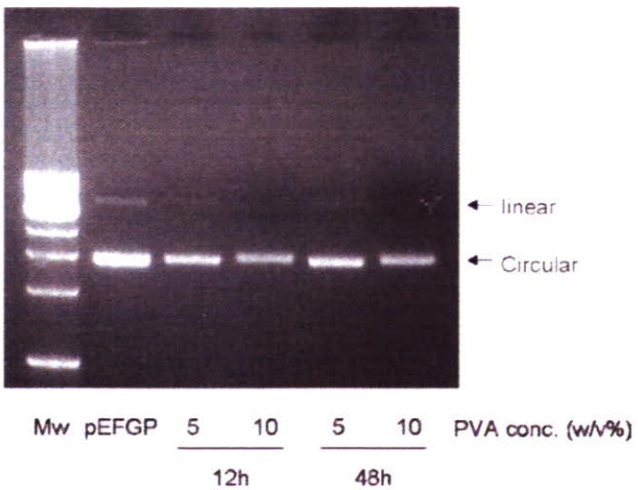

Fig. 4. Agarose gel electrophoresis of plasmid DNA (pDNA) released from PVA/pDNA hydrogels with PVA concentrations of 5% and 10% w/v produced by pressurization at 10000 atm and 37°C for 10 min after immersion in phosphate-buffered saline for 12 and 48 h

from the PVA/DNA hydrogels was stable. Two bands of linear and circular plasmid DNA were observed with 5% w/v PVA/DNA hydrogel, while circular plasmid DNA was released from the 10% w/v PVA/DNA hydrogel, indicating that the linear form of plasmid DNA tends to interact more strongly with PVA than the circular plasmid DNA.

Conclusions

Novel PVA/DNA hydrogels crosslinked physically by hydrogen bonds were developed using UHP technology. DNA released from the hydrogels was controlled by varying the PVA concentration and pressurization conditions, such as the level and duration of pressure used to form the hydrogels. The demonstrated stability of the DNA released from the hydrogels suggests that PVA/DNA hydrogels have potential as a candidate for gene delivery.

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Physical and biological properties of collagen-phospholipid polymer hybrid gels

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Abstract

We successfully developed a novel method for immobilizing poly(2-methacryloyloxyethyl phosphorylcholine) [Poly(MPC)] polymer onto collagen using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as cross-linkers. In order to obtain the highest possible molar ratio of immobilized MPC moieties on the collagen gel, a collagen-phospholipid polymer hybrid gel was prepared by repeating the cross-linking process up to three times to create a dense network of collagen and PMA. Network formation by repeating the immobilization process was successful, resulting in decreased free amine group content and a low swelling ratio. The hybrid gel displayed very high stability against degradation by collagenase and possessed high hydrophilicity. Fibrinogen adsorption and cell adhesion were reduced and demonstrated less cell proliferation as compared to that by uncross-linked collagen gel. The collagen-phospholipid polymer hybrid gel did not exhibit toxicity, and the cell morphology remained intact (round); this implies that the interaction between the cell and the collagen-phospholipid polymer hybrid gel is safe and mild.

Keywords: Collagen; Phospholipid polymer; Immobilization; Protein adsorption; Cell adhesion

1. Introduction

In order to use collagen as a biomaterial product, cross-linking of collagen and/or immobilizing synthetic polymers onto collagen are indispensable measures. Non-treated natural collagen cannot be directly applied to a biological system due to drawbacks such as poor mechanical strength, calcium deposition, and high thrombogenicity. However, collagen is biocompatible, non-antigenic, synergic with bioactive components, easily modifiable, and abundantly available; these attributes render it suitable for medical application [1]. Hence, the undesirable properties of collagen should be eliminated while retaining its desirable properties.

When cross-linking the collagen gel, it should be ensured that the cross-linker is not toxic and does not affect biocompatibility. Preparing a cross-linked collagen gel does not necessarily require chemical cross-linking. Diverse methods such as chemical and physical cross-linking, UV irradiation, and blending have been used to cross-link collagen [2–6]. Among these, cross-linking using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) was chosen for this study [7–9]. Cross-linked collagen with EDC and NHS results in "zero-length" amide cross-links between the carboxylic acid groups from aspartic and glutamic acid residues, and the ε-amino groups from (hydroxy-)lysine residues [9]; these form intra- and interhelical cross-links to provide an EDC/NHS cross-linked collagen gel. A 2-methacryloyloxyethyl phosphorylcholine (MPC)-based copolymer, namely, poly(MPC-*co*-methacrylic acid) (PMA), which is also a well-known hemocompatible material [10], was used to cross-link the microfibrils of collagen to produce a collagen-polymer hybrid gel [11].

In our previous study, we discovered that the collagen-polymer hybrid gel could be prepared efficiently under alkaline pH conditions. Immobilization of PMA onto collagen would cover the entire collagen surface, increase the mechanical strength, reduce water absorption, and

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impart durability against collagenase degradation. However, a high percentage of MPC did not immobilize on the collagen gel surface. A longer coupling time or use of larger amount of EDC, NHS, or PMA did not result in an increase in the amount of immobilized PMA. This is believed to be attributable to spatial problems. For immobilization of heparin, low adoption of the polymer with the EDC/NHS coupling reaction was considered a problem. With regard to heparin, it is known that approximately 5.5% immobilization can be achieved [12]. However, activation of the carboxylic group can be triggered at any time point [7]. The coupling reaction continues when EDC/NHS is re-added to collagen gel. Based on this, we developed a novel technique to immobilize the polymer—the "repeat immobilization process."

The repeat immobilization process comprises simply repeating the process of immobilization on the surface of the pre-activated polymer, and a polymer-immobilized collagen gel is thus obtained. The rationale for this process is based on the following: (1) the carboxyl groups can be activated at any time point, and (2) a high percentage of unreacted amine groups are available. Re-activation may promote formation of additional amide bonds, which could not be achieved by a longer coupling time or the use of a higher amount of EDC, NHS, or PMA. In this study, we repeated the immobilization process until a collagen gel with the highest possible number of phospholipid head groups was obtained. Using this gel, we characterized the physical and biological properties of the collagen-polymer hybrid gel. To distinguish the collagen-polymer hybrid gel prepared by the repeat immobilization process from that prepared by time control, we named the former as "collagen-phospholipid polymer hybrid" (a CoPho gel).

2. Experimental methods

2.1. Preparation

2.1.1. Synthesis of PMA

PMA was synthesized according to a previously published method [11,13]. In brief, MPC and methacrylic acid (MA) were dissolved in an ethanol solution. Subsequently, a specific amount of 2,2-azobisisobutyronitrile (AIBN) was added to the ethanol solution. Polymerization was performed in a completely sealed round-bottom flask for 16 h at 60 °C. The solution was precipitated into diethyl ether, freeze-dried, and stored in vacuo until further use. The molar ratio of PMA was MPC:MA = 3:7, and the average molecular weight was 3×10^5.

2.1.2. Preparation of EDC and NHS cross-linked collagen gel (EN gel)

Cross-linked collagen gel was prepared by a previously reported method [11]. Instead of the 0.5 wt% collagen type I solution (pH 3; KOKEN, Tokyo, Japan), 2 wt% collagen type I solution was prepared and used for the film preparation. The collagen solution was dropped onto the polyethylene film and allowed to dry at room temperature. The collagen film (thickness = 56±3 μm) was immersed in a 0.05 M 2-morpholinoethane sulfonic (MES) acid buffer (pH 9.0) (Sigma, St Louis, USA) containing EDC (Kanto Chemicals, Tokyo, Japan) and NHS (Kanto Chemicals). The molar ratio of the constituents was EDC:NHS:-collagen-carboxylic acid groups = 10:10:1. The cross-linking procedure was allowed to proceed at 4 °C for 4 h to produce a cross-linked gel (EN gel). After 24 h, the reaction was terminated by removing the gel from the solution. Subsequently, the gel was first washed with a 4 M aqueous Na_2HPO_4 solution for 2 h to hydrolyze any remaining O-acylisourea groups and subsequently with distilled water over a duration of 3 days to remove traces of salts from the gel.

2.1.3. Preparation of MPC-immobilized gel (MiC gel, MdC gel, and MtC gel; CoPho gel)

MPC-immobilized collagen gel (MiC gel) was prepared using uncross-linked collagen gel (immersed in an alkaline solution at pH 9.0 for 30 min) or EN gel [11]. PMA was added to the MES buffer (pH 9.0) along with EDC and NHS and was activated for 10 min before the uncross-linked collagen or EN gel was immersed. The molar ratios of each chemical was fixed; EDC:NHS:collagen-carboxylic acid groups = 10:10:1. The immobilization of PMA to collagen continued for 48 h at 4 °C. Subsequently, the gel was first washed with 4 M aqueous Na_2HPO_4 solution for 2 h and then with distilled water for 1 day to remove traces of salts and thus prepare a salt-free MiC gel. Fig. 1 illustrates the basic scheme for activation of PMA by EDC and NHS immobilization on collagen. To increase the number of MPC moieties on the collagen-polymer hybrid gel, a MPC-double immobilized collagen (MdC) gel was prepared by immobilizing PMA on the MiC gels by using the same procedure as earlier. To investigate the possibility of further immobilization, we prepared a MPC-triple immobilized collagen (MtC) gel using MdC as the base collagen for PMA immobilization. MiC-0, MdC-0, and MtC-0 were prepared from uncross-linked collagen gels; MiC-1, MdC-1, and

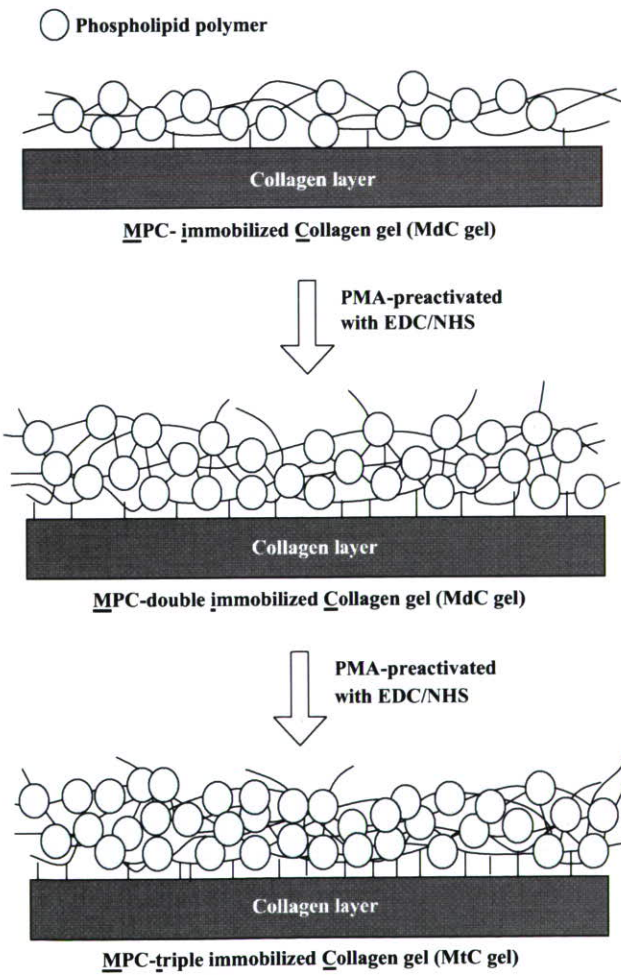


Fig. 1. Schematic diagram of the immobilization process of PMA on collagen.

MtC-1, from EN gels. The terminology used for the gel samples is listed in Table 1.

2.1.4. Preparation of glutaraldehyde cross-linked collagen gel (G-gel)

Cross-linking collagen gel to glutaraldehyde was performed by a previously reported method [14]. In brief, a 25% glutaraldehyde solution (Merck, Damstadt, Germany) was diluted to 0.5 wt% in phosphate buffer solution (PBS). The collagen film was immersed in the glutaraldehyde/PBS solution and was cross-linked for 3 h at room temperature. After cross-linking, the sample was first rinsed in running tap water for 30 min and then in 4 M NaCl for 2 h. In order to eliminate NaCl, the sample was rinsed with distilled water for 1 day to yield a glutaraldehyde cross-linked collagen gel (G-gel). The physical and biological properties of this gel were compared with those of the CoPho gels.

2.2. Characterization

2.2.1. Surface analysis

Surface analysis was executed using X-ray photoelectron spectroscopy (XPS; AXIS-HSi, Shimadzu/KRATOS, Kyoto, Japan) and static contact angle (SCA; ERMA-G1, Tokyo, Japan). The samples that had been cut into small pieces were lyophilized overnight. The chemical composition of the gel surface was determined by the releasing angle of the photoelectrons fixed at 90°. SCA measurement was performed by using a contact angle goniometer (ERMA-G1, Tokyo, Japan) and a Bil-mont syringe. The contact angle of the drop on the surface was measured at room temperature. The SCA experiment was repeated 7 times, and the average was calculated together with the standard deviation.

2.2.2. Determination of the reacted amine group content

The concentration of the primary amine group in tissue samples was determined using a colorimetric assay [15,16]. Three to four milligrams of each sample was prepared. These samples were placed in a 4 wt% aqueous $NaHCO_3$ solution (Kanto Chemicals, Tokyo, Japan) and 2,4,6 trinitrobenzene sulfonic (TNBS) acid. Subsequently, 0.5 wt% aqueous TNBS solution was added (Wako chemicals, Osaka, Japan). The reaction was allowed to proceed for 2 h at 40 °C; the samples were rinsed with saline solution in a vortex mixer to remove unreacted TNBS. Subsequent to freeze-drying the samples overnight, the dry mass was determined. The dry samples were immersed in 2 mL of 6 M aqueous HCl until fully dissolved. The resultant solution was subsequently diluted with distilled water (8 mL) and absorbance was measured at 345 nm (V-560, Jasco, Tokyo, Japan). The concentration of reacted amine groups was calculated using the following equation [16]:

$$[NH_2] = \frac{A \times V}{\varepsilon \times l \times m}, \quad (1)$$

where $[NH_2]$ denotes the reacted amine group content (mol/g collagen gel), ε the molar absorption coefficient of trinitrophenyllysine (1.46×10^4 mL/mmol cm), A the absorbance, V the volume of the solution (mL), l the path length (cm), and m the weight of the sample (mg). The reacted amine group contents of respective collagen gels were all compared with Uc gel.

2.2.3. Swelling test

The swelling test of the samples was executed by cutting the lyophilized gels into small pieces and placing them in a neutral pH aqueous solution at 37 °C. The pH of the aqueous solution was adjusted to 7.4. The gels were gently shaken for 24 h and were measured for assessing the change in weight of the sample. Swelling ratio was calculated in order to define the swelling phenomenon accomplished by water absorption. The experiment was repeated 5 times and the average was calculated along with standard deviation. The following equation was used to calculate the swelling ratio.

$$\text{Swelling ratio, } S(\%) = \frac{W_h - W_d}{W_d} \times 100,$$

where W_h denotes hydrated weight of the gel and W_d the dry weight of the gel.

2.2.4. Fibrinogen adsorption test

Bioresponse was evaluated in terms of protein adsorption by using bovine plasma fibrinogen. The concentration was adjusted to 1 mg/mL. First, the collagen gels were equilibrated by immersing them in PBS. Subsequently, the gels were transferred to the fribrinogen solution, and the solution was incubated for 3 h. After rinsing with PBS, the adsorbed fibrinogen was recovered by dipping the samples in 1 wt% *n*-sodium dodecyl sulfate (SDS) for 60 min [17]. The concentration of recovered fibrinogen was determined at 490 nm by using a Micro BCA kit (Bio-rad, Model 680, Tokyo, Japan).

2.2.5. Cell adhesion test

The interaction between the L929 cells (mouse fibroblast) and the collagen gels was evaluated. The fibroblasts were cultured in Eagle's Minimum Essential Medium (E-MEM, Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA) at 37 °C in 5% CO_2 atmosphere. After treatment with 0.25% trypsin, the cell density was adjusted to 5×10^3 cells/mL and the cells were seeded on the gel surface [18,19]. The collagen gels were sterilized by placing the gels first in an ethanol:water (50:50) solution for 2 h, then in a 70:30 solution for 2 h, and overnight in a 100:0 solution before lyophilization. The lyophilized gels were hydrolyzed with E-MEM for 5 min, and the E-MEM was disposed immediately prior to cell seeding. After 24- and 48-h cycles, the number of adhering cells was measured using UV–vis spectrophotometer (V-560, Jasco, Tokyo, Japan) at 560 nm by lactate dehydrogenase (LDH) assay. All experiments were repeated 3 times and the average was calculated together with the standard deviation.

The morphology of the L929 cells after the 48-h incubation period was observed using scanning electron microscopy (SEM). The cells attached to the samples were rinsed with PBS and fixed with 2.5% glutaraldehyde. Subsequently, the samples were dehydrated for 15 min using an ethanol dilution series (10%, 30%, 50%, 70%, and 90%) before the final dehydration with 100% ethanol. After dehydration, the samples were first dried at room temperature and then in vacuum. In order to avoid deformation of the cells, all samples were fixed onto the glass cover prior to vacuum drying.

Table 1
Terminology of collagen gels used in this study

Terminology		Composition
Uc-gel		Uncross-linked gel (stabilized under pH 9.0)
EN gel		EDC/NHS-cross-linked collagen gel under pH 9.0
CoPho gel	MiC-0	PMA immobilized on Uc-gel under pH 9.0
	MdC-0	PMA immobilized on MiC-0 gel under pH 9.0
	MtC-0	PMA immobilized on MdC-0 gel under pH 9.0
	MiC-1	PMA immobilized on EN gel under pH 9.0
	MdC-1	PMA immobilized on MiC gel under al pH 9.0
	MtC-1	PMA immobilized on MdC gel under pH 9.0

2.2.6. Cell viability test

The cell viability test was executed using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, USA) assay. The cells were cultured on each test specimen (5000 cells/well) for 48 h and were washed twice with PBS. Subsequently, 200 μl of the MTT solution (0.5 mg/mL in medium, filter sterilized) was added to the culture wells. After incubation for 4 h at 37 °C in a 10% CO_2 atmosphere, the MTT reaction medium was removed and blue formazan was solubilized by the addition of 100 μL dimethylsulfoxide (DMSO). The optical density readings were subsequently performed at 570 nm by using the Micro BCA kit.

2.2.7. Statistical analysis

All the experiments were repeated at least 3 times and the values were expressed as mean ± standard deviation. In several figures, the error bars are not visible because they are included in the plot. Statistical analysis was performed using Student's *t*-test with the significant level set as $P<0.05$.

3. Results

Atomic concentration percentage of phosphorus is shown in Table 2. Phosphorus was not detected in the case of Uc gel and EN gel, while phosphorus was detected in the case of CoPho gels. The phosphorus concentration increased for MdC gels, but significant increase in the phosphorus atomic concentration was not shown for MtC gels.

SCA of the respective collagen gels was measured and is illustrated in Fig. 2. The SCA for Uc and EN gels was approximately 70°. SCA decreases as a result of repeating the immobilization process, and was 20° for MtC-0 and -1 gels, thus indicating that the CoPho gel was acquiring a hydrophilic nature.

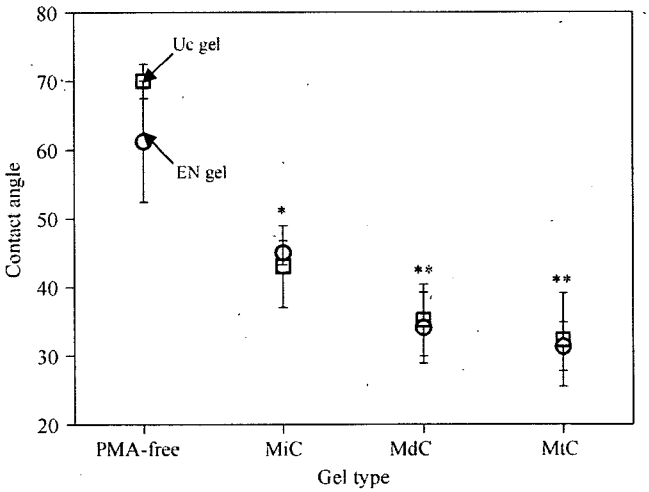
Fig. 3 illustrates the results of the reacted amine group content of the respective collagen gels. It decreases from approximately 60–30% as the PMA is immobilized compared to Uc gel. The lowest reacted amine group content was observed for G-gel, which was approximately 15% of Uc gel.

Fig. 4 illustrates the swelling ratio of the collagen gels under acidic and neutral pH conditions. Uc gel dissolved in acidic pH conditions (pH 2.4) and swelled to approximately 350% in neutral pH conditions (pH 7.4). The swelling ratio decreased rapidly on execution of cross-linking. In terms of the swelling ratio, the two gels did not differ substantially under neutral pH conditions, contrary to the situation under acidic pH conditions. However, a decrease in the swelling ratio was observed; after re-immobilization it decreased from 130% for MiC-1 gel to 95% for MtC-1 gel.

Degradation by collagenase demonstrated that cross-linking decreases the degradation rate of collagen gels (Fig. 5). Uc gels, which completely degraded within 6 h, remained undegraded for 24 h when cross-linked with EDC/NHS alone. Immobilization stabilized the gels against degradation by collagenase.

Fig. 6 illustrates the results of fibrinogen adsorbed on the surface of the collagen gel. It can be clearly seen that fibrinogen adsorption decreased as cross-linking proceeded. The amount of adsorbed fibrinogen further decreases as the MPC polymer is immobilized.

Fig. 7 illustrates the results of the cell adhesion test. Here, we discovered that repeated immobilization of PMA suppressed cell adhesion. Comparison of cell adhesion on completion of 24- and 48-h cycles revealed that the number of adhered cells for in the case of the Uc gel after 48-h cycle had increased by approximately 2 times; the rate of

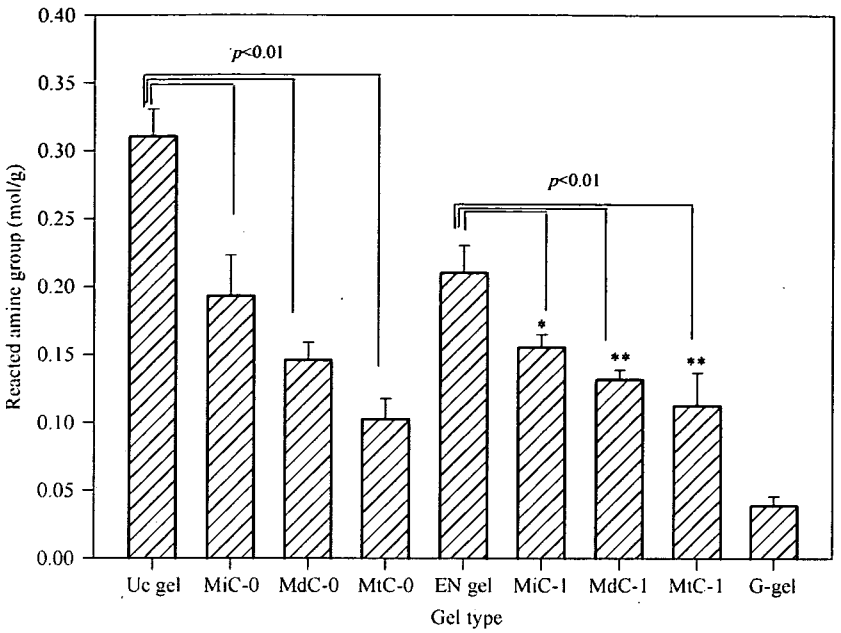


Uc gel and EN gel $p < 0.01$ vs. * and **
* $p < 0.01$ vs. **

Fig. 2. The static contact angle of the collagen gels. Each value represents the mean ± SD ($n = 5$).

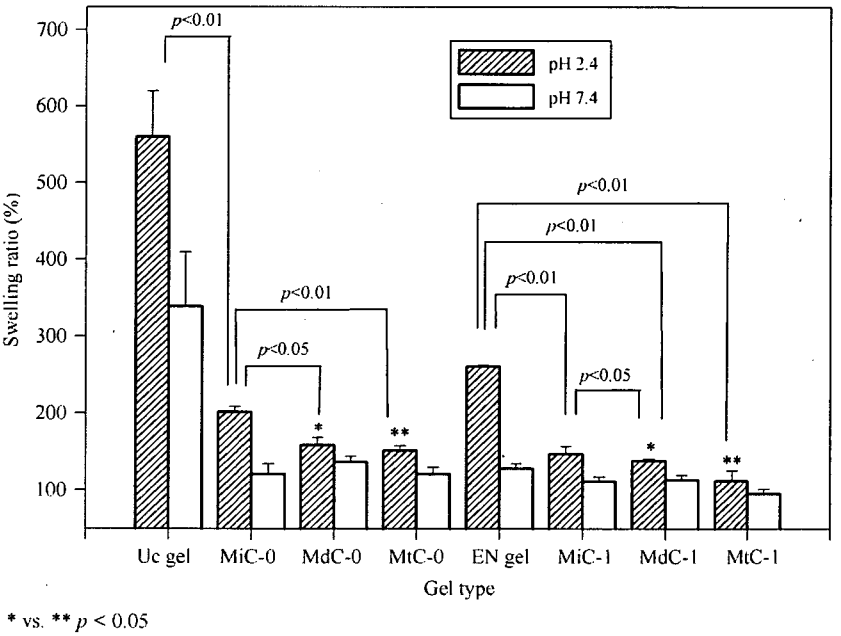
Table 2
Atomic phosphorus concentration of respective collagen gels

Gel type		Atomic phosphorus concentration (%)
Uc gel		0
EN gel		0
CoPho gel	MiC-0	0.21 ± 0.06
	MdC-0	0.36 ± 0.05
	MtC-0	0.37 ± 0.06
	MiC-1	0.18 ± 0.02
	MdC-1	0.37 ± 0.09
	MtC-1	0.36 ± 0.05



* vs. ** $p < 0.05$

Fig. 3. The percentage of free amine group contents of respective collagen gels. Each value represents the mean ± SD ($n = 5$).



* vs. ** $p < 0.05$

Fig. 4. Swelling ratio of the collagen gels under acidic pH conditions (pH 2.1) (hatched bar) and under neutral pH conditions (pH 7.4) (empty bar). Each value represents the mean ± SD ($n = 5$).

increase decreases as the cross-linking of the collagen gels is proceeded. When assessing the suppression of cell adhesion in terms of intra- and interhelical cross-links, we observed that higher suppression was considerably higher in gels with intra- and interhelical cross-link. MtC-1 gel displayed cell adhesivity that was similar to that of the G-gel.

Cell morphology observed using SEM (Fig. 8) demonstrated that the L929 cells were deformed on the non-MPC surface. On the other hand, the cells remained intact (round) on the CoPho gel surface. An increase in the density of the MPC head group resulted in a decrease in the distribution of L929 cells.

Fig. 9 illustrates the viability of the L929 cells after 48 h. It reveals that cell viability ranges from 96% to 115% (TCPS as 100%) [20]. Cell viability was approximately 70% for the glutaraldehyde cross-linked collagen gel. Immobilization of PMA did not induce any toxicity.

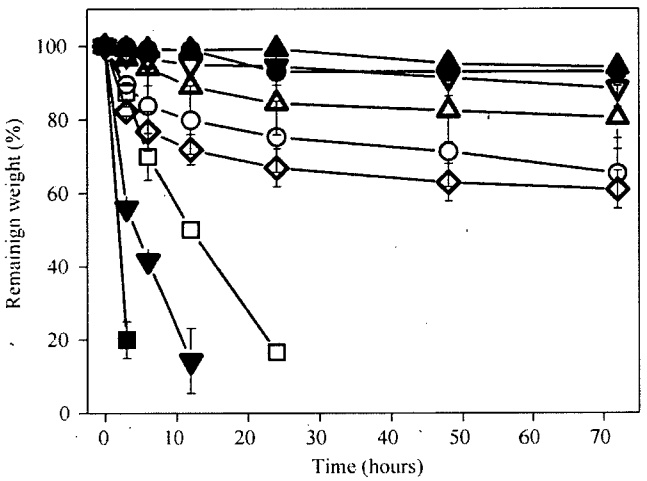


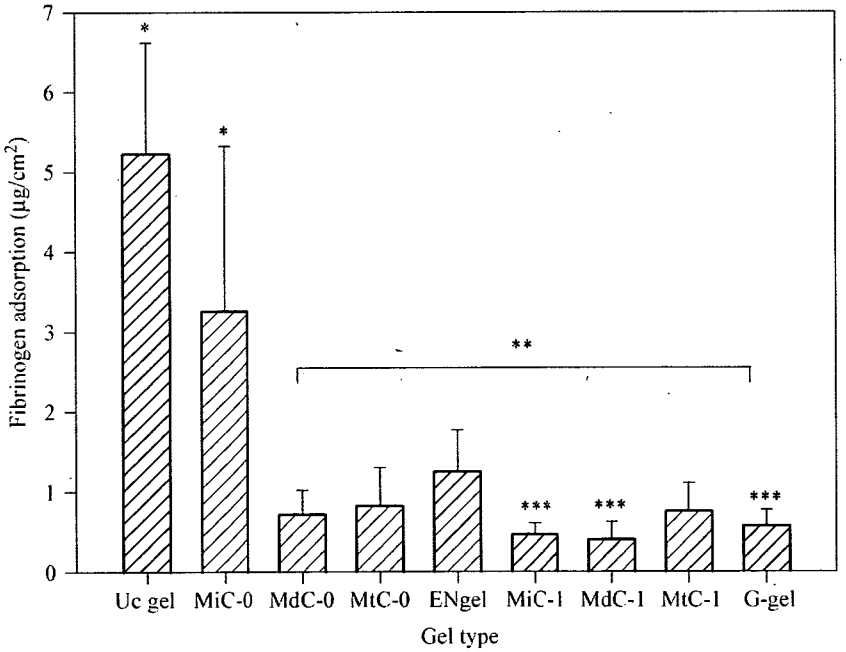
Fig. 5. Degradation of collagen gels by collagenase in Tris–HCl buffer (pH 7.4) at 37 °C. (■) Uc gel, (●) MiC-0 gel, (▲) MdC-0 gel, (▼) MtC-0 gel, (□) EN gel, (○) MiC-1 gel, (△) MdC-1 gel, (▽) MtC-1 gel, and (◇) G-gel. Closed symbols indicate gels without interhelical cross-links while open symbols indicate gels with interhelical cross-links. Each value represents the mean ± SD ($n = 5$).

4. Discussion

4.1. Physical properties of the EN and CoPho gels

The collagen gel that was prepared from a 2 wt% aqueous collagen solution differed from that prepared from a 0.5 wt% collagen solution [11]. A considerably thicker film was obtained (≈ 50 μm), and this film displayed tougher mechanical strength, suppressed swelling, and it slowed collagenase degradation. However, thermodynamic conditions such as shrinkage temperature remained unaltered.

XPS signals displayed a phosphorus peak and a nitrogen peak [$N^+(CH_3)_3$] at 134 and 403.2 eV, respectively; this indicates that PMA was effectively adopted [10,11]. This implies that PMA was successfully immobilized on the surface of the collagen gels. The phosphorus concentration would increase when the MPC is immobilized on the collagen, but did not increase significantly for MtC gels (Table 2). This implies that the immobilization would not occur when the PMA is immobilized for the third time. The increase in the density of the PMA chains is interfering further immobilization process. This can be supplemented by SCA result. The phospholipid head groups on the surface of the collagen gel decreased in the SCA, implying that the surface of the CoPho gel was acquiring a hydrophilic nature (Fig. 2). The hydrophilicty of the CoPho gel was due to the MPC head group, which was located on the surface [18]. The hydrophilic nature of the MPC polymer is thought to be one factor that can suppress



* vs. ** $p < 0.01$
** vs. *** $p < 0.01$

Fig. 6. Fibrinogen adsorption by the collagen gels. Each value represents the mean ± SD ($n = 5$).

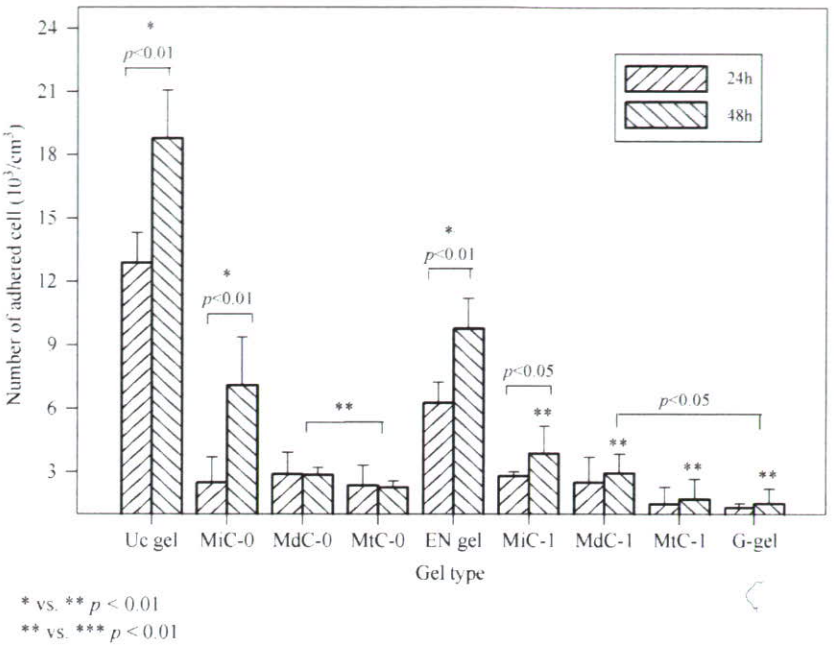


Fig. 7. Cell adhesion property of the respective collagen gels at a seeding density of 5000 cells/cm^2. Each value represents the mean ± SD ($n = 5$).

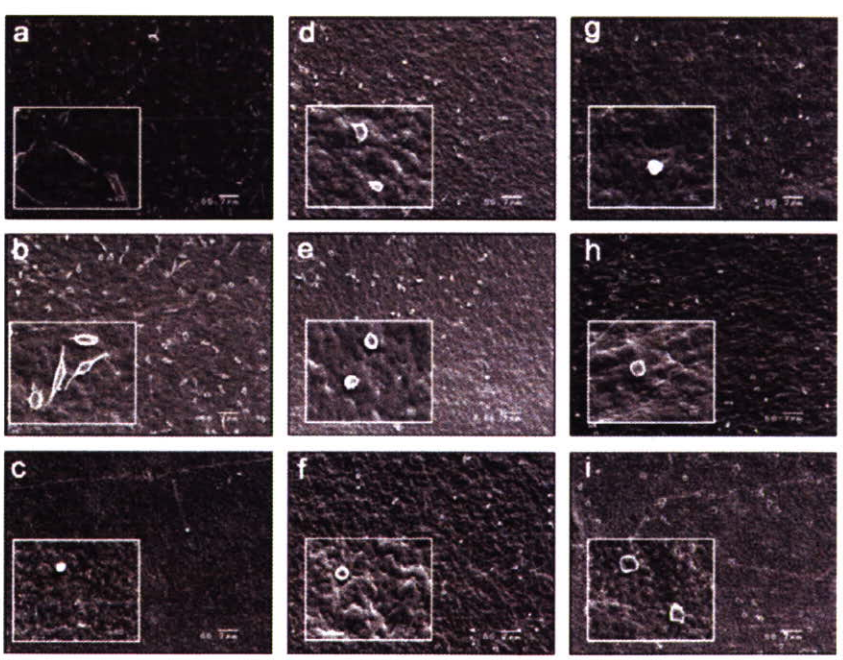


Fig. 8. SEM images of adhered fibroblast cells on respective collagen gels after 48 h of incubation. (a) Uc gel, (b) EN gel, (c) G-gel, (d) MiC-0, (e) MdC-0, (f) MtC-0, (g) MiC-1, (h) MdC-1, and (i) MtC-1 gel. SEM images in the large frame are shown at a magnification of × 150 and the small frame at a magnification of × 1000.

the protein adsorption. It is because the wet condition of the surface is inducing the increase in the mobility of the MPC polymer head group. SCA further decreases as a result of the re-immobilization process, indicating an increase in the density of phospholipid head groups on the surface of the CoPho gels. However, third immobilization process did not decrease the contact angle further.

Did the increase in PMA on the surface of the collagen gel result in a change in the structure of the collagen gels as indicated in Fig. 1? We attempted to characterize the network structure by investigating the reacted amine group content and the swelling ratio (Figs. 3 and 4). Cross-linking collagen gels with EDC/NHS leads to a decrease in the number of reacted amine groups because NH_2 from

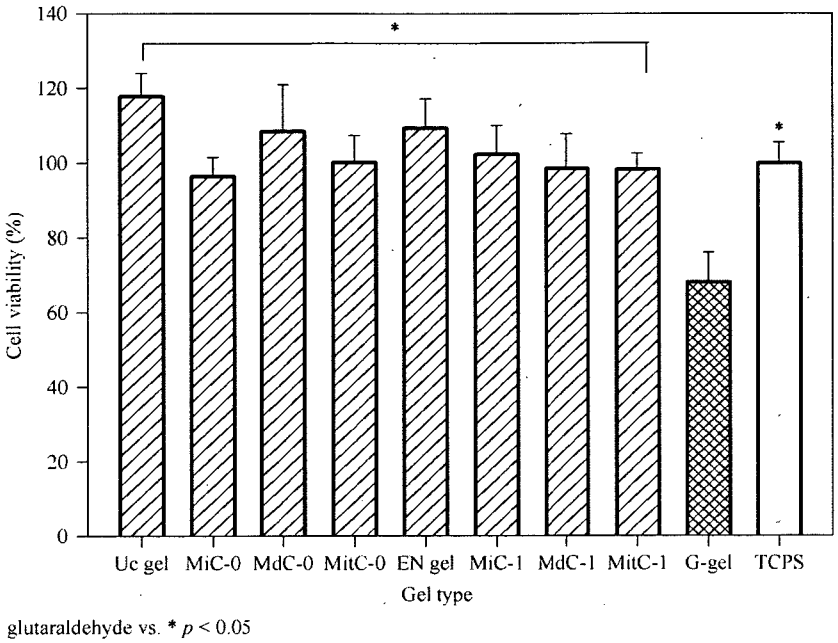


Fig. 9. Cell viability of L929 at 48 h of incubation with at a seeding density of 5000 cells/cm^2 on the respective collagen gels. Each value represents the mean ± SD ($n = 3$).

(hydroxy-)lysine residues is consumed on amide bond formation and NH_3^+ is not formed [7,21]. However, the percentage of the unreacted amine group content was higher than the expected number. Approximately, 60% of the NH_2 remained unreacted compared to Uc gel. Immobilization of PMA on the collagen gel consumed approximately 40% of the amine groups. With regard to PMA immobilization, it is believed that the amine groups may be consumed only on the surface of the collagen gel because PMA cannot penetrate the collagen α-helices [11]. The re-immobilization process decreases the reacted amine group content by up to 40% of Uc gel; immobilization process repeated three times, by up to 30% of Uc gel. The increase in the phosphorus concentration and the decrease in SCA were not observed, but the unreacted amine group content decreased for MtC gels. This implies that the immobilization is stopped, but few intra- and interhelical cross-links have occurred. This decrease is considered high when compared to that of G-gel, which demonstrates approximately 15% of Uc gel. Since the immobilization process occurs only on the surface of collagen gel, lowering the free amine content any further was not possible. An immobilization period of 48 h was the longest period of immobilization that showed a decrease in the number of unreacted amine group, and addition of a higher amount of EDC, NHS, and PMA during the cross-linking process did not cause a significant change in the reacted amine group content (data not shown).

The EN gel is formed by intra- and interhelical cross-links, whereas the CoPho gels are formed by a polymer-helix network. The formation of the cross-link network leads to a decrease in the swelling ratio. In a previous study, we have mentioned that the swelling ratio of the collagen gels is expected to decrease with the progress in cross-linking [11]. The network formed by cross-linking would be dense, and this renders water absorption difficult for the gels. The swelling ratio under acidic and neutral pH conditions varies due to the repulsion force amongst the NH_3^+ and COO^- groups. The swelling ratio is higher for collagen gels immersed in acidic pH condition because they stabilize under neutral pH conditions. With regard to collagen gels prepared with a 0.5 wt% collagen solution, highly acidic pH conditions causes the uncross-linked collagen gels to dissolve [11]; however, none of the collagen gels used in this study dissolved because the α-helices were packed more tightly and were stabilized during gel preparation. Under neutral pH conditions, the collagen film would stabilize by forming a lattice network of fibrils comprising hydrophobic and electrostatic bonds [11,22–24]; hence, the swelling ratio would be less than that observed under acidic pH conditions. The difference in the swelling ratio between the collagen gels with and without inter- and intrahelical cross-links (under acidic pH conditions) is approximately 20–50%. The reacted amine group shows a difference of only 5–10% because stabilization of α-helices by intra- and interhelical cross-links causes a decrease in the swelling ratio. The consumption of carboxyl groups and amine groups is eliminating the site for the protein binding. Arg-Gly-Asp (RGD) site is consumed for the immobilization process, which is making the protein more difficult to adsorbed [25,26]. This would be discussed in Section 4.2.

Formation of a denser network leads to difficulties in the degradation by collagenase (Fig. 5). Activation of collagenase requires adsorption on the collagen gel surface [11,27]. Subsequently, the collagenase penetrates the collagen gel and begins to cleave the helices [28]. However, a low swelling ratio does not permit collagenase absorption by the CoPho gels. The degradation rate is considerably slower due to an increase in PMA density. A high density of phospholipid head groups is believed to prevent collagenase adsorption on the surface. Eventually, the CoPho gels would be more stable against collagenase.

4.2. Biological properties of EN and CoPho gels

As previously mentioned, the hydrophilicity of the CoPho gel increases with immobilization of PMA due to the presence of phospholipid head group on the CoPho gel. High hydrophilicity is known to be one of the factors that lead to difficulties in protein adsorption [29]. Increase in the density of PMA results in a decrease in the adsorption rate of fibrinogen (Fig. 6). This implies that the immobilized MPC polymer leads to difficulties in the interaction of proteins with the gel surface. In addition to this, the cross-links also decrease protein adsorption. The ε-amino groups from (hydroxy-)lysine residues of collagen are blocked by the cross-linking process [30]. Thus, it is believed that hydrophilicity of the hybrid gel and the blocked ε-amino group renders it difficult for the hybrid gel surface to adsorb fibrinogen. A similar phenomenon was observed during the cell adhesion test (Fig. 7). We observed that repeated immobilization of PMA suppressed cell adhesion. Comparison of cell adhesion after 24- and 48-h cycles revealed that the number of adhered cells in the case of the Uc gel after 48 h cycle had increased by approximately 2 times; this rate of increase would decrease as collagen gels more phospholipids is immobilized. Immobilization of PMA did not induce any toxicity. Decrease in cell attachment on CoPho gels was entirely attributable to the surface property, i.e., the ability to regulate cell adhesion and protein adsorption. These results indicate that the immobilization of the PMA would induce almost the same effect as that of G-gel but without toxicity. As mentioned in Section 4.1, the formation of the cross-linking is eliminating the site for the protein binding. Same affect can be expected for the G-gels. Consumption of RGD for the cross-linking is making the gels to resist against protein adsorption and cell adhesion. Improper cross-linking by glutaraldehyde would induce the high protein adsorption [25]. However, in our case, G-gel showed low protein adsorption and cell adhesion, indicating that the RGD is effectively cross-linked. The formation of the cross-link is bringing the difficulty in the adsorption of proteins.

When assessing suppression of cell adhesion in terms of intra- and interhelical cross-links, we observed that suppression was considerably higher in gels with intra- and interhelical cross-links. The MtC-1 gel displayed cell adhesivity that was similar to that of the G-gel. This implies that intra- and interhelical cross-links also constitute an important parameter in suppression of cell adhesion. This can be reaffirmed by the fact that the number of cells adhered onto the EN gel is less than that in the case of the Uc gel. Much higher affect can be seen for G-gel, but we could not detect high suppression of cell adhesion by EDC/NHS cross-link, indicating that the functional groups still exist largely on the surface. Comparing EN gel and MiC-0 gel, the reacted amine group content is almost the same but the biological property is different. This is due to the difference in the surface property of the EN gel and MiC-0 gel. Investigation of cell morphology revealed that the L929 cells were deformed on the non-MPC surface (Fig. 8). On the other hand, the cells remained intact (round) on the surface of the CoPho gels, indicating a weak interaction between cells and the surface [19]. However, the adsorption of protein and the adhesion of the cell were still higher compared to other materials that used MPC polymer [18,31–33]. Ishihara et al. pointed out that 30 mol% of MPC polymer is required for fibroblast suppression [29]. Repeated immobilization increased the number of phosphorylcholine moieties on the surface of collagen gel surface. However, it is believed that the increase in the number of phospholipids moieties is no longer possible, and no significant decrease was observed in the amount of adsorbed fibrinogen and adhered cells.

5. Conclusion

Repeated immobilization of PMA can increase its immobilization rate, resulting in an increase in the number of MPC head groups; hence, unreacted amine group content and the swelling ratio decreased and the degradation by the collagenase was delayed. The cell morphology remained round indicating a weak interaction between the cells and the gel surface. Thus, the CoPho gel can be used as an alternative collagen-based gel for an implantable biomedical device. Furthermore, we expect that co-immobilization with different polymer-possessing carboxyl groups such as heparin is possible. In the near future, we look forward to reporting on the use of the CoPho gel *in vivo*.

Acknowledgments

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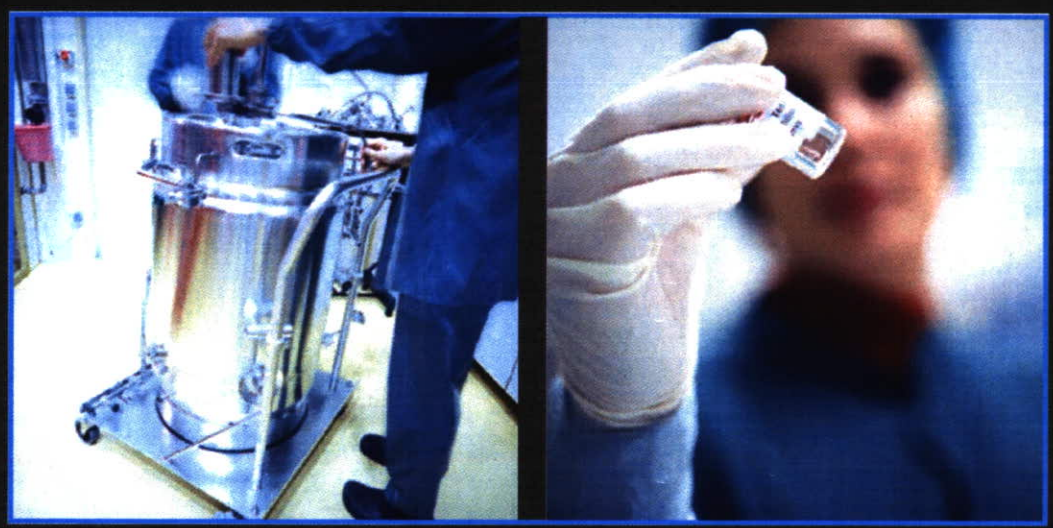

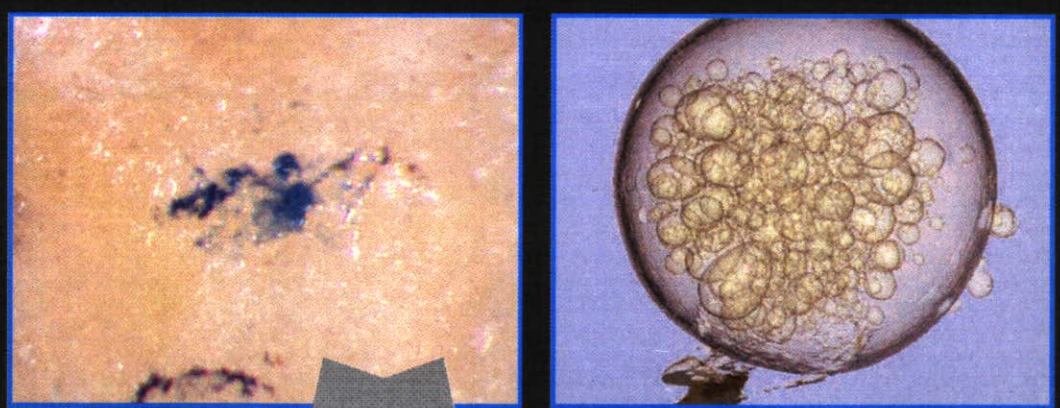

Scientifically Speaking

Gene Transfection on Tissue Engineered Bone Decellurized by Ultra-High Hydrostatic Pressurization

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Introduction

The development of scaffold, which contributes to adhesion and expansion of cells that can regenerate tissue lost to disease, is one of the key factors in tissue regeneration. Many researchers have investigated polymeric scaffolds, such as poly(lactic acid) (1), poly(glycolic acid) (2), hyaluronic acid (3), and collagen (4). It has been reported that the shape and microscopic structure of these scaffolds, such as porous, fibrous, and gel, plays an important role in tissue formation, as does the physical and physicochemical nature of the scaffold (5). However, it is difficult to obtain the same shape and structure as the biological tissue. Therefore, there is an alternate approach for preparing scaffold that is similar to the natural scaffold that uses decellularized tissues from which the cells and antigen molecules have been removed to diminish the host immune reaction. The decellularized scaffold is thought to have the same structure and composition as the natural tissue, and the regeneration within the scaffold is expected to be regulated by donor cells. Detergents, such as Triton® X-100 (6), sodium dodecyl sulfate (7), and sodium cholate (8), generally are used to remove the donor cells and their components. The remainder of the detergents, the residual cellular component in the scaffold, and the denaturing of tissue are reported to be important problems. We have also reported on the development of tissue engineered bone by novel physical decellularization process using ultra-high pressure (UHP) technology without surfactant (9). This decellularization method involves two processes. As a first step, cells, bacteria, and viruses in the tissue are disrupted by ultra-high pressurization. Subsequently, the residues of disrupted cells are removed by washing (Figure 1).

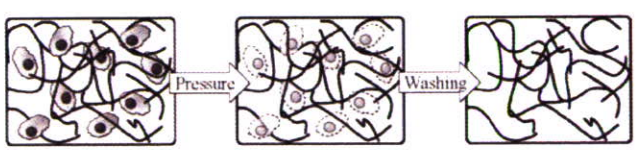

Figure 1. Preparation procedure for decellularizing tissue using ultra-high pressure treatment.

Recently, the focus has been the combination of tissue engineering scaffold and gene therapy, which provide the physical support for cell adhesion and cellular functioning by delivering the gene (10). For *in vitro* gene delivery, non-viral vectors, such as cationic polymers, cationic lipids (11), and calcium phosphate (12), have been used for stabilization of DNA, resulting in effective gene transfection. On the other hand, when they are applied in a living body, their cytotoxicity and low transfection efficiency likely will become considerable problems. For bone regeneration, it is thought that calcium phosphate, which is one component of bone, is suitable as a gene carrier because it is able to form a co-precipitate with DNA for gene transfection and to become bone by itself.

In this study, we demonstrated the preparation of decellularized bone by pressurization and gene transfection to reseeded cells on the decellularized bone with co-precipitates of calcium phosphate with plasmid DNA *in vitro*.

Results

Porcine bones (femur and costa) were cut and shaped and then pressurized at 25°C and 10,000 atm (980 MPa) for 10 min (UHP treatment). After UHP treatment, they were washed by culture medium containing DNase I at 37°C for 2 weeks. The decellularization of bone was evaluated by hematxylin and eosin (H-E) staining. Figure 2 shows that the removal of cells in bone and bone marrow of femur was completely achieved by UHP treatment. The porous structure of bone and the fibrous structure of collagen, along with lipid droplets in bone marrow, were well maintained. The decellularized costa also was prepared by UHP treatment. MC3T3 cells (1×10^5 cells) were reseeded on the decellularized bone *in vitro*. After cultivation for 3 days, the

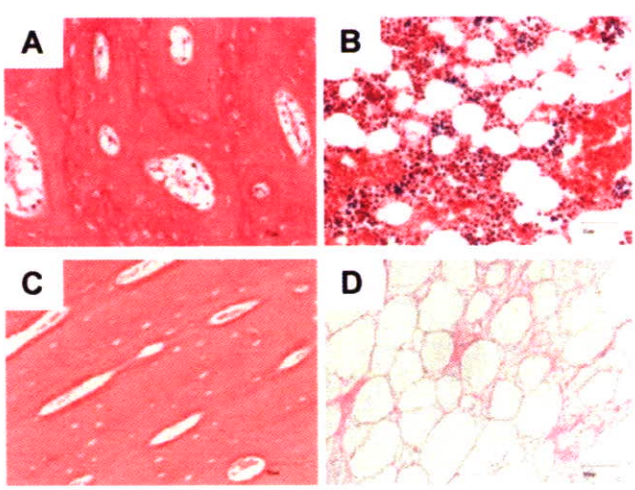

Figure 2. Hematoxylin eosin staining of (A) native cortical bone, (B) native bone marrow, (C) decellularized cortical bone, and (D) decellularized bone marrow by pressurization at 10,000 atm (980 MPa) for 10 min.

adhesion and extension of cells on the surface of the decellularized bone was observed at the outside and the inside of the bone under scanning electronic microscopy (SEM). The decellularized femur was implanted subcutaneously in rats to investigate their biocompatibility. After 2- and 4-weeks implantation, they were explanted and subjected to histological study (H-E staining). Light microscopic observation confirmed that a strong inflammatory response was observed on native bone after 2 weeks. Fibrous encapsulation and gradual collapse of bone marrow occurred after 4 weeks. On the other hand, very thin fibrous encapsulation was observed around the decellularized femur. The re-construction of tissue by infiltration of cells in decellularized bone marrow also was observed after 4 weeks, suggesting the capability of the decellularized bone as a bio-scaffold.

Plasmid DNA encoding beta-galactositase gene under cytomegarovirus promoter (pCMV-beta: clonetech) was used. A solution of pCMV-beta was mixed with $CaCl_2$ solution (2*M*) and added to 2× HBS solution to form the co-precipitate of pCMV-beta and calcium phosphate. The decellularized bone was immersed in the mixture at 37°C for 30 min. MC3T3 cells (5×10^4 cells) were reseeded on the decellularized bone and cultivated for 3 days. The gene transfection was evaluated by X-gal staining. Without co-precipitation, there was no change in cells reseeded on the decellularized bone with only DNA, whereas blue-stained cells were observed on the decellularized bone with calcium/DNA co-precipitate (Figure 3), indicating effective gene expression by the combination of the calcium phosphate co-precipitate method and tissue engineered bone. This result indicated that decellularized tissue was significantly useful in the novel combination of the tissue engineered scaffold and gene delivery.

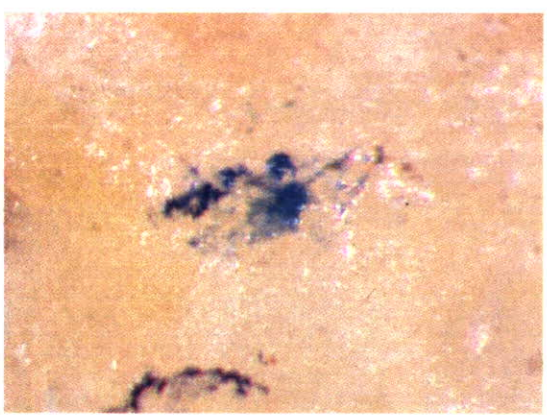

Figure 3. X-gal staining of cells reseeded on decellularized costa with calcium phosphate/DNA co-precipitate.

Conclusions

Porcine bones (femur and costa) were decellularized successfully using UHP and washing processes. The decellularized tissue would be useful in bone tissue regeneration. The decellularized bone also acted as a gene delivery/transfectioning matrice for the cells incorporated to the bone. Combining a decellularized tissue and gene delivery system is expected to be a useful technology for regenerating tissue, not only bones but also other tissues, such as blood vessels, skin, and heart muscles.

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Controlling Coupling Reaction of EDC and NHS for Preparation of Collagen Gels Using Ethanol/Water Co-Solvents

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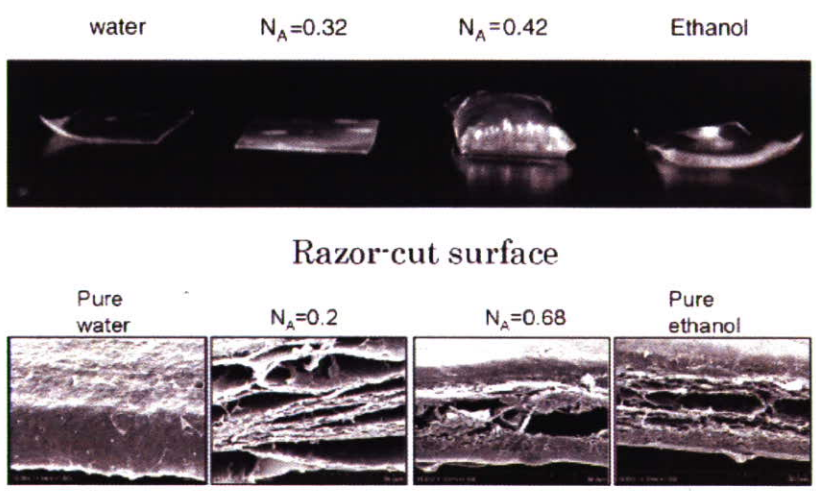
To control the crosslinking rate of the collagen gel, ethanol/water co-solvent was adopted for the reaction solvent for the collagen microfibril crosslinking. Collagen gel was prepared by using EDC and NHS as coupling agents. Ethanol did not denaturate the helical structure of the collagen and prevented the hydrolysis of EDC, but showed the protonation of carboxylate anions. In order to control the intra- and interhelical crosslink of the collagen triple helix, variations of the mole ratio of carboxyl group/EDC/NHS, and of the ethanol mole concentration were investigated. Increase in the EDC ratio against the carboxyl group increased the crosslinking rate. Furthermore, an increase in the ethanol mole concentration resulted in an increase of the crosslinking rate until ethanol mole concentration was 0.12, but showed gradual decrease as the ethanol mole concentration was further increased. This is because the adsorption of solvent by the collagen gel, protonation of carboxylate anion, and hydrolysis of EDC is at its most optimum condition for the coupling reaction when the ethanol mole concentration is 0.12. The re-crosslinking of the collagen gel showed an increase in the crosslinking rate, but did not show further increase when the coupling reaction was executed for the third time. This implied that the highest possible crosslinking rate for the intra- and interhelical is approximately 60% when EDC/NHS is used.



Introduction

The construction of an extracellular matrix (ECM) using natural products has been performed by many researchers worldwide. Based on the fact that an ECM is mainly composed of collagen and elastin, many researchers have

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attempted to prepare a collagen- or elastin-based material to construct an ECM. Ever since Weinberg and Bell succeeded in preparing a blood vessel using collagen,[1] diverse approaches using collagen gel to prepare an ECM had been executed. However, the critical aspect in using collagen gel is that its mechanical strength is too small and easily deforms its triple-helix structure into a random coil structure when heated. The low mechanical strength and easy deformability make collagen shrink easily due to external stimuli. These aspects make it difficult to use collagen as an ECM. The use of crosslinkers to overcome these problems was investigated and is well reviewed by Khor.[2] By crosslinking collagen triple-helices, it is possible to maintain its mechanical strength and suppress any deformation caused by external stimuli. However, it is very important to consider biological responses in the designing stage of a crosslinking process because of the possibilities of severe problems such as toxicity, inflammatory response or the alteration of protein structure.

A crosslinking method using 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in aqueous condition is a one of the best methods to produce a non-toxic collagen product. This reaction mixture induces the formation of an amide bond by activation of the side chain carboxylic acid groups of aspartic and glutamic acid residues, followed by aminolyis of the *o*-isoacylurea intermediates by the ε-amino groups of (hydroxy-)lysine residues, forming intra- and interhelical crosslinks.[3–5] A coupling reaction that involves EDC depends on the amount of EDC and on the EDC/NHS ratio.[4–6] A higher EDC and NHS mole ratio against the carboxylic groups increases the coupling reaction rate. The pH of the solvent for the coupling reaction should be higher than the pK_a value, which is 5.8 for collagen. This is because the carboxylate anions otherwise exhibit a higher coupling rate than that exhibited by the carboxyl groups.[6] The coupling reaction using EDC is one of the most widely used crosslinking methods in the biomaterials field; however, it is regarded as an inappropriate method, especially in tissue engineering, owing to its extremely low coupling efficiency. This is because EDC tends to hydrolyze rather rapidly under aqueous conditions.[3–7] The use of NHS to suppress the hydrolysis does not function to the desired extent. Furthermore, since collagen consists of triple helices, the efficiency of the coupling reaction is lower than that of crosslinkers such as diol-related crosslinkers or glutaraldehyde because the only possible reactions are the intra- and interhelical coupling reactions. Hence, the question of whether it is possible to control the coupling reaction rate of EDC for collagen crosslinking was brought up.

Our research group attempted to control the coupling reaction of EDC/NHS using the collagen gel. We found out that in order to obtain a crosslinked collagen gel that is mechanically tough and possesses a low swelling ratio, collagen should be crosslinked under neutral or alkaline pH conditions with the EDC/NHS/carboxylate anions in a ratio of 10:10:1.[4] The swelling ratio in pH 7.4 was less than 150%, which is approximately 1/5 of that of the uncrosslinked collagen gel. It was shown that no denaturation of the triple helix had occurred. The elastic modulus increased to approximately 4.8 times that of the uncrosslinked collagen gel. However, when we investigated the free amine group contents, the lowest value of that we could obtain was approximately 60%. Glutaraldehyde crosslinking on the same collagen gel revealed that the free amine group content was less than 15% and the diol-related crosslinker exhibited an approximate free amine group content of 30%.[7] We concluded that this is the lowest possible coupling reaction rate for the collagen microfibrils under aqueous conditions. Thereafter, we started to search for new conditions for collagen crosslinking using EDC and NHS. In this study, we attempted to control the EDC/NHS coupling reaction rate by making the reaction environment highly hydrophobic. To achieve the more hydrophobic environment, we used ethanol, which is miscible with water. Ethanol/water mixed solvents were prepared in different mole concentrations to control the hydrophobicity of the solvent. There are a number of research papers on the reaction of EDC/NHS with collagen in ethanol, but it is not completely clear as to how the EDC and NHS coupling reaction would be affected when the alcohol percentage in aqueous conditions changes; hence, different ethanol concentrations are being used without characterization of the coupling rate.[8–11]

Experimental Part

Preparation of Collagen Gel

The preparation of the collagen film was performed by the same method as that reported previously.[5,7] A 0.5 wt.-% solution of collagen type I (I-AC, KOKEN, Tokyo, Japan) was concentrated into a 2 wt.-% collagen type I solution and used for the film preparation. The collagen solution was dropped onto a polyethylene film and dried at room temperature. A transparent film with a thickness of 56 ± 3 μm was obtained. The films were stored in a dry environment.

To investigate the effect of the solvent, the collagen film was immersed into an ethanol/water mixed solvent containing EDC and NHS (both from Kanto Chemicals, Tokyo, Japan). Each chemical was added in the mole ratio of EDC/NHS/collagen-carboxylic acid group = 10:10:1. The ethanol mole concentration (N_A) was changed from 0 to 1 [ethanol/water ratio from 10:0 to 0:10 (v/v)]. The crosslinking procedure was allowed to continue for 24 h at 4 °C to produce a crosslinked gel (EN gel). After 24 h, the reaction was terminated by removing the gel from the solution. The gel was then washed with distilled water for 3 d in order to remove any unreacted chemicals from the collagen gel. For the

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re-crosslinking process, the same procedure as above was repeated using water, $N_A \approx 0.12$, $N_A \approx 0.42$ and 100% ethanol as the reaction solvent. Crosslinking of the collagen gel to glutaraldehyde was performed by using a 0.5 wt.-% glutaraldehyde solution (Merck, Darmstadt, Germany) in a phosphate buffer solution (PBS).[12] The collagen film was immersed in the glutaraldehyde/PBS solution and was crosslinked for 3 h at room temperature. After crosslinking, the sample was first rinsed under running tap water for 30 min and then in 4 M NaCl for 2 h. In order to eliminate NaCl, the sample was rinsed with distilled water for 1 d to yield a glutaraldehyde-crosslinked collagen gel. The 1,4-butanediol diglycidyl ether (BDDGE)-crosslinked collagen was prepared by immersing a collagen film in a 4% BDDGE/PBS solution and reacting for 5d.[13] The BDDGE-crosslinked collagen was left under running tap water for 15 min to wash off the unreacted BDDGE. The washing process was repeated several times. The glutaraldehyde-crosslinked collagen gel and the BDDGE-crosslinked collagen gel were used for the characterization of the free amine group content.

Characterization of the Collagen Gel

A solubility test was performed in the ethanol/water mixed solvents. The collagen films (3–4 mg) and collagen chunks obtained from lyophilization (7–10 mg) were immersed in ethanol/water mixed solvents. The collagen solutions were left at room temperature until complete dissolution occurred. The triple-helix structure was characterized using a circular dichroism (CD) spectrometer (J-720W, Jasco, Tokyo, Japan). Collagen solution was prepared at a concentration of 1×10^{-7} M and characterized 5 times for each sample to obtain the average spectra. Surface analysis was performed by scanning electron microscopy (SEM, SM-200, Topcon, Tokyo, Japan). The same solubility test was repeated using the collagen film. The diffusion coefficient D was calculated using a collagen gel that was prepared in a 2-(*N*-morpholino)ethansulfonate (MES) buffer. The collagen gels were immersed in the ethanol/water mixed solvents at pH 9.0. The gels were then removed at 10, 60, 120, 240, 360, 1 440, and 4 320 min (3 d) and the adsorbed amounts of the solvent were measured. The following equation was used for the calculation of D:

$$M_t/M_\infty = 4(Dt/\pi l^2)^{1/2}, \quad (1)$$

where M_t and M_∞ are the amounts of the solvent adsorbed at time t and at infinity, respectively and l is the thickness of the collagen gel.[14,15]

The primary amine group concentrations in the tissue samples were determined using a colorimetric assay.[16,17] From each sample a 2–4 mg specimen was prepared. These samples were immersed in a 4 wt.-% aqueous $NaHCO_3$ solution (Kanto Chemicals, Tokyo, Japan) and a 0.5 wt.-% aqueous solution of 2,4,6-trinitrobenzene sulfonic acid (TNBS; Wako chemicals, Osaka, Japan) was added. The reaction was allowed to continue for 2 h at 40 °C, after which the samples were rinsed in saline solution using a vortex mixer to remove the unreacted TNBS. The samples were freeze-dried overnight, after which the dry mass was determined. The dry samples were immersed in 2 mL of 6 M aqueous HCl until fully dissolved. The obtained solution was then diluted with distilled water (8 ml) and the absorbance was measured at 345 nm (V-560, Jasco, Tokyo, Japan). The concentration of the reacted amine groups was calculated using the following equation:[16,17]

$$[NH_2] = (A \cdot V)/(\varepsilon \cdot l \cdot m) \quad (2)$$

where $[NH_2]$ denotes the reacted amine group content [in mol/g of collagen gel]; ε, the molar absorption coefficient of trinitrophenyl lysine (1.46×10^4 l·mol^{-1}·cm^{-1}); A, the absorbance; V, the volume of the solution [mL]; l, the path length [cm]; and m, the weight of the sample [mg]. The free amine group contents were calculated by assuming that the uncrosslinked collagen gel has 100% free amine groups.[7,8] The experiment was repeated five times and the average along with the standard deviation was calculated.

All the experiments were repeated at least thrice and the values were expressed as mean ± standard deviation. In several figures, the error bars are not visible because they are included in the plot. A statistical analysis was performed using the student's t test with the significance level set at $p < 0.05$.

Results and Discussion

We started by setting up three hypotheses: 1) ethanol does not denaturate the triple helix, 2) ethanol prevents the hydrolysis of EDC, and 3) the carboxyl groups are reactive with EDC in ethanol. These three hypotheses are important in the aspect that the failure of one hypothesis implies that the collagen crosslinking is meaningless. Hence, the experiment was conducted by proving the hypotheses one by one. We first started with the characterization of the triple helix of the collagen. The exposure of the collagen triple-helices to ethanol induces hydrophobic interactions, which may lead to a change in the conformation of the collagen microfibrils. Using a CD spectrometer, we observed the conformation structure of collagen in the range of $N_A \approx 0$–0.42 (ethanol/water = 0/10–7/3, v/v). The increase in ethanol concentration against water did not bring about any distinguishable change in the triple helical structure (Figure 1). The positive band and the cross-band seen in the CD spectra were the same for all the tested samples ($N_A \approx 0$–0.42). The negative band exhibited a slight red-shift as the ethanol concentration was increased. However, no signs of denaturation, such as a decrease in the peak intensity of positive and negative band, were detected.[18,19] Hence, it is assumed that ethanol up to $N_A \approx 0.42$ does not change the triple helices into random coils.[20] The main forces that hold the helical structure of collagen are hydrogen bonds, electrostatic interactions, and hydrophobic interactions. In water, the hydrogen bonds and electrostatic interactions within collagen contribute to the stabilization of the helices, but they are not the dominant factors.[20] The structure of collagen depends on the concentration of the alcohols. This

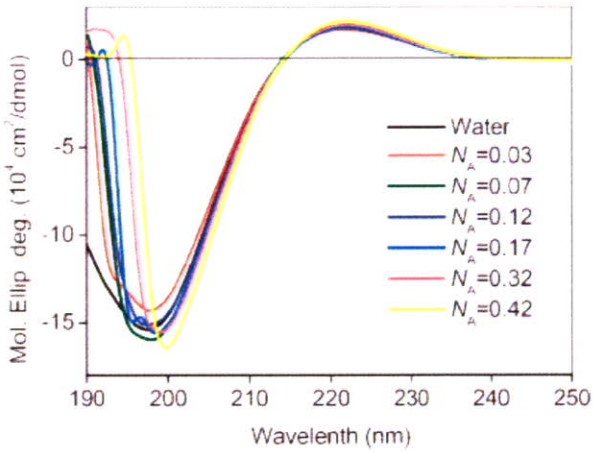

Figure 1. CD spectra of the collagen microfibrils under various ethanol mole concentrations.

is because an increase in the hydrophobic interactions between the solvent and collagen stabilized the structure of collagen.[21] The hydrophobic interactions between the non-polar amino acid side chains are also very important factors that contribute to the stabilization of the helices. Exposure of the non-polar amino acid side chains to the outer side would induce hydrophobic interactions, which were not observed under aqueous conditions. This causes a hydrophobic shielding effect.[22] However, it is generally assumed that this tendency is strongly influenced by the type of alcohol used. Thus, polyhydric alcohols such as sorbitol or glycerol favour the native structure, while monohydric alcohols enhance the native structure.[23] In the case of ethanol, the secondary and tertiary structures of collagen would be affected.[22,24] As result, it is assumed that the transformation 'triple helix — random coil' does not occur, and the use of ethanol for the amide coupling reaction for collagen crosslinking is preferable. The triple-helix structure at $N_A > 0.55$ was measured indirectly. That is, since the random coil is not reconverted to the triple-helix structure,[22] we resolubilized collagen in water and observed the CD spectra and concluded that the collagen structure would remain a triple helix even at extremely high ethanol mole concentrations.

However, it should be noted that the use of ethanol is not a solution for the control of the coupling reaction. The surface of collagen is too hydrophobic and rigid, in which the fibrillar structure disappears. The solubility test showed that the ethanol mole concentration should be at least 0.42 to dissolve collagen. The same phenomenon was observed for the collagen film. The collagen film, which is un-crosslinked, could be dissolved at $N_A \approx 0.42$, but would remain undissolved in higher hydrophobic conditions. Expectedly, the time required for complete dissolution was different, where high-hydrophobic conditions delayed the dissolution time. Figure 2 shows the morphology of collagen microfibrils observed by SEM. It is seen that the microfibril structures disappear as the hydrophobicity increases. The disappearance of the fibrillar structure decreases the absorptivity of the solvent. This suggests that for the collagen film, the adsorption of ethanol by the collagen gel would be extremely low. To prove this, we have calculated the diffusion coefficients D for various mole concentrations of ethanol, as shown in Figure 3, using the collagen gel crosslinked with EDC/NHS in a MES buffer that was prepared by the method reported previously.[5] This shows that the D of the solvent decreases rapidly when $N_A \geq 0.55$ (ethanol/water = 8/2,

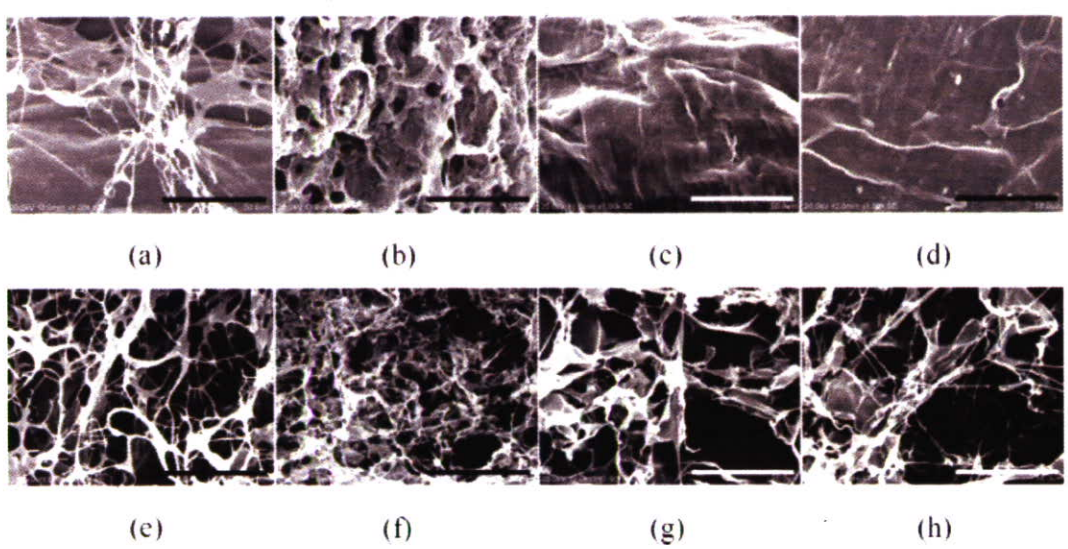

Figure 2. Morphology of collagens after immersing in ethanol/water mixed solvents of different concentrations. (a) Water, (b) $N_A \approx 0.07$, (c) $N_A \approx 0.17$, (d) $N_A \approx 0.32$, (e) $N_A \approx 0.42$, (f) $N_A \approx 0.55$, (g) $N_A \approx 0.73$, and (h) ethanol. Single bar indicates 50 μm.

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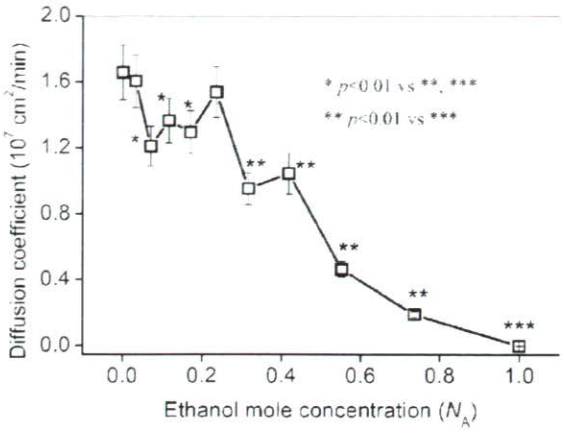

Figure 3. Change in the diffusion coefficient of ethanol in collagen gel according to ethanol mole concentrations.

v/v); furthermore, the D value of pure ethanol (1.2×10^{-10} cm$^2 \cdot$ min^{-1}) is approximately 1 400 times lower than that of pure water. This directly affects the crosslinking ability. The solvent adsorption ability in pure ethanol and at $N_A \approx 0.74$ (ethanol/water = 9/1, v/v) is about 50% of that of pure water and 80% at $N_A \approx 0.55$ after 24 h of solvent adsorption. This implies that ethanol could not completely reach the interior of the collagen gel throughout the crosslinking procedure.

Using EDC and NHS, we obtained crosslinked collagen gels under various ethanol concentrations (Figure 4). When EDC and NHS are used for the crosslinking process, the lowest value of the free amine group content was approximately 45% (60% when crosslinked in MES buffer). This can be achieved when the crosslinking was executed for 24 h at $N_A \approx 0.07$–0.17 (ethanol/water = 2/8–4/6, v/v) with 51 mmol of EDC. This range is assumed to be the most proficient range for the coupling reaction, where the suppression of hydrolysis and fast solvent absorption has occurred. The addition of ethanol is thought to have prevented the hydrolysis of EDC. On the other hand, when

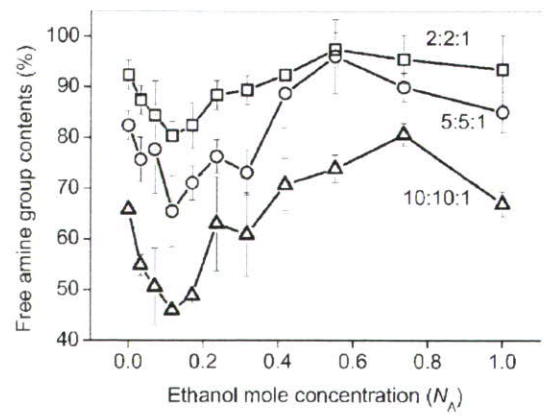

Figure 4. Change in the free amine group contents of collagen gel according to ethanol mole concentrations.

$N_A \approx 0.24$ (ethanol/water = 5/5, v/v), the free amine group content increases again, and from $N_A \approx 0.42$ and above, the free amine group content increases to higher than that of pure water. This is because of the decrease in the number of carboxyl groups reacting with EDC.[25,26] The reactivity of the carboxyl groups decreases as the ethanol concentration increases because EDC reacts with the carboxylate anions. The increase in the number of neutral carboxyl groups would lead to relatively low *O*-isoacylurea formation.[6] Furthermore, when $N_A \geq 0.42$, the crosslinking is assumed to be mainly concentrated on the surface of the collagen gel. The decrease in D causes heterogeneous coupling reactions in the collagen gel. That is, the partly crosslinked network of the collagen gel could be mainly located on the surface of the gel. This can be confirmed when the collagen gels prepared at $N_A \geq 0.42$ are placed in pure water. The sudden change in the environment causes the gel to adsorb a large amount of water, which makes the uncrosslinked collagen microfibrils dissolve and expand to the maximum extent by an increase in the free energy. The expansion of the collagen microfibrils is obstructed by the crosslinked part, which is mainly located on the surface. For the collagen gel prepared at $N_A \approx 0.42$, D is approximately the same as that of the gel prepared at $N_A \approx 0.32$, but it is thought that the protonation of the carboxyl groups prevents the formation of *O*-isoacylurea. The reactivity between the carboxyl groups and D alters the formation of the collagen gel. When the morphology of the razor-cut surface was observed, the monolithic morphology of the collagen gel was found to form a layered structure as the hydrophobicity increased, which eventually collapses. The collapse of the inner part of the collagen gel is due to the dissolution of the uncrosslinked collagen microfibrils. This implies that the crosslinking of the collagen gel would start from the surface and then occur inside the collagen gel. Furthermore, it is possible to crosslink only the surface of the collagen gel to obtain a phase-separated collagen gel when the ethanol concentration is controlled.

An extended reaction time under high-hydrophobic conditions ($N_A \geq 0.42$) did not cause any significant difference in the free amine group content. The crosslinking rate is much higher after 24 h, as compared to 4 h; however, no significant change is observed after 48 h. When crosslinking was performed in MES buffer, we observed a decrease in the free amine group content;[7] however, in the case of ethanol, the formation of the *O*-isoacylurea does not occur due to the slow adsorption and protonation of the carboxyl groups.

Is it possible to obtain a collagen gel with a smaller number of free amine groups? To answer this question, we have re-crosslinked the collagen gel by repeating the same procedure (Figure 5). The activation by EDC can be triggered when EDC is introduced into the reaction solvent.[4] We

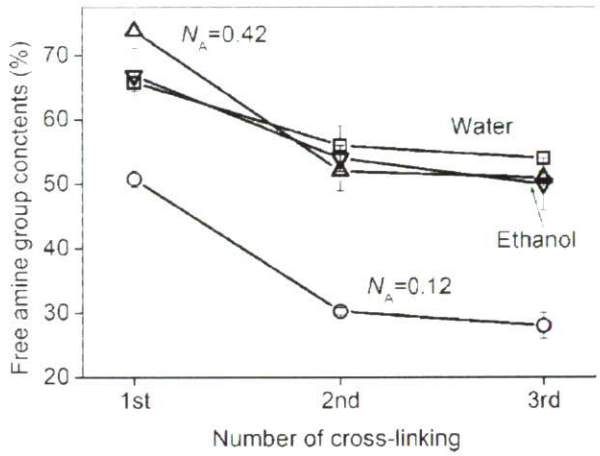

Figure 5. Change in the free amine group content of collagen gel by the re-crosslinking procedure in different solvents.

have proved in our previous report that the carboxyl groups can be activated at any point of time during the course of the reaction.[6,7] Thus, by re-crosslinking the collagen gel, we attempted to evaluate the highest coupling rate possible using this process. The re-crosslinking was possible and the least value of the free amine group content was 30% ($N_A \approx 0.12$). This value is still high as compared with the glutaraldehyde-crosslinked collagen gel (≈12% using the same collagen gel) and the BDDGE-crosslinked collagen (≈25% using the same collagen gel). This is thought to be the lowest limit of the EDC/NHS crosslinker. Unlike glutaraldehyde and BDDGE, which can interconnect the microfibrils of the collagen, EDC/NHS can only induce intra- and interhelical crosslinks. It is difficult to assume that the microfibrils are crosslinked via the EDC/NHS crosslinker due to distal problem. Hence, it is not possible to achieve a free amine group content that is lower than ≈30%. The crosslinking may still occur when a different crosslinker or a polymer is added to this collagen gel.

Conclusion

We have proposed a new method for controlling the coupling reaction rate using EDC and NHS for collagen crosslinking. The collagen triple-helix was stable in ethanol/water mixed solvent, but the properties of the collagen gel prepared in the above solvent could be altered by the ethanol mole concentration. The highest reaction rate was achieved at $N_A \approx 0.07$–0.17 with 51 mmol of EDC in 24 h. This is the optimum concentration range that balances the reactivity of EDC and the formation of carboxyl groups. We also discovered that the coupling reaction begins from the surface of the collagen gel. The coupling reaction was limited to the surface of the collagen when $N_A > 0.55$; this was because of the slow penetration of EDC and NHS caused by the high-ethanol environment and the decrease in the number of carboxylate anions. It is thought that the same procedure could be repeated not only in collagen but also in collagen-based materials such as body tissue and proteins.

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Keywords: collagen gel; crosslinking; EDC; ethanol

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