

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ABSTRACT: In this study, freezing was used to separate a solute (polymer) and solvent (deionized water). The polymer in the ice crystals was then crosslinked with solvents, and this diminished the linear pores to form a porous structure. Gelatin and chitosan were blended and frozen, after which crosslinking agents were added, and the whole was frozen again and then freeze-dried to form chitosan/gelatin porous bone scaffolds. Stereomicroscopy, scanning electron microscopy, compressive strength testing, porosity testing, *in vitro* biocompatibility, and cytotoxicity were used to evaluate the properties of the bone scaffolds. The test results show that both crosslinking agents, glutaraldehyde (GA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, were able to form a porous structure. In addition, the compressive strength increased as a result of the increased crosslinking time. However, the porosity and cell viability were not correlated with the crosslinking times. The optimal porous and interconnected pore structure occurred when the bone scaffolds were crosslinked with GA for 20 min. It was proven that crosslinking the frozen polymers successfully resulted in a division of the linear pores, and this resulted in interconnected multiple pores and a compressively strong structure. The 48-h cytotoxicity did not affect the cell viability. This study successfully produced chitosan/gelatin porous materials for biomaterials application. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41851.

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INTRODUCTION

Bone scaffolds require a three-dimensional, porous, interconnected pore structure; compressive resistance; good biocompatibility; and nontoxicity; therefore, the combination of a freeze-drying technique and biocompatible polymers is ideal for the preparation of bone scaffolds.[1–3] Such a technique sublimates the ice crystals composed of the frozen solvent and leaves the solute (e.g., polymer) and, thereby, directly produces bone scaffolds with the aforementioned required structure.[4] In addition, the production is convenient and fast.

As has been proven by a previous study, the pores appear to be horizontal and linear; this is caused by the heat transfer direction during the freezing process and the direction the ice crystals are formed.[5] However, the linear pores caused by the freeze-drying technique are a defect for bone scaffolds in use. The existence of linear pores prevents the bone scaffolds from possessing an interconnected pore structure.[6,7] A porous and interconnected pore structure is essential for bone scaffolds as it allows for cell migration and nutrient flows.[3] The relationship between the pore size and MG-63 osteoblast cell attachment has been confirmed.[8] The linear pores possess a large size, and this prohibits cell attachment. Inorganic particles, such as hydroxyapatite powders or pore-making agent, such as NaCl powders, are thus added to the polymer solution to prevent the formation of linear pores.[9,10] Inorganic particles can replace the ice crystals and also diminish their size so as to decrease the size of the pores. The pore-making agent can be removed by rinsing after the freezing process, and this also increases the corresponding pore size of itself. The implementation of both methods can yield a porous structure.

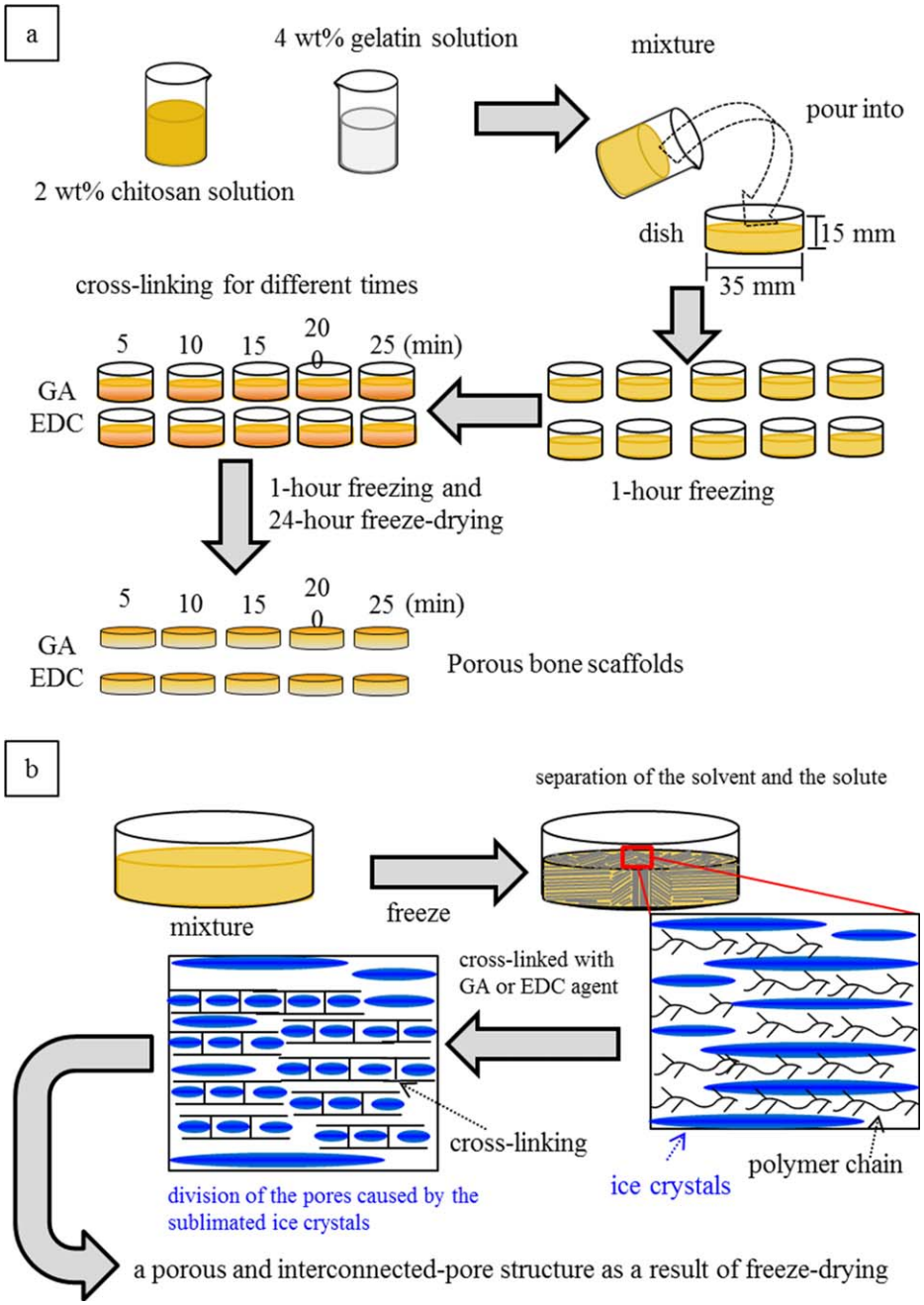

Figure 1. (a) Processing procedure for the bone scaffolds and (b) mechanism of a porous structure by crosslinking with the solute (polymer). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The combination of hydroxyapatite powder and pore-making agent affects the interconnected pore structure, which is highly essential to cells.[3] The nutrient transport and metabolism of cells are negatively influenced by bone scaffolds without interconnected pores. Therefore, in this study, we aimed to produce a porous and interconnected pore structure; the frozen polymers were crosslinked to divide the linear pores and to thereby attain ideal interconnected pores.

Collagen, gelatin, and chitosan have been studied and applied commonly.[3,11–13] Collagen can be synthesized with glycosaminoglycans to form porous sponges that are applied in bone tissue engineering because these materials can simulate the artificial extracellular matrix and render good infiltration to the cells.[14–16] Chitosan and gelatin are also commonly blended for use in bone tissue engineering, as an artificial extracellular matrix contains glycosaminoglycans, which have a structure similar to that of chitosan.[15] However, collagen possesses antigenicity because it is extracted from animal origin. Gelatin is a hydrolysate product of collagen and, thus, has a relatively lower antigenicity than collagen, and gelatin also has a low cost.[16,17] In addition, gelatin also retains some information signals, such as arginine–glycine–aspartic acid (RGD), which is conducive to

the acceleration of cell differentiation, proliferation, and attachment to materials. In the study of the effects of the products and production costs, the combination of chitosan and gelatin has been used commonly in bone tissue engineering.[17] Both chitosan and gelatin are biodegradable, biocompatible, and nontoxic, and they are beneficial for cell attachment and proliferation. Thus, the combination of inorganic particles, such as β-tricalcium phosphate and hydroxyapatite, can further facilitate the repair of bones. Furthermore, they both have water as their basic solvent, and this benefits freeze-drying preparation.[18–23]

Glutaraldehyde (GA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) are two crosslinking agents that are commonly used with chitosan and gelatin. The amines of chitosan and gelatin can be formed into amine–amine bonds via GA, whereas the amines of chitosan and gelatin and the activated carboxylic acid groups of gelatin, can be formed into new isopeptide bonds via EDC.[24,25] Thus, in this study, we used GA and EDC to crosslink chitosan and gelatin to divide the linear pores.

When used in studies for a porous and interconnected pore structure, the freeze-drying technique is mostly adjusted with the temperature and the combination of inorganic particles and pore-making agents.[6,9,10,26] The methods used to form a porous structured samples by Yuan *et al.*[26] and Wu *et al.*[6] were different from that used in this study. Yuan *et al.* created their samples with a freeze-gelatin method and then dehydrated the samples in a stepwise process, whereas Wu *et al.* used a frozen gelatin solution in conjunction with the evaporation of liquid nitrogen and then freeze-dried the resulting samples. In addition, this study also used a similar premix procedure for chitosan and gelatin solutions, but a different crosslinking treatment was used in the studies by Peter *et al.*[9] and Alizadeh *et al.*[10] In this article, we propose a novel method for producing chitosan/gelatin porous bone scaffolds with a porous and interconnected pore structure. By taking advantage of the separation conditions of the solvent (deionized water) and solute (polymer) when the chitosan/gelatin mixture was frozen, we crosslinked the polymer with GA and EDC, respectively, for various durations. Finally, the variations in structural formation, compressive strength, porosity, and cytotoxicity of the different chitosan/gelatin porous bone scaffolds were compared in terms of their various crosslinking agents and times.

EXPERIMENTAL

Materials

Chitosan (Global Technology Co., Taiwan, Republic of China) had a deacetylation of 85%. Gelatin from porcine skin (Type A, Sigma-Aldrich Co, Ltd.) possessed a gel strength of 300. Acetic acid (Shimakyu's Pure Chemicals, Japan) was a reagent chemical and had a 99–100% concentration. GA (Choneye Pure Chemicals, Taiwan, Republic of China) had an assay of 25%. EDC was purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Minimum essential medium (MEM) was purchased from Gibco, Inc. Dimethyl sulfoxide was purchased from Applichem, Inc. 3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS) was purchased from Sigma, Uni-Onward Corp. MG-63 was purchased from the Food Industry Development Institute (Taiwan, Republic of China).

Preparation of the Bone Scaffolds

The acetic acid and deionized water were made into a 1 v/v% acetic acid solution, which was then blended with chitosan powder at 60°C for 24 h to form a 2 wt % chitosan solution. The gelatin powder and deionized water were blended at 60°C for 24 h to form a 4 wt % gelatin solution. Figure 1(a) illustrates the preparation process of the bone scaffolds. The chitosan solution and gelatin solution were mixed at a 1:1 volume ratio at 60°C for 1 h to form a chitosan/gelatin mixture. A volume of 10 mL of the mixture was added to dishes and frozen at −20°C for 1 h to separate the solvent and solute. The frozen samples were removed and placed in a hood at 37°C. Next, 2 mL of the two 0.5 v/v% crosslinking agents (GA and EDC) were added and crosslinked with the solute for 5, 10, 15, 20, and 25 min; this divided the pores to yield a porous structure. The mechanism of the porous structure is shown in Figure 1(b). Then, the samples were frozen at −20°C for 1 h and then freeze-dried for 24 h to form the chitosan/gelatin porous bone scaffolds. The notation of the samples consisted of the crosslinking agent (GA or EDC) and the time (digit). For example, GA5 refers to bone scaffolds crosslinked with GA for 5 min.

Tests

Surface Observation. For surface observation, the surface structures of various samples were compared as related to various crosslinking agents and times. The samples were placed and observed on the platform of a stereomicroscope (SMZ-10A, Nikon Instruments, Inc., Japan). Motic Images Plus 2.0 software (Motic Group Co., Ltd.) was used to produce a picture, the scale bar of which was then calculated by the pixel and ruler unit with Image-Pro 6.2 software (Total Smart Technology Co., Ltd., Taiwan, Republic of China).

Scanning Electron Microscopy (SEM) Observation. The porous and interconnected pore structures of the samples were examined as related to the crosslinking agents and times. After they were coated with a thin layer of gold for 30 s on an Ion Sputter instrument (E-1010, Hitachi, Japan), samples were cut randomly into sections and then affixed with their surface facing upward on the sample holder with carbon paste. Samples were observed with an SEM instrument (S3000, Hitachi, Japan) with an accelerating voltage of 15 kV to analyze their structures caused by various crosslinking agents; meanwhile, the influences of the crosslinking agents and times on the interconnected pore structure were examined.

Compressive Strength. An Instron 5566 instrument (Instron) was used to measure the compressive strength of the samples, which were adhered vertically to the plane of a compressive clamp, as specified in ASTM D 6641M-09.[27] The space between the compressive clamps was 1.5 cm, and the test velocity was set at 1.3 mm/min. The compressive strength of the bone scaffolds as related to the various crosslinking agents and times was examined. There were a total of six samples for each specification.

Porosity Analyses. The samples were measured with a vernier caliper for their radius and height, both of which were then

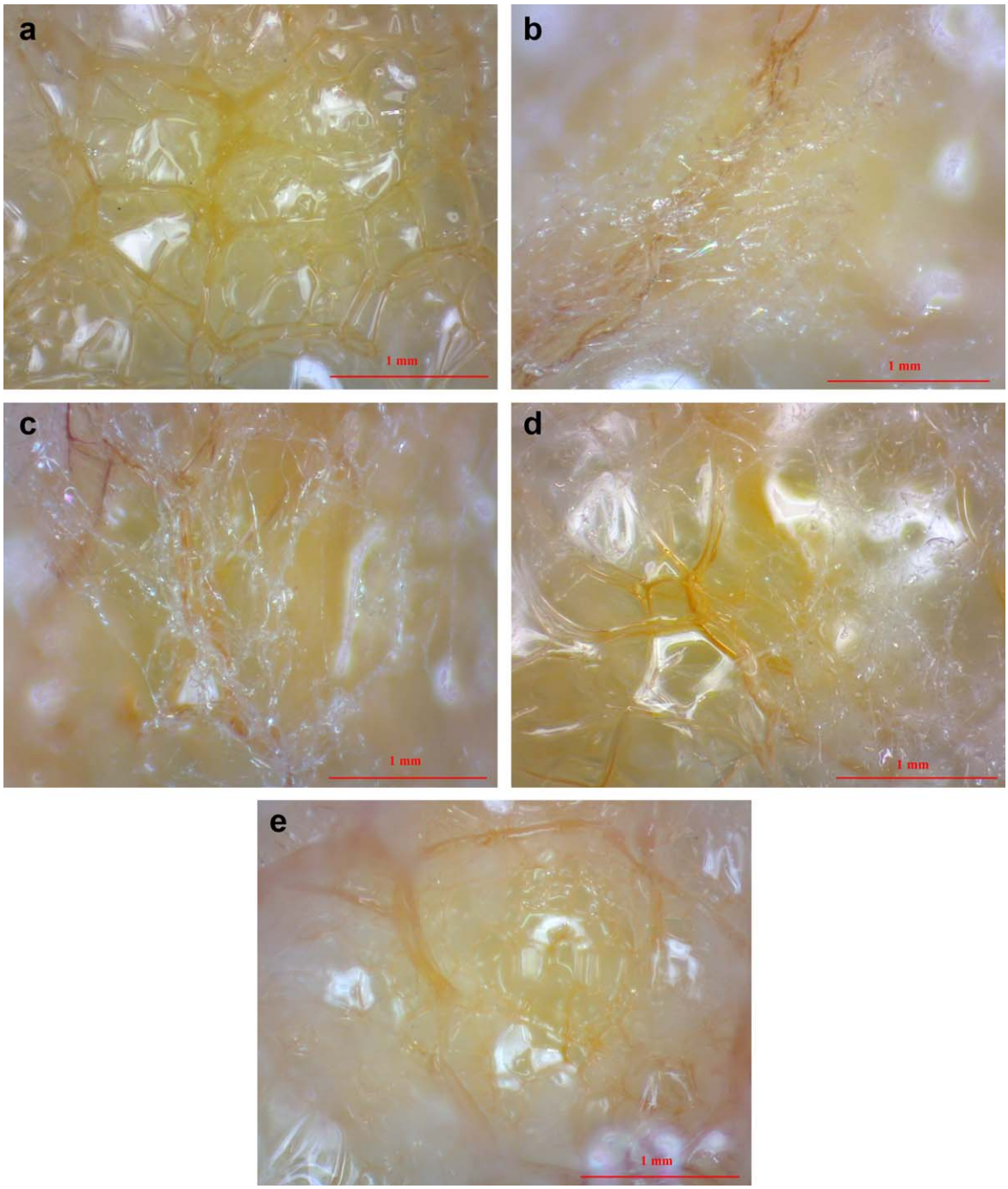

Figure 2. Images (30×) of the chitosan/gelatin porous bone scaffolds that underwent GA crosslinking: (a) GA5, (b) GA10, (c) GA15, (d) GA20, and (e) GA25. The scale bar represents 1 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

used to calculate the total volume of the sample (V_{total}). Then, the samples were immersed in a graduated cylinder containing deionized water to determine the height that the water increased and also to calculate the real volume of the sample (V_{real}). Next, eq. (1) was used to calculate the pore volume (V_p), and eq. (2) was used for porosity.[6] Finally, the variations in the porosity between the different samples were compared in terms of the various crosslinking agents and times. There were a total of 10 samples for each specification.

$$V_{total} - V_{real} = V_p \quad (1)$$

$$\text{Porosity}(\%) = [(V_{total} - V_{real}/V_{total}) \times] 100\% = [V_p/V_{total}] \times 100\% \quad (2)$$

***In Vitro* Biocompatiblity.** This test followed ISO 10993-5. The samples were rinsed with deionized water three times and then sterilized by 12-kGy γ rays.[28] MG-63 osteoblast cells were cultured in a Petri dish, from which the MEM was removed. Then, PBS was used to rinse the dish and was then removed. A volume

of 0.5 mL of trypsin was added to the dish, which was then placed in an incubator at 37°C with 5% CO_2 for 3–5 min. A volume of 0.5 mL of PBS was used to dilute the concentration of trypsin to form the final cell suspension, which was taken with the required density (5×10^4 cells/well) by means of a hemocytometer and added to a 96-well culture plate to coculture with samples for 24 and 48 h. The biocompatibility of the samples was observed with an optical microscope (IX71, Yuan Li Instrument Co., Ltd., Taiwan, Republic of China). The control group was MG-63 osteoblast cells cultured with MEM only. There were a total of six samples for each specification.

Cytotoxicity. This test followed ISO 10993-5. All of the samples were sterilized by 12-kGy γ rays and tested with an MTT assay to determine their cellular viability (%).[28] The sample extracts were infused to the 96-well culture plate at 10 μL with a concentration 1.6 mg/1 mL, to which the MG-63 osteoblast cell suspension was added at 90 μL with a density of 10^4 cells/well. The plate was placed in an incubator at 37°C with 5% CO_2 for 24 and 48 h and then placed on a horizontal laminar flow table. MEM was then removed by Pasteur pipette suction, and 70 μL of the MTT reagent and MEM were added to the plate at a volume ratio of 1:39. The plate was kept in darkness for 4 h; then, the MTT/culture medium mixture was removed, and 70 μL of dimethyl sulfoxide solvent was added to the plate. An ELISA reader was used to measure the optical densities at 570 nm, the results of which were used to determine the cellular viability (%) with eq. (3). There were a total of six samples for each specification.

$$\text{Cellular viability } (\%) = OD_e/OD_m \quad (3)$$

where OD_e is optical density of the medium containing the extract solution and OD_m is the optical density of the fresh medium.

Statistical Analyses

In this study, we used one-way analysis of variance from SPSS Statistics 17 for statistical analyses. When p was less than 0.05, the test results had significant deviations and were marked with an asterisk. When p was less than 0.01, the test results had highly significant deviations and were marked with two asterisks.

RESULTS AND DISCUSSION

Surface Observation

Figures 2(a) and 3(a) show that GA5 and EDC5 both exhibited significant porous structures. When crosslinking time was extended to 10 and 15 min, the porous structures of GA10 and GA15 were absent [Figure 2(b,c)], but the porous structures of EDC10 and EDC15 still remained [Figure 3(b,c)]. Crosslinking for 20 or 25 min allowed the GA20 and GA25 to regain porous structures [Figure 2(d,e)] and EDC20 and EDC25 to turn the porous structure into a compact structure [Figure 3(d,e)].

The structures of GA10 and GA15 both had loose layers, which were proven to be able to withstand two different freezing temperatures in the freeze-drying technique; these temperatures, in turn, created ice crystals of two different sizes.[29] However, such a loose-layer structure disappeared in GA20 and was replaced by a porous structure. This loose-layer structure occurred when the chitosan/gelatin mixture underwent crosslinking with polymers located in the linear ice crystal and started to become crosslinked, and it did not occur because of the ice crystals. Therefore, the polymers crosslinked to a greater level with increasing crosslinking time; this caused the loose-layer structure to disappear. This result indicates that the crosslinking time helped the polymers between ice crystals to crosslink and contributed to the division of the linear pores, a phenomenon that occurred solely for the GA series.

EDC5, EDC10, and EDC15 were all formed with porous structures, and EDC20 and EDC25 exhibited compact structures rather than porous structures; this indicated that the crosslinking mechanism of the polymer groups affected the division of linear pores. Furthermore, the crosslinking agent of EDC led to the amine of gelatin or chitosan and the glutamic acid of the gelatin, or it may also have led to the amine of gelatin or chitosan and the activated carboxylic acid groups by the aspartic acid residue of gelatin, both of which could form new isopeptide bonds.[25] The crosslinking agent of GA crosslinked with the amide groups of the chitosan and the amide groups of the gelatin to form —C═N compounds via —COH and —NH_2. A short crosslinking time of 5 min did not differentiate GA from EDC for a porous structure; however, the difference between GA and EDC was that the former could crosslink with polymers between ice crystals and thus divide the linear pores when the crosslinking time was 10 or 15 min, whereas the latter could result in a compact structure from a porous one when the crosslinking time increased.

SEM Observation

Figures 4(a) and 5(a) show that GA5 and EDC5 both had horizontal linear pores without being distinctly interconnected. GA10 and GA15, indicated by red circles in Figure 4(b,c), exhibited a loose-layer structure composed of polymers; this extended to link each other by crossing the linear pores. The linear pores diminished as a result of the linking of the polymers and were, thus, divided to form a porous and interconnected-pore structure. By contrast, EDC10 and EDC15 also had porous structures but without significantly interconnected pores. GA20 and GA25 [Figure 4(d,e)] showed that in addition to the divided linear pores caused by the crosslinking of polymers, they also demonstrated complete walls of pores as a result of the linking of polymers. The distinct porous and interconnected pore structure is indicated by red arrows in Figure 4(d). Figure 5(d,e) shows that the porous structure of EDC20 and EDC25 was gone and replaced with a less porous and more compact structure. Such results indicate that the crosslinking mechanism between the crosslinking agents and polymers influenced the polymers to divide the linear pores and the final structure after freeze drying. The use of GA as a crosslinking agent resulted in amine–amine bonds between molecular chains of chitosan and gelatin, whereas the use of EDC as a crosslinking agent created gelatin–gelatin, gelatin–chitosan, and chitosan–chitosan molecular-chain isopeptide bonds. These occurrences of crosslinking reactions formed a reticular structure between those molecular chains. Compared to isopeptide bonds formed by crosslinking with EDC, the —NH_2 crosslinking

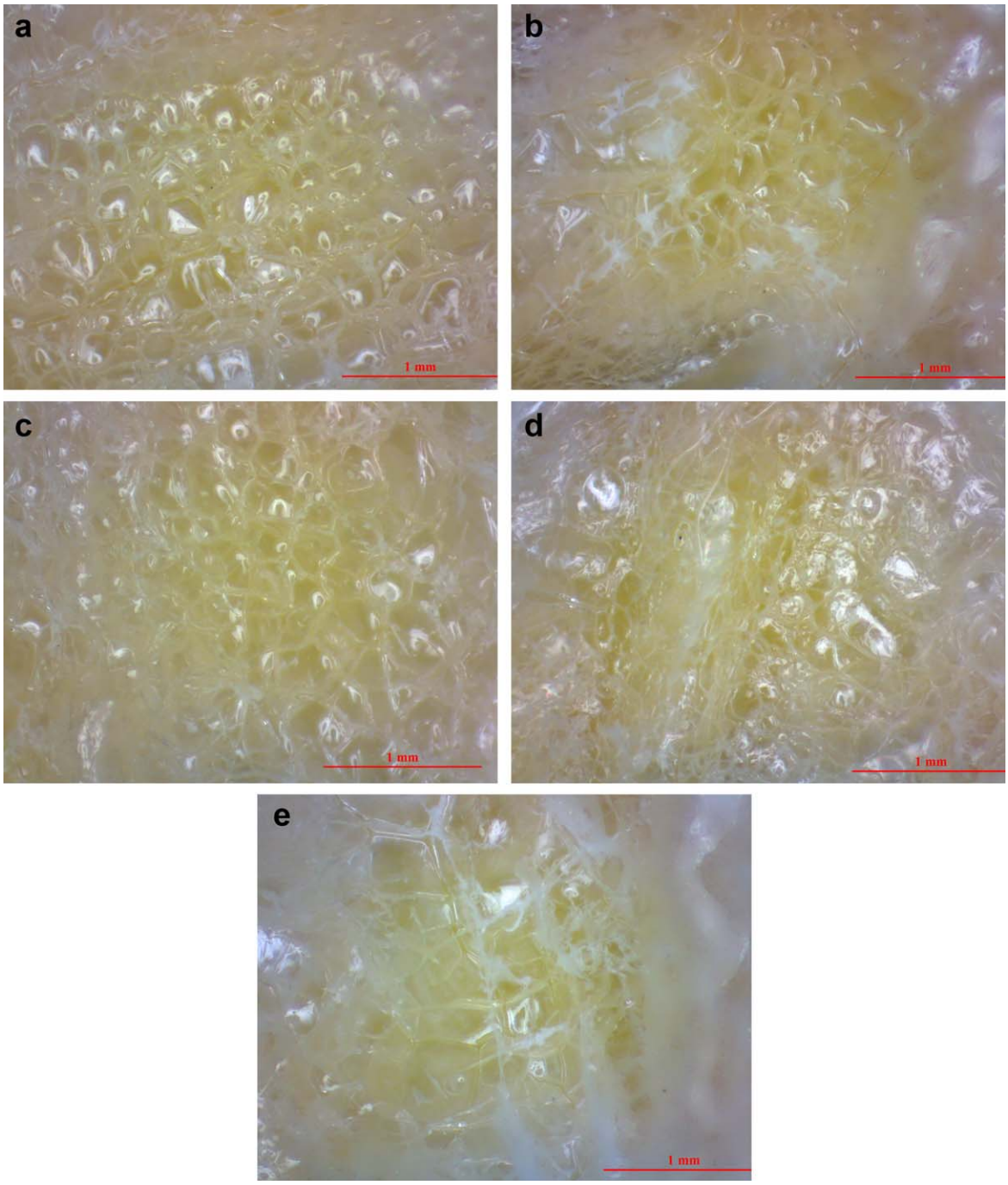

Figure 3. Images (30×) of the chitosan/gelatin porous bone scaffolds that underwent EDC crosslinking: (a) EDC5, (b) EDC10, (c) EDC15, (d) EDC20, and (e) EDC25. The scale bar represents 1 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

between GA and chitosan and/or gelatin demonstrated a better division of the linear pores.

In comparison with Figure 2, Figure 4 is more capable of showing the porous structure of GA10 and GA15. SEM instruments can photograph and provide images with a greater depth of field than a stereomicroscope. Therefore, the synthesis of stereomicroscopic images and SEM images could be used to compare the superficial pore morphology with the interconnected pore structure.

The test results show that the linear pores created bonding and then loose layers as a result of crosslinking, and such a phenomenon was correlated with the duration of crosslinking. GA5 was crosslinked for a short time (i.e., 5 min), and thus failed to create loose layers. Continuous GA crosslinking resulted in a complete porous structure, in which the pores were interconnected. However, the ongoing formation of a porous structure caused the interconnected pore structure to disappear because the polymers divided the linear pores as a result of crosslinking, after

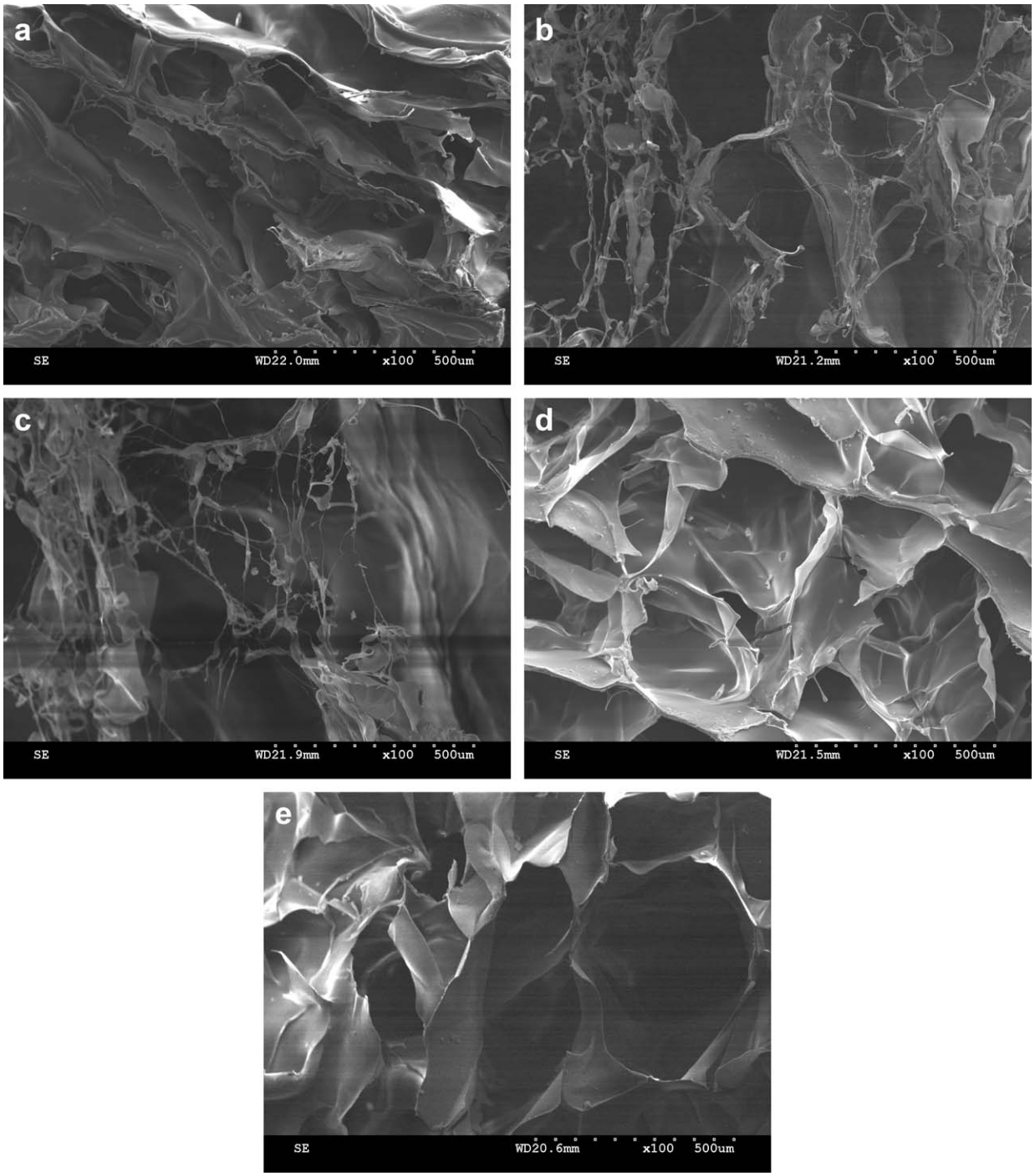

Figure 4. Images (100×) of the chitosan/gelatin porous bone scaffolds that underwent GA crosslinking for (a) 5, (b) 10, (c) 15, (d) 20, and (e) 25 min. The scale bar represents 500 μm.

which the pores again joined each other to create closed areas, as shown in Figure 4(e). The interconnected pore structure was dependent on the duration of the freezing process, in which the nucleation of crystals created the bonding between them, and the bonding could be further secured by the application of the freeze-drying process. However, this structure could not be yielded with certainty, and a crosslinking treatment was thus used for the frozen samples to create bonding between materials, and thereby, we obtained an interconnected pore structure.

Compressive Strength of Chitosan/Gelatin Porous Bone Scaffolds

The concentration and blending ratio of a chitosan/gelatin mixture have proven to be influential to the compressive strength of the resulting bone scaffolds.[30,31] He *et al.*[30] created bone scaffolds with an interconnected pore structure. Their compressive strengths were between 48 ± 4 and 264 ± 10.1 kPa when the bone scaffolds were composed of 0.5–3 wt % chitosan and 1.7–10 wt % gelatin at a 1:1 volume ratio and crosslinked with a

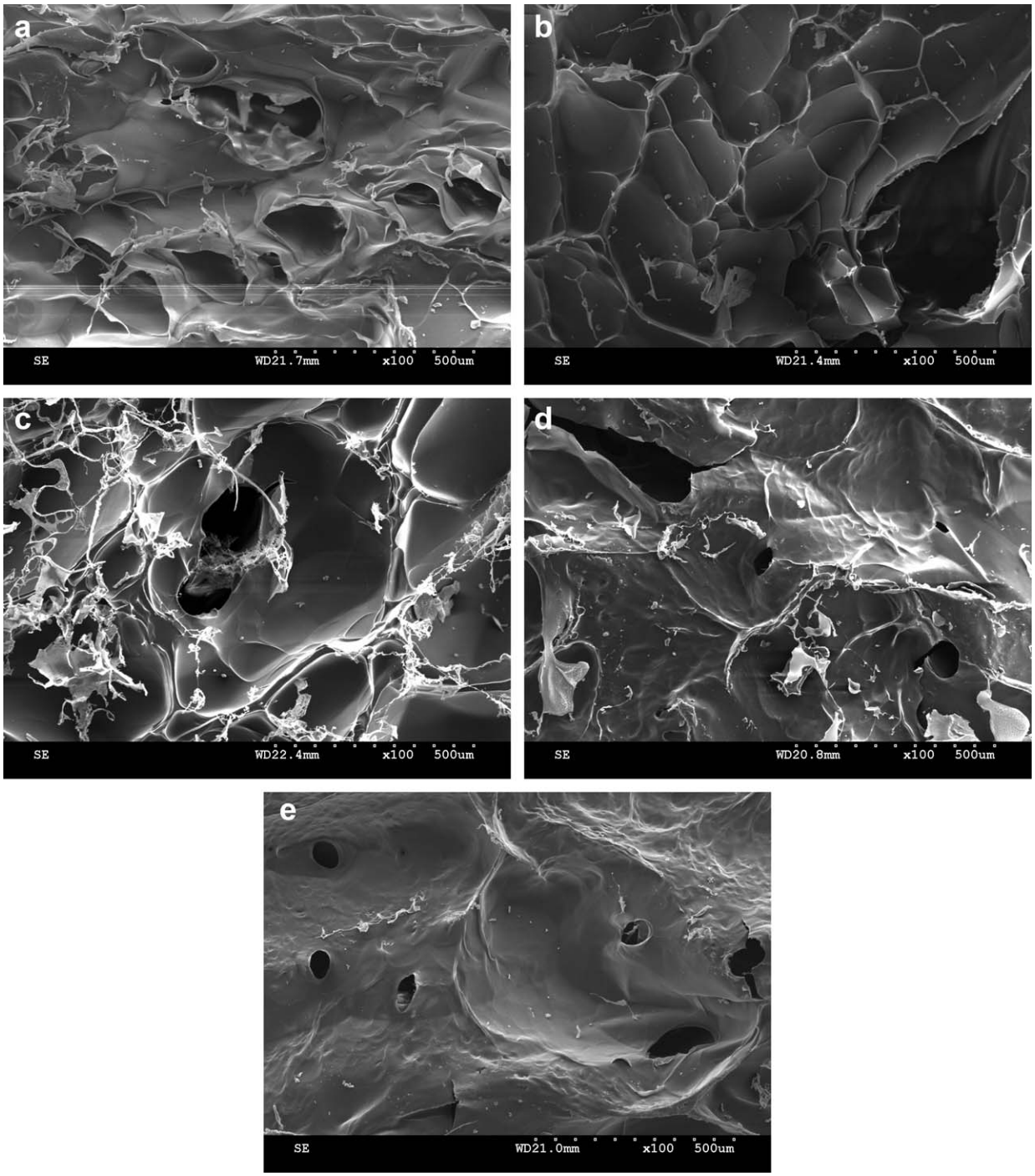

Figure 5. Images (×100) of chitosan/gelatin porous bone scaffolds that underwent EDC crosslinking for (a) 5, (b) 10, (c) 15, (d) 20, and (e) 25 min. The scale bar represents 500 μm.

0.25% GA solution.[30] Wu *et al.*[6] proposed bone scaffolds with a linear pore structure by blending 3% w/v gelatin solution and a 5% v/v GA solution. Their compressive strength along the longitudinal direction was 102 kPa, and that along the transverse direction was 25 kPa. In this study, the compressive strengths were between 92.38 ± 7.47 and 134.09 ± 8.49 kPa (Figure 6); this was ascribed to the concentration of the polymers. An increasing solute ratio (i.e., polymers) fortified the unit area of the bone scaffolds so that it could bear a greater force after freeze drying. In comparison with an interconnected pore structure, the linear pore structure provided the resulting bone scaffolds with a compressive strength that was dependent on the direction of the compressive force.

The test results also show that with a specified content of solute, a specified concentration of crosslinking agent, and a specified blending ratio of chitosan and gelatin, an increase in the crosslinking times fortified the compressive strength of the bone

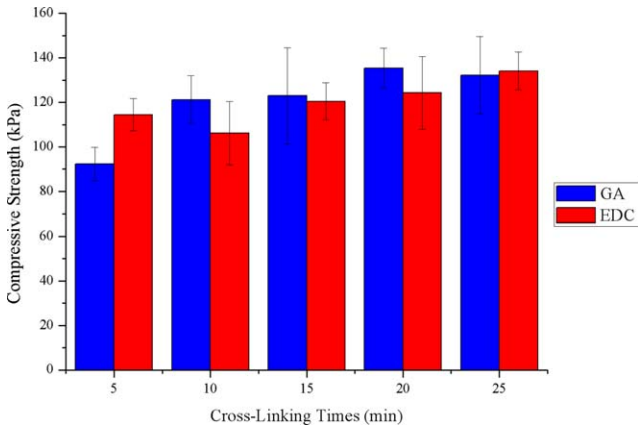

Figure 6. Compressive strength of chitosan/gelatin porous composite bone scaffolds with respect to various crosslinking times with GA and EDC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

scaffolds. The crosslinking time was correlated with the linking of the polymers in the ice crystals and the division of the linear pores. The solute constructed a complete porous structure and decreased the number of pores with the crosslinking time, which in turn affected the compressive strength. Nevertheless, the porosity was not influenced by the crosslinking time and remained between 83 ± 7.31 and $92.45 \pm 1.82\%$. This was due to a high volume of solvent in the samples and freeze-drying sublimating the ice crystals and leaving the pores. In addition, regardless of the crosslinking that caused the division of the linear pores, the porosity of the bone scaffolds did not change distinctly. Such results conformed to the fact that the crosslinking duration only influenced the forms of the pores and, thus, increased the compressive strength relatively.

Biocompatibility and MTT Results

Chitosan and gelatin both have good biocompatibility.[19–22] Figure 7 shows that the cell counts per unit area of a 57×57 μm^2 area after 24-h of culturing for the control group, the GA series, and the EDC series were 23–39, 47–65, and 30–51, respectively, whereas the cell counts per unit area after 48 h of culturing for the control group, the GA group, and the EDC group were 49–52, 55–80, and 48–75, respectively. Such results indicate that the residual amount of the crosslinking agents, (i.e., GA and EDC) did not affect the biocompatibility of the experimental groups. Figure 7 shows that the MG-63 osteoblast cells appeared to be a long, narrow spindle apparatus; this showed that the cells grew and attached well.[8,9] Compared with the control group, the GA series and EDC series also exemplified the same spindle apparatus, the results of which confirm that the crosslinking times of GA or that EDC did not influence the cell growth of the chitosan/gelatin porous bone scaffolds.

Figure 8 shows that after 24 h of coculturing, the cell viability of the GA series bone scaffolds was between 96 and 111% and that of the EDC series bone scaffolds was between 84 and 96%. The EDC series had a cell viability beneath 100%, which was ascribed to the residual crosslinking agent in the samples, as demonstrated by its more drastic influence on the viability of MG-63 osteoblast cells than GA did. However, with a 48-h coculture, regardless of GA crosslinking or EDC crosslinking, the viabilities of the samples all reached 100%. Namely, when the experimental group had a cell viability that exceeded 100%, it likewise meant that the experimental group had a greater amount of cells than the control group and also indicated that experimental group was conducive to cell proliferation. In other words, a 48-h coculture had little influence on cell proliferation. A similar result was found in a previous study, in which the cell viability was also proportional to the culture durations, but the cell viability was beneath 100% for both

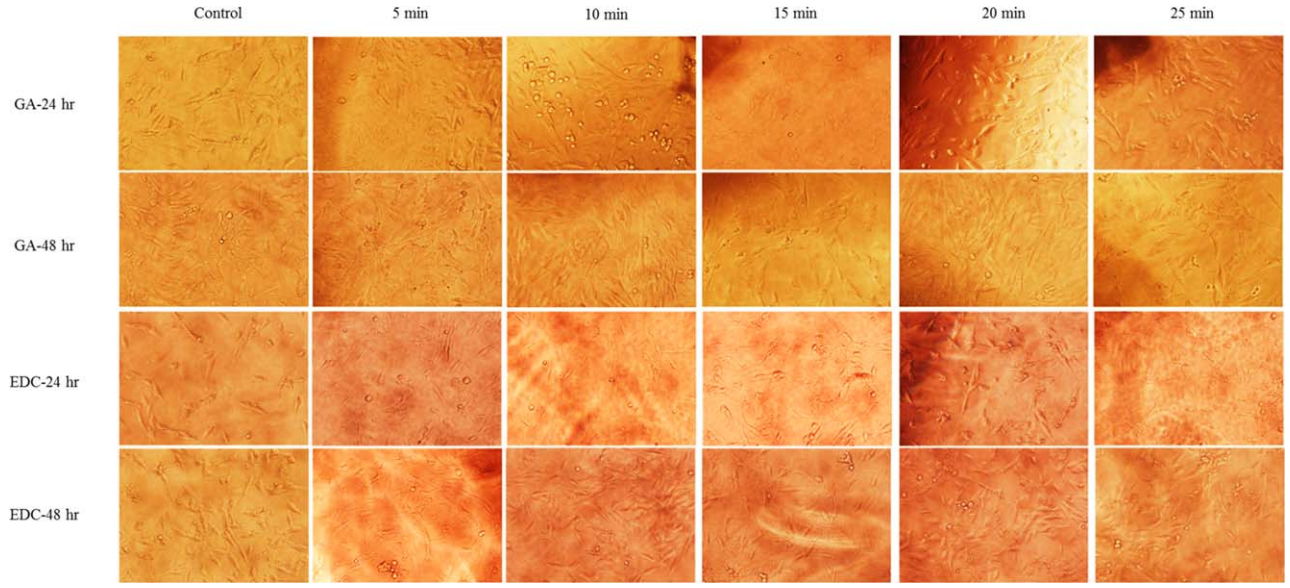

Figure 7. Biocompatibility of the chitosan/gelatin porous bone scaffolds with respect to various crosslinking agents and durations. The optical microscopic images have a magnification of 300×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

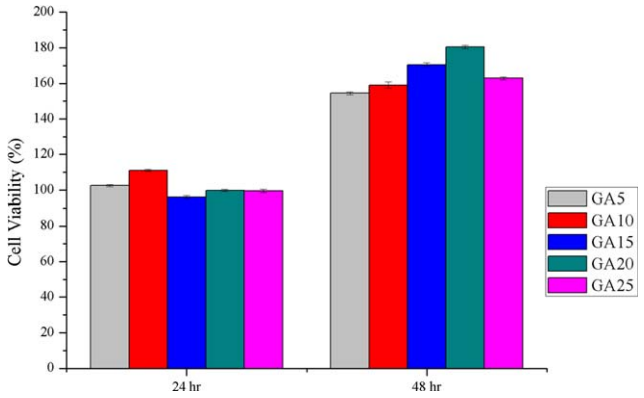

Figure 8. MTT assay of the chitosan/gelatin porous bone scaffolds that underwent (a) GA and (b) EDC crosslinking. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

culture durations.[9] The possible explanation for such a result is the unreacted aldehyde groups of GA, the exposure of which to the cells resulted in cell death. Meanwhile, it also proved that the untreated groups of crosslinking agents used in this study did not last longer than 48 h. In addition, GA20 possessed a good interconnected structure. However, given the cell viability data that was obtained from a 24-h culture, the cell viability of GA20 did not pertain to its interconnected structure.

CONCLUSIONS

In this study, we used GA and EDC to crosslink with solute (polymers) between ice crystals; this divided the linear pores and, thereby, successfully produced chitosan/gelatin porous bone scaffolds. A freeze-drying technique was used to effectively prepare bone scaffolds with linear pores and a porous structure. However, the yield of a porous, interconnected pore structure was random. The presence of linear pores was inevitable, and thus, the crosslinking of the frozen samples was used to divide the large size of pores by turning them into a porous, interconnected-pore structure. This was optimally exemplified by GA20. The division of linear pores did not influence the porosity of the chitosan/gelatin porous bone scaffolds. The compressive strength exclusively depended on the crosslinking times, which were 43% greater for GA25 than for GA5 and 17.1% greater for EDC25 than for EDC5. The porosity was influenced by the volume of the solvent, which was beyond 83% for all of the samples. Even though the crosslinking agents were used and the crosslinking times were varied, after a 48-h coculture, the viability of the GA series was improved by 77.239% and that of the EDC series was improved by 59.996%. The porous bone scaffolds made in this study possessed compressive strength to maintain their three-dimensional, porous structure, which could be used for a small segment of an impaired bone.

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