Biodegradability and cell-mediated contraction of porous collagen scaffolds: The effect of lysine as a novel crosslinking bridge

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Abstract: A novel crosslinking method was adopted to modify the porous collagen scaffolds by using a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) in the presence of lysine, which functions as a crosslinking bridge. In vitro biodegradation tests proved that in the presence of lysine the biostability of the EDAC crosslinked scaffolds was greatly enhanced. The biostability of the resultant scaffolds was also elucidated as a function of the concentrations of lysine and EDAC/NHS. Compared to the Col-DHT, the ability of the Col-EDAC and the Col/Lys to resist cell-mediated contraction (CMC) was greatly enhanced. Yet no obvious difference between the Col-EDAC and the Col/Lys was found with respect to CMC. SEM observations showed that the microstructure of the crosslinked scaffolds could be largely preserved after fibroblast seeding. As a result, MTT assays proved that the fibroblasts in the Col/Lys scaffolds proliferated faster compared to the DHT-treated one on the assumption that the cell viability was preserved to a similar level. Histological section results indicated that the Col/Lys scaffolds had the ability to accelerate the cell infiltration and proliferation. All these results demonstrated that this novel crosslinking method is an effective way to achieve a collagen scaffold with improved biostability and a more stable structure, which can resist cell-mediated contraction. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 71A: 334–342, 2004

Key words: collagen; scaffold; lysine; crosslink; contraction; tissue engineering

INTRODUCTION

Because of the excellent biocompatibility of collagen, the use of porous collagen scaffolds as a regeneration template for tissues such as skin, cartilage, bones, and nerve has been widely researched. However, the fast biodegradation rate and low mechanical strength of the untreated collagen cannot meet the demands of in vitro or in vivo applications in many cases, and have been the crucial factors that limit the further use of this material.

Crosslinking of the porous collagen scaffolds is an effective way to slow the biodegradation rate and optimize the mechanical properties. Currently, two kinds of crosslinking methods are frequently employed to improve the properties of the porous collagen scaffolds—physical methods and chemical methods. The former includes the use of photooxidation, dehydrothermal treatments (DHT), and ultraviolet irradiation, which can avoid introducing potential cytotoxic chemical residuals and sustain the excellent biocompatibility of collagen materials. However, most of the physical treatments cannot yield enough crosslinking to meet the demands of tissue engineering. Therefore, treatments by chemical methods are still necessary in most cases. Several crosslinking agents, such as glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), polyglycidyl ether, and polyepoxides have been used widely. Among them, the EDAC/N-hydroxysuccinimide (NHS) crosslinking system, which has shown better biocompatibility than GA, is used frequently. This crosslinking takes place by reaction between carboxyl groups of glutamic or aspartic acid residues and amine groups to form amide bonds. However, the crosslinking degree cannot match the applications of tissue engineering in many cases under the EDAC/NHS treatment. Therefore, diamine agents such as putrescine, hexane diamine, and so forth, have...
been used to assist the crosslinking. Up to now the use of amino acids as crosslinking bridges to enhance the biostability of porous collagen scaffolds has rarely been reported. Amino acids are the basic components of proteins and naturally have good biocompatibility. A preliminary study has found that amino acids with neutral, acidic, or basic properties have different effects on the crosslinking efficiency. Basic amino acids are favorable for the improvement of biostability.

The contraction of cell-seeded matrices and in vivo implants is a key factor that influences the construction of tissue engineering organs. Because of the contraction of cell-seeded scaffolds, changes in the size and microstructure of the implants take place frequently. A change in size will cause difficulty in fitting a specific implant site, or cause separation from the surrounding tissue host. The crucial effect of the contraction is that the pore size and porosity reduction of the scaffolds will impede the migration of cells from the surrounding tissue into the scaffolds and affect the free exchange of the nutriments and the metabolites. Contraction of collagen-based matrices, such as gels and scaffolds, caused by many types of connective tissue host. The crucial effect of the contraction is that the pore size and porosity reduction of the scaffolds will impede the migration of cells from the surrounding tissue into the scaffolds and affect the free exchange of the nutriments and the metabolites. Contraction of collagen-based matrices, such as gels and scaffolds, caused by many types of connective tissue host.23,24 In order to resist the contraction, crosslinking treatments are applied to enhance the mechanical properties of the scaffolds.

In this study, a novel crosslinking strategy, that is, EDAC/NHS in the presence of lysine, was employed to improve the biostability and the ability to resist cell-mediated contraction of porous collagen scaffolds. The factors controlling the biostability of the crosslinked scaffolds (Col/Lys) and the effect on the cell-mediated contraction were researched in detail. Changes to the microstructure and the cytocompatibility of the scaffolds were evaluated as well.

**Preparation of porous collagen scaffold**

Collagen was dissolved in 0.5M acidic acid solution to obtain a 0.5% (w/v) solution. After being deoxygenated under reduced pressure to remove entrapped air bubbles, the collagen solution was injected into a homemade mold (diameter: 16 mm, depth: 2 mm), frozen in 70% ethanol bath at −20°C for 1 h, and then lyophilized for 24 h to obtain a porous collagen scaffold.

**Crosslinking treatments**

All the scaffolds were dehydrated at 105°C under vacuum (less 0.2 mbar, DHT) for 24 h prior to any additional chemical crosslinking. Collagen scaffolds weighted 2.5 mg were incubated in 1 mL 50 mM MES (pH 5.5) solution containing (Col/Lys) or not containing (col-EDAC) 2.5 μM lysine for 1 h at room temperature. Then 1 mL MES solution containing 40 mM EDAC and 20 mM NHS was added. After incubation for 24 h at room temperature, the scaffolds were washed with double-distilled water (10 min × 6 times) and lyophilized.

The porous collagen scaffolds were also crosslinked with 20 mM GA in the presence or absence of lysine in order to compare the crosslinking efficiency of these two kinds of crosslinking agents.

**Characterization of collagen scaffolds**

The microstructure of the scaffolds before or after crosslinking with EDAC/NHS in the presence or absence of lysine was observed under scanning electron microscopy (SEM, Cambridge stereoscan 260). The mean pore size of the scaffolds was determined by analysis of the SEM images from the cross-section positions. The porosity of each kind of scaffold was measured by immersing the scaffolds (initial weight, \( W_0 \)) into ethanol at room temperature for 24 h (wet weight, \( W \)) and calculated by the formulas below.

\[
\text{porosity} (%) = \frac{(W - W_0)p_1}{p_1W + (p_2 - p_1)W_0} \times 100\%,
\]

where \( p_1 \) and \( p_2 \) represent density of collagen (1.21 g/mL) and ethanol (0.79 g/mL), respectively.

The swelling test was performed by incubating the scaffolds in distilled water at room temperature and determining the wet weight of the scaffolds 24 h later. The swelling ratio of the scaffolds was defined as the ratio of weight increase to the initial weight. Each value was averaged from three parallel measurements.

**In vitro collagenase degradation**

The biological stability of the crosslinked collagen scaffolds was evaluated by in vitro collagenase biodegradation test. Each kind of scaffold was incubated in phosphate-
buffered saline (PBS, pH 7.4) containing a given concentration of collagenase (type I, Sigma) at 37°C. The degradation was terminated at the given time interval by incubating the assay mixture in an ice bath immediately. Following centrifugation at 1000 rpm for 10 min, the clear supernatant was hydrolyzed with 6M HCl at 120°C for 12 h. The amount of hydroxyproline released from the collagen molecules in the scaffolds was measured by absorbance spectroscopy (CARY 100 BIO, America) at a wavelength of 560 nm.27 The biodegradation degree is defined as the percentage of the released hydroxyproline from the scaffolds to the completely degraded one with the same composition and weight.

**Fibroblast isolation and scaffold seeding**

Fibroblasts used in this study were isolated from human dermis by collagenase digestion.28 The cell suspensions were cultured at 37°C, 5% CO₂, and 96% humidity in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), and 10% fetal bovine serum (FBS) (complete medium), which was changed every 3 days. Cells were passaged at confluence and the fourth–eighth passage fibroblasts were used for the in vitro seeding. The scaffolds were sterilized in 75% ethanol for 12 h, followed with solvent exchange by PBS six times. Each scaffold was then placed on a 24-well polystyrene plate (Falcon) and seeded with 200 μL human dermal fibroblast suspension at a density of 5 × 10⁶ cells/mL. After incubation for 4 h, the cell-seeded scaffold was moved to another well in the 24-well plate with 1 mL complete medium. Then the scaffolds were cultured in a 5% CO₂ incubator at 37°C with medium change every 3 days. Cultures were terminated 3, 7, 14, and 21 days after seeding. The unseeded scaffolds were incubated in the same culture conditions and employed as controls.

**Measurement of the scaffold contraction**

The diameters of the seeded and unseeded scaffolds were determined with a ruler with 0.5-mm intervals (n = 3). The contraction of the scaffolds was calculated by dividing the diameter change of each scaffold at the termination of the culture time by its original diameter. Herein, cell-mediated contraction (CMC) was determined by subtracting the contraction of the unseeded scaffolds from the contraction of the seeded scaffolds.23,29

\[
CMC = \left( \frac{\text{original diameter} - \text{diameter}}{\text{original diameter}} \right)_{\text{Seeded}} - \left( \frac{\text{original diameter} - \text{diameter}}{\text{original diameter}} \right)_{\text{Unseeded}}
\]

**Morphology of the fibroblast-seeded scaffold**

At 7 and 21 days after seeding, the culture was discontinued and the scaffolds were washed with PBS and fixed with 2.5% glutaraldehyde at room temperature for 24 h. After being washed with PBS to remove the remaining glutaraldehyde, the scaffolds were dehydrated with a graded series of ethanol. Then the scaffolds were further dehydrated with acetone and treated with isoamyl acetate. After being dried by the critical point dry method, the scaffolds were coated with an ultrathin gold layer and observed under scanning electron microscopy (SEM, Cambridge stereoscan 260).

**Behavior of cell proliferation**

The cell proliferation as a function of culture time was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay according to the methods of Mosmann with minor modification.30 At each culture interval, the media in the plates were removed and 200 μL MTT (5 mg/mL, Sigma)/complete medium (volume ratio 1:4) solution was injected into the scaffolds. After being incubated for 4 h, the scaffolds were transferred into a flask, where dimethylsulfoxide (DMSO) was added up to 500 μL. Then the solution was mixed thoroughly with a vortex mixer. Finally, the absorbance of violate substance was measured at 570 nm by using a microplate reader (Biorad, model 550).

**Histological observation**

At the termination of the cultures, the cell-seeded Col/Lys scaffolds were fixed with 10% neutral buffered formalin at room temperature for 24 h and embedded in paraffin. The specimens were cross sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H&E). The morphology and distribution of cells in the scaffolds were observed with a light microscope (Axiovert 200, Zeiss)

**RESULTS**

**Microstructure and swelling property**

Figure 1 shows the cross-section morphology of the scaffolds before and after crosslinking treatment. The cross-section images reveal that the porous structure of the collagen scaffolds was largely preserved after crosslinking either in the presence or absence of lysine. However, more sheet-like structures could be observed after crosslinking. Table I lists the analyzing data on the scaffold pore size, porosity, and PBS swelling ratio. As seen in the table, the typical pore size is smaller than 100 μm in the Col-DHT scaffolds. In either the presence or absence of lysine, the mean pore size became slightly enlarged under the EDAC/NHS treatment. No significant changes in the porosity were observed before or after the crosslinking treatment. All the scaffolds have porosities larger than 99%.
However, the crosslinking leads to an obvious decrease in swelling ratios. The value was lowered from 105.9 ± 6.2 for the Col-DHT, to 79.3 ± 4.6 for the Col-EDAC, and further to 68.8 ± 1.9 after crosslinking in the presence of lysine (Col/Lys).

**In vitro** biodegradation

Figure 2 compares the biodegradation degree of the scaffolds by the collagenase digest test. The figure shows that the Col-DHT has been fully degraded after incubation in the collagenase solution for 12 h. After

<table>
<thead>
<tr>
<th></th>
<th>Pore Size (µm)</th>
<th>Porosity (%)</th>
<th>Swelling Ratios</th>
</tr>
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<tbody>
<tr>
<td>Col-DHT</td>
<td>99 ± 19</td>
<td>99.46 ± 0.02%</td>
<td>105.9 ± 6.2</td>
</tr>
<tr>
<td>Col-EDAC</td>
<td>106 ± 17</td>
<td>99.35 ± 0.03%</td>
<td>79.3 ± 4.6</td>
</tr>
<tr>
<td>Col/Lys-EDAC</td>
<td>107 ± 16</td>
<td>99.44 ± 0.03%</td>
<td>68.8 ± 1.9</td>
</tr>
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**Figure 1.** SEM images to show the cross sections of the collagen scaffolds treated with EDAC/NHS in the presence/absence of lysine (original magnification ×200). (a) Col-DHT, (b) Col-EDAC, (c) Col/Lys.

**Figure 2.** The effect of lysine on the biodegradation degree of the collagen scaffolds crosslinked with EDAC/NHS or GA. All scaffolds were incubated in 100 µg/mL (30 units) collagenase for 12 h. Values are mean ± standard deviation (n = 3).
being treated with EDAC/NHS, the crosslinked collagen scaffold (Col-EDAC) was more resistant to collagenase digestion, and the biodegradation degree was decreased from 100% to 23.3 ± 4.3%. With the addition of lysine (Col/Lys), a lower biodegradation degree, 9.1 ± 2.4%, was achieved. Compared to the value of Col-EDAC, the decrease in the biodegradation degree was highly significant ($P = 0.007$). The biodegradation degree of the Col-GA and the Col/Lys-GA was 7.7 ± 4.6% and 6.7 ± 2.8%, respectively, after the same digestion procedure. No significant difference was detected between these two scaffolds ($P = 0.76$). The Col-GA showed a lower biodegradation degree compared to Col-EDAC ($P = 0.01$). The addition of lysine to the scaffolds crosslinked with EDAC/NHS resulted in biostability similar to that of the GA crosslinked material.

The effect of the lysine concentration on biostability is illustrated in Figure 3. The biodegradation degree decreased as a function of the concentration of lysine when the concentration was lower than 20 μM. From 0 to 10 μM, the biodegradation degree decreased sharply with the increase of lysine concentration. Then the biodegradation degree had a slight decrease when the lysine concentration was increased to 20 μM. With further increase of the lysine concentration, the biodegradation degree was kept nearly constant up to 100 μM.

Figure 4 shows the biodegradation behavior of the scaffolds crosslinked with different concentrations of EDAC/NHS in the presence of 2.5 μM lysine. When the EDAC and NHS concentrations were 5 and 2.5 μM, respectively (abbreviated as 5/2.5), the scaffold had been degraded 26.4% over the 12-h digestion period. After 96 h of digestion, the biodegradation degree was increased to 60%. The degradation rate became slower with increasing EDAC/NHS concentration. Only 9.9% of scaffolds were degraded after digestion for 96 h when the concentrations of EDAC and NHS were 50/25. It is worth noting that concentration increase higher than 30/15 has no significant effect on the decrease of the biodegradation degree.

**Scaffold contraction**

The cell-mediated contraction (CMC) of the scaffolds as a function of culture time is shown in Figure 5. For the Col-DHT, the CMC value had increased to 0.34 after culture for only 3 days. Twenty-one days...
later, the CMC value was 0.7, meaning that the scaffold had been largely contracted. In contrast to the Col-DHT, no obvious contraction was observed for the Col-EDAC and Col/Lys scaffolds during the 21-day culture period. The addition of lysine has no significant enhancement to resist the cell-mediated contraction.

The cross-section images of the scaffolds after 21 days of culture are presented in Figure 6. In comparison to Figure 1, the pores in the Col-DHT are hardly distinguished at the same magnification [Figure 6(a)]. It indicates that the pore size was reduced to a large extent because of the cell-mediated contraction. However, the three-dimensional structure of the Col/Lys was still preserved with a pore size similar to that of the unseeded one because of the crosslinking treatment [Figure 6(b)].

Cytocompatibility

Figure 7 shows the cell-proliferation behavior by MTT assay. For the Col-DHT, the cell viability in the scaffold increased in the first week. Then, with prolonged culture time, the cell viability decreased. However, the cell viability of the Col/Lys increased over the whole culture period, and the Col/Lys always behaved with higher viability than the Col-DHT ($P < 0.005$ at all intervals).

The SEM images of the cell-seeded Col/Lys scaffolds are displayed in Figure 8. After being cultured for 7 days, several fibroblasts could be detected in the scaffold with a typical shuttle-like morphology [Figure 8(a)]. When the culture time was prolonged, more cells were observed in the scaffold. The cells spread well along the collagen fibers and had been confluent to some extent [Figure 8(b)]. Histological results show that the cells only existed at the edge of the scaffold during the initial culture stage. After being cultured for 21 days, many fibroblasts could be observed both on the surface and in the inside of the scaffold and distributed evenly (Figure 9). This proves that the cells had infiltrated into the Col/Lys and proliferated there.

**DISCUSSION**

The effects of the lysine used as a novel crosslinking bridge on enhancing the ability of collagen scaffolds to resist the collagenase biodegradation and the cell-mediated contraction are demonstrated. It is well known that the three-dimensional porous structure of a scaffold is rather important for cell infiltration and proliferation. Therefore, the preservation of the porous structure is a crucial aspect to evaluate a crosslinking method. In general, the crosslinking treatment always
results in some extent of collapse because of the rehydration process. After crosslinking by EDAC/NHS under the assistance of lysine, the porous structures were largely preserved, with a slight reduction in porosity compared to the Col-DHT one. Meanwhile, the crosslinking treatments have some effect on enlarging the pore size. These results can be explained by the recombination of the collagen fibers during the rehydration and refreeze–drying procedures of the crosslinking treatment.28,32

Swelling ratio is another key factor of a scaffold, representing the ability to hold the nutrients to accelerate cell infiltration and proliferation as well as to transfer the metabolites through the scaffold.33 The crosslinking treatment led to about 30% shrinkage in swelling ratio. This decrease should be mainly attributed to the partial diminishing of the hydrophilic groups and the collapse of the scaffold after crosslinking. The initially existing hydrophilic groups, such as amino groups and carboxyl groups, were partially transformed into hydrophobic amide groups under the EDAC/NHS treatment. Nevertheless, the swelling ratio of the crosslinked scaffolds is still big enough to meet the demand of the matrices used in tissue engineering.

To the best of the authors’ knowledge, a higher crosslinking efficiency can be achieved under GA treatment than under EDAC/NHS treatment. However, EDAC/NHS has been used more frequently in the treatment of porous collagen scaffolds, because of better biocompatibility. In this study, the collagen scaffolds crosslinked by EDAC/NHS in the presence of lysine had biostability similar to that of the GA crosslinked one. The added lysine could function as a crosslinking bridge between collagen molecules. Previous study has proved that the biodegradation degree depends on the NH2/COOH ratio in the EDAC/NHS crosslinking system. In the presence of amino acids, there are two complete reactions. One is the functional group’s blocking reaction by amino acids; the other is the bridging reaction between two collagen molecules. The predominant reaction can be tailored by controlling the total NH2/COOH ratio in the crosslinking system. Below an optimal ratio, the biodegradation degree decreases with augmented NH2/COOH ratios; then a reversed function can be obtained. Therefore, the further addition of lysine results in the NH2/COOH ratio closing to a constant, for which the biodegradation degree tends to be a constant with increasing lysine concentration. It is easy to understand that the biodegradation degree will decrease with increasing EDAC/NHS concentration at the beginning. When EDAC/NHS is too excessive, further addition will have little effect on the crosslinking degree.

Along with enhanced biostability, the crosslinking treatment yielded a more stable structure that can
better resist cell-mediated contraction. The scaffold’s contraction may be due to the contraction of the myofibroblasts, a major contractile phenotype of fibroblasts that expresses a smooth muscle actin isoform, α-smooth muscle actin (SMA). In general, the contractile cells are inevitable in the seeding population, because of the process of the cell isolation and the two-dimensional culture conditions. Hence, the obvious contraction always occurred in the Col-DHT collagen matrix implanted in vitro or in vivo for its low mechanical properties. Although the addition of lysine could bring a great enhancement in biostability, there is no obvious difference in the ability to resist contraction between the Col-EDAC and the Col/Lys. This would mean that the contractile force caused by SMA is not big enough to distinguish the difference of CMC between these two kinds of scaffolds.

The effects of the contraction will reduce the pore diameter of the scaffolds and compress the space for cell proliferation and the diffusion of the nutrients. It is important to determine the successive proliferation of the seeding fibroblasts. In order to evaluate cytocompatibility of the scaffolds, the MTT assay was applied to analyze the cell proliferation because the absorbance value in 570 nm can be correlated to the cell number in the scaffolds on the assumption that the cell viability was preserved to a similar level. When the culture time was prolonged, the viability of the Col/Lys increased gradually. It can be observed that the violating substance was only on the surface in the first week, then on the entire scaffold after 21 days of culturing. The low CMC of the Col/Lys resulted in the three-dimensional structure being largely preserved. Therefore, the Col/Lys scaffolds have enough space for the cell proliferation. In contrast, the Col-DHT scaffolds contracted 50% in the first week, so that the cell viability did not increase further, but decreased with increasing culture time. This can be explained by the limited amount of available nutrients and the limited amount of available space caused by the contraction. Meanwhile, both the SEM images and the histological sections indicated that the fibroblasts could spread along the wall of the pores and proliferate well in the scaffolds. It is attributed to the original cytocompatibility of lysine, the addition of which does not deteriorate the excellent cytocompatibility of collagen. All these results prove that this novel crosslinking strategy is an effective way to modify the porous collagen scaffold.

CONCLUSIONS

Herein lysine is applied as a novel crosslinking bridge to enhance the biostability of the porous collagen scaffolds. Under the crosslinking treatment, the microstructures of the scaffolds were largely preserved. Biostability was much improved after crosslinking in the presence of lysine, resulting in a similar crosslinking degree as the GA crosslinked one. There exists an optimal concentration of lysine and EDAC/NHS to increase the biostability. Compared to the Col-DHT, the ability of the Col-EDAC and the Col/Lys to resist the cell-mediated contraction was greatly enhanced. Yet no obvious difference between the Col-EDAC and the Col/Lys was found with respect to CMC. A porous structure could remain after 21 days of culture, which could accelerate the cell infiltration. Cell MTT assay and histological results proved that the cells could proliferate well and distribute evenly in the Col/Lys scaffolds. In conclusion, crosslinking by EDAC/NHS in the presence of lysine is an effective method to fabricate collagen scaffolds with enhanced biostability and good cytocompatibility.

References


