Preparation and Cell Compatibility Evaluation of Chitosan/Collagen Composite Scaffolds Using Amino Acids as Crosslinking Bridges

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ABSTRACT: In this study, a novel freeze-gelation method instead of the conventional freeze-drying method was used to fabricate porous chitosan/collagen-based composite scaffolds for skin-related tissue engineering applications. To improve the performance of chitosan/collagen composite scaffolds, we added 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and amino acids (including alanine, glycine, and glutamic acid) in the fabrication procedure of the composite scaffolds, in which amino acid molecules act as crosslinking bridges to enhance the EDC-mediated crosslinking. This novel combination enhanced the tensile strength of the scaffolds from 0.70 N/g for uncrosslinked scaffolds to 2.2 N/g for crosslinked ones; the crosslinked scaffolds also exhibited slower degradation rates. The hydrophilicity of the scaffolds was also significantly enhanced by the addition of amino acids to the scaffolds. Cell compatibility was demonstrated by the *in vitro* culture of human skin fibroblasts on the scaffolds. The fibroblasts attached and proliferated well on the chitosan/collagen composite scaffolds, especially the one with glutamic acid molecules as crosslinking bridges, whereas cells did not grow on the chitosan scaffolds. Our results suggest that the collagen-modified chitosan scaffolds with glutamic acid molecules as crosslinking bridges are very promising biomaterials for skin-related tissue engineering applications because of their enhanced tensile strength and improved cell compatibility with skin fibroblasts. J Appl Polym Sci 105: 1774–1785, 2007

Key words: amino acid; biocompatibility; biomaterials; chitosan; crosslinking

INTRODUCTION

Skin substitutes such as xenografts, allografts, and autografts have been employed for therapeutic treatments of severe burns and nonhealing wounds. Tissue engineering applies both life science and engineering principles to develop functional substitutes for damaged tissues.[1,2] Engineered skin products have the potential to assist wound healing and reduce scarring after skin cancer surgery. To find suitable biomaterials for artificial skin development, a combination of chitosan, collagen, and amino acids was employed to prepare composite scaffolds in this study.

Chitosan-based biomaterials are receiving increased attention in tissue engineering-related applications. Chitosan is a polysaccharide derived from the N-deacetylation of chitin. The molecular structure of chitosan is a copolymer comprised of glucosamine and *N*-acetylglucosamine. The term, degree of deacetylation, represents the molar ratio of glucosamine units to all repeating units on the chitosan molecule.[3] Because of the presence of amino groups, the chitosan polymer is positively charged and solubilized by protonation at environmental pH values of <6. Therefore, the solubility of chitosan is influenced by its degree of deacetylation. Usually a 1–3% acetic acid solution is used as a solvent for chitosan. The presence of amino groups, due to their chemical reactivity, also provides the possibility for the chemical modification of chitosan. Chitosan can be enzymatically degraded by chitinase, chitosanase, and pectinase. It can also be degraded by lysozyme *in vivo*. Its mechanical properties can be improved by crosslinking.[4] The degradation rate of chitosan is relatively slow, but this can be modified by the degree of deacetylation. A higher degree of deacetylation produces a lower rate of degradation.[5,6] Because of its antiseptic, biocompatible, and degradable properties, chitosan has been widely used in various biomedical applications,[7,8] including wound dressings,[9] drug delivery,[10] and tissue engineering.[11–14]

Collagen scaffolds have also been widely used as a dermal equivalent to induce fibroblast infiltration and

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dermal regeneration.[9] Collagen is a major component of the human extracellular matrix and connective tissues such as dermis, bone, cartilage, tendons, ligaments, and basement membrane. Because of its excellent biocompatibility, low antigenicity, and high availability, collagen is widely used as a biomaterial. Various forms of collagen materials, such as films, sponges, gels, have been developed for medical applications. However, collagen scaffolds generally degrade quickly *in vivo*, so some treatments, such as glutaraldehyde crosslinking,[15] thermal dehydration,[16] and UV radiation,[17] are used to increase the mechanical strength of collagen by introducing intra- and intermolecular linkages as well as to decrease its degradation rate. Even though collagen is a major component of skin, it is relatively expensive.

Scaffolds composed of collagen and chitosan may create an appropriate environment for the regeneration of skin. Our previous studies have indicated that skin fibroblasts are not very compatible with chitosan.[18,19] The addition of collagen should facilitate the attachment and proliferation of skin fibroblasts. Chitosan/collagen composite scaffolds have been fabricated by many researchers.[20–24] Nevertheless, some studies did not use crosslinking agents, and thus the scaffolds, especially collagen ones, quickly degraded. A few studies used glutaraldehyde as a crosslinking reagent, and some studies mentioned that glutaraldehyde did not have harmful effect to cells,[25,26] but some studies reported that glutaraldehyde was toxic even at a concentration of 3.0 ppm.[17,27] To improve the cell compatibility of chitosan and the biological stability of collagen, this study utilized the crosslinking reagent, 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide/*N*-hydroxyl-succinimide (EDC/NHS),[28,29] and amino acids including alanine, glycine, and glutamic acid as crosslinking bridges to fabricate novel composite scaffolds (Fig. 1). EDC is a zero-length crosslinking agent used to conjugate carboxyl to amino groups. NHS can improve the efficiency of EDC coupling reactions. Alanine has a nonpolar, hydrophobic side chain [Fig. 1(a)]. Glycine is the simplest amino acid and is nonpolar [Fig. 1(b)]. The number ratio of carboxyl to amino groups in alanine or glycine is 1 ($COOH/NH_2 = 1:1$). Alanine differs from glycine in that a hydrophobic methyl group rather than hydrogen atoms is attached to its α-carbon. Glutamic acid has an acidic carboxyl group on its side chain [Fig. 1(c)]. The number ratio of carboxyl to amino groups in glutamic acid is 2 ($COOH/NH_2 = 2:1$).[30] Amino acids with positively charged side chains like lysine were not used in this study because chitosan provides a large amount of amino groups and also many positive charges. Thus for increasing the possibility of crosslinking in the EDC/NHS system, we should increase the amount of carboxyl groups, and that led to the use of alanine, glycine, and especially glutamic acid in this study. Our previous research utilized polyglutamic acid to modify chitosan scaffolds, and the results were promising.[31]

In the present study, we prepared composite scaffolds containing chitosan as a framework and collagen as a cell-recognizing component plus amino acids as crosslinking bridges, and the proposed crosslinking scheme is shown in Figure 1. Additionally, a novel freeze-gelation method[32] instead of the conventional freeze-drying method was used to fabricate porous composite scaffolds. This method saves time and energy, and is suitable for fabricating large-sized scaffolds.[32] The structure, mechanical strength and elongation, hydrophilicity of the scaffolds, and the ability of fibroblasts to attach to and proliferate on these composite scaffolds were examined.

EXPERIMENTAL

Materials

Chitosan (with a degree of deacetylation of 90% and a molecular weight of ~ 300,000) was purchased from Kiotek (Taipei, Taiwan). EDC, NHS, phosphate-buffered saline (PBS), alanine, glycine, glutamic acid, sodium dodecyl sulfate (SDS), Tergitol (type NP-40), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO). Collagen was purified from fresh pig skin by pepsin digestion and an acetic acid dissolution method.[33] The purity of the type I collagen was confirmed by SDS-PAGE analysis with Coomassie brilliant blue staining. Distilled and deionized water was used throughout this study.

Minimum essential medium (MEM) was purchased from Sigma-Aldrich, and fetal bovine serum (FBS) and penicillin–streptomycin (10,000 U/mL) from Biological Industries (Kibbute, Israel). Trypsin-EDTA was purchased from Biochrom AG (Berlin, Germany), and sodium pyruvate was purchased from GIBCO Invitrogen (Grand Island, NY). Tissue culture flasks and 12- and 24-well plates were obtained from Corning (Big Flats, NY). Cells used for the cell compatibility study were WS1 human embryonic skin fibroblasts (ATCC CRL-1502; American Type Culture Collection, Manassas, VA).

Preparation of scaffolds

Porous scaffolds were prepared by the freeze-gelation method.[32] Briefly, chitosan was dissolved in a 0.2*M* acetic acid aqueous solution to form a 3.5 wt % chitosan polymer solution. Afterwards, EDC, NHS, amino acid, and collagen acetic acid solutions were added to form a viscous polymer solution, which was continually stirred at 4°C for 6 h. The polymer solution was centrifuged for 15 min at 3000 × *g* to remove the

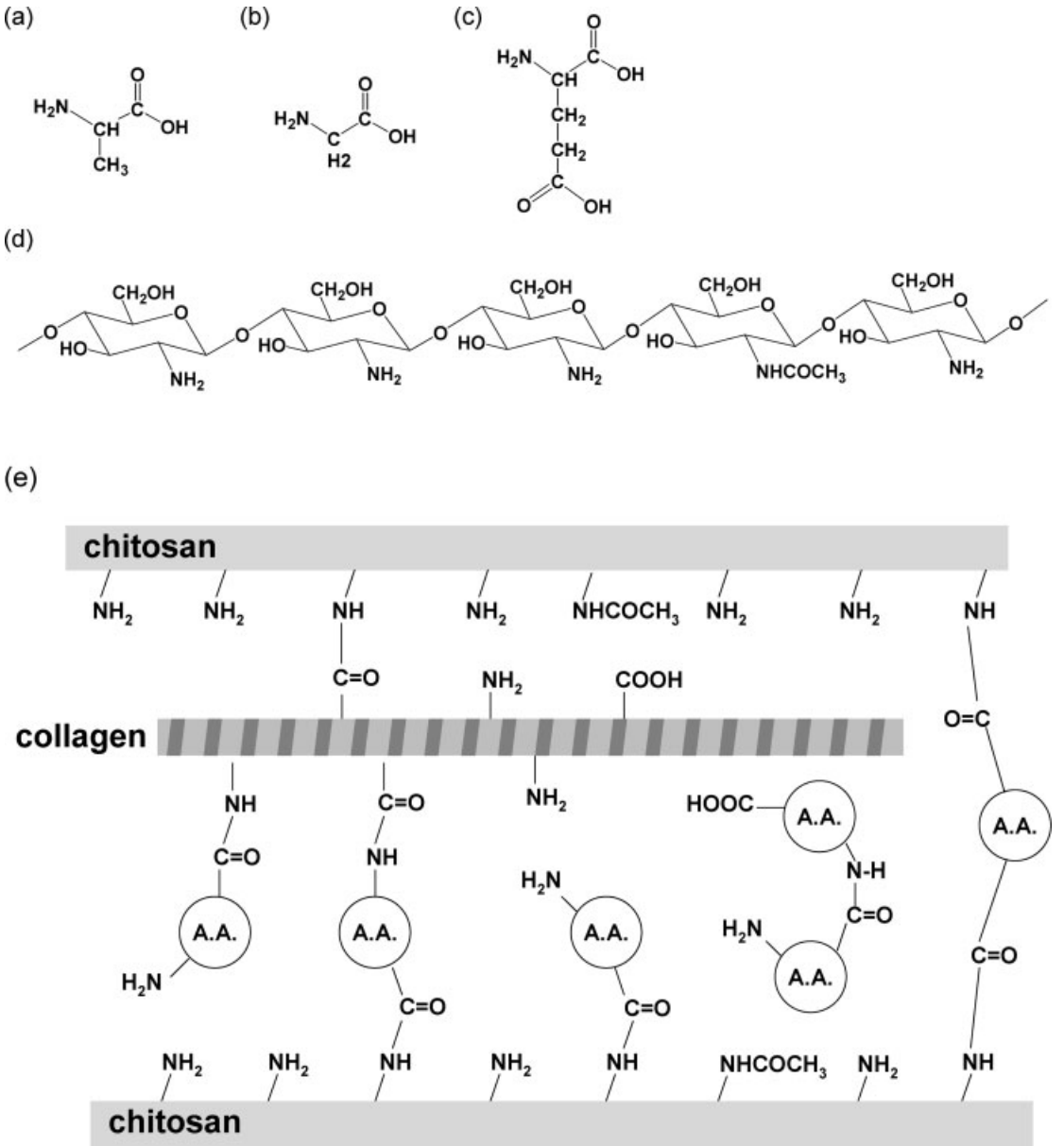

Figure 1 Structural formula of amino acids utilized as crosslinking bridges. (a) Alanine; (b) glycine; (c) glutamic acid; (d) structure of chitosan; (e) proposed crosslinking diagram of chitosan, collagen, and amino acids. A.A.: amino acid.

insoluble impurities, and then the polymer solution was poured into a square stainless steel plate (150 × 150 mm^2) with a specially made mold and frozen at −80°C for 6 h. The frozen chitosan solution was immersed in a precooled NaOH aqueous solution for 24 h, and gelation occurred below the freezing point of the polymer solution. Subsequently the scaffolds were washed by 95% ethanol and PBS buffer. Finally the scaffolds were kept in a moist condition at 4°C until further experiments were carried out.

To observe the fluorescence staining of cells, nonporous films were made from the same polymer solution. Each solution was prepared in a 0.2*M* acetic acid solution, continually stirred for 12 h, and then centrifuged for 15 min at 3000 × *g*. The film solution was then poured into dishes, and dried in an oven for 24 h with the temperature maintained at 40°C. The dehydrated films were then immersed in a 3*M* NaOH solution for 12 h, followed by rinsing with ethanol and a PBS solution. At the end, the films were washed with distilled water six times and then stored at 4°C for further cell compatibility assays. For comparison between the freeze-gelation method and lyophilization, we also made lyophilized samples. Briefly, the polymer solution was poured into the same square stainless steel plate (described in the preceding paragraph) and then frozen at −80°C for 6 h. Finally, the frozen solutions were lyophilized for 72 h in a freeze-

TABLE I
Compositions and Abbreviations of the Five Types of Scaffold Solutions

Scaffold solution	Chitosan (wt %)	Collagen (wt %)	Alanine (m*M*)	Glycine (m*M*)	Glutamic acid (m*M*)	Total (m*M*)
chi	3.5 (190, 0)[a]	–	–	–	–	(190, 0)
chi-col-non	3.5 (190, 0)	0.0188 (1.8, 1.8)	–	–	–	(191.8, 1.8)
chi-col-ala	3.5 (190, 0)	0.0188 (1.8, 1.8)	150 (150, 150)	–	–	(341.8, 151.8)
chi-col-gly	3.5 (190, 0)	0.0188 (1.8, 1.8)	–	150 (150, 150)	–	(341.8, 151.8)
chi-col-glu	3.5 (190, 0)	0.0188 (1.8, 1.8)	–	–	150 (150, 300)	(341.8, 301.8)

The concentrations of acetic acid, EDC, and NHS were 0.2*M*, 21.5 m*M*, and 10.75 m*M*, respectively, in all scaffold solutions. According to the average molecular weight of the chitosan repeating units being 165.2, the amino group concentration of chitosan in the scaffold solution was about 190 m*M*. The amino and carboxyl group concentrations of collagen in the scaffold solution were about 1.8 m*M*.

[a] Values within parentheses are the concentrations of amino (NH_2) and carboxyl (COOH) groups, respectively.

drier (Heto LyoLab 3000, Allerød, Denmark) to obtain lyophilized samples.

We divided the scaffold solutions into five groups according to their compositions. Three different amino acids [alanine (Ala), glycine (Gly), and glutamic acid (Glu)] were used in this study. The weight percentage of chitosan in the scaffold solution was 3.5%. Chitosan is comprised of two kinds of repeating units, glucosamine (M_w: 161) and *N*-acetyl-glucosamine (M_w: 203). The degree of deacetylation of chitosan we used was 90%, so the average molecular weight of the repeating units was 165.2. By using the value of the molecular weight, the number of amino groups in the chitosan solution could be calculated. The amount of amino acid (150 m*M*) used was 70% of the amount of the amino groups on the chitosan in the solution. The compositions of all five types of scaffold solutions are summarized in Table I.

Thermal properties analysis by differential scanning calorimetry

Differential scanning calorimetry (DSC) was employed to investigate the endothermic peak temperature shift among the scaffolds. Scaffolds were cut into small pieces (about 2 mm^3), and then four to six small pieces, weighing about 5 mg, were placed in an aluminum pan. The aluminum pan was pressed to seal it, and thus the porous scaffolds were crushed to ensure complete contact with the aluminum pan. Analysis was carried out in a differential scanning calorimeter (DSC2010; TA Instruments, New Castle, DE) from 50 to 250°C at a controlled heating rate of 5°C/min. Three samples from each composition were measured.

Analysis of the scaffold structure by scanning electron microscopy

The surface and cross-section morphologies of the porous scaffolds were examined by scanning electron microscopy (SEM; Hitachi JSM-6300, Tokyo, Japan). Samples were placed on a Cu mount and coated using a gold-coating apparatus. *In vitro* cell samples were fixed with 2.5% glutaraldehyde in a 0.1*M* PBS solution. Samples were dehydrated through a graded series of ethanol, supercritical carbon dioxide-dried, and then gold-coated.

Determination of water uptake

Hydrophilicity is an important characteristic property of biomaterials. To determine the hydrophilicity of the porous scaffolds, the bulk water absorption of the scaffolds was determined to reveal their hydrophilic behavior. To determine the water uptake,[34] scaffolds were immersed in PBS at 37°C to obtain the change in water uptake with respect to time. Six samples were measured for each type of scaffold. The percentage of water uptake was calculated using the following equation:

$$\text{Percentage of water uptake} = (W_{\text{wet}} - W_{\text{dry}})/W_{\text{dry}} \times 100\%,$$

where W_{dry} and W_{wet} are the weights of the scaffolds before and after immersion in PBS, respectively.

Analysis of scaffold tensile strength and elongation

The tensile strength and elongation to fracture of the porous scaffolds were examined using a universal testing machine (model LRX; LLOYD, Paoli, PA) at a constant speed of 10 mm/min with a preload of 0.5 N. Samples were cut into a typical dog-bone shape similar to the American Society for Testing Material (ASTM) D 4762-04 standard, and then each sample weight was measured. The measured maximum tensile strength (load to fracture) was normalized by weight for each scaffold. Before measuring the tensile strength and elongation, all test specimens were immersed in PBS for 30 min at room temperature. At least eight samples were measured for each composition of scaffold.

In vitro enzymatic degradation studies

In vitro biodegradation test of the scaffolds with and without the addition of amino acids as crosslinking bridges was performed by lysozyme digestion. The scaffolds were immersed in PBS (pH 7.4) containing 0.5 mg/mL lysozyme (L6876; Sigma-Aldrich) at 37°C on an orbital shaker with speed of 100 rpm for 1, 2, 3, and 4 weeks. At the end of each period, the scaffolds were dried at 50°C for 12 h, and then the weights were measured. Five samples were measured for each type of scaffold. The remaining weight for the scaffolds is defined by the following equation:

$$\text{Remaining weight } (\%) = W/W_i \times 100\%,$$

where W_i is the initial dry weight and W is the dry weight after enzymatic degradation.

Determination of cell compatibility

WS1 human embryonic skin fibroblasts were cultured in the MEM supplemented with 10% FBS at 37°C in a 5% CO_2 incubator. Scaffolds were sterilized with a 70% ethanol solution and ultraviolet light prior to cell culture. WS1 skin fibroblasts were seeded onto 12-well plates at a concentration of 1×10^4 cells/well. Cell morphology and attachment were monitored using an image analysis system connected to an Olympus (Tokyo, Japan) IX70 inverted phase-contrast microscope. Images were captured with a high-resolution digital camera, and processed using image analysis software. For fluorescence microscopy, the culture medium was removed and then PBS supplemented with a fluorescein diacetate (FDA) and propidium iodide (PI) working solution was added and incubated for 10 min at 37°C in the dark. FDA is a nonpolar and nonfluorescent compound that is able to enter cells and is hydrolyzed to fluorescein and acetate by nonspecific esterases in the cytoplasm.[35] Fluorescein is retained by the cell if the plasma membrane is intact. Fluorescein can be monitored by excitation at 495 nm and emission at 535 nm (green fluorescence). PI intercalates into double-stranded nucleic acids and can be monitored by excitation at 530 nm and emission at 610 nm (red fluorescence).[36] PI cannot pass through intact cell membranes and is used to stain nucleic acids in dead or dying cells with injured membranes. The fluorescence images were obtained using a digital camera mounted on an Olympus inverted microscope with fluorescence accessories.

To determine the cell numbers on various surfaces, a standard curve of cell number versus the respective protein concentration was first established. Then it was possible to indirectly determine the cell number by measuring the protein concentration of a cell lysate. To carry out the measurement, the medium was removed, and then cultured cells were washed with PBS three times. Lysis buffer was introduced to the cells with a reaction time of 10 min. Lysis buffer contained 0.25 wt % sodium deoxycholate, 1 wt % NP-40, 0.2 wt % SDS, and 4 m*M* PMSF. The resulting cell lysate was recovered for the protein concentration assay using the Pierce BCA protein assay kit (Rockford, IL). At least three samples were measured for each group.

Statistical analysis

Significant differences in the mechanical properties and cell compatibility were determined utilizing a one-way analysis of variance (ANOVA test) and Newman–Keuls *post hoc* comparisons. The independent variable was the composition. Significant differences in the water uptake and *in vitro* enzymatic degradation were determined utilizing a two-way ANOVA test and Newman–Keuls *post hoc* comparisons. The independent variables were composition and time. Differences were considered to be statistically significant at $P < 0.05$. All statistical analyses were undertaken using the SPSS statistical software (Chicago, IL).

RESULTS AND DISCUSSION

Thermal properties of chitosan/collagen composite scaffolds

The thermal properties of the scaffolds were analyzed by DSC as shown in Figure 2. There was an endothermic peak around 95°C for the chitosan/collagen (chi-col-non) scaffold, 120°C for the chi-col-ala scaffold, 122°C for the chi-col-glu scaffold, and 128°C the for

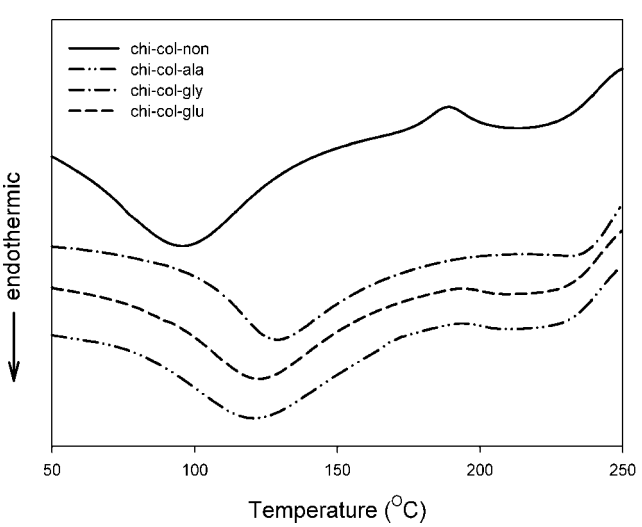

Figure 2 Differential scanning calorimetric (DSC) curves from chitosan/collagen composite scaffolds with or without the addition of different amino acids as crosslinking bridges. See Table I for the composition of each scaffold. The heating rate was 5°C/min.

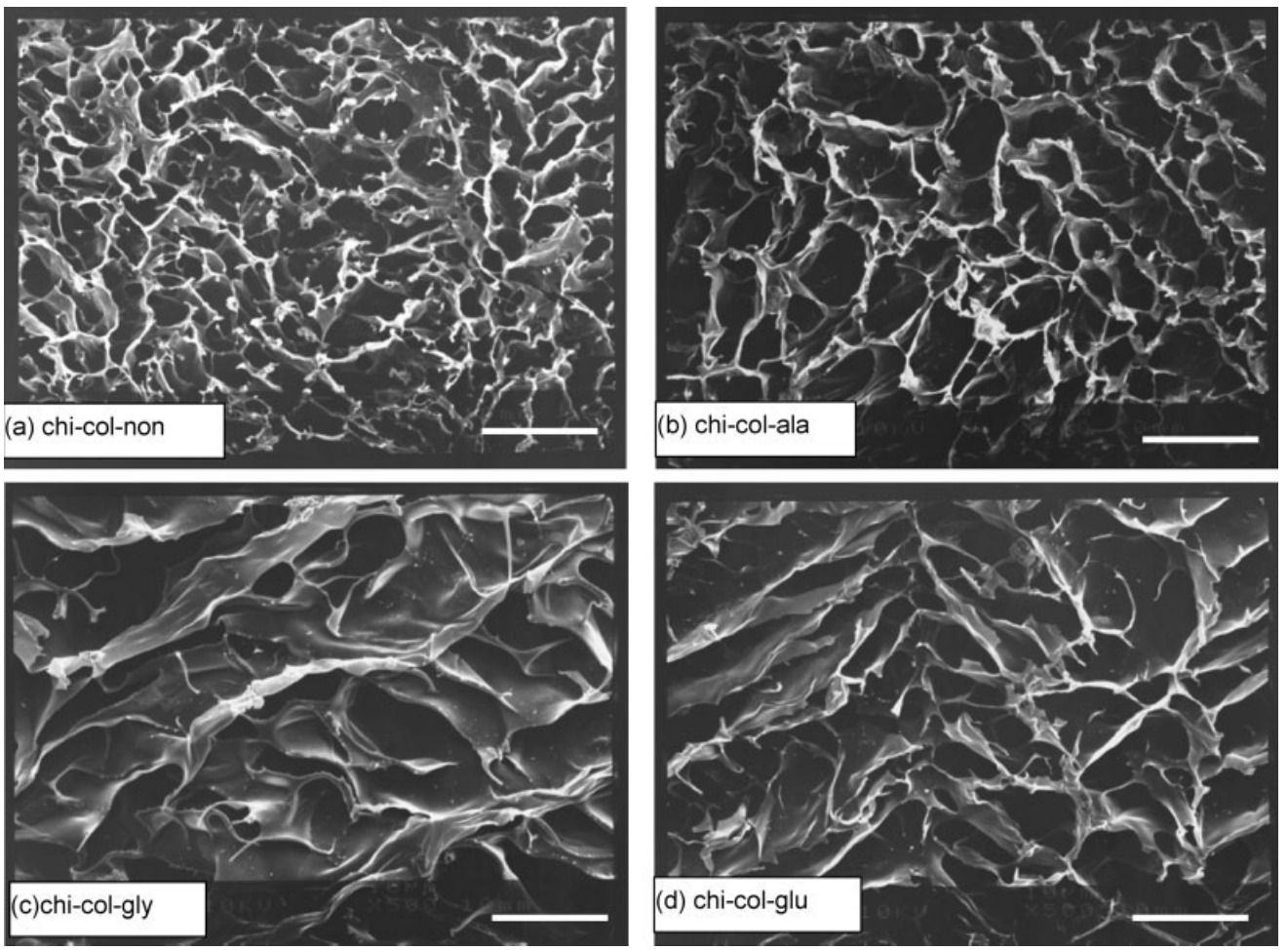

Figure 3 Scanning electron microscopic (SEM) images (magnification, 500×) showing the surface morphology of chitosan/collagen composite scaffolds with or without the addition of amino acids. (a) chi-col-non scaffold; (b) chi-col-ala scaffold; (c) chi-col-gly scaffold; (d) chi-col-glu scaffold. Scale bar = 50 μm.

chi-col-gly scaffold. The scaffolds with amino acids as crosslinking bridges showed higher peak temperatures than did the scaffold without amino acid addition. Each composite scaffold had only one obvious endothermic peak, indicating that there was no noticeable phase separation in the composite scaffolds. Shanmugasundaram et al.[37] reported that chitosan/collagen has a transition band between 63 and 168°C. The endothermic peak could result from loss of bound water retained within the scaffold.[38] Zhang et al. reported that the peak temperature for the chi-col-non composite material shifts to a higher temperature because of the rigidity of the collagen and chitosan molecular chains,[39] and our results demonstrate that the addition of amino acids to the chi-col-non scaffolds caused the peak temperature to shift to a higher temperature. Our data thus suggest that covalent bonds are formed among the chitosan, collagen, and amino acids. The presence of covalent bonds could change the microstructure and porosity of the scaffold, and that may affect the amount of bound water retained within the scaffold.

SEM analysis of porous scaffolds

The surface morphology and interior cross-sectional structure of the chitosan/collagen composite scaffold were examined by SEM. The surface pore structures of the scaffolds are shown in Figure 3. All scaffold surfaces were porous, demonstrating that the use of the freeze-gelation method could construct double-sided porous scaffolds. In contrast, a dense layer at the surface is produced by the traditional freeze-drying procedure.[40]

Figure 4 displays the cross-sectional structures of the chitosan/collagen composite scaffolds. The entire scaffold shows an interconnected porous structure that is confirmed by the surface and cross-sectional SEM images (Figs. 3 and 4). The pores are continuous and uniformly distributed. These results demonstrate that the use of the freeze-gelation method resulted in a uniformly distributed pore structure even with the addition of amino acids. We also observed that using different amino acids as crosslinking bridges produced pores with different sizes in the composite

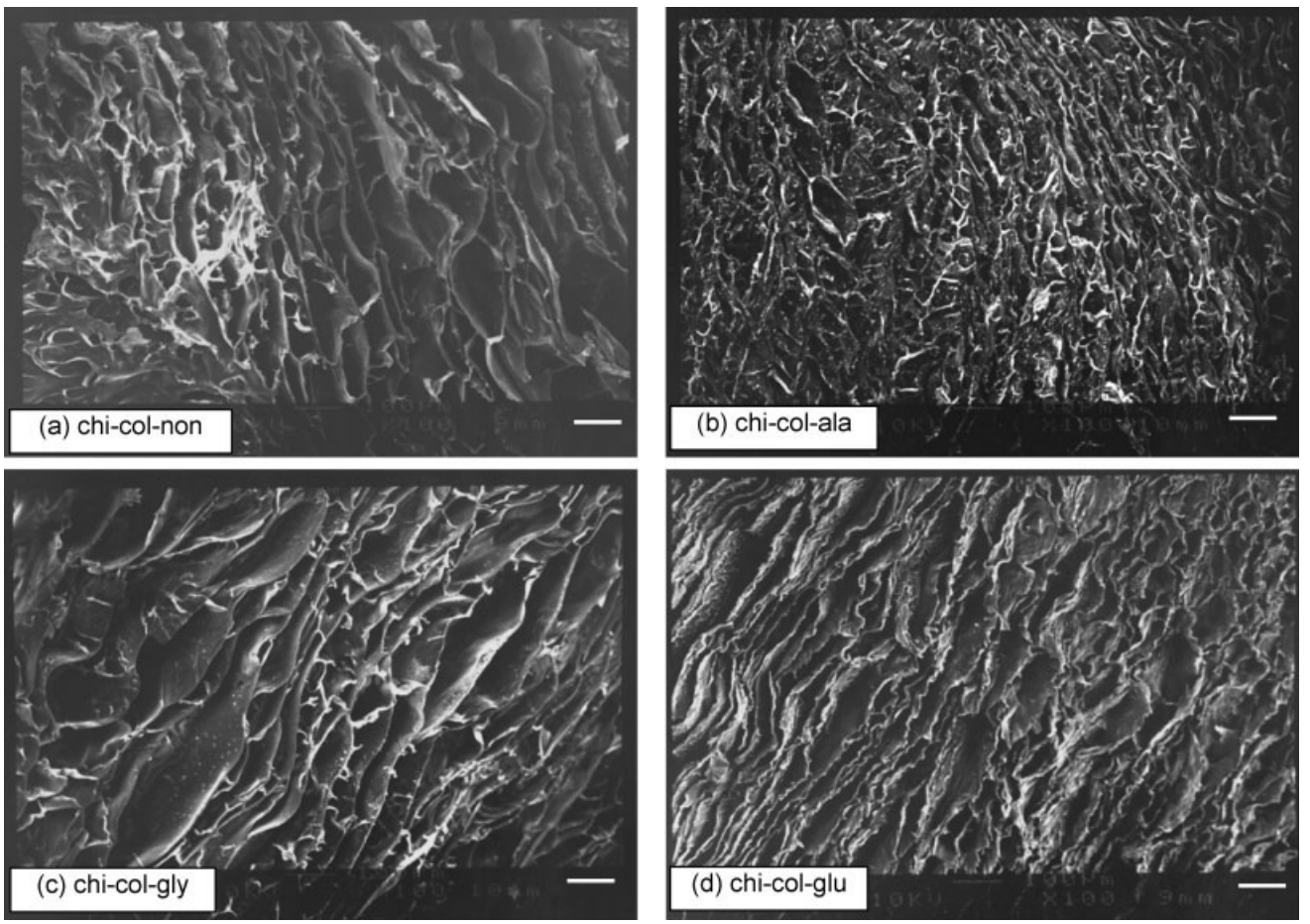

Figure 4 Scanning electron microscopic (SEM) images (magnification, 100×) showing the cross-sectional morphology of chitosan/collagen composite scaffolds with or without the addition of amino acids. (a) chi-col-non scaffold; (b) chi-col-ala scaffold; (c) chi-col-gly scaffold; (d) chi-col-glu scaffold. Scale bar = 100 μm.

scaffold. From Figure 4, we observed that the chi-col-non and chi-col-gly scaffolds had comparatively larger pores than did the chi-col-ala and chi-col-glu scaffolds, but most pores were in the range of 50–250 μm. The pore size was determined by direct measurement of 10 randomly chosen areas on the SEM images. Scaffolds used for skin tissue engineering should have pore sizes of from 100 to 200 μm.[41] Thus, our chitosan/collagen composite scaffolds with amino acids as crosslinking bridges had appropriate pore sizes for skin tissue engineering applications.

Water uptake of the scaffolds

Figure 5 shows the water uptake percentages of the chitosan and chitosan/collagen composite scaffolds with or without the addition of amino acids. The water uptake was comparatively low for the chi and chi-col-non scaffolds and significantly increased for the chi-col-gly, chi-col-ala, and chi-col-glu scaffolds. The final water uptake reached 371% for the chi-col-ala, 473% for the chi-col-gly, and 337% for the chi-col-glu scaffolds; however, water uptake was only 135% for the chi-col-non and 108% for the chi scaffolds. The *post hoc* Newman–Keuls test showed that the composition (independent variable) had four significantly different groups: chi and chi-col-non, chi-col-ala, chi-col-gly, and chi-col-glu. Thus, we concluded that the addition of amino acids to chitosan/collagen composite scaffolds significantly increased the water uptake ability and made them more hydrophilic. In addition to the above *post hoc* Newman–Keuls test, the effect of time on water uptake was analyzed, and we found that after 20 min, the water uptake values of the chi-col-gly, chi-col-ala, and chi-col-glu scaffolds was significantly higher than those of the chi and chi-col-non scaffolds. The water uptake percentages of the chi and chi-col-non scaffolds had similar results compared with previous research.[42] With the addition of amino acids, the water uptake of the scaffolds increased by more than 2 to 2.5 times. This might have been due to the hydrophilic nature of the amino acids. For tissue engineering applications, it is very important for nutrients to be able to infiltrate into porous scaffolds; for this reason, a hydrophilic scaffold is more desirable.[26] Previous research also showed that a scaffold

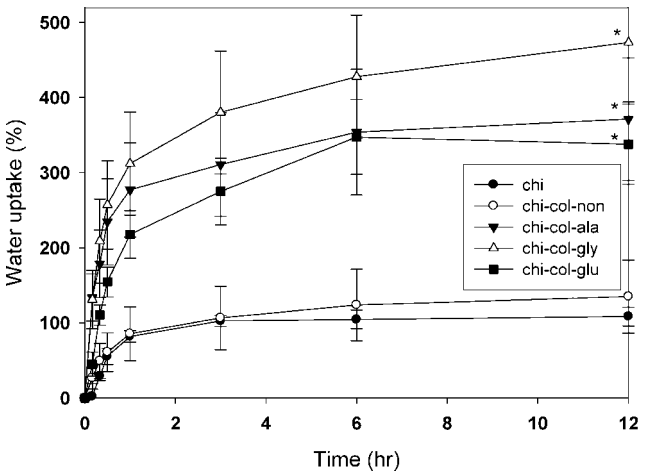

Figure 5 Water uptake of chitosan and chitosan/collagen composite scaffolds with or without the addition of amino acids. (△) chi-col-gly, (▼) chi-col-ala, (■) chi-col-glu, (○) chi-col-non, and (●) chi. Error bars represent the mean ± SD for $n = 6$; *$P < 0.05$ compared with the chi scaffold.

with water uptake of about 350% can be used in artificial skin application,[43] and our prepared scaffolds also fall in that range. This suggests that the addition of amino acids enhances the hydrophilicity of the scaffolds and thus increases the percentage of water uptake.

Tensile strength and elongation of porous scaffolds

Tensile strength and elongation of the scaffolds are important for their application in tissue engineering.[44] Soft scaffolds are suitable for brain cells, and stiff scaffolds are suitable for skin cells.[45] Because the weights of scaffolds of different compositions varied slightly, we decided to report the load to fracture normalized by the weight (normalized maximum tensile strength). Figure 6 displays the normalized maximum tensile strength and maximum tensile elongation of the chitosan/collagen scaffolds with different amino acids as crosslinking bridges. The normalized maximum tensile strength of the chitosan/collagen scaffolds without the addition of amino acids was low (0.7 N/g), but with the addition of amino acids as crosslinking bridges, the normalized maximum tensile strength of the scaffolds increased by more than 1.5–2 times [Fig. 6(a)]. We found that by adding alanine or glycine to the scaffolds, the normalized maximum tensile strength was 1.9 N/g (chi-col-ala) and 1.8 N/g (chi-col-gly). Furthermore, by adding glutamic acid to the chitosan/collagen scaffold, the normalized maximum tensile strength reached 2.2 N/g. These results indicated that adding amino acids to the chitosan/collagen scaffolds significantly increased the normalized maximum tensile strength compared with chi-col-non scaffolds. Our data imply that the ratio of carboxyl groups to amino groups significantly affects the normalized maximum tensile strength of scaffolds. According to the molecular structure, the amino groups on the chitosan and carboxyl and amino groups on the amino acids and collagen all provide possibilities for crosslinking (Fig. 1). Our group also tried to use the conventional freeze-drying method to fabricate porous chi-col-glu scaffolds (FD), and the normalized maximum tensile strength of the FD scaffolds was around 2.3 N/g [Fig. 6(a)], similar to the normalized maximum tensile strength of scaffolds prepared by the freeze-gelation method. The statistical tests showed that there was no significant difference in the normalized maximum tensile strength between chi-col-glu and FD scaffolds [Fig. 6(a)].

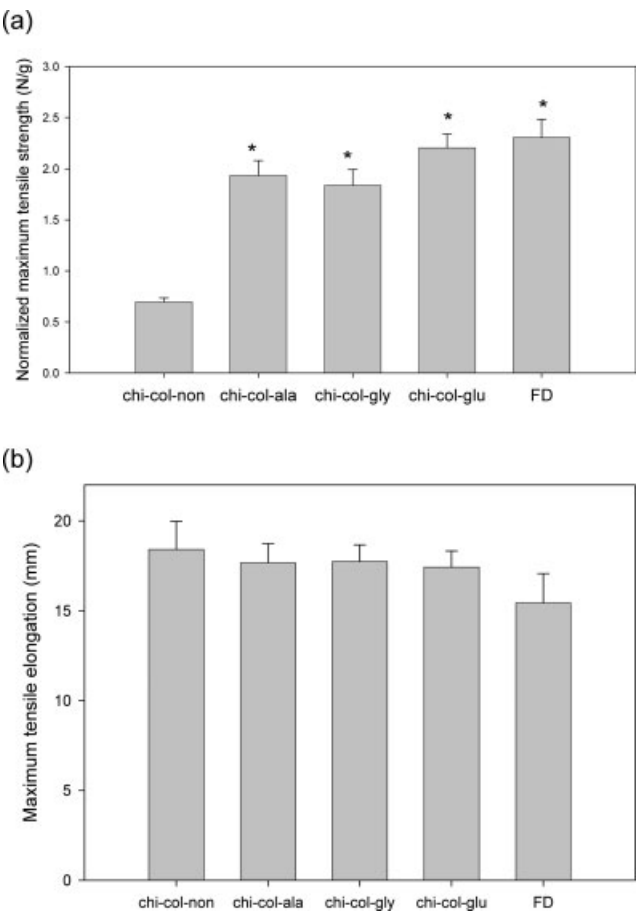

Figure 6 (a) Normalized maximum tensile strength of scaffolds with or without the addition of different amino acids as crosslinking bridges. The normalized maximum tensile strength is defined as the load to fracture (maximum load) divided by the weight of scaffold. The freeze-dried (FD) group contains chi-col-glu scaffolds fabricated by the conventional freeze-drying method. *$P < 0.05$ versus chi-col-non scaffold. (b) Maximum tensile elongation (strain at maximum load) of composite scaffolds with or without the addition of different amino acids as crosslinking bridges. The FD group contains chi-col-glu scaffolds fabricated by the conventional freeze-drying method. Error bars represent the mean ± SD for $n \geq 8$. *$P < 0.05$ versus chi-col-non scaffold.

Crosslinking using EDC/NHS consumes one carboxyl and one amino group each time. The concentration of amino groups ([NH_2]) was 341.8 m*M*, including [NH_2] in chitosan of 190 m*M* plus that of the amino acids of 150 m*M* and that in collagen of 1.8 m*M*, whereas the concentration of carboxyl groups ([COOH]) was 151.8 m*M* in the chi-col-ala and chi-col-gly systems in which the amino acids had the number ratio of COOH/NH_2 of 1 (Table I). With the COOH/NH_2 = 1 amino acid system, in the case where the carboxyl group on the amino acid first forms a covalent bond with the amino group on the chitosan, the amino acid has only one free amino group, and is thus unable to form covalent bonds with amino groups on the chitosan to continue the crosslinking reaction. With the COOH/NH_2 = 2 amino acid system (such as chi-col-glu with [NH_2] = 341.8 m*M* and [COOH] = 301.8 m*M*) (Table I), one carboxyl group on the amino acid can first be used to form a covalent bond with chitosan, and the amino acid still has one free carboxyl group that can form a covalent bond with another amino group. Because in the COOH/NH_2 = 2 system, the amino acid has a higher probability of continuing the crosslinking reaction, scaffolds prepared using the COOH/NH_2 = 2 amino acid system would be expected to have increased values for the normalized maximum tensile strength.

Although there are twice as many carboxyl groups on glutamic acid than on alanine and glycine, the normalized maximum tensile strength of chi-col-glu scaffold, however, was only 15–20% higher. One possible reason may be that the EDC/NHS reagent mediates the crosslinking between the carboxyl and amino groups, whether they are on chitosan, collagen, or amino acids. Because the numbers of chitosan molecules and amino acid molecules far exceeded those of collagen molecules, most crosslinking occurred between the amino acid and chitosan, and between amino acids. However, other crosslinking reactions still existed, and thus it is difficult to quantitatively predict the increment in the normalized tensile strength of scaffolds when using glutamic acid.

Both alanine and glycine belong to the COOH/NH_2 = 1 system. The difference between alanine and glycine is that one hydrogen atom of glycine is replaced by a methyl group to become alanine. The normalized maximum tensile strengths of the two systems were similar. This indicates that the number ratio of carboxyl to amino groups significantly influences the normalized maximum tensile strength. However, the substituted group, like the methyl group, which cannot react with EDC/NHS, does not significantly influence the normalized maximum tensile strength of the scaffolds.

The elongation data of chitosan/collagen composite scaffolds with or without the addition of amino acids as crosslinking bridges are shown in Figure 6(b). The highest and lowest groups were within the range of one standard deviation. Therefore, statistically, there was no significant difference in the elongation properties of these scaffolds. Because the FD group contained scaffolds fabricated by the traditional freeze-drying method, Figure 6(b) also demonstrates that the elongation properties of scaffolds prepared by the freeze-gelation method and those prepared by the freeze-drying method were similar.

Our data also indicate that the tensile strength of composite chitosan/collagen scaffolds with the addition of amino acids is significantly higher than that of scaffolds prepared in a previous study,[46] and thereby these composite scaffolds are suitable for skin tissue engineering applications.[47]

In vitro enzymatic degradation

The *in vitro* enzymatic degradation of the scaffolds by lysozyme is shown in Figure 7. It can be seen that the remaining weight for the chi and chi-col-non scaffolds was about 77% after 4 weeks of degradation. In contrast, the remaining weights for the chi-col-ala, chi-col-gly, and chi-col-glu scaffolds were about 85% after 4 weeks of degradation. The *post hoc* Newman–Keuls test showed that the chi-col-non scaffold degraded significantly more slowly than the chi scaffold in weeks 1 and 2, but exhibited no difference with the chi scaffold in weeks 3 and 4. In addition, the percent weights remaining of the chi-col-ala, chi-col-gly, and chi-col-glu scaffolds were significantly higher than those for the chi and chi-col-non scaffolds from weeks

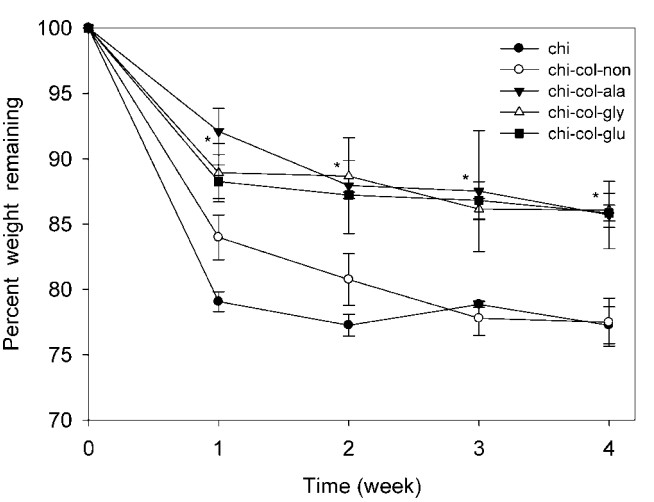

Figure 7 Percent weight remaining of chitosan/collagen composite scaffolds subjected to *in vitro* enzymatic degradation. The percent weights remaining of the chi-col-ala, chi-col-gly, and chi-col-glu scaffolds were significantly higher than those of the chi and chi-col-non scaffolds from weeks 1 to 4. Error bars represent the mean ± SD for $n = 5$. *$P < 0.05$ compared with the chi and chi-col-non scaffolds.

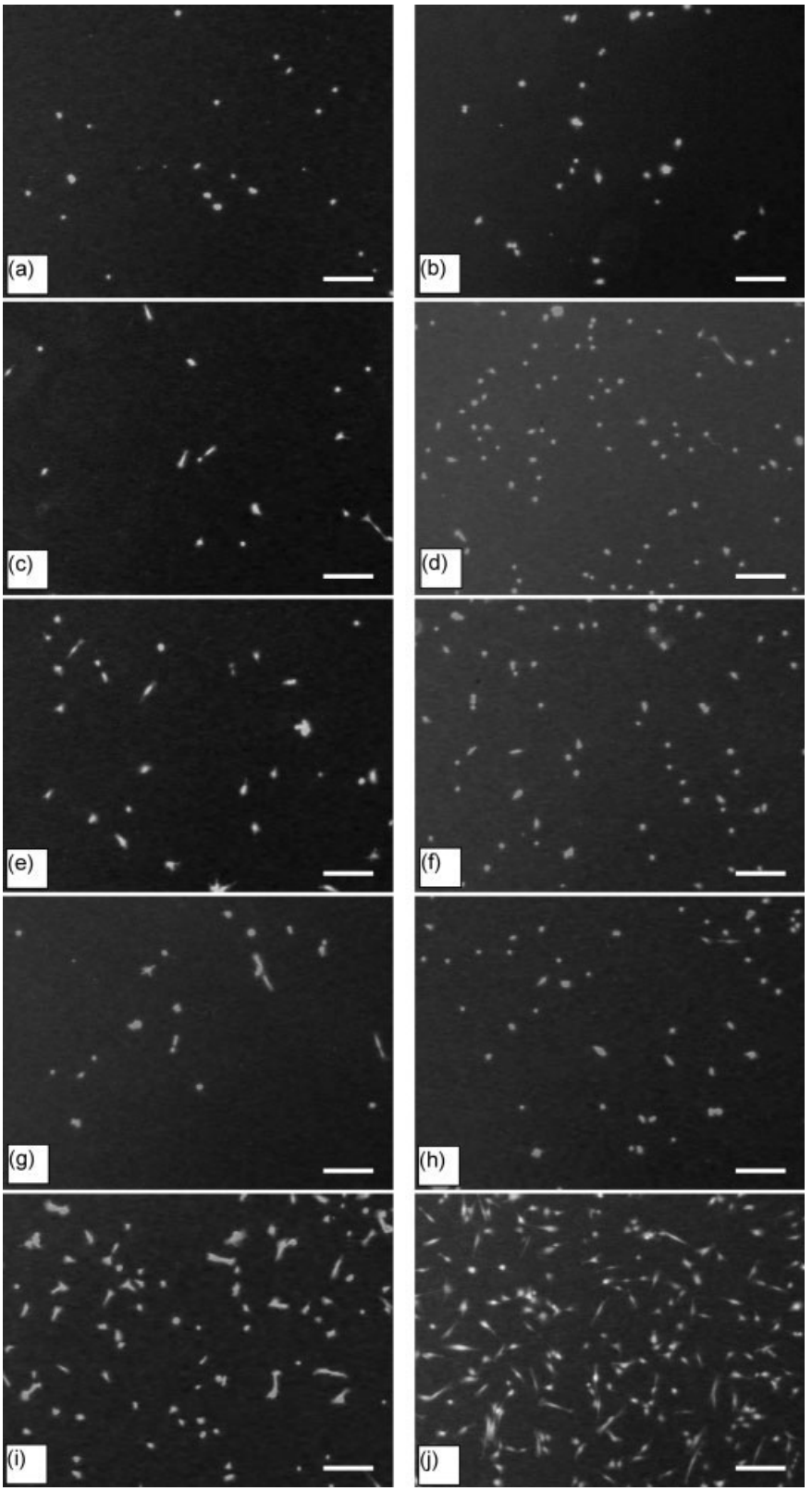

Figure 8 Morphology of WS1 skin fibroblasts on various surfaces visualized by fluorescence staining. (a) chi, day 3; (b) chi, day 7; (c) chi-col-non, day 3; (d) chi-col-non, day 7; (e) chi-col-ala, day 3; (f) chi-col-ala, day 7; (g) chi-col-gly, day 3; (h) chi-col-gly, day 7; (i) chi-col-glu, day 3; (j) chi-col-glu, day 7. Scale bar = 200 μm.

1 to 4. The effect of time on degradation according to the Newman–Keuls test demonstrated that there were significant differences among week 1, week 2, and weeks 3 and 4, but no difference between weeks 3 and 4.

It has been determined that chitosan is most susceptible to hydrolysis by lysozyme at pH 5.2, and that the optimum range of pH is from pH 5.2 to 8.0.[48,49] The lysozyme solution (pH 7.4) used for the degradation study was also in that range. However, the degradability of chitosan scaffolds by lysozyme depends on the fabrication methods, lysozyme concentration, etc.[50] Our results clearly demonstrated that the presence of amino acids for forming crosslinking bridges greatly improved the stability of the scaffolds when exposed to lysozyme degradation. It has been mentioned that prolonging the lives of scaffolds can facilitate implantation in patients and replacement by patients' tissues.[51]

Cell compatibility of scaffolds

The extracellular matrix *in vivo* is composed of glycosaminoglycans, collagens, laminin, etc., which control cell survival, proliferation, differentiation, and migration, as well as the cell cycle, polarity, and apoptosis.[52] To examine the cell compatibility of scaffolds, fluorescence images of WS1 human embryonic skin fibroblasts cultivated on various nonporous films were analyzed (Fig. 8). The nonporous films facilitated fluorescence observation. WS1 skin fibroblasts did not spread well on the chitosan surfaces even after 7 days in culture [Fig. 8(a,b)]. Cell proliferation was evident on the chitosan/collagen surface with EDC/NHS crosslinking treatment but without adding any amino acid (chi-col-non) [Fig. 8(c,d)]. On chitosan/collagen surfaces with amino acids (alanine or glycine) as the crosslinking bridges (chi-col-ala and chi-col-gly), cells exhibited better proliferation than those on the chitosan surfaces [Fig. 8(e–h)]. More importantly, WS1 skin fibroblasts showed much better proliferation on the chitosan/collagen surface with glutamic acid (chi-col-glu) as the crosslinking bridge [Fig. 8(i,j)].

Cell proliferation on different surfaces over the culture time was further quantified and is presented in Figure 9. On day 1, the cell number on the chi-col-glu surface was obviously larger than those on the other surfaces, suggesting that the cell attachment was better on the chi-col-glu surface. After 7 days in cultivation, the cell number on the chi-col-glu surface (35,600 cells/well) was 4.7-fold of that on the chi-col-gly surface (7500 cells/well).

We speculated that the use of glutamic acid molecules to form crosslinking bridges might cause the chi-col-glu surface to adsorb more serum proteins in the culture medium,[31] thus attracting more WS1 skin fibroblasts onto the surface than onto the chi-col-ala and chi-col-gly surfaces. Recently, other studies have demonstrated that the use of polyglutamic acid to modify the surface of a biomaterial has a positive effect on cell growth.[53] Meanwhile, the finding that polyglutamic acid can promote cell proliferation has been reported,[31] and our results are consistent with these studies.

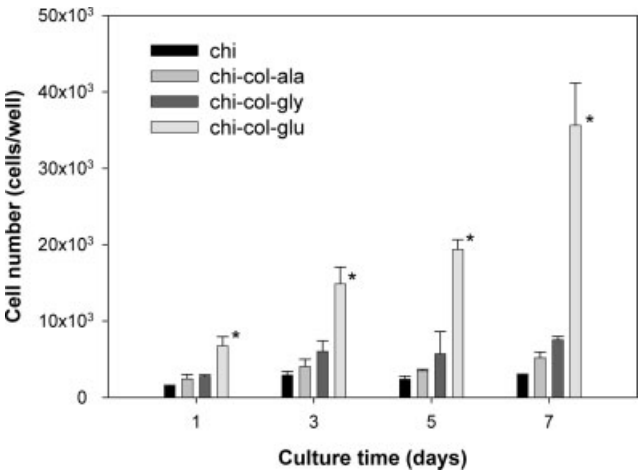

Figure 9 Proliferation of WS1 skin fibroblasts on various surfaces. Error bars represent the mean ± SD for $n = 3$. *$P <$ 0.05 compared with the other three surfaces.

CONCLUSIONS

We successfully fabricated porous chitosan/collagen composite scaffolds with the addition of amino acids as crosslinking bridges using a novel freeze-gelation method. The DSC results demonstrated that collagen and amino acids were well-mixed with the chitosan. The freeze-gelation method can save much time and energy, and large-sized porous scaffolds can be fabricated by this method. In other studies using the freeze-drying method, small-sized porous scaffolds were usually prepared. We concluded that the freeze-gelation process is a promising way for fabricating various chitosan-based composite biomaterials. We also found that the use of amino acids for crosslinking bridges in the chitosan/collagen composite scaffolds significantly increased their tensile strength and hydrophilicity. Furthermore, these novel composite scaffolds also possess enhanced cell compatibilities and reduced degradation rates. Our results also demonstrated that adding glutamic acid to enhance the performance of chitosan/collagen composite scaffolds is especially effective and thus these scaffolds should have great potential in skin-related tissue engineering applications.

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