Bull. Mater. Sci., Vol. 35, No. 5, October 2012, pp. 913–918. © Indian Academy of Sciences.

Enhanced physicochemical properties of collagen by using EDC/NHS-crosslinking

CHUNRONG YANG
Department of Materials Science and Engineering, Fujian University of Technology, Fuzhou 350108, China

MS received 4 July 2011; revised 21 December 2011

Abstract. **Collagen-based scaffolds are appealing products for the repair of cartilage defects using tissue engineering strategies. The present study investigated the collagen scaffolds with and without 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS)-crosslinking. Crosslinking density, matrix morphology, swelling ratio shrinkage temperature and resistance against collagenase digestion were determined to evaluate the physicochemical properties of the collagen matrices with and without crosslinking. The results conformed that the porous structure of collagen was largely preserved and adjusted by crosslinking treatment. Furthermore, crosslinked collagen samples showed significantly reduced swelling ratio and increased resistance against thermal treatment and enzymatic degradation compared to non-crosslinked samples. An *in vitro* evaluation of MC3T3-E1 cells seeded onto the crosslinked and non-crosslinked collagen matrix indicated that crosslinked collagen was nontoxic and improved cell proliferation. Through this work, it was shown that an osteoconductive collagen matrix with optimized properties used as bioactive and bioresorbable scaffolds in bone tissue engineering could be fabricated through the EDC/NHS-crosslinking method.**

Keywords. **Crosslinking; collagen; microstructure; tissue engineering; degradation.**

1. Introduction

The necessity of bone substitutes for wound healing has promoted development of the biomimetic bone biomaterials. Collagen, therefore, becomes a popular biomaterial for this purpose due to its compatibility. The desired properties of collagen which facilitates wound-healing processes are stimulation of cell migration and infiltration and support of cell proliferation.

The disadvantages of using collagen as a biomaterial for tissue repair are its low biomechanical stiffness and rapid biodegradation. The high enzymatic turnover rate of natural collagen *in vivo* makes stabilization of collagen-based biomaterials necessary. This can be achieved by chemical crosslinking methods, which provide biomaterials with desired mechanical properties for implantation and defect repair. Several chemical agents have been used to achieve this goal (Kurashina *et al* 2002; Tsai *et al* 2002; Sell *et al* 2008). Glutaraldehyde (GA), a bifunctional reagent for bridging amino groups, is the most widely used reagent for crosslinking collagen. However, GA is associated with cytotoxicity *in vitro* and *in vivo*, caused by the presence of unreacted functional groups or by the release of those groups during enzymatic degradation of the crosslinked biomaterials (Lee *et al* 2001; Pieper *et al* 2002).

Methods have been developed that allow the crosslinking of collagen materials directly without incorporation of the crosslinking reagent. For example, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) or acylazide were used to generate peptide-like bonds in biomaterials (Peter *et al* 2004; Vickers *et al* 2006; Barnes *et al* 2007; Cao and Xu 2008; Everaerts *et al* 2008; Rafat *et al* 2008). Use of EDC and *N*-hydroxysuccinimide (NHS) to crosslink collagen seems to yield collagen matrix with good biocompatibility, higher cellular differentiation potential and with increased resistance against enzymatic degradation.

In this study, collagen has been crosslinked with various concentrations of EDC/NHS. The physicochemical properties of collagen after crosslinking were characterized and evaluated.

2. Experimental

2.1 *Materials*

Collagen was purchased from Chunger Ltd. (Guangzhou, China). It was type I, prepared from bovine tendon in the form of acetic acid solution with a content of 5·8 mg/ml and pH 5.

1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and morpholine ethanesulfonic acid (MES) were all of chemical grade.

(lambmeo@163.com)

2.2 *Crosslinking of collagen*

Crosslinking test of collagen was carried out in MES buffer (pH 5·5) with variable concentrations of EDC. NHS was added in an EDC:NHS ratio of 4:1. After crosslinking with a reaction time of 4 h the samples were washed twice with 0·1 m Na_2HPO_4 for 1 h and 4 times with deionized water for 30 min. Finally, collagen was dried in vacuum.

2.3 *Characterization of collagen crosslinking*

2.3a *Crosslinkage degree*: To assess the degree of collagen crosslinking, free amino group content was determined. This was done by the reaction of the samples with 2,4,6-trinitrobenzenesulfonic acid (TNBS), according to an assay described by Bubis and Ofner (1992). The amount of non-crosslinked amino groups can be obtained by detecting the absorbance value. Finally, crosslinking degree can be calculated via the absorbance value before and after crosslinking.

2.3b *Morphology observation*: Scanning electron microscopy (SEM) (30XLFEG, Philips, The Netherlands) was used to observe the morphology of collagen with or without crosslinking. The samples were sputter-coated with a layer of gold for observation at 10 kV and varying levels of magnification.

2.3c *Shrinkage temperature*: The shrinkage temperature was determined by differential scanning calorimetry (DSC) (204F1, NETZSCH Corporation, Germany). Heating was carried out at a rate of 10°C/min in the temperature range 0∼200 °C with an empty aluminum pan as the reference probe. Shrinkage temperature was determined as the onset value of the occurring endothermic peak.

2.3d *Swelling kinetics measurements*: Swelling kinetics measurements were based on the determination of the volume of retained low-molecular penetrating agent (0·5 M solution of acetic acid) dependent on the time of swelling at a temperature 25°C. Disk samples with a size of 1·84 mm (length) ×13·2 mm (diameter) were used, and each weighed 0·5 g.

2.3e *Enzymatic stability*: Enzymatic stability of collagen samples was tested with *in vitro* collagenase digestion experiments. Films with an accurately weighed amount of collagen were incubated at 37°C with 2 ml of DMEM culture medium containing 7 μg/ml of collagenase for different times. Thereafter, residues of non-digested collagen were rinsed with PBS, dried and weighted.

2.3f *Cell morphology and proliferation*: To study the osteoblast biocompatibility of the crosslinked collagen matrices in bone formation, MC3T3-E1 cells (Japan Riken Cell Collection) were chosen to test the material. The matrices containing the cells were suspended in DMEM–10% FBS medium. The cell density was adjusted to 1×10^4 cells·ml^{-1}, and 2 ml of cell suspension was added to each dish. The cells were then allowed to attach to the matrices and remained undisturbed in a humidified incubator (37°C and 5% CO_2)

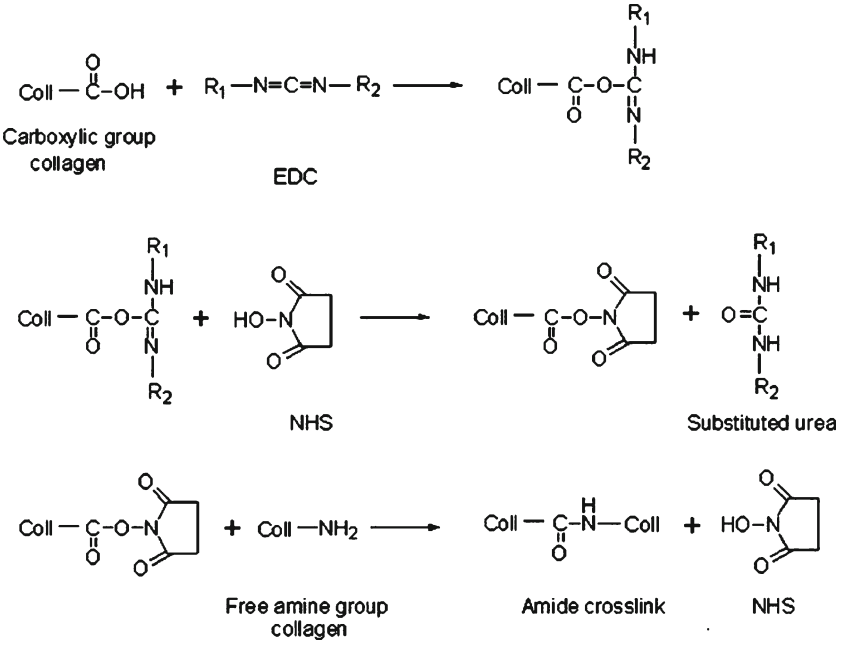

Figure 1. Chemical reactions of EDC/NHS-crosslinking of collagen.

for 1 day. After incubation, the specimens were washed twice by phosphate-buffered saline (PBS) and immersed in 2·5% glutaraldehyde (pH 7·4) for 2 h. They were then dehydrated in increasing concentrations of ethanol (from 30, 50, 70, 90, 95 to 100%), followed by lyophilization. They were then coated with gold and examined under SEM.

Proliferation measurements were performed using 96-well plates pre-coated with crosslinked and non-crosslinked collagen substrata. The cell density was adjusted to 8×10^4 cells/ml, and 2 ml of cell suspension was added to each dish. The cells were then allowed to attach to the materials, and to remain undisturbed in a humidified incubator (37°C and 5% CO_2) for 1, 3 and 5 days. After incubation, the dishes were rinsed twice with PBS and 200 μLMTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrasodium bromide), 1·8 ml medium was added to each dish for incubation at 37°C for 4 h. Then the MTT solution was removed and insoluble blue formazan crystal was dissolved in dimethyl sulfoxide (DMSO). One hundred microlitres of solution from each dish was aspirated and poured in a 96-well plate for absorbance measurement. The absorbance was directly proportional to cell proliferation.

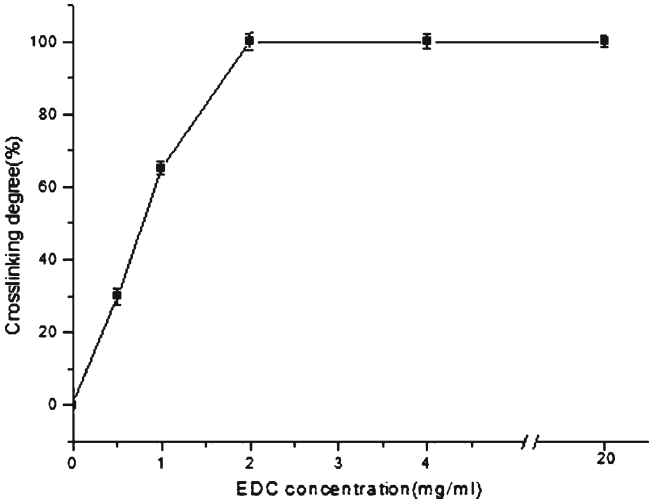

Figure 2. Crosslinking degree vs EDC concentration. With increase of EDC concentration, crosslinking degree increased.

3. Results and discussion

3.1 *EDC/NHS-crosslinking of collagen*

The chemical reactions of EDC/NHS-crosslinking are outlined in figure 1.

Crosslinking of the collagen matrices could be controlled by variation of EDC/NHS concentration. The degree of crosslinking is inversely proportional to the amount of free amino groups. With increasing EDC concentration, the amount of free amino groups decreased and the degree of crosslinking increased (figure 2). At concentrations of EDC higher than 2 mg/ml, no further increase in crosslinking was obtained.

3.2 *Microstructure of collagen matrices before and after crosslinking*

The microstructure of collagen matrices with and without EDC/NHS-crosslinking (0, 2 mg/ml-EDC/NHS) was analysed by scanning electron microscopy (figure 3). The collagen origin showed highly interconnective pores (figure 3a). EDC/NHS-collagen crosslinking was able to dramatically change the matrix microstructure. After crosslinking treatment, the collagen matrices revealed parallelly aligned and interconnected pores with size ranging from 33 μm up to 220 μm and a median pore size of about 120 μm.

In biological process, self-assembly process is an essential feature that leads to the creation of specific shapes and conformations in macromolecular structure. Self-assembly process alone usually leads to polydomain structures with short-range order and an overall random orientation. The driving force for preferred orientation or structural order may operate over multiple molecular lengths to perpetuate the growth of self-organizing materials (Geutjes *et al* 2006; Place *et al*

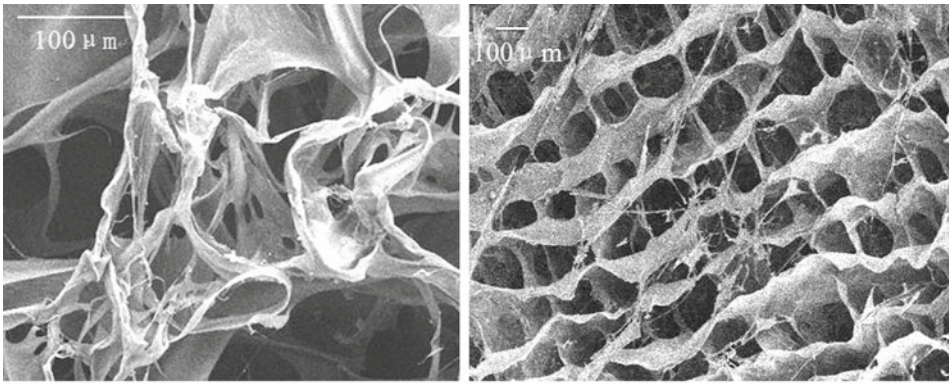

Figure 3. SEM micrographs of uncrosslinked (**a**) and crosslinked (**b**) collagen matrices.

2009). Collagen fibres are a case in point and have a strong driving force for self-assembly to make well organized fibril network. Crosslinking due to EDC/NHS greatly potentiates the ability for self-assembly and self-organization of collagen into more ordered structure.

3.3 *Thermal stability*

The influence of crosslinking was also reflected on thermal stability of collagen. The results of the present study demonstrate that EDC/NHS-crosslinked collagen matrices increased in shrinkage temperature compared with the uncrosslinked samples, as shown in figure 4.

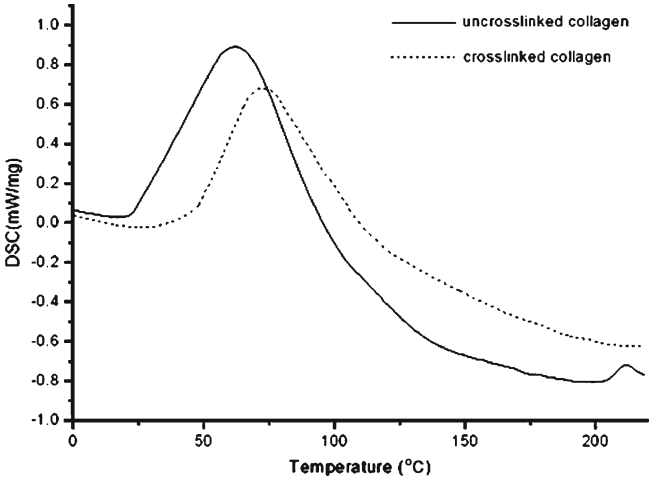

Figure 4. DSC analysis of uncrosslinked and crosslinked collagen matrices. Shrinkage temperature is determined as the onset values of the occurring endothermic peak.

EDC reacts with carboxyl group of the aspartic and glutamic acid residues existing in the collagen matrix to form an activator, viz. the unstable derivative of urea. The use of NHS can improve the crosslinking yield of carbodiimides by forming a more stable ester (Olde Damink *et al* 1996). On the other hand, in the process of crosslinking, carboxyl and amino groups that exist within collagen would react to yield hydrogen bond. It was the production of stable ester and hydrogen bond that made the thermal stability of crosslinked collagen higher than uncrosslinked collagen.

3.4 *Macroscopic form stability*

The different collagen matrices were compared morphologically with respect to their form stability using their swelling ratios after being soaked with fluid. EDC/NHS-crosslinking resulted in a significant decrease in swelling ratios compared to uncrosslinked collagen, and with the increase of the degree of EDC/NHS-crosslinking, a highly significant reduction in swelling ratios during contact with fluid could be detected.

Swelling is the process where macromolecular material absorbs the liquid and the volume spontaneously expands. We can see from figure 5 that uncrosslinked collagen saturated after 15 min immersing in acetic acid solution, and completely collapsed after 30 min. In contrast, longer duration of the collapse can be observed for crosslinked samples. With the increase of EDC concentration, collagen showed

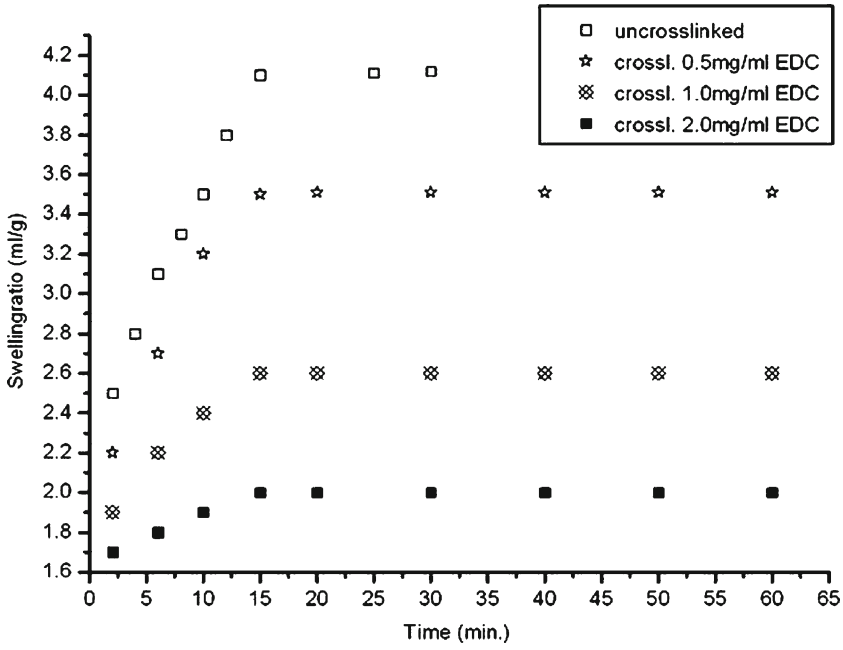

Figure 5. Swelling ratio of collagen with different degrees of crosslinking. EDC/NHS crosslinking resulted in a significant decrease of swelling ratio. With increase of EDC concentration, swelling ratio of collagen decreases.

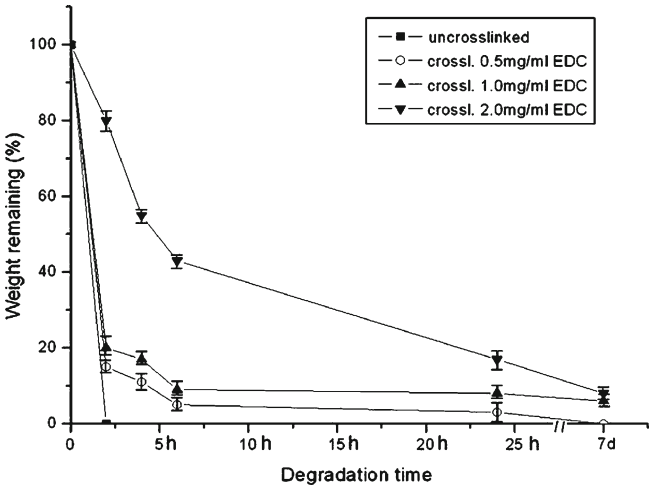

Figure 6. Relative collagen weight (%) after different time points of collagenase digestion. Values indicated represent mean ±SD, where $n = 6$.

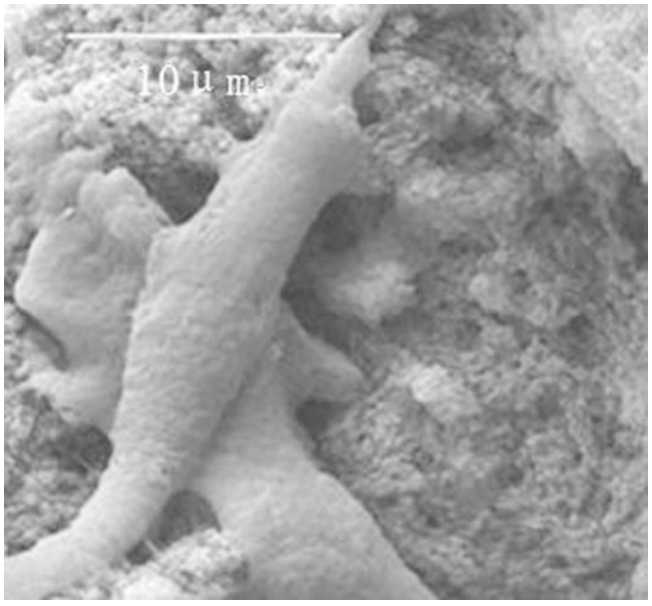

Figure 7. SEM of MC3T3-E1 cells cultured on crosslinked collagen matrix for 1 day after inoculation.

lower swelling ratio. At an EDC concentration of 2 mg/ml, collagen matrix still remained close structured after 1 h of immersion.

For similar reasons, the production of stable ester due to reaction of EDC/NHS with collagen and hydrogen bond due to internal interaction of collagen matrices during crosslinking brought collagen more stable structure.

3.5 *Enzymatic stability*

Another effect of crosslinking is increased resistance to enzymatic degradation by bacterial collagenase. In the present study, a significant increase in resistance to enzymatic degradation could be shown after crosslinking (figure 6). Even a low degree of EDC/NHS-collagen crosslinking (0·5 mg/ml) was able to dramatically change the rate of enzymatic degradation by preserving the matrix ultrastructure under short term-collagenase treatment. Collagen matrices with 2 mg/ml EDC/NHS-crosslinking revealed a partial degradation (of up to about 83%) after six days of collagenase treatment, whereas collagen origin samples showed complete degradation after only 2 h of collagenase treatment.

One possible explanation is that collagenase cleavage sites are more effectively masked by the crosslinked collagen samples. Bacterial collagenase catalyses hydrolytic cleavage of collagen in non-polar regions, either in a single alpha-chain or simultaneously across three chains of the triple helix in lateral fashion (Harpers *et al* 1972). EDC/NHS-crosslinking may occur within an alpha chain, between alpha chains, or as intermolecular or interfibrillar linkages, which may have been effective in blocking collagenase's specific ability to cleave alpha-chain linkages.

Another factor that may also affect the enzymatic degradation susceptibility of crosslinked collagen matrices is their reduced swelling ratio and lower surface area. Because the enzymatic activity mainly affects the matrix surface, collagenase may degrade less collagen per time unit in matrices of crosslinked collagen compared to collagen origin.

3.6 *Cell morphology and proliferation*

Scanning electron microscopy was used to observe the cells growing on the crosslinked collagen. Figure 7 showed that

Table 1. Effect of different collagen matrices on MTT production by MC3T3-E1 cells in culture (OD value).

Materials	1d	3d	5d
Culture	0·156 ± 0·003	0·261 ± 0·002	0·374 ± 0·003
Uncrosslinked collagen matrices	0·211 ± 0·004	0·354 ± 0·002	0·433 ± 0·002
Crosslinked collagen matrices	0·305 ± 0·003	0·499 ± 0·003	0·568 ± 0·004

Note: In every experiment, quadruplicate wells were set up for each condition. Values from all four measurements were averaged to calculate a single value; the differences among wells were routinely within 5% of each other. Values presented are means ±SD for number of experiments indicated.

MC3T3-E1 cells attached and spread on the surface of crosslinked collagen matrix after 1 day of culture.

Successful cell attachment is beneficial to cell proliferation. Table 1 displays the proliferation rate of MC3T3-E1 cells cultured on uncrosslinked and crosslinked collagen matrices for 1, 3 and 5 days after inoculation. Compared to culture, the increased absorbance on all surfaces of collagen matrices at the end of the culture period showed that proliferation occurred on two types of collagen matrices. In addition, cell proliferation appeared to be significantly higher on collagen matrices with 2 mg/ml EDC/NHS-crosslinking when compared to uncrosslinked collagen matrices after 1, 3 and 5 days of culture.

The cell attachment, spreading, and growth are important criteria to judge the biocompatibility of a biomaterial. This process is influenced by many properties of biomaterials, including the surface chemical states and morphology. The process of cell attachment usually occurs in two steps. The first one is the random cell adhesion on the surface of biomaterials, which is mainly directed by physical and chemical interaction between materials and cells. The interaction of the polar carboxyl and amino groups of collagen with the cells is one possible mechanism to explain MC3T3-E1 cell affinity. The second step is specific cell adhesion on the surface. As discussed earlier, specific conformations and higher structure stability of collagen after EDC/NHS-crosslinking are concerned with the specific attachment and proliferation of cells on material surface.

4. Conclusions

In summary, the physicochemical properties of collagen could be effectively improved by using EDC/NHS-crosslinking method. At an EDC concentration of 2 mg/ml, collagen matrix with maximum crosslinking and best biostability could be achieved. The porous structure of collagen was largely preserved and adjusted after crosslinking. A further study found that less distinct matrix swelling after contacting with fluid was observed and increased resistance against thermal treatment and enzymatic degradation was achieved after crosslinking. For tissue engineering purposes, the more appropriate porous structure, the higher form and thermal stability, the stronger ability to resist enzymatic degradation as well as the more favourable cell affinity make the case for considering EDC/NHS-crosslinked collagen.

Acknowledgement

This work was financially supported by Science and Technology Plan Project of Fujian Department of Education, China (Grant no. JK2009021).

References

Barnes C P, Pemble C W, Brand D D, Simpson D G and Bowlin G L 2007 *Tissue Eng.* **13** 1593
Bubis W A and Ofner C M 1992 *Anal. Biochem.* **207** 129
Cao H and Xu S Y 2008 *J. Mater. Sci. Mater. Med.* **19** 567
Everaerts F, Torrianni M, Hendriks M and Feijen J 2008 *J. Biomed. Mater. Res.* **A85** 547
Geutjes P J, Daamen W F, Buma P, Feitz W F, Faraj K A and van Kuppevelt T H 2006 *Adv. Exp. Med. Biol.* **585** 279
Harpers E, Berger A and Katchaiski E 1972 *Biopolymers* **11** 1607
Kurashina K, Kurita H and Wu Q 2002 *Biomaterials* **23** 407
Lee C R, Grodzinsky A J and Spector M 2001 *Biomaterials* **22** 3145
Olde Damink L H, Dijkstra P J, van Luyn M J, van Wachem P B, Nieuwenhuis P and Feijen J 1996 *Biomaterials* **17** 679
Peter A, Jochen A and Richard K 2004 *Biomaterials* **25** 2831
Pieper J S *et al* 2002 *Biomaterials* **23** 3183
Place E S, Evans N D and Stevens M M 2009 *Nature Mater.* **8** 457
Rafat M, Li F, Fagerholm P, Lagali N S, Watsky M A, Munger R, Matsuura T and Griffith M 2008 *Biomaterials* **29** 3960
Sell S A, Francis M P, Garg K, McClure M J, Simpson D G and Bowlin G L 2008 *Biomed. Mater.* **3** 45001
Tsai C L, Hsu S H and Cheng W L 2002 *Artif. Organs* **26** 18
Vickers S M, Squitieri L S and Spector M 2006 *Tissue Eng.* **12** 1345