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# Mechanical and Biological Properties of Bio-Inspired Nano-Fibrous Elastic Materials from Collagen

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## 1. Introduction

Collagen-based biomaterials have been widely used in medical applications, because of its many advantages, including low antigenicity, abundant availability, biodegradability, and biocompatibility [1]. Collagen represents the major structural protein, accounting for nearly 30% of all vertebrate body protein. The collagen molecule comprises three polypeptide chains ( $\alpha$ -chains) which form a unique triple-helical structure (Fig. 1) [2]. Each of the three chains has the repeating structure glycine-X-Y, where X and Y are frequently the imino acids proline and hydroxyproline (Fig. 1b). The collagen molecules self-aggregate through fibrillogenesis into microfibrils forming extracellular matrix (ECM) in the body [3-5]. The fibrils provide the major biomechanical matrix for cell growth (Fig. 1a), allowing the shape of tissues to be defined and maintained. The main application of collagen for biomaterials is as a scaffold for tissue engineering and a carrier for drug delivery [2, 6-9]. Many different forms of collagen biomaterials, such as film [10, 11], gel [12-17], sponge [18-20], micro-/nano-particle [21, 22], and fiber [23], have been fabricated and used in practice. However, most collagen biomaterials become brittle and fail under quite low strains, which limit their application to biomedical engineering fields that need larger mechanical properties, especially elasticity [16].

Recently, we reported a novel crosslinking method of improving the mechanical properties and thermal stability of collagen [24]. The method mimics actual biological events to form collagen matrix in the body; monomeric collagens extruded from cells into extracellular environment initially form microfibrillar aggregates, then lysyl oxidase crosslinking during their assembly to form fibrils (Fig. 1). The *in vitro* crosslinking during collagen fibrillogenesis, namely "bio-inspired crosslinking", creates a crosslinked collagen fibrillar gel with high mechanical properties at certain crosslinking agent concentrations [25, 26]. Fibril formation involves the aggregation and alignment of collagen molecules, and helps increase the collagen's thermal stability. The introduction of crosslinking during fibril formation further increases the thermal stability of collagen. The synergistic effects of crosslinking and fibril formation are found to enable an increase in the thermal stability of

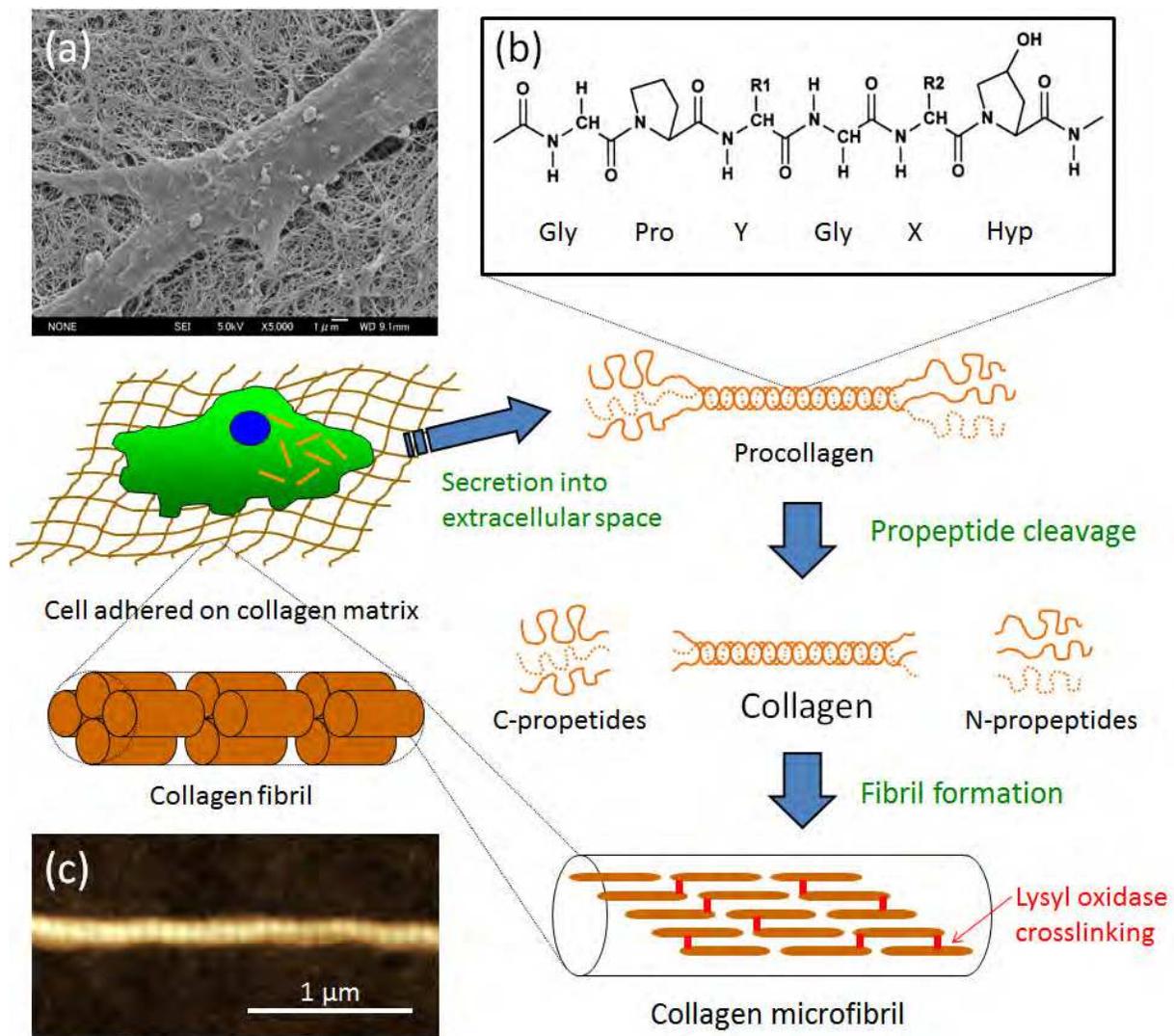


Fig. 1. Schematic representation of collagen synthesis process. Procollagen consists of a 300 nm long triple helical domain (comprised of three alpha-chains each of approximately 1000 residues) flanked by a trimeric globular C-propeptide domain and a trimeric N-propeptide domain. Procollagen is secreted from cells and is converted into collagen by the removal of the N- and C-propeptides by proteases. The collagen spontaneously self-assembles into cross-striated fibrils that occur in the extracellular matrix of connective tissues. The fibrils are stabilized by covalent crosslinking, which is initiated by oxidative deamination of specific lysine and hydroxylysine residues in collagen by lysyl oxidase. (a) Scanning electron microscopy image of a human fibroblast adhered on reconstituted salmon collagen fibrillar matrix [17]. (b) Chemical structure of a collagen alpha-chain. (c) Atomic force microscopy image of reconstituted salmon collagen fibril [17]. The repeating broad dark and light zones (where  $D=67$  nm, the characteristic axial periodicity of collagen) can be seen in the fibril.

salmon-derived collagen (SC) [24]. Additionally, heat denaturation of the bio-inspired crosslinked SC gel provides elastic materials, which has the elongation of over 200% at break point [24]. So far, the elasticity of the collagens that had been crosslinked “after” fibrillogenesis is not as high as that crosslinked “during” fibrillogenesis (bio-inspired crosslinking) [25]. This may indicate that the bio-inspired crosslinking confer intrafibrillar

crosslinking as well as interfibrillar one, allowing for homogenous crosslinking sites and larger mechanical properties. Therefore, the bio-inspired crosslinking may provide more wide range of applications of collagen biomaterials. However, the bio-inspired crosslinking has not yet been applied to bovine collagen (BC), which is widely used for medical applications for past decades, because the bio-inspired crosslinking has been developed for thermal stabilization of “fish collagens” with low-denaturation temperature to develop marine-derived collagen biomaterials [13-17, 24-26]. In this study, we studied the crosslinking condition to create bio-inspired crosslinked BC gel and prepared the elastic material from the BC gel (e-BC gel) by heat treatment. The mechanical properties (tensile strength and elongation rate) and biological properties (biodegradability, cell culture, and blood compatibility) of the e-BC gel were evaluated. Herein, we report the fabrication of the bio-inspired elastic material from BC and demonstrate its applicability for biomaterials, especially in vascular tissue engineering.

## 2. The bio-inspired crosslinking conditions for BC

Acid-soluble collagen molecules self-assemble and form fibrils under physiological conditions. The pH, NaCl concentration, and temperature are important factors to provide a successful reconstituted collagen fibrillar gel. First, we evaluated the effect of NaCl concentrations on fibril formation of BC at constant pH of 7.4 and temperature of 37°C. The fibril formation of BC was monitored by a turbidity change observed at 310 nm [26, 27]. Figure 2 shows that a rapid rise in turbidity was observed in the mixture of BC solution and 30 mM Na-phosphate buffer at NaCl concentration from 50mM to 100 mM. Then, the rise in turbidity increase was gradually decreased at over 140 mM NaCl. The fibril formation rate of collagen is known to be reduced by addition of salts [4], which appears to reduce electrostatic interaction among collagen molecules. The bio-inspired crosslinking needs active fibrillogenesis during crosslinking (see below) [17, 26]. Therefore, the optimum range of NaCl concentration for BC fibril formation was determined to be 50-100 mM.

Cross-linking generally reinforces the biomaterials composed of collagen fibrils for further improvement of mechanical properties. Various techniques for stabilizing collagen have been developed and reported. These techniques are divided into chemical treatments and physical treatments. Glutaraldehyde is one of the most widely used chemical agents [28, 29]; it is known, however, that there are side effects to its use in cross-linking [30], for example, cytotoxicity, enhancement of calcification, and a mild inflammatory response compared with using other reagents. The water soluble condensing agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) has been reported to be significantly less cytotoxic than glutaraldehyde because EDC reagents do not remain in the linkage and are simply washed away during the cross-linking process [28, 29]. On the other hand, physical cross-linking methods such as UV irradiation [31, 32] and dehydrothermal treatment [33, 34] do not introduce any additional chemical units. These methods may therefore be more biocompatible than chemical treatments. However, the mechanical properties of materials cross-linked by physical treatments are lower than those cross-linked by chemical treatments. Therefore, EDC was used for a crosslinking agent in this study.

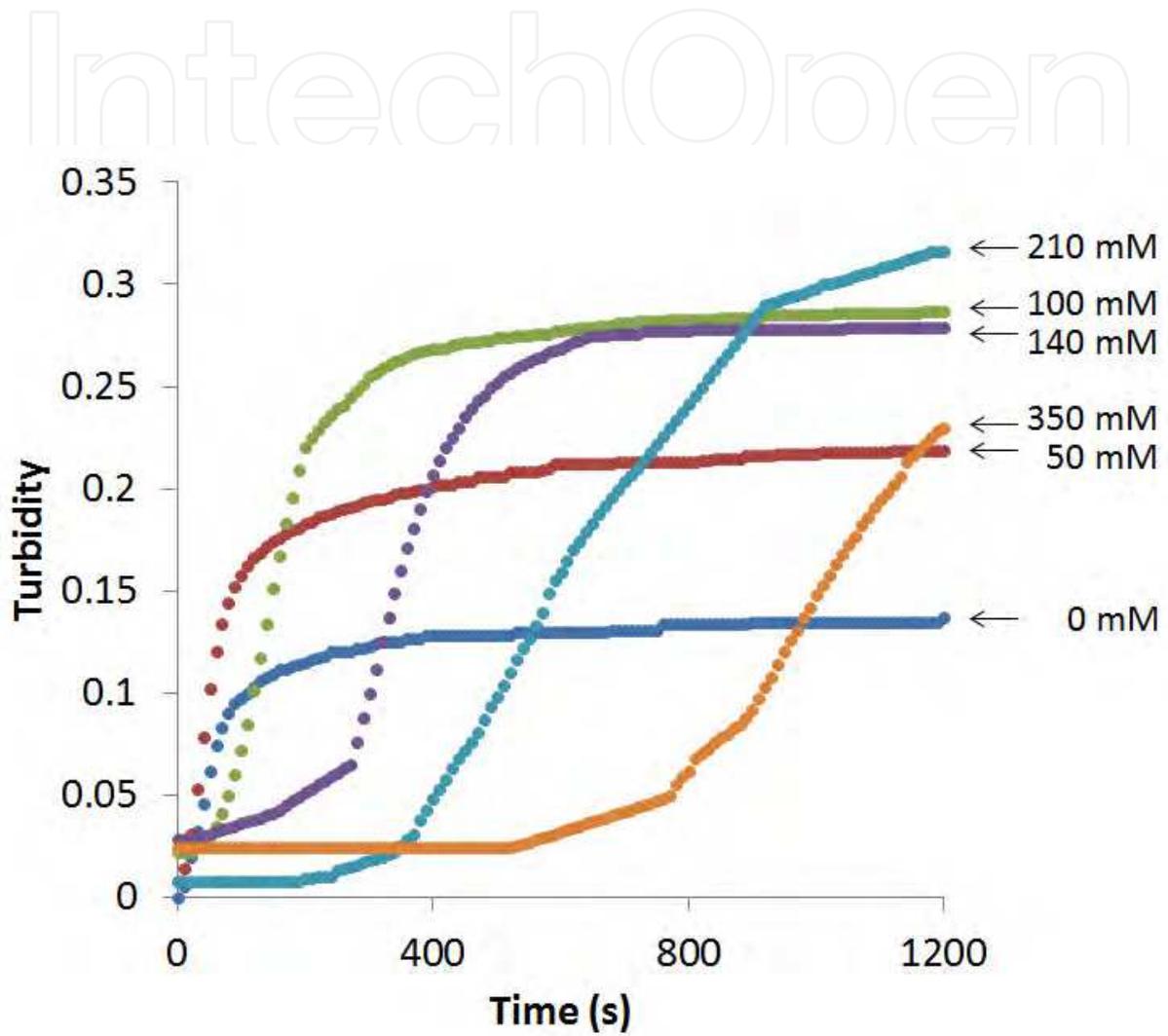


Fig. 2. Effect of NaCl concentration on fibril formation curve. The values in the graphs indicate the final NaCl concentrations in the gels.

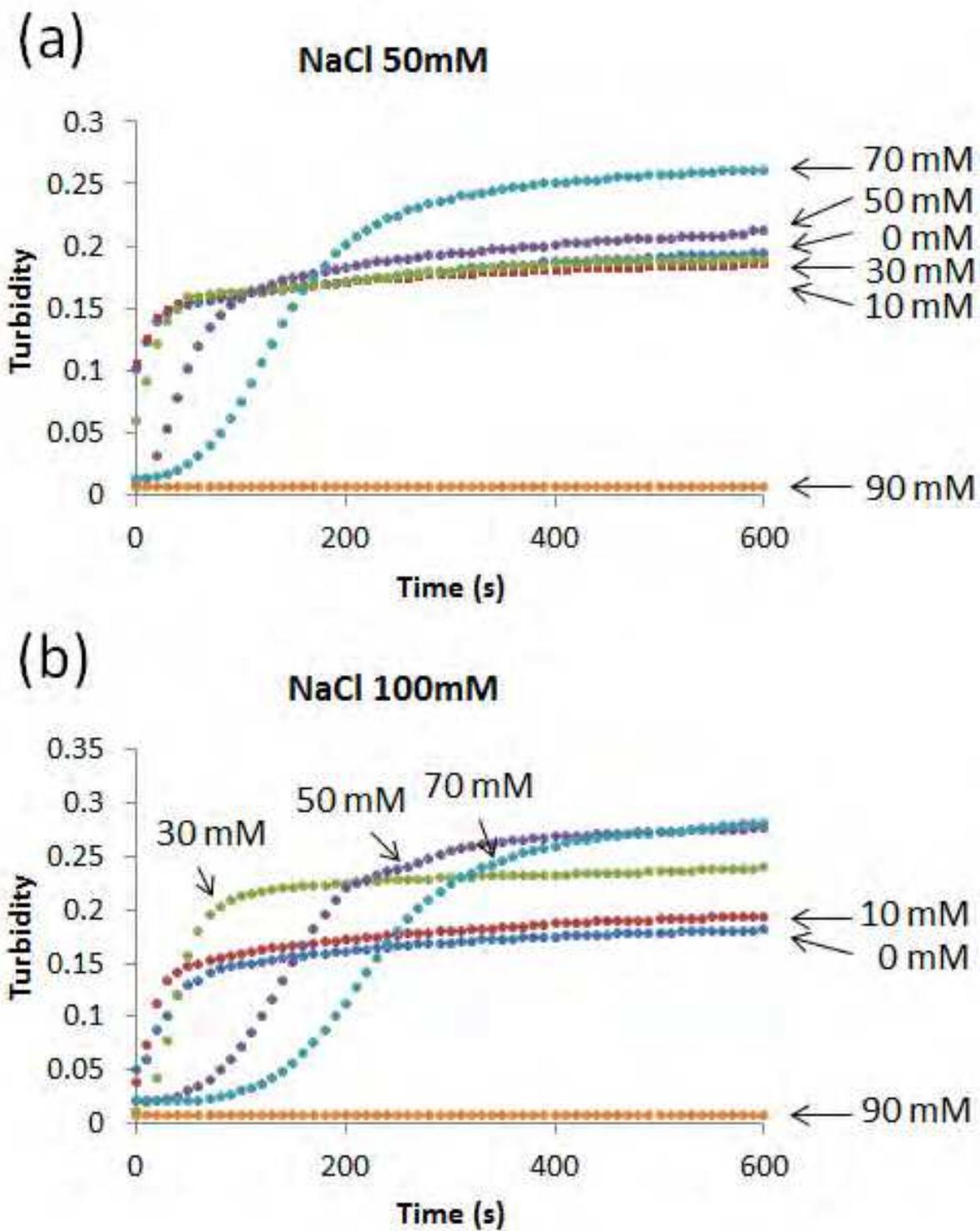


Fig. 3. Effect of EDC concentrations on fibril formation curve in the presence of 50 mM NaCl (a) and 100 mM NaCl (b). The values in the graphs indicate the final EDC concentrations in the gels.

The turbidity changes in BC solution mixed with Na-phosphate buffer including various EDC concentration at 50 mM or 100 mM NaCl were monitored using a spectrophotometer

(Fig. 3a, b). The rate of fibril formation decreased with increasing EDC concentration, which indicates EDC exhibited an inhibitory effect on collagen fibril formation. These inhibitory effects were probably the results of the rapid reaction of EDC to monomeric collagens upon mixing of the EDC containing buffer and acidic collagen solution, by which the ability to form fibrils was reduced or lost through random and nonfibrous aggregation of monomeric collagens. Further increased EDC concentration above 90 mM completely suppressed fibril formation irrespective of NaCl concentrations. Based on these results, it appeared that a buffer that would enable a faster fibril formation rate would be desirable. According to a previous report, EDC is sufficiently stable and active under such conditions [35].

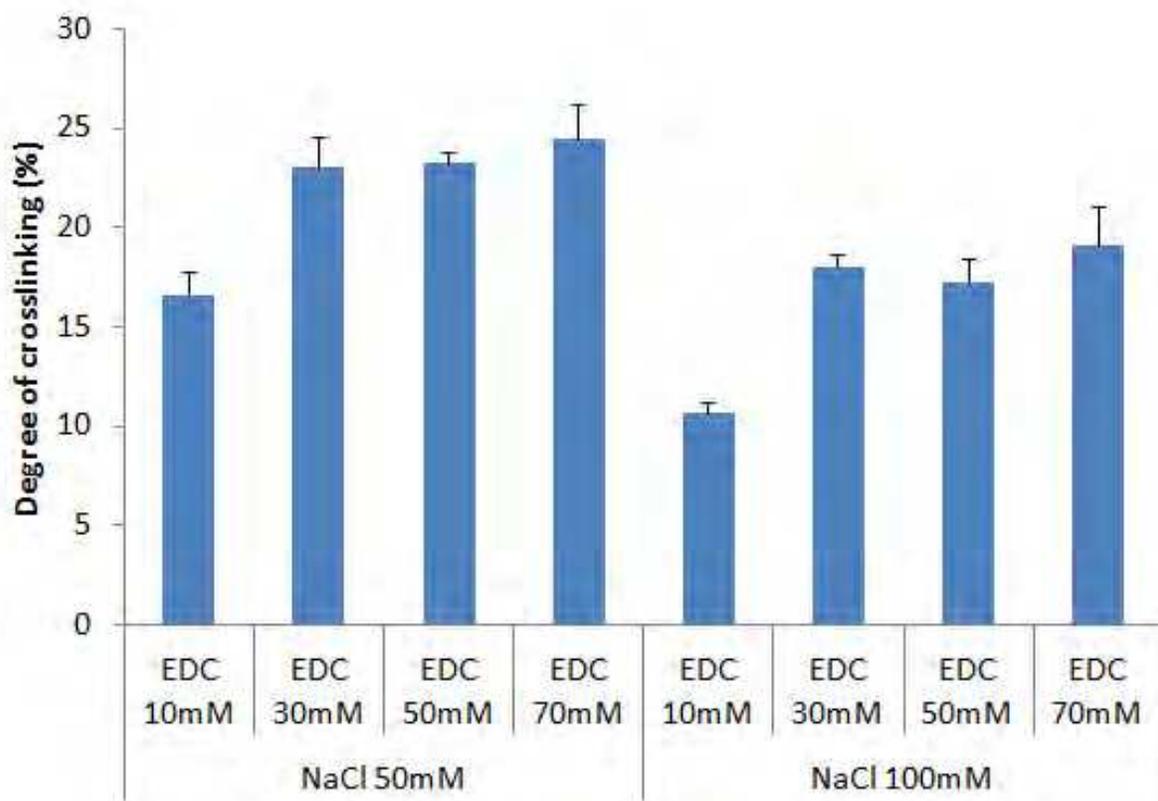


Fig. 4. Degree of crosslinking in the collagen. Values are mean  $\pm$  SD (n=4).

The degree of crosslinking was determined as the decrease in the free amino group content of the collagen molecules [19, 26]. The free amino group content was measured spectrophotometrically after the reaction of the free amino groups with 2,4,6-trinitrobenzenesulfonic acid and was expressed as the decrease in the ratio of the free amino group content of the crosslinked sample to that of the uncrosslinked sample. An increase in the degree of crosslinking with increase in the EDC concentration was observed (Fig. 4). The degree of crosslinking was slight lower at NaCl concentration of 100 mM compared to the NaCl concentration of 50 mM. This may be attributed to lower ability of fibril formation observed at NaCl concentration of 50 mM. Fig 2 shows that the plateau level in turbidity at 50 mM NaCl was lower than that at 100 mM NaCl. Lower fibril formation may result in an increase of nonfibrous aggregates, which can lead to high degree of crosslinking due to increased sites of crosslinking in monomeric collagens. The synergistic effects of crosslinking and fibril formation were thought to be complete at EDC and NaCl concentrations of 70 mM and 100 mM, respectively.

### 3. Fibril formation of BC

To produce an elastic material from bio-inspired crosslinked BC gel (e-BC gel), heat denaturation process is needed. By heat treatment, the cross-linked collagen fibrils shrink, maintaining the cross-linkage among the collagen molecules and fibrils through the denaturation of triple-helical collagen molecules to the random-coil form [24]. At the same time, uncross-linked collagen molecules and fibrils may be lost through dissolution to water. In fact, the original BC gels crosslinked with the EDC concentrations of 30-70 mM showed drastic shrinkage (Fig. 5) and rubber-like elasticity after heat treatment at 60°C for 5 min. The BC gels crosslinked with EDC concentrations of 0-10 mM dissolved away after heat treatment due to incomplete crosslinking.

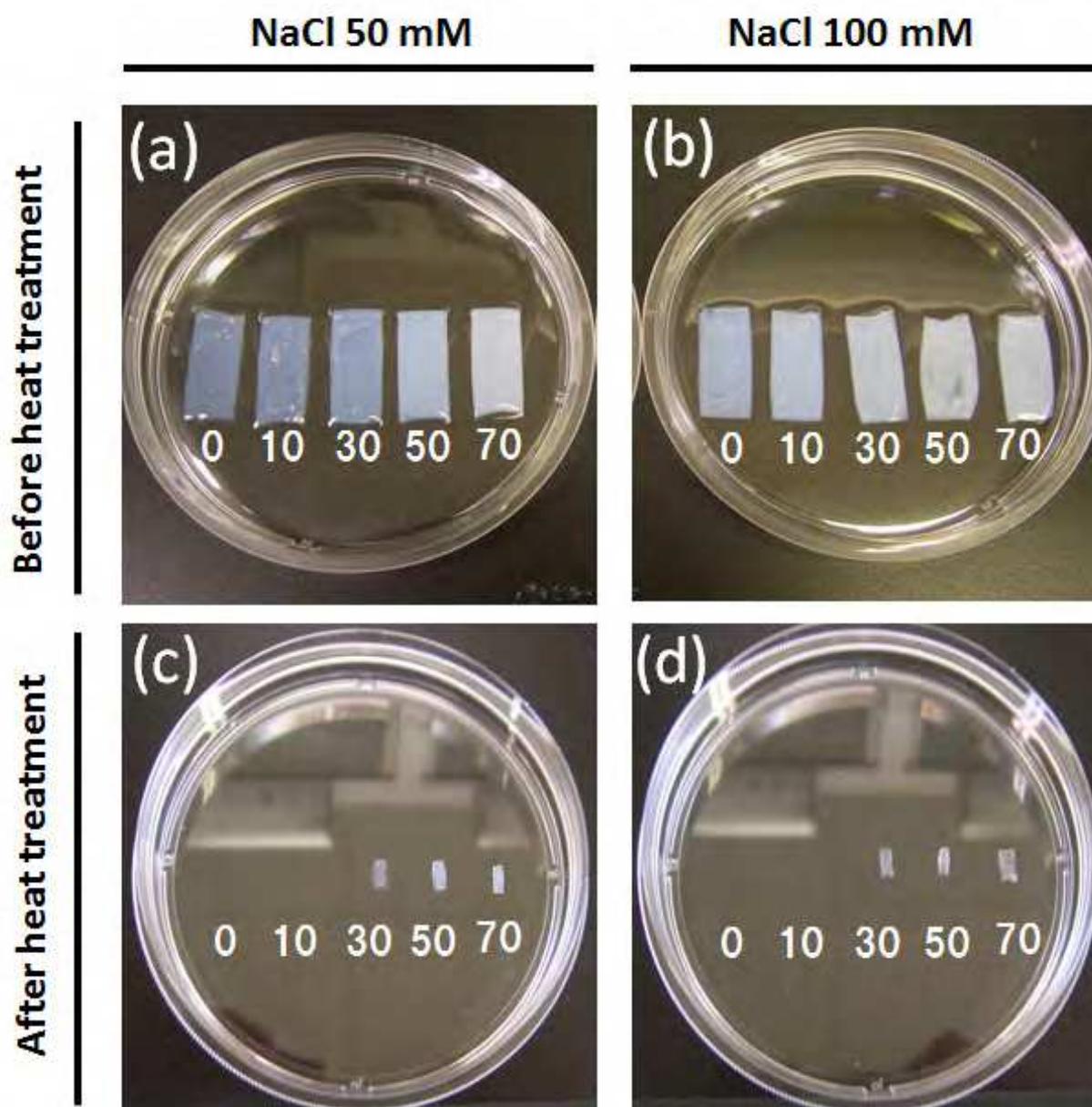


Fig. 5. Appearances of BC gels (a, b) and e-BC gels (c, d). The values in the graphs indicate the final EDC concentrations (mM) in the gels.

The collagen fibrils were observed by high-resolution scanning electron microscopy (SEM). The preparation of the specimen was performed according to a previous report [16, 24]. Figure 6 shows the well-developed networks of nano-fibrils on the BC gel and e-BC gel. The width of the fibrils on the BC-gel was in the range of 50–100 nm (Fig. 6a). However, a wider (width; >200 nm) and winding fibril-like structure was observed on the e-BC gel (Fig. 6b), indicating that the fibril structure of collagen was deformed through the heat treatment. The wide and winding fibril-like structure of the e-BC gel should be directly derived from the collagen nano-fibrils of the original BC gel through swelling of the fibrils by comparison of both surface structures.

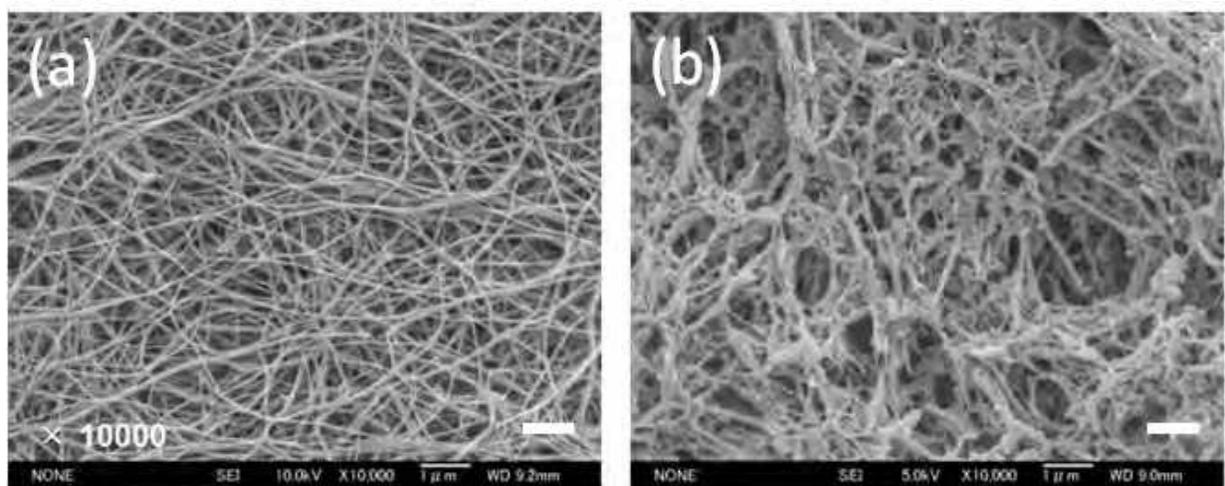


Fig. 6. SEM images of BC gels (a) and e-BC gels (c). Bars: 1  $\mu\text{m}$ .

#### 4. Mechanical properties of e-BC gel

The mechanical properties of the e-BC gel were evaluated by tensile tests. The original BC gel rarely had elasticity and stretchability similar to the usual collagen materials. However, the e-BC gel showed rubberlike elasticity and high stretchability. Figure 7 shows the stress-strain curves to the breaking point obtained in the strain rate of 0.1 mm/s ( $n = 5$ ). Salmon-derived elastic collagen gels (e-SC gel) were used as controls [24]. The mean values  $\pm$  standard deviation (SD) of elongation at the break of the e-BC gel and e-SC gel were  $201 \pm 47\%$  and  $260 \pm 59\%$ , respectively (Fig. 7c). At the early stage of loading, stress was almost linearly increased depending on the strain. Above a strain of ca. 100%, an increase in strain hardening was observed. There was no significant difference in elongation between the two e-gels. According to the report by Koide and Daito [31], collagen films reinforced by traditional cross-linking reagents, glutaraldehyde and tannic acid, showed only small elongation at the breaking point (6.6% and 12.4%, respectively). Weadock et al. showed small ultimate strains (approximately 40% and 30%) of collagen fibers cross-linked by UV irradiation and dehydrothermal treatment, respectively [36]. Even a purified skin with an intact fibrous collagen network gives elongation at a breaking point of 125% [36]. Recently, it was reported that a chemically cross-linked collagen-elastin-glycosaminoglycan scaffold, which are the contents analogous to the actual tissue/organs, demonstrated good stretchability (150% strain) [37]. To the best of our knowledge, this is the first report of a material from bovine collagen with elongation at a breaking point over 200%. Although the mechanism of the high stretchability was not well understood, the denaturation of the

collagen molecules probably plays an important role in the elongation, i.e., the bend structure of denatured collagen fibrils observed in Fig. 6b is considered to provide its rubber-like stretchability.

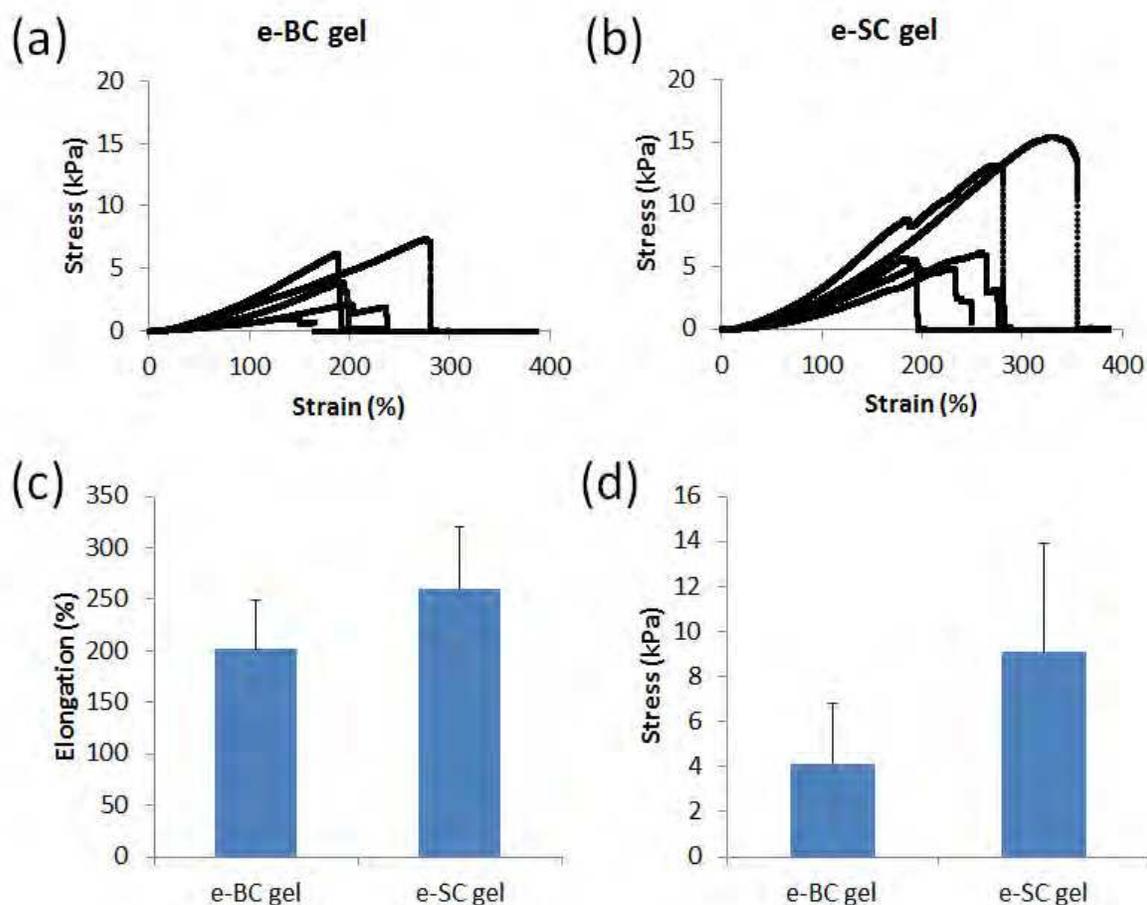


Fig. 7. Stress-strain curves generated from tensile testing of e-BC gels (a) and e-SC gels (b). The specimen ( $5 \times 3 \times 12$  mm) was gripped to achieve a gauge length of 8 mm and stretched in the strain rate of 1.25%/s. (c) Elongation at the break of the e-BC gel and e-SC gel. (d) Ultimate strength at the break of the e-BC gel and e-SC gel. Values are mean  $\pm$  SD (n=5).

The mean values  $\pm$  SD of ultimate strength at the break of the e-BC gel and e-SC gel were  $4.1 \pm 2.6$  kPa and  $9.0 \pm 4.8$  kPa, respectively (Fig. 7d). The strength of the e-BC gels was lower than that of e-SC gels, although there was no significant difference between the two. Collagen exhibits a limited mechanical resistance so that collagen requires an additional skeletal material such as inorganic materials [38]. Bio-inspired crosslinking can provide a collagen scaffold with high mechanical strength as well as stretchability. It is useful without the addition of any other material. Because a collagen solution is a precursor for fabrication of various collagen forms, bio-inspired crosslinking would be a useful fabrication process of widely various collagen biomaterials.

## 5. Biological properties of e-BC gel

To investigate the potential of the e-BC gel for use as a cell culture scaffold, we measured the growth rates of human umbilical vein endothelial cells (HUVECs) cultured on the e-BC gel.

The cell number was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test [13]. The MTT test is an established method to determine viable cell number by measuring the metabolic activity of cellular enzymes. Figure 8 shows steady increases in cell number with culture time on both the e-BC gel and tissue culture plate (TCP). There was a lag time before steady increase in cell number on the e-BC gel. Semler et al. reported that cells shows high growth rate on matrices with high mechanical compliance such as TCP, whereas the cells aggregate three-dimensionally on matrices with low mechanical compliance such as collagen gel [39]. Therefore, the difference in the mechanical properties of the surface of the culture substrate might affect the initial rate of cell growth.

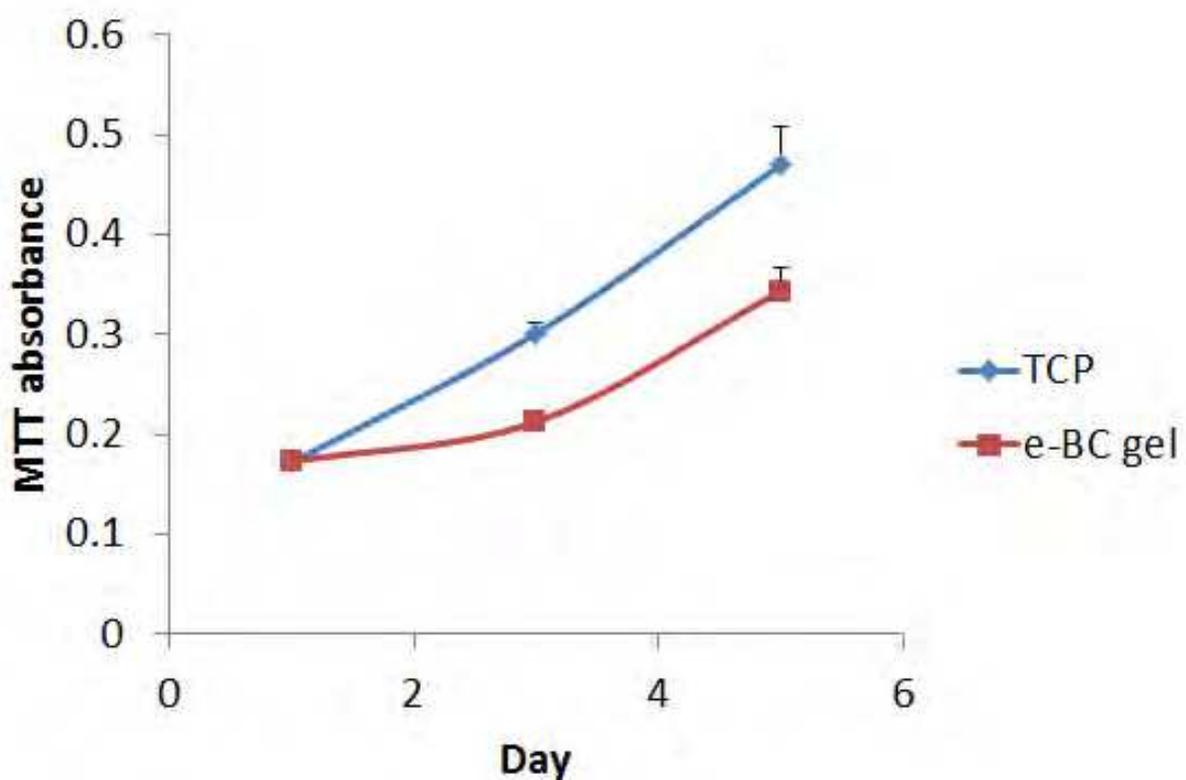


Fig. 8. Growth curves of HUVECs cultured on e-BC gels and plastic tissue culture plate. Values are mean  $\pm$  SD (n=4).

The SEM images show the distribution and spreading morphology of the HUVECs cultured on the e-BC gel at day 1 and day 5 after cultivation (Fig. 9). Apparently, the cells at 5d cultivation were confluent and showed cobblestone-like morphologies (Fig 9b). HUVECs also grow better on the e-SC gel and shows confluent at day 6 after cultivation [13]. Therefore, the e-BC gel could be used as a cell culture scaffold as well as the e-SC gel. EDC cross-links collagen molecules by the formation of isopeptides without being incorporated itself, thus precluding depolymerization and the possible release of potentially cytotoxic reagents. Furthermore, the by-product of the cross-linking reaction and un-reacted EDC in the e-BC gel should be completely removed by the drastic shrinkage in hot water. It is expected that the e-BC gel has good biocompatibility and no cytotoxicity.

To assess the degradability of the e-BC gel in collagenase solution (50 U/ml), protein content measurement was performed using a bicinchoninic acid protein assay kit as

previously described [40]. Figure 10 shows that e-BC gel was completely digested for 24 h as well as the e-SC gel. The e-BC gel degraded slightly later than the e-SC gel. This may be due to the low denaturation temperature of SC (19°C). The physiological concentration range of collagenase is approximately 1 U/ml [41], therefore, the e-BC gels might show the slow degradation profile *in vivo* than the *in vitro* results. Additionally, e-SC gel gradually degrades 1 month after implantation in rat subcutaneous pouches [16], e-BC gel, therefore, would show the same or later biodegradation profile *in vivo*.

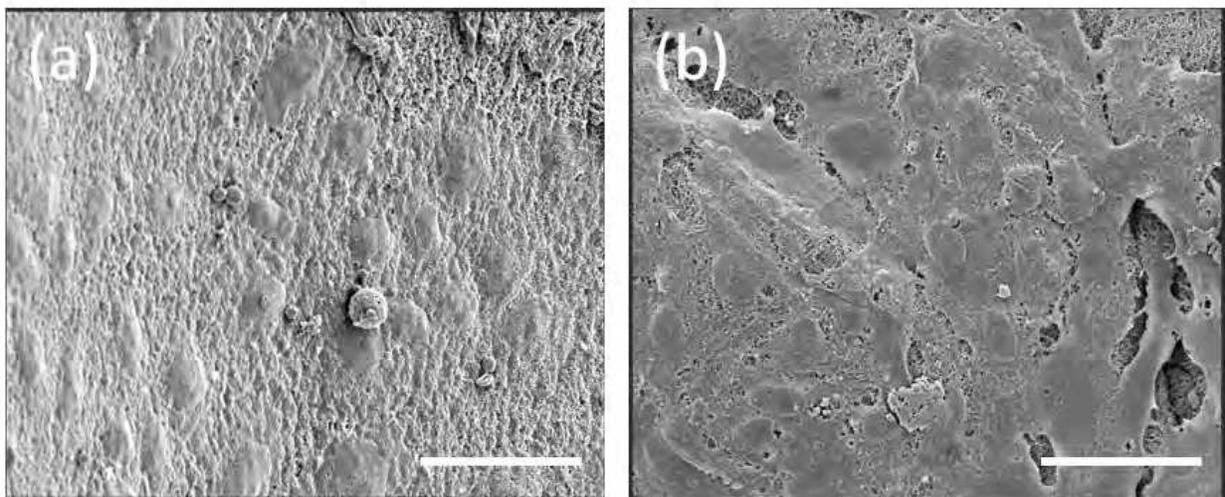


Fig. 9. SEM images of HUVECs cultured on e-BC gel for 1d (a) and 5d (b). Bars: 50 μm.

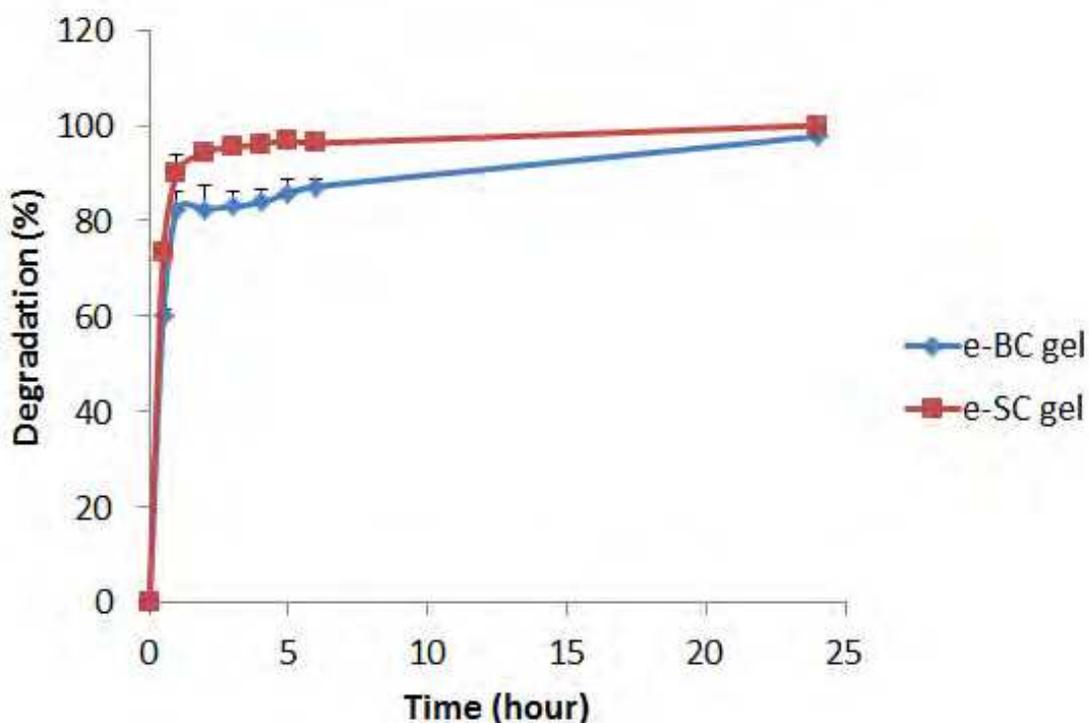


Fig. 10. In vitro degradation rate of e-BC gel and e-SC gel in collagenase solution. Values are mean  $\pm$  SD (n=4).

Owing to the mechanical, biological, and biodegradable properties of the e-BC gel, it could potentially be used to engineer blood vessels *in vivo*. Synthetic materials such as polyethylene terephthalate (Dacron) and expanded polytetrafluoroethylene (ePTFE) have been clinically applied as vascular grafts for a long time to replace or bypass large-diameter blood vessels. However, when used in small-diameter blood vessels (inner diameter < 6 mm), the patency rates are poor compared to autologous vein grafts. These failures are due to early thrombosis and gradual neointimal hyperplasia, and the pathological changes occurred due to the lack of blood or mechanical compatibility of the synthetic grafts [42]. To address this problem, tissue engineering approach is promising. A variety of biodegradable polymers and scaffolds have been evaluated to develop a tissue-engineered vascular graft [43-46]. These approaches depend on either the *in vitro* or *in vivo* cellular remodeling of a polymeric scaffold. For successful *in vivo* cellular remodeling, the biocompatibility, biodegradability, and mechanical properties of the scaffold must be suitable to the dynamic environment of the blood vessel. Therefore, the ideal scaffold should employ a biocompatible and biodegradable polymer with elastic properties that interact favorably with cells and blood. Therefore, the interaction of the e-BC gel with rat whole blood and plasma was investigated to assess their blood compatibility for use in vascular-tissue engineering.

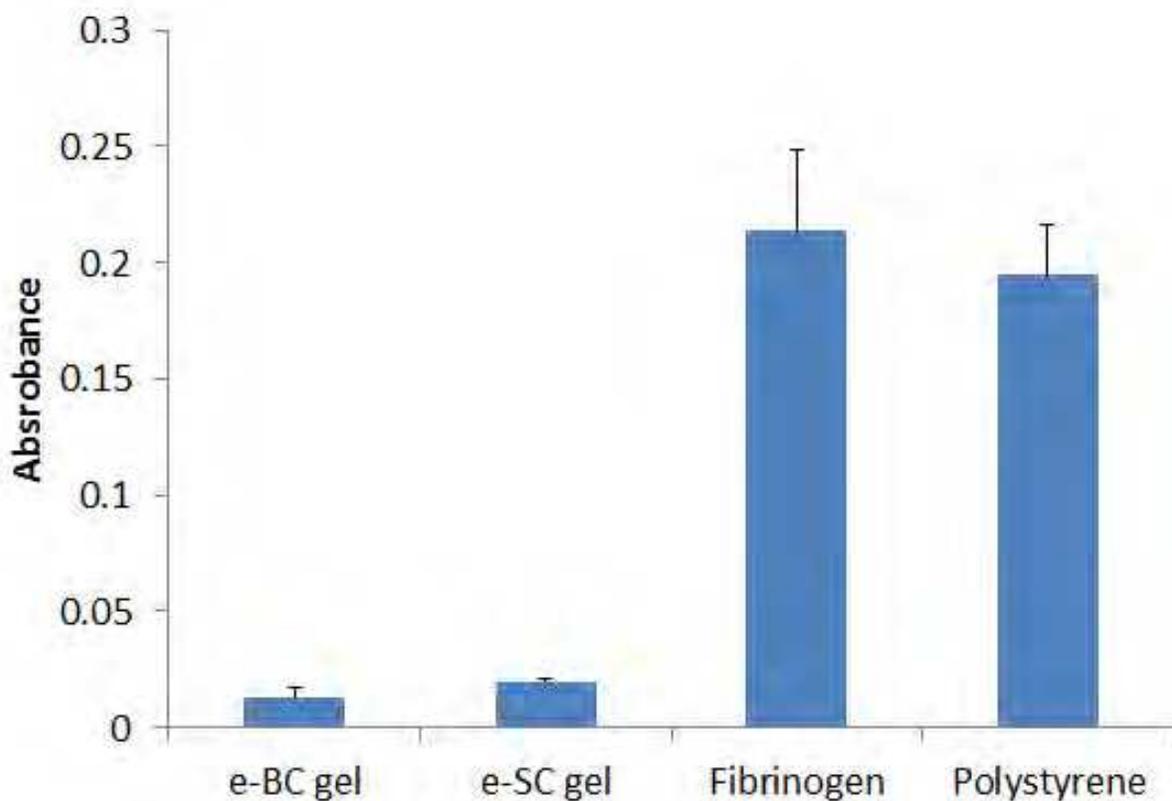


Fig. 11. Platelet adhesion rates on the e-BC gel, e-SC gel, and the control samples. Values are mean  $\pm$  SD (n=4).

After incubation of the e-BC gel with platelet-rich plasma (PRP) collected from rat blood, the colored *p*-nitrophenol produced by the acid-phosphatase reaction of the platelets was measured with a microplate reader at an absorbance of 405 nm. The percentage of adherent

platelets was calculated according to the methods reported previously [14]. Figure 11 shows the number of platelets that adhered to the samples. The platelet number was estimated from the acid phosphatase reaction [47]. There was a linear relationship between the PRP concentration and the absorbance values at 405 nm, indicating that the acid phosphatase reaction of the platelets may be considered a reliable indicator of platelet number (data not shown). The results demonstrate that the platelet adhesion rates were markedly low on the e-BC gel when compared to the fibrinogen-coated or polystyrene surfaces. The e-SC gel also showed an adhesion rate as low as the e-BC gel. We are separately studying the fabrication of a vascular graft using the e-SC gel [16]. Consequently, the e-BC gel can potentially be used for the fabrication of tissue-engineered vascular graft.

Considering that the platelets adhered better to the collagen-coated than to the gelatin-coated surface [14], the anticoagulant ability of the e-BC gel may have been due to heat denaturation. The e-BC gel was prepared by heat treatment at 60°C resulting in collagen denaturation (gelatinization). Polanowska-Grabowska and coworkers reported that the platelet adhesion rate on a gelatin-coated surface was lower than on collagen-coated or fibrinogen-coated surfaces [48]. However, blood coagulation is known to depend on material properties, such as surface-free energy, surface charge, and wettability; these properties govern protein adsorption involving platelet adhesion [49, 50]. Experiments using human whole blood are needed to test the clinical applications of the gels. Further examinations are necessary to ensure the blood compatibility of the e-BC gel.

## 6. Conclusion

In conclusion, we successfully fabricated an elastic collagen material from EDC cross-linked BC fibrillar gel by heat treatment. "Bio-inspired crosslinking" used in this study involves collagen fibril formation in the presence of EDC as a crosslinking reagent, which was developed in an attempt to mimic the *in vivo* simultaneous occurrence of fibril formation and crosslinking. We successfully prepared the bio-inspired crosslinked BC gels by adjusting the NaCl and EDC concentrations during collagen fibril formation. An advantage of bio-inspired crosslinking is the achievement of homogenous intrafibrillar crosslinking as well as interfibrillar one, providing higher mechanical properties compared to the traditional sequential crosslinking in which monomeric collagen initially forms fibril, then subsequently crosslinked using chemical or physical methods. Another advantage is the elastic properties of bio-inspired crosslinked BC gels after heat treatment. Although common collagen materials dissolved in water at a temperature above their denaturation temperature, we found that the bio-inspired cross-linked BC gel drastically shrank at a high temperature without remarkable dissolution. The collagen gel obtained interestingly showed rubber-like elasticity and high stretchability. The human cells showed good attachment and proliferation on this elastic material, suggesting its potential to be utilized in biomaterials for tissue engineering. Additionally, the elastic material demonstrated excellent blood compatibility. Our future work will focus on fabrication of small-caliber tubes (inner diameter < 6 mm) for small-caliber vascular grafts and preclinical animal studies to further assess the safety and effectiveness of the collagen-based vascular grafts.

## 7. Acknowledgment

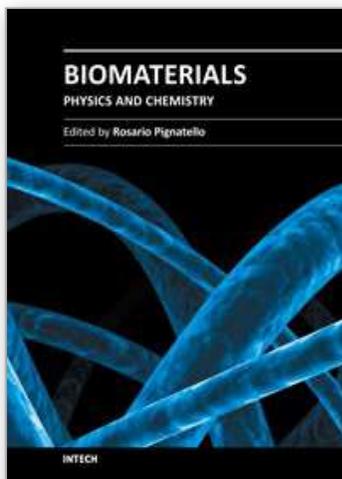
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