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Preparation and characterization of cross-linked collagen–phospholipid polymer hybrid gels

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Abstract

2-methacryloyloxyethyl phosphorylcholine (MPC)-immobilized collagen gel was developed. Using 1-ethyl-3-(3-dimethyl aminopropyl)-1-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), we cross-linked a collagen film in 2-morpholinoethane sulfonic acid (MES) buffer (EN gel). EN gel was prepared under both pH 4.5 and pH 9.0 in order to observe changes in cross-linking ability. To cross-link MPC to collagen gel, poly(MPC-*co*-methacrylic acid) (PMA) having a carboxyl group side chain was chosen. E/N gel was added to the MES buffer having pre-NHS activated PMA to make MPC-*i*mmobilized *c*ollagen gel (MiC gel). MiC gel was prepared under both acidic and alkaline conditions to observe the changes in the cross-linking ability of PMA. X-ray photoelectron spectroscopy showed that the PMA was cross-linked with collagen under both acidic and alkaline conditions. Differential scanning calorimetry (DSC) results showed that the shrinkage temperature increased for the MiC gels and that the increase would be greater for the MiC gel prepared under alkaline conditions. The data showed that swelling would be less when the MiC gel was prepared under alkaline conditions. The biodegradation caused by collagenase was suppressed for the MiC gel prepared under alkaline conditions due to stable inter- and intrahelical networks.

Keywords: Collagen; Phospholipid; Cross-linking; Surface modification

1. Introduction

Collagen is an extracellular-matrix protein that plays an important role in the formation of tissues and organs and is involved in various functional expressions of cells [1]. Collagen is non-toxic, non-antigenic, favors cell adhesion, proliferation, and differentiation to mimic the natural cell environment. However, favoring cell adhesion can be both advantageous and disadvantageous, for its strong affinity to cells and blood is uncontrollable, which may soon lead to blood coagulation and mineralization when applied for use as artificial blood vessels. Furthermore, the collagen that is prepared in a matrix form such as a gel for tissue reconstruction is mechanically insufficient [2]. Without modification, the collagen gel cannot be applied for bioprosthesis [3].

To overcome the disadvantages of collagen while maintaining its biological performance, a prosthesis-tissue complex, or bioartificial polymeric material, was developed by blending or mixing biomolecules and synthetic materials. The chief purpose for developing such a bioartificial polymer material is to overcome the poor biological performance of synthetic polymers and to enhance the mechanical characteristics of biomolecules [4].

To control cell adhesiveness and to increase mechanical strength simultaneously, collagen must be modified by cross-linking or mixing with synthetic polymers. Polymers such as poly(vinyl alcohol), poly(acrylic acid), poly(vinyl pyrrolidone), and polyethylene are used as bioartificial polymer materials because of their favorable chemical reactivity with collagen, absence of toxicity, and good mechanical performance [4–8].

However, it is very important to consider biological response in the adoption of a cross-linker or synthetic polymer because of the possibilities of severe problems

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such as toxicity, inflammatory response, or alteration of protein structure. Furthermore, some synthetic polymers that are known to be 'biocompatible' degrade in biological fluids, making the collagen structure unstable. Adoption of natural cross-linkers such as glutaraldehyde [9], genipin [10], or transglutaminase [11], and natural polymers like hyaluronic acid [12], heparin [13], or chondroitin-6-sulfate [14,15] is used as direct cross-linker or immobilizer to overcome the problems presented by the use of synthetic polymers, but cannot fully solve the problems.

To overcome these problems, we developed a biosynthetic hybrid material by cross-linking collagen with a 2-methacryloyloxyethyl phosphorylcholine (MPC) based copolymer using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as cross-linkers by activating the MPC polymer with EDC and NHS to cross-link the microfibrils and polymer chain using amide bond [3,16–18].

MPC is a blood compatible product developed in the early 1990s [19]. Design of the MPC polymer took into account the surface structure of the biomembrane. Recently, phospholipid-accumulated surfaces have been prepared by various methods, and it has been reported that the phosphorylcholine group plays an important role showing excellent blood compatibility and anti-protein adsorptivity [20–23]. The MPC units can then be introduced to conventional polymers by various methods of modification. They effectively reduce protein adsorption and denaturation and inhibit cell adhesion even when the polymer is exposed to whole blood in the absence of any anticoagulants [24]. By adopting the MPC polymer with the collagen gel, it is possible to expect a biocompatible collagen–polymer hybrid gel that is stable, has its molecular weight controlled, has no cross-linker leaking, and is mechanically tough.

In the present study, cross-linking ability between poly(MPC-*co*-methacrylic acid) (PMA) and collagen using EDC and NHS was investigated by altering several parameters, and the physical properties of PMA-immobilized matrices were characterized. In this article, the terms interchain cross-linking and immobilization are used synonymously.

2. Experimental method

2.1. Preparation of collagen–phospholipid polymer hybrid gel

2.1.1. Synthesis of PMA

PMA was synthesized by a method that has already been published [19]. In short, desired amount of MPC and MA was dissolved in ethanol in an ampoule. Then 2,2'-azoisobutyronitrile (AIBN) was added to the ethanol solution. The argon gas was bubbled into the ethanol solution to eliminate the oxygen. The ampoule was sealed and heated to 60 °C for 16 h. The solution was precipitated into diethyl ether, freeze-dried, and kept in vacuum until use. The mole ratio of PMA was controlled to MPC:MA = 3:7, and the number average molecular weight $\overline{M}_n$ of the PMA was approximately 300,000. The chemical structure of PMA is shown in Fig. 1.

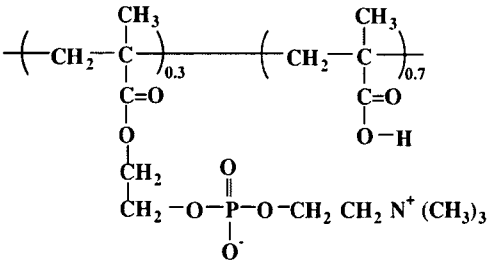


Fig. 1. Chemical structure of PMA.

Table 1
Terminology of collagen gels used in this study

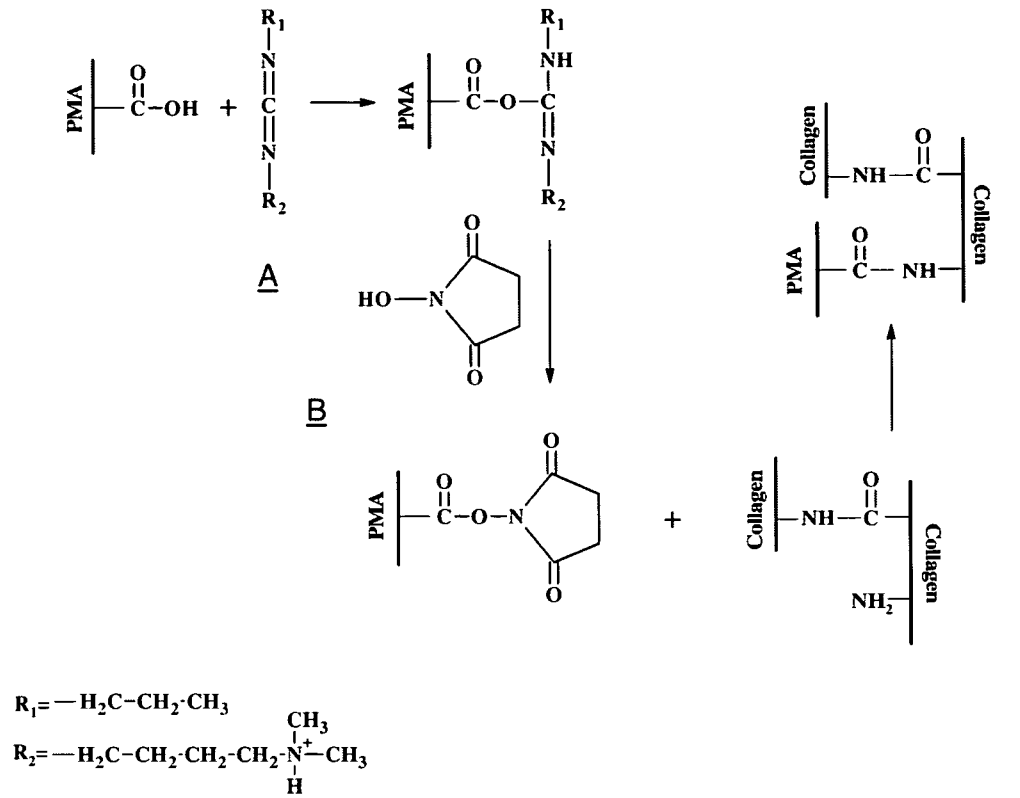
Terminology	Composition
Uc-gel	Uncross-linked collagen gel (immersed in alkaline pH conditions)
EN-1	EDC/NHS-cross-linked collagen gel under acidic pH conditions
EN-2	EDC/NHS-cross-linked collagen gel under alkaline pH conditions
MiC-11 gel	PMA immobilized to EN-1 gel under acid pH conditions
MiC-12 gel	PMA immobilized to EN-1 gel under alkaline pH conditions
MiC-21 gel	PMA immobilized to EN-2 gel under acid pH conditions
MiC-22 gel	PMA immobilized to EN-2 gel under alkaline pH conditions

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

Cross-linked collagen gel was prepared by using 0.5 wt% collagen type I solution (pH 3, KOKEN, Tokyo, Japan). Conventional film fabrication method was used for the film fabrication. The collagen solution was dropped onto the polyethylene film and dried in room temperature. The collagen film (thickness = 36 ± 2 μm) was immersed into a 0.05 M 2-morpholinoethane sulfonic acid (MES) buffer (pH 4.5) (Sigma, St. Louis, USA) containing 1-ethyl-3-(3-dimethyl aminopropyl)-1-carbodiimide hydrochloride (EDC) (Kanto Chemicals, Tokyo, Japan) and NHS (Kanto Chemicals, Tokyo, Japan). Each chemical was added at the mole ratio of EDC:NHS:collagen-carboxylic acid groups = 5:5:1 [11,13]. The cross-linking procedure was allowed to continue for 4 h at 4 °C to produce a cross-linked gel (EN-1 gel). After 4 h, the reaction was stopped by removing the gel from the solution. The gel was then washed with 4 M of Na_2HPO_4 aqueous solution for 2 h to hydrolyze any remaining *O*-acylisourea groups and then with distilled water for 3 days to remove any salts from the gel. Same preparation process was repeated under alkaline conditions (pH 9.0; adjusted with NaOH) to prepare an EN-2 gel.

2.1.3. Preparation of MPC-immobilized collagen gel (MiC gel)

Preparation of the MiC gel was done by using the EN-1 and EN-2 gels. PMA was added with EDC and NHS to the MES buffer (pH 4.5 and pH 9.0) and was pre-activated for 10 min before immersion of the EN-1 or EN-2 gel. The immobilization of PMA to the collagen was allowed to continue for 4 h at 4 °C. The gel was then washed with 4 M of Na_2HPO_4 aqueous solution for 2 h and then with distilled water for 1 day to remove any salts from the gel to prepare a salt-free MiC gel: MiC-11 gel (PMA immobilized under acidic conditions using the EN-1 gel), MiC-12 gel (PMA immobilized under alkaline conditions using the EN-1 gel), MiC-21 gel (PMA immobilized under acidic conditions using the EN-2 gel), and MiC-22 gel (PMA immobilized under alkaline conditions using the EN-2 gel). The terminology of the samples is listed in Table 1. PMA cross-linking with the collagen is shown in Fig. 2. Collagen film was immersed



$R_1 = -H_2C-CH_2\cdot CH_3$

$R_2 = -H_2C-CH_2\cdot CH_2-CH_2-\overset{\overset{CH_3}{|}}{\underset{\underset{H}{|}}{N^+}}-CH_3$

A: 1-ethyl-3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC)
B: *N*-hydroxysuccinimide (NHS)

Fig. 2. Schematic picture of immobilization of MPC polymer with collagen.

into the MES buffer pH 9.0 for 1 day to obtain a non cross-linked collagen gel (Uc-gel) and was used as a reference.

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 scanning electron microscopy (SEM, SM-200, Topcon, Tokyo, Japan). The samples, which had been cut into small pieces, were lyophilized overnight. The chemical composition of the surfaces of the gel was determined by the take-off angle of the photoelectrons fixed at 90°. The morphologies of the gels were observed with SEM after gold coating with an ion coater (IB-3, Eiko Co., Ibaraki, Japan). The razor blade-cut surfaces of the respective gels were observed.

2.2.2. Shrinkage temperature

The shrinkage temperatures of the gels were determined using differential scanning calorimetry (DSC, DSC6000, Seiko, Chiba, Japan) in the range 0–150 °C at a scanning rate 5 °C/min. The samples were incubated with small amounts of phosphate buffer solution for 1 h at room temperature before being measured [9]. Instead of an empty container, a container of PBS was used for reference.

2.2.3. Mechanical properties

The stress–strain curves of the respective collagen gels were determined by uniaxial measurements using a universal testing machine (Orientec STA-1150, Tokyo, Japan). The sizes of the samples used for measurement were 4 cm × 1 cm. Each sample was strained at the rate of 10 mm/min. The obtained data were fitted to the stress–strain curves of the samples and the elongational modulus at 1% and 8% was calculated.

2.2.4. Swelling test

A swelling test of each sample was executed by cutting the lyophilized gels into small pieces and putting them into pH-controlled aqueous solutions at 37 °C. The pH of the aqueous solution was controlled to 2.1 or 7.4. The gels were gently shaken for 24 h and then removed for weighing. The swelling ratio was calculated in order to define the exact amount of swelling caused by water absorption. The equation used for the swelling ratio was

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

where W_h is hydrated weight and W_d is dried weight of the gel.

2.2.5. Enzymatic degradation test

Degradation tests of the gel samples were executed using collagenase from Clostridiopeptidase histoluticum (EC 3.4.24.3, *Sigma*, St. Louis, USA) with collagenase activity of 300 units/mg. In this experiment, 30 ± 2 mg of collagen gels were immersed into 2 mL of 0.1 M Tris-HCl buffer (pH 7.4) with 5×10^{-3} M of calcium chloride (Kanto Chemical, Tokyo, Japan) and 8×10^{-4} M of sodium azide (Kanto Chemicals, Tokyo, Japan) and was shaken for 1 h at 37 °C. Then, 2 mL of collagenase Tris-HCl buffer solution with a concentration of 1.32 mg/mL was added to the solution containing the gel to determine the total concentration of collagenase at 100 units/mL. The container was returned to the shaking water bath. The remaining weights of the samples were measured for 72 h.

2.2.6. Statistical analysis

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

3. Results and discussion

3.1. Basic characteristics of collagen gels

The reaction between EDC and the carboxyl groups are shown elsewhere; the mechanism is well known [25,26]. According to Nakajima and Ikada [26], proton and ionized carboxyl groups are required for the reaction with EDC. The excess amount of EDC against the carboxyl groups should be used up, and no reaction occurred when the molar ratio of EDC to the carboxyl groups was below 0.5. Using EDC for cross-linking might cause hydrolysis, which makes the carboxyl groups return to the original carboxyl groups.

The use of NHS is to prohibit the hydrolysis of the carboxyl groups. NHS would lead to formation of NHS-ester, which prevents the side reaction of the *O*-acylisourea groups [25,26]. This is because the reactive species relative to the neucleophilic attack of the free amine group of collagen are the NHS-activated carboxyl groups rather than the *O*-acylisourea groups.

Fig. 3 shows the XPS result of Uc-gel, EN-2 gel, MiC-21 gel, and MiC-12. All gels showed XPS signals attributed to carbon in CH_3– or –CH_2–, –COC–, C(=O)–, and nitrogen in –CONH– was observed at 285, 286.6, 288.5, and 400.8 eV, respectively. The phosphorus peak and one nitrogen peak in $-N^+(CH_3)_3$ were observed at 134 and 403.2 eV, respectively, indicating that PMA was a properly cross-linked collagen [21,24].

SEM images of the outer surfaces and razor blade-cut surfaces (vertical cross-section) of the respective collagen gels are shown in Fig. 4. The razor blade-cut surfaces of the Uc-gel and the EN-1 and EN-2 gels are porous. For MiC, the non-porous layer is shown to be deposited on the porous layer. Non-porosity can be seen for the pure PMA film prepared using same method (image not shown). This implies that PMA covers the collagen gel instead of being blended, making it a heterogeneous phase. However, the outer surface of the gel is entirely one phase showing no defects, indicating that PMA is immobilized on the collagen surface and is distributed homogeneously. This is because the high molecular weight of the PMA causes the polymer to be located primarily on the surface of the collagen gel. When the PMA and collagen are premixed and gelled, the razor blade-cut surface shows that the porous and non-porous structures coexist (picture not shown). In the case of MiC-11 and MiC-12, the non-porous outer layer is very thin and the pore size is bigger, indicating that a sizeable amount of swelling had occurred.

3.2. Network structure of collagen gels

Shrinkage temperature T_s is considered as the rupture of the inter-chain bonds bringing the fusion of the oriented peptide chains [27], which is responsible for the shrinkage, and the cross-linking will result in the stabilization of the triple helix structure and an increase in the shrinkage temperature [28]. Table 2 lists T_s of the respective collagen gels. The result indicates that T_s would increase when the gels are cross-linked. Because PMA is immobilized, T_s of the gels would shift to a higher temperature, eventually reaching approximately 85 °C, which is about a 30 °C increase from that of uncross-linked collagen gel. The EN-1 gel and the MiC-11 and MiC-12 gels showed that T_s is lower than the EN-2 gel and the MiC-21 and MiC-22 gels. This implies that formation of inter- and intrahelical cross-links, which prevent the fusion of the peptide chains, is very important for stabilization of the network. The immobilization of PMA made the extra cross-link, that is, the bond between the PMA chain and collagen microfibril by the amide bond, eventually increasing T_s further. Comparing T_s of MiC-11 and MiC-12, we can see that the numeric value is almost the same. The same phenomenon can be seen for MiC-21 and MiC-22, implying that the immobilization of PMA would be affected by the pH of the MES buffer. Under pH 4.5, the carboxyl groups of PMA would be protonated, leading to the formation of COO–NHS, because the pK_a of PMA is known to be 2.7 [29].

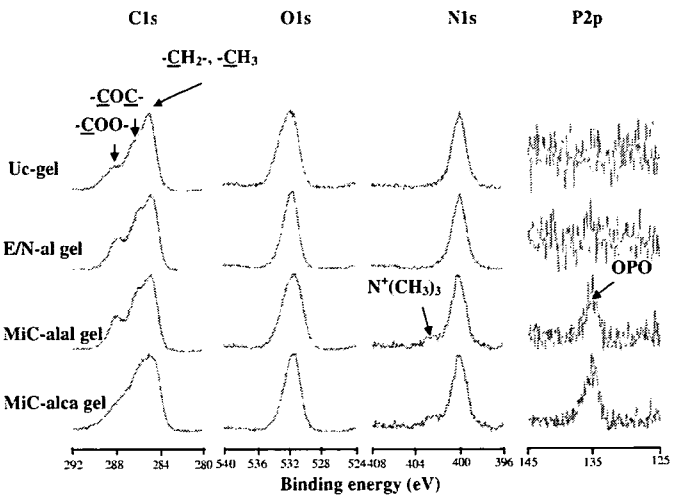


Fig. 3. XPS chart of Uc-gel, E/N-2l gel, MiC-22 gel, and MiC-21 gel. The takeoff angle of photoelectron was 90°.

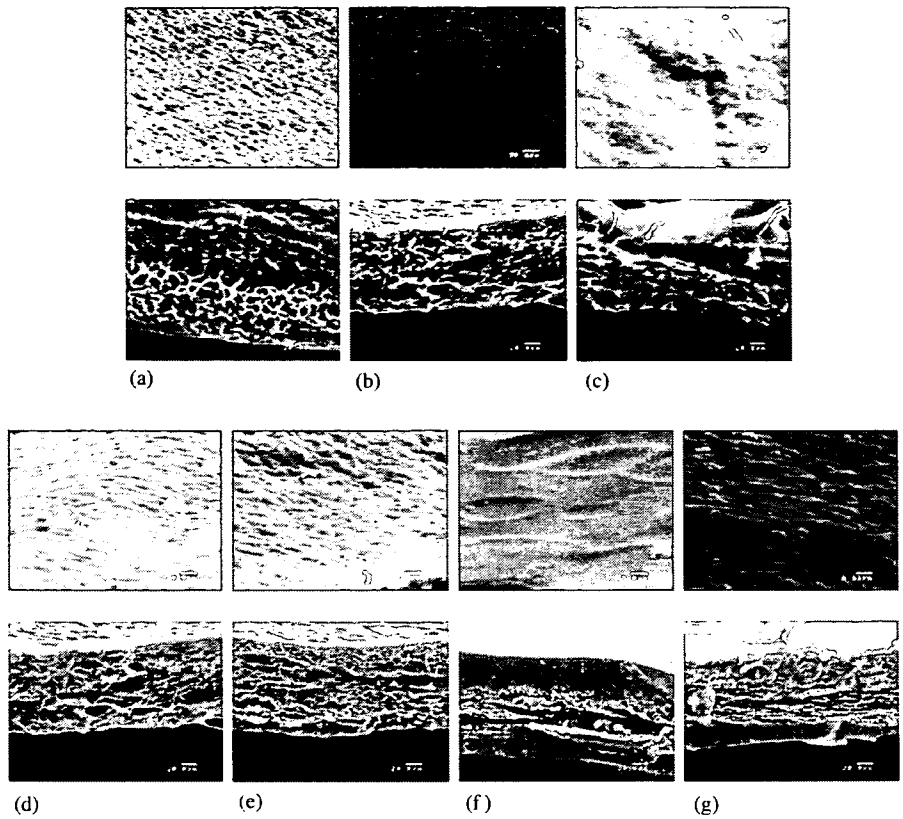


Fig. 4. Outer surface morphology (upper) and razor blade cut morphology (below) of respective gels: (a) Uc-gel, (b) E/N-1 gel, (c) E/N-2 gel, (d) MiC-11 gel, (e) MiC-12 gel, (f) MiC-22 gel, and (g) MiC-21 gel.

Table 2
Shrinkage temperatures of collagen and collagen gels

Sample	T_s (°C)
Uncross-linked	56.4±8.1
EN-1	67.4±0.9
EN-2	76.5±2.9
MiC-11	74.1±3.9
MiC-12	75.1±2.0
MiC-21	84.8±2.0
MiC-22	84.1±3.9

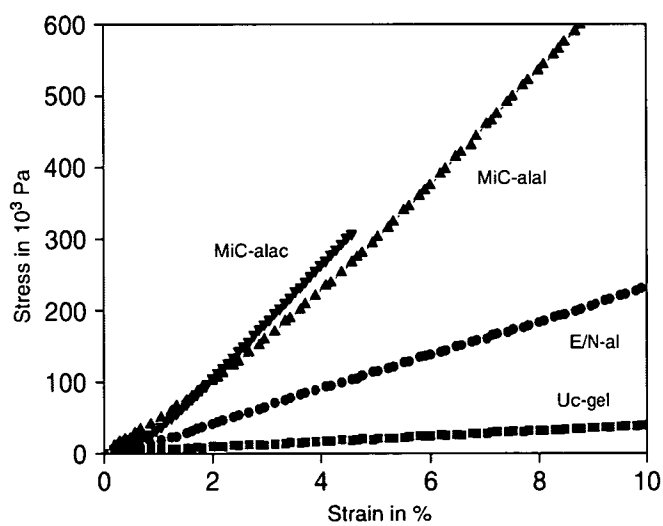


Fig. 5. Stress–strain curve of respective collagen gels.

Fig. 5 shows the strain–stress curve of the Uc-gel, EN-2 gel, MiC-22, and MiC-21 gels. It can be seen that all collagen gels are J-shaped. This shape indicates that, after the cross-linking and immobilization processes, the collagen maintains its soft tissue viscoelastic behavior, which is soft and tough [30]. Table 3 shows the results of the elongational strain modulus of the respective gels at 1% and 8% of strain. Cross-linking with EDC/NHS increased the elongational strain modulus approximately five times and immobilization of PMA increased the elongational strain modulus about 12.5 times that of the uncross-linked collagen gel. The cross-linking process and immobilization of PMA made the collagen gel much tougher. This strongly suggests that the PMA must be immobilized onto the surface of the collagen gel in order to maintain its biomolecular property and stronger mechanical property simultaneously. The EN-1 gel, MiC-21, and MiC-11 gels were too fragile to measure the strain modulus.

Fig. 6 shows the swelling of the respective gels under pH 2.4 and pH 7.4. For uncross-linked gels, the gel dissolved under pH 2.4, while it swelled approximately 1400% under pH 7.4. When collagen gels absorb water, the triple helix structure is known to turn into a random coil conformation because the collagen peptide chains increase the accessibility to hydration. In the neutral and alkaline

Table 3
Mechanical strength of the collagen gels

Sample	Strain modulus at 1% (MPa)	Strain modulus at 8% (MPa)
Uncross-linked	0.4±0.1	0.6±0.1
EN-2 gel	2.1±0.1	2.9±0.2
MiC-21	5.6±1.1	8.7±1.6
MiC-21	5.1±0.6	8.0±1.0

Mechanical strength of EN-1, MiC-11 and MiC-12 was not measured due to fringe nature of the samples.

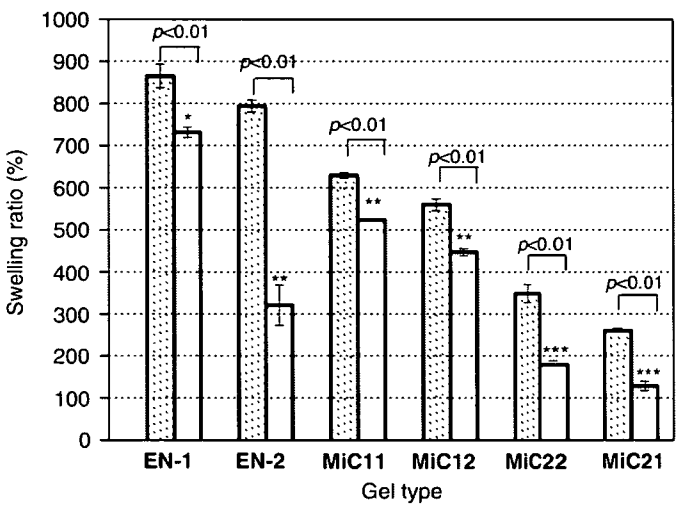


Fig. 6. Swelling ratio of respective collagen gels under pH 2.1 (hatched bar) and under pH 7.4 (empty bar) aqueous solutions. Each value represents the mean±SD ($n = 5$).

conditions, collagen film would be stabilized by forming an entanglement of fibrils formed by hydrophobic and electrostatic bonds [31–33]. Since the pK_a of collagen type I is known to be approximately 5.5 [34,35], a stable gel without any cross-linker can be formed under neutral and alkaline conditions.

The EN-1 and EN-2 gels showed a high swelling ratio under pH 2.1, but had different swelling ratios under neutral pH conditions. The EN-1 gels showed a swelling ratio of about 870% under pH 2.4 and 730% under pH 7.4, while the EN-2 gels showed 800% under pH 2.4 and 320% under pH 7.4. The swelling ratio was relatively higher for the EN-1 gel than the EN-2 gel because the network density was much higher for the EN-2 gel. The EN-2 gel, for which cross-linking was executed under alkaline conditions, is thought to possess a denser cross-linking network. As mentioned earlier, EDC and NHS are known to bring inter- and intrahelical cross-links, holding the α-helices together tightly. [36,37].

Immobilization of PMA on the collagen gels brought different swelling ratios according to the conditions of preparation. For the MiC-11 and MiC-12 gels, the swelling ratio was lower than that for the EN-1 gel, implying that a network between collagen and PMA is formed by the interchain cross-links. However, their swelling ratio under pH 2.4 was lower than that for the EN-2 gel, but was higher under pH 7.4. PMA could not penetrate into the collagen gel during the immobilization process, leaving much of the amine groups unreacted. In contrast, MiC-21 and MiC-22 showed that the swelling ratio under pH 2.4 and pH 7.4 would be lowest among all collagen gels. As mentioned earlier, the formation of a denser network brought a lower swelling ratio. The low swelling ratio of the MiC-11 and MiC-12 gels under pH 2.4 and pH 7.4 implies that the intra- and interhelical cross-links play important roles in the stabilization of the collagen gels.

3.3. Degradation of collagen gels by collagenase

Fig. 7 shows the degradation of collagen gels caused by the activation of collagenase in Tris-HCl buffer. The collagenase absorbed into the collagen gel would cleave the helical segment, hydrolyzing the collagen gels. Collagenase is known to be adsorbed onto the collagen fibers once it penetrates into the fiber [36–39]. Therefore, it is thought that the swelling ratio is related to this biodegradation process.

Our study shows that the collagen gel that is not cross-linked would degrade within 2 or 3 h. Cross-linking the collagen with EDC and NHS would strongly maintain the helical structure, extending the time of complete degradation from 6 to 24 h according to the cross-linking conditions. Low swelling collagen gels lead to slow degradation. For the MiC-22 and MiC-21 gels, almost 80% of the original collagen gel remained after 24 h. The E/N gels have only intra- and interhelical cross-links while the MiC gels possess interchain cross-links. For the E/N

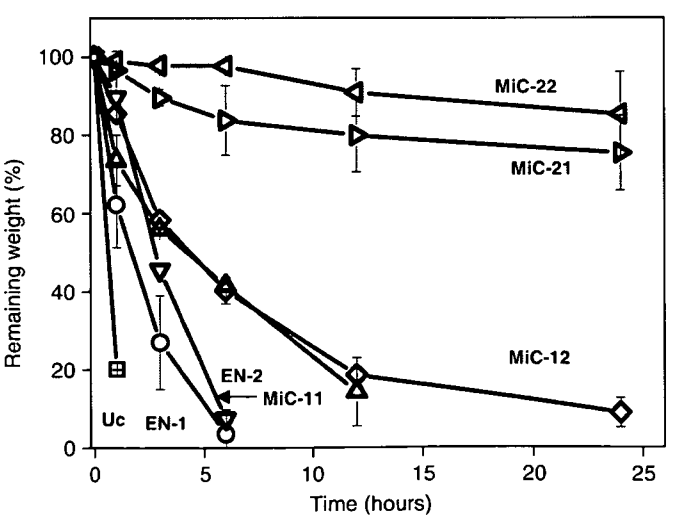


Fig. 7. Degradation of collagen gels by collagenase in Tris-HCl buffer (pH 7.4) at 37 °C. Each value represents the mean±SD ($n = 4$).

gels, the intra- and interhelical cross-links maintained the helical structure after cleavage by collagenase [39]. However, the absorption of water eventually made the E/N gels dissociate within 24 h, with slightly faster degradation for the EN-1 gel. In contrast, the MiC gels possess interchain cross-links that link the microfibrils and the PMA chains, making the degree of swelling much lower [38]. The cleavage by collagenase would be prevented by the PMA-collagen network, which links microfibrils together, shielding the helices.

4. Conclusion

We were able to successfully immobilize MPC to collagen and prepare a stable gel. By using collagen film prepared from 0.5 wt% collagen solution, MiC gels were prepared under MES buffer. EDC/NHS and PMA polymer could form a cross-link with the collagen film. The physical behaviors of the gels changed according to the preparation conditions such as the pH of the MES buffer. Inter- and intrahelical cross-links were formed by EDC/NHS. Higher cross-link efficiency can be obtained under an alkaline condition because the pK_a of collagen is approximately 5.5. A pre-NHS activated PMA polymer chain could be located on the collagen gel and cross-linked with the amine collagen group, forming an interchain cross-link. Since the pK_a of PMA carboxyl groups is 2.7, the immobilization of PMA was successful at any pH. The coexistence of intra- and interhelical cross-links and intermolecular cross-links make the network much denser, which leads to difficulty in either penetration or hydrolyzation by the collagenase. Mechanical and enzyme stability enable this gel to be applied as a biosynthetic hybrid biomaterial.

We will report on the biological properties of the collagen–phospholipid polymer hybrid gel in the near future.

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References

[1] Fujioka K, Maeda M, Hojo T, Sano A. Protein release from collagen matrices. Adv Drug Deliv Rev 1998;31:247–66.

[2] Kato YP, Silver FH. Formation of continuous collagen fibers: evaluation of biocompatibility and mechanical properties. Biomaterials 1990;11:169–75.

[3] Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. Crosslinking of dermal sheep collagen using a water-soluble carbodiimide. Biomaterials 1996;17:765–73.

[4] Cascone MG, Barbani N, Cristalli C, Giusti P, Ciardelli G, Lazzeri L. Bioartificial polymer materials based on polysaccharides. J Biomater Sci Polym Edn 2001;12:267–81.

[5] Barbani N, Lazzeri L, Cristalli C, Cascone MG, Polacco G, Pizzirani G. Bioartificial materials based on blends of collagen and poly(acrylic acid). J Appl Polym Sci 1999;72:971–6.

[6] Cascone MG, Martinti S, Barbani N, Laus M. Effect of chitosan and dextran on the properties of poly(vinyl alcohol) hydrogels. J Mater Sci Mater Med 1999;10:431–5.

[7] Sionkowska A. Interaction of collagen and poly(vinyl pyrrolidone) in blends. Eur Polym J 2003;39:2135–40.

[8] Dascălu MC, Vasile C, Silvestre C, Pascu M. On the compatibility of low density polyethylene/hydrolyzed collagen blends. II: new compatibilizers. Eur Polym J 2005;41:1391–402.

[9] Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. Glutaraldehyde as a crosslinking agent for collagen-based biomaterials. J Mater Sci Mater Med 1995;6:460–72.

[10] Sung HW, Chang WH, Ma CY, Lee MH. Crosslinking of biological tissue using genipin and/or carbodiimide. J Biomed Mater Res 2003;64A:427–38.

[11] Orban JM, Wilson LB, Kofroth JA, El-Kurdi MS, Maul TM, Vorp DA. Crosslinking of collagen gels by transglutaminase. J Biomed Mater Res 2004;68:756–62.

[12] Segura T, Chung PH, Shea LD. DNA delivery from hyaluronic acid–collagen hydrogels via a substrate-mediated approach. Biomaterials 2005;26:1575–84.

[13] Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, et al. Immobilization of heparin to EDC/NHS-crosslinked collagen, characterization and in vitro evaluation. Biomaterials 2001;22:151–63.

[14] Perper JS, Hafmans T, Veerkamp JH, van Kuppevelt TH. Development of tailoir-made collagen-glycosaminoglycan matrices: EDC/NHS crosslinking, and ultrastructural aspects. Biomaterials 2000;21:581–93.

[15] Jaworski J, Klapperich CM. Fibroblast remodeling activity at two- and three-dimensional collagen–glycosaminoglycan interfaces. Biomaterials 2006;23:4212–20.

[16] van Luyn MJA, van Wachem PB, Olde Damink LHH, Dijkstra PJ, Feijen J. Relations between in vitro cytotoxicity and crosslinked dermal sheep collagens. J Biomed Mater Res 1992;26:1091–110.

[17] van Wachem PB, van Luyn MJA, Olde Damink LHH, Dijkstra PJ, Nieuwenhuis P. Biocompatibility and tissue regenerating capacity of crosslinked dermal sheep collagen. J Biomed Mater Res 1994;17:353–63.

[18] van Wachem PB, Zeeman R, Dijkstra PJ, Feijen J, Hendriks M, Cahalan PT, et al. Characterization and biocompatibility of epoxy-crosslinked dermal sheep collagens. J Biomed Mater Res 1999;47:270–7.

[19] Ishihara K, Ueda T, Nakabayashi N. Preparation of phospholipid polymers and their properties as polymer hydrogel membranes. Polym J 1990;22:355–60.

[20] Iwasaki Y, Mikami A, Kurita K, Yui N, Ishihara K, Nakabayashi N. Reduction of surface-induced platelet activation on phospholipid polymer. J Biomed Mater Res 1997;36:508–15.

[21] Ishihara K, Nomura H, Mihara T, Kurita K, Iwasaki Y, Nakabayashi N. Why do phospholipid polymers reduce protein adsorption? J Biomed Mater Res 1998;39:323–30.

[22] Kitano H, Imai M, Mori T, Gemmei-Ide M, Yokoyama Y, Ishihara K. Structure of water in the vicinity of phospholipid analogue copolymers as studied by vibrational spectroscopy. Langmuir 2003;19:10260–6.

[23] Ueda H, Watanabe J, Konno T, Takai M, Saito A, Ishihara K. Asymmetrically functional surface properties on biocompatible phospholipid polymer membrane for bioartificial kidney. J Biomed Mater Res A 2006;77:19–27.

[24] Watanabe J, Ishihara K. Phosphorylcholine and poly(D,L-lactic acid) containing copolymers as substrates for cell adhesion. Artif Organs 2003;27:242–8.
[25] Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. In vitro degradation of dermal sheep collagen crosslinked using a water-soluble carbodiimide. Biomaterials 1996;17:679–84.
[26] Nakajima N, Ikada Y. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. Bioconjugate Chem 1995;6:123–30.
[27] Flory PJ, Garrett RR. Phase transitions in collagen and gelatin systems. J Am Chem Soc 1958;80:4836–45.
[28] Khor E, Li HC, Wee A. Animal tissue-polypyrrole hybrid biomaterials: shrinkage temperature evaluation. Biomaterials 1996;17:1877–9.
[29] Nam K, Watanabe J, Ishihara K. Modeling of swelling and dissociation mechanism, and release behavior of spontaneously forming hydrogel composed of phospholipid polymers for oral delivery carrier. Int J Pharma 2004;275:259–69.
[30] Gentleman E, Lay AN, Dickerson DA, Nauman EA, Livesay GA, Dee KA. Mechanical characterization of collagen fibers and scaffolds for tissue engineering. Biomaterials 2003;24:3805–13.
[31] Ripamonti A, Roveri N, Briga D. Effects of pH and ionic strength on the structure of collagen fibrils. Biopolymers 1980;19:965–75.
[32] Wallace D. The relative contribution of electrostatic interactions to stabilization of collagen fibrils. Biopolymers 1990;29:1015–26.
[33] Rosenblatt J, Devereux B, Wallace D. Dynamic rheological studies of hydrophobic interactions in injectable collagen biomaterials. J Appl Polym Sci 1993;50:953–63.
[34] Luescher M, Ruegg M, Schindler P. Effect of hydration upon the thermal stability of tropocollagen and its dependence on the presence of neutral salts. Biopolymers 1974;13: 2489–503.
[35] Zhang J, Senger B, Vautier D, Picart C, Schaaf P, Voegel J-C, et al. Natural polyeletrolyte films based on layer-by-layer deposition of collagen and hyaluronic acid. Biomaterials 2005;26: 3353–61.
[36] Zeeman R, Dijkstra PJ, van Wachem PB, van Luyn MJ, Hendriks M, Cahalan PT, et al. Successive epoxy and carbodiimide crosslinking of dermal sheep collagen. Biomaterials 1999;20:921–31.
[37] Vizárová K, Bakos D, Rehákova M, Petríkova M, Panáková E, Koller J. Modification of layered atelocollagen: enzymatic degradation and cytotoxicity evaluation. Biomaterials 1995;16: 1217–21.
[38] Park S-N, Park J-C, Kim HO, Song MJ, Suh H. Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking. Biomaterials 2002;23:1205–12.
[39] Ma L, Gao C, Mao Z, Zhou J, Shen J. Enhanced biological stability of collagen porous scaffolds by using amino acids as novel cross-linking bridges. Biomaterials 2004;25:2997–3004.

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ORIGINAL ARTICLE

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Preparation of poly(vinyl alcohol)/DNA hydrogels via hydrogen bonds formed on ultra-high pressurization and controlled release of DNA from the hydrogels for gene delivery

Abstract Poly(vinyl alcohol) (PVA) hydrogels interacting with DNA mediated by hydrogen bonds (PVA/DNA hydrogel) were developed using ultra-high pressure (UHP) technology. The goal was to create a new method of gene delivery by controlled release of DNA. Mixed solutions of DNA and PVA at various concentrations were pressurized at 10000 atmospheres at 37°C for 10 min. PVA/DNA hydrogels with good formability were produced at PVA concentrations of more than 5% w/v. The presence of DNA in the obtained hydrogels was confirmed by spectroscopic analysis and nucleic acid dye staining. DNA release from the hydrogels was investigated using PVA/DNA hydrogel samples of 5% and 10% w/v formed by UHP treatment or by conventional freeze–thaw methods. The DNA release curves from both types of samples showed a rapid phase in the initial 15 h followed by a sustained release phase. However, there was a difference in the amount of DNA released. Less DNA was released by the pressurized hydrogels than by the freeze–thaw hydrogels. Also, the cumulative amount of DNA released decreased as the PVA content in the hydrogels increased. These results indicate that DNA release from the hydrogels can be modulated by changing the preparation method and the PVA content. Furthermore, it was demonstrated that DNA release could be controlled by varying the amount and duration of pressurizing used to form the hydrogels. Intact fractions of plasmid DNA released from the hydrogels were separated by agarose gel electrophoretic analysis. These results suggest that, using controlled release, DNA from PVA/DNA hydrogels formed by UHP treatment can be transfected into cells.

Key words Controlled release · Ultra-high pressure · DNA · Hydrogel · Poly(vinyl alcohol)

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Introduction

Safe and biocompatible synthetic materials have been developed as biomaterials.[1] In gene therapy, nonviral synthetic gene carriers have been the focus of attention due to their biological safety advantages over viruses.[2] In many cases, cationic synthetic materials, such as cationic lipids, liposomes,[3] polyethyleneimine,[4] polyamideamine dendrimer,[5] poly-L-lysine (PLL), PLL derivatives,[6] and other cationic peptides,[7] have been used as nonviral vectors. It is possible to form complexes between these materials and DNA using the electrostatic interaction between their cationic groups and the anionic groups of DNA, making the DNA robust against nuclease degradation and enabling effective transfection into mammalian cells.[8,9] However, the cytotoxicity of cationic materials was reported to be a significant problem.[10,11] For safer and more efficient gene delivery, it is necessary to develop a noncationic or less cationic gene carrier through nonelectrostatic interaction with DNA. Sakurai et al. reported that a triple helical complex of single-strand DNA and double-strand schizophyllan, which is a kind of polysaccharide (β-1,3 glucan), was formed through hydrogen bonding.[12] In addition, we previously reported that nanoparticles of poly(vinyl alcohol) (PVA) bonded to DNA via hydrogen bonds were obtained when mixed solutions of PVA (less than 0.01% w/v) and DNA were treated under ultra-high pressure (UHP) at

10000 atmospheres (980MPa) and 40°C for 10min.[13] It is well known that intra- and intermolecular hydrogen bonding increases in these conditions.[14] The PVA/DNA nanoparticles could be internalized into mammalian cells, suggesting that they have utility as a novel nonviral vector that uses nonelectronic interactions.

Recently, controlled release of DNA was also investigated as a possible method of enhancing transfection efficiency using various biomaterials such as poly (lactide-co-glycolide) (PLGA),[15] hyaluronic acid,[16] atelocollagen,[17] and gelatin.[18,19] Shea et al. reported that the sustained delivery of DNA from PLGA led to effective transfection of a large number of cells in vitro and in vivo.[15] However, it was difficult to regulate the release of DNA owing to the lack of interaction forces, such as covalent, electrostatic, and hydrogen bonding, with which DNA molecules are loaded into PLGA with polymer molecules. Tabata et al. reported enhancement and prolongation of gene expression using a cationized gelatin hydrogel interacting with DNA electrostatically.[18,19] The controlled release of DNA depended on hydrogel degradation, but the cationized gelatin hydrogel was crosslinked by glutaraldehyde, which has generally cytotoxic properties, to obtain different degrees of cationization.

In the present study, we report the preparation of a novel PVA hydrogel with DNA crosslinked physically by hydrogen bonds using UHP technology and its application to the controlled release of DNA. The goal is to develop an effective, low-cytotoxic and gene-releasable biomaterial. PVA/DNA hydrogels were obtained for various pressurization conditions, temperatures, and processing times. DNA release from the hydrogels was investigated in vitro. PVA is widely used for biomedical applications because of its biocompatibility and neutrally charged nature.[20] It is also known that PVA hydrogel is formed by physical crosslinking with hydrogen bonds when PVA solution is frozen and thawed several times, which is called the freeze–thaw method.[21]

Materials and methods

Materials

In our experiments, we used PVA samples with an average molecular weight of 74800 and a degree of saponification of 99.8%, as supplied by Kuraray (Osaka, Japan). We also used salmon sperm DNA purchased from Wako (Osaka, Japan), plasmid DNA encoding enhanced green fluorescence protein under a cytomegalovirus promoter (pEGFP-N1, BD Science, Palo Alto, CA, USA), and nucleic acid staining dye solution (Mupid Blue) obtained from Advance (Tokyo, Japan).

Preparation of PVA/DNA hydrogels by UHP

Aqueous PVA solutions of 6%, 8%, 10%, 14%, and 20% w/v were prepared by autoclaving three times for 30min at 121°C. Salmon sperm DNA was dissolved in a Tris-EDTA buffer (TE, pH = 7.8) at a concentration of 16.3mg/ml. The DNA solution was mixed with PVA solutions of 10%, 14%, and 20% w/v at a ratio of 1:1. The 0.7-ml samples were transferred in silicon tubes (9 × 25mm) with both ends capped by silicon plugs. The tubes were pressurized under various UHP conditions, using different pressures, temperatures, and durations, in a high-pressure machine (Kobe Steel, Kobe, Japan).

Confirmation of the presence of DNA in the PVA/DNA hydrogels

The presence of DNA in the PVA/DNA hydrogels produced by UHP treatment was confirmed by nucleic acid dye staining and UV-visible spectroscopy. For the former method, the PVA/DNA hydrogels were immersed in nucleic acid dye solution for 1 min and then transferred to 70% ethanol. After 1min, they were immersed in ion-exchanged water for 1min. For the latter method, after the PVA/DNA hydrogels were melted at 90°C for 10min, their DNA concentration was measured by a spectrophotometer (V-560, JASC, Tokyo, Japan).

DNA release from hydrogels

The PVA/DNA hydrogels prepared by UHP were immersed in 5ml of phosphate-buffered saline (PBS) for 144h at 37°C. At 0.25, 0.5, 2, 3, 15, 27, 48, 111, and 144h, 20μl of the samples in the outer part of the PBS solution was collected and the DNA concentration was measured spectrophotometrically at 260nm (Gene Quant Pro S, Amersham, Tokyo, Japan).

Stability of plasmid DNA released from hydrogels

Plasmid DNA (pDNA) was used instead of salmon sperm DNA and the mixed solutions of pDNA (100μg/ml) and PVA (5% or 10% w/v) were treated by UHP under the conditions described above. The obtained PVA/pDNA hydrogels were immersed in PBS for 12 and 48h, and then the samples in the outer part of the solution were collected and analyzed by agarose gel electrophoresis at 100V for 45min.

Results and discussion

Aqueous solutions of PVA at concentrations ranging from 3% to 10% w/v were hydrostatically pressurized at 10000atm at 37°C for 10min. With a PVA solution of 3% w/v, the clear solution was transformed into a turbid and viscous solution by pressurization (Fig. 1A). An aggregation of PVA particles with an average diameter of 1μm was observed in the PVA solution on scanning electron microscopy (SEM, data not shown). For PVA concentrations of more than 4% w/v, hydrogels were produced on pressuriza-

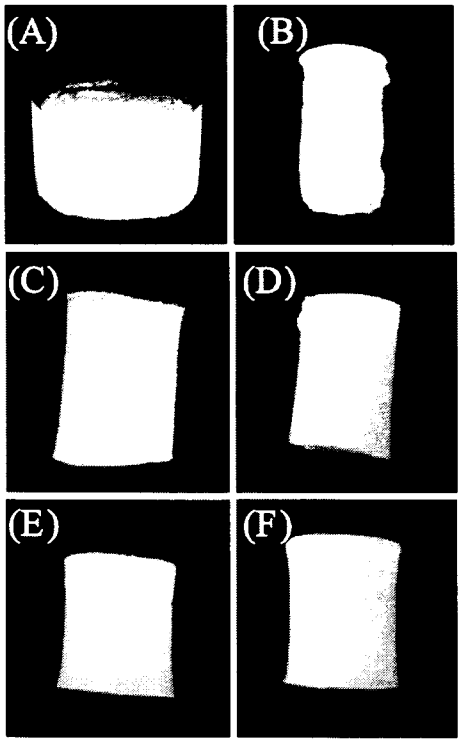

Fig. 1. Photographs of poly(vinyl alcohol) (PVA) hydrogels (**A–D**) and PVA/DNA (**E,F**) hydrogels at concentrations of **A** 3% w/v, **B** 4% w/v, **C,E** 5% w/v, and **D,F** 10% w/v obtained by ultra-high pressure treatment

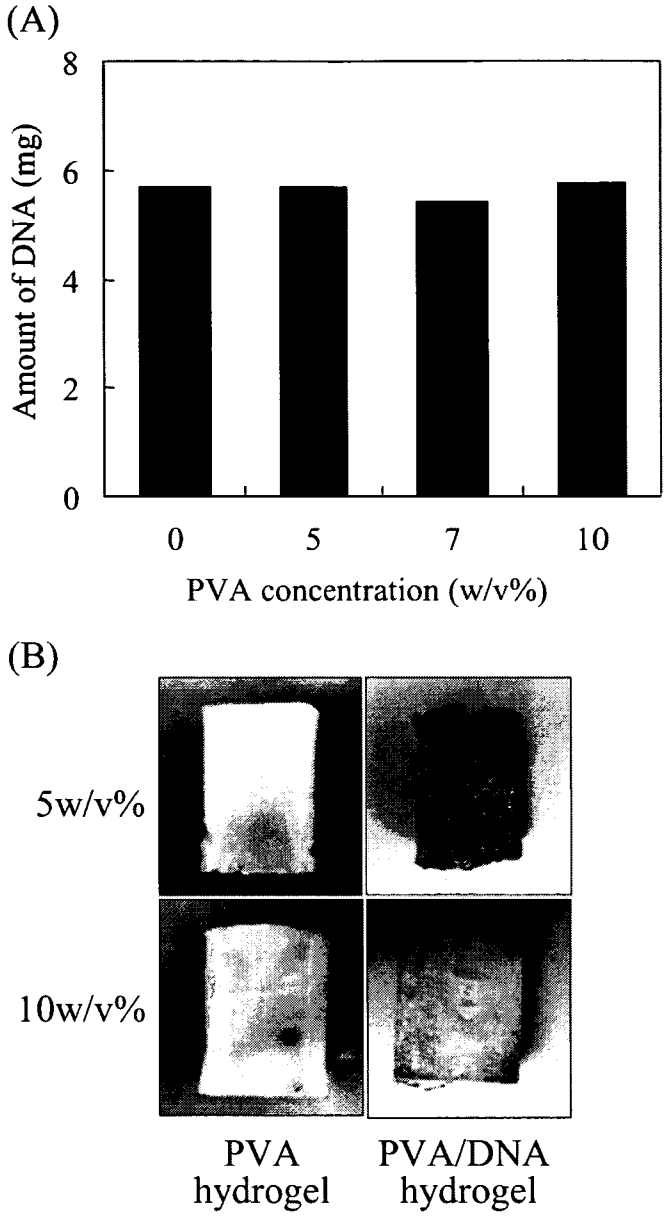

Fig. 2A,B. Presence of DNA in PVA/DNA hydrogels. **A** Amount of DNA in solution obtained by melting PVA/DNA hydrogels prepared using ultra-high pressure processing. **B** Photographs of PVA hydrogels and PVA/DNA hydrogels stained with nucleic acid dye

tion (Fig. 1B–D). The PVA hydrogel of 4% w/v was fragile (Fig. 1B), but increasing the PVA concentration enhanced hydrogel formability, and hard hydrogels were obtained at a PVA concentration of 10% w/v (Fig. 1D). These results indicate that pressurization induced physical cross-linking of PVA molecules and that the degree of cross-linking increased as the PVA concentration increased. To investigate whether the PVA molecules were physically cross-linked by hydrogen bonding, a PVA solution of 5% w/v with urea (3.3 M), which was used as a hydrogen bond inhibitor, was treated under the above pressurizing conditions. The solution remained translucent (data not shown), indicating that the PVA hydrogel obtained by pressurization was mediated by hydrogen bonding.

The gelation of mixed solutions of DNA and PVA (5% and 10% w/v) was achieved by pressurization in the conditions described above (Fig. 1E,F). To confirm the presence of DNA in the hydrogels obtained, they were heat treated at 90°C for 10 min and then the DNA concentration of the solutions obtained was measured spectrophotometrically at 260 nm. Roughly equal amounts of DNA were contained in each hydrogel (Fig. 2A). Also, when the hydrogels were immersed in nucleic acid dye solution, which interacts electrostatically with the phosphate groups of DNA, the PVA hydrogel with DNA was stained, whereas the PVA hydrogel without DNA was not (Fig. 2B). These results indicate that a PVA hydrogel that sustains DNA (PVA/DNA hydrogel) was formed on pressurization. On the other hand, when urea was introduced, PVA/DNA hydrogel was not obtained on pressure treatment. This result suggests that hydrogen bonding between PVA and DNA took place in the pressurized PVA/DNA hydrogel.

DNA release from the PVA/DNA hydrogel formed by pressurization at 10000 atm at 37°C for 10 min was investigated. PVA/DNA hydrogels produced by the freeze–thaw method, a common method of forming PVA hydrogels,[21] were used as control samples. Figure 3A shows DNA release profiles from the PVA/DNA hydrogels at PVA concentrations of 5% and 10% w/v obtained by pressurization and the freeze–thaw method. Each release curve of DNA from a hydrogel consisted of a rapid phase in the initial 15 h followed by a sustained release phase. However, the amount

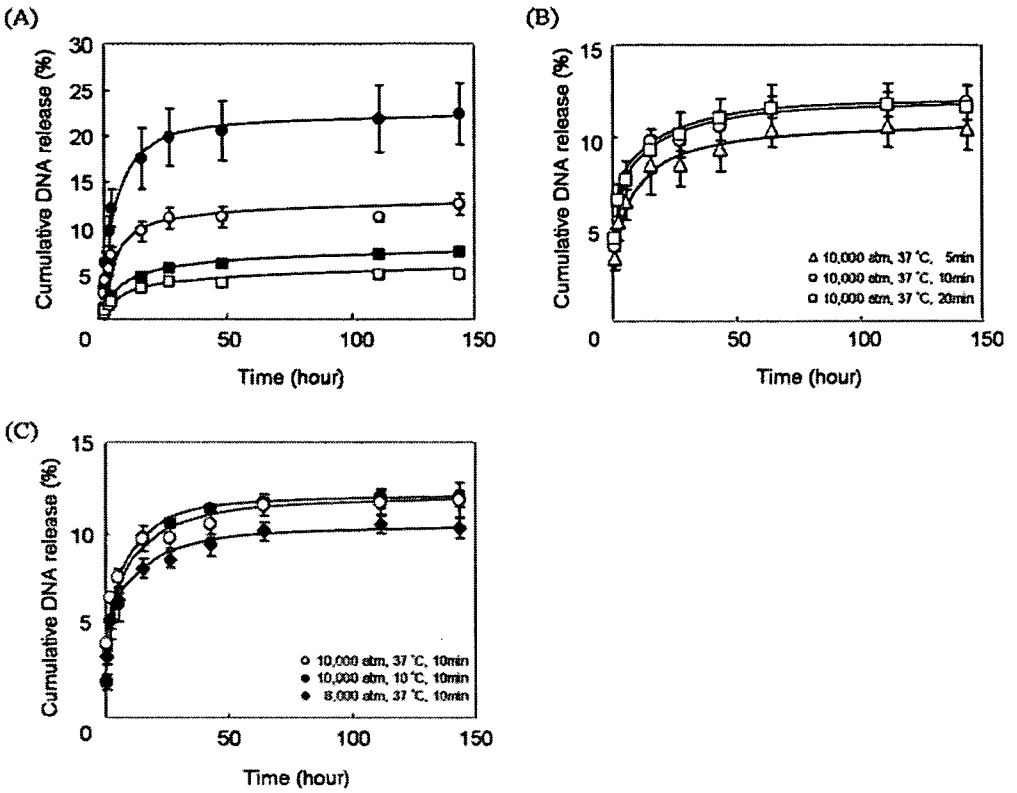

Fig. 3A–C. DNA release test from PVA/DNA hydrogels produced by pressurization under various conditions or by the freeze–thaw method. **A** Release profiles of DNA from hydrogels at PVA concentrations of 5% w/v (○, ●) and 10% w/v (□, ■) PVA concentration. *Open* and *solid* symbols indicate DNA from hydrogels obtained by pressurization (at 10000atm and 37°C, 10min) and the freeze–thaw method, respectively. **B** Release profiles of DNA from hydrogels of 5% w/v obtained by pressurization at 10000atm and 37°C for 5min (□), 10min (○), and 20min (□). **C** Release profiles of DNA from hydrogels of 5% w/v obtained by pressurization at 10000atm and 37°C (○), 10000atm and 10°C (●), and 8000atm and 37°C (□) for 10min

of DNA released was dependent on PVA content and on which procedure was used to prepare the hydrogels. The DNA release from the 10% w/v PVA/DNA hydrogels was lower than that from the 5% w/v PVA/DNA hydrogels, irrespective of the preparation methods. This is consistent with the fact that the 5% w/v samples were more easily stained by nucleic acid dye than the 10% w/v samples. We suppose that the increased crosslinking in the hydrogel caused by the increase in the PVA content contributed to the reduction of DNA released from the hydrogel. On the other hand, at the same PVA concentrations, DNA was more effectively released from the freeze–thaw hydrogels than from the pressurized hydrogels. Fibrous structures with large spaces (larger than 1μm) were observed on SEM in the hydrogels made from 5% w/v PVA obtained by the freeze–thaw method, while many porous structures with diameters of 300μm were observed in the pressurized hydrogels (data not shown). We believe that this difference in internal structure between sample types affected the interaction of PVA and DNA, resulting in the larger release of DNA from the freeze–thaw hydrogels.

To investigate the influence of the pressure conditions used to form hydrogels on DNA release, PVA/DNA hydrogels of 5% w/v were prepared by different levels of pressurization at different temperatures and for different durations. First, with pressure processing periods varying from 5 to 20min at 10000atm and 37°C, similar DNA release profiles were exhibited for the hydrogels obtained at pressurizing times of 10 and 20min, but the amount of DNA released by hydrogel samples pressurized for 5min (Fig. 3B) was less than that released by samples with longer pressurizing times. Second, the DNA release curves of the PVA/DNA hydrogel produced on pressurization at 10000atm and 10°C for 10min were the same as those for hydrogels produced on pressurization at 10000atm and 37°C for 10min. However, less DNA was released by hydrogels produced at pressures of 8000atm and 37°C for 10min than by hydrogels produced at 10000atm and 37°C for 10min (Fig. 3C). These results indicate that DNA release from pressurized hydrogels is dependent on the level and duration of pressure used in the hydrogel formation process. We previously reported that PVA gelation was promoted by increasing the pressure and by prolonging the pressurization time, by which close hydrogen bonds between PVA molecules are formed.[22] It seems that DNA was easily released from PVA/DNA hydrogels pressurized under conditions of more than 10000atm for longer than 10min because the hydrogen bonding interaction between PVA and DNA was more unstable than that between PVA molecules under more intense pressure conditions.

It is important for DNA to be released from hydrogels without structural change or degradation.[2,23] Plasmid DNA (pDNA), which is generally used as the DNA delivered by a nonviral vector, was used instead of salmon sperm DNA. PVA/pDNA hydrogels at PVA concentrations of 5% and 10% w/v were obtained by pressurization at 10000atm at 37°C for 10min and then immersed in 5ml PBS. After 12 and 48h of immersion, the outer part of the solution was collected and analyzed by agarose gel electrophoresis at 100V for 30min to investigate the stability of released pDNA from the hydrogels (Fig. 4). No degradation of DNA was observed, indicating that the plasmid DNA released

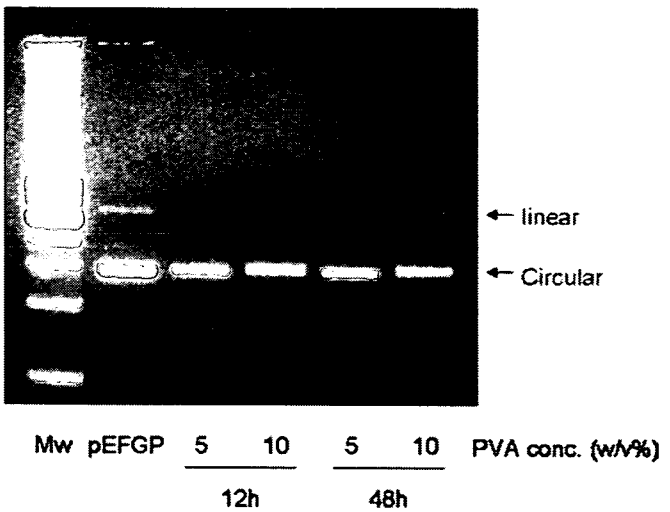

Fig. 4. Agarose gel electrophoresis of plasmid DNA (pDNA) released from PVA/pDNA hydrogels with PVA concentrations of 5% and 10% w/v produced by pressurization at 10000 atm and 37°C for 10 min after immersion in phosphate-buffered saline for 12 and 48 h

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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References

1. Nowak T, Nishida K, Shimoda S, Konno Y, Ichinose K, Sakakibara M, Shichiri M, Nakabayashi N, Ishihara K. Biocompatiblility of MPC: in vivo evaluation for clinical application. J Artif Organs 2000;1:39–46
2. Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. Nat Rev Genet 2005;6:299–310
3. Zhang S, Xu Y, Wan B, Qiao W, Liu D, Li Z. Cationic compounds used in lipoplexes and polyplexes for gene delivery. J Control Release 2004;100:165–180
4. Lungwitz U, Breunig M, Blunk T, Göpferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm 2005;60:247–266
5. Dufes C, Uchegbu IF, Scatzlein AG. Dendrimers in gene delivery. Adv Drug Deliv Rev 2005:57:2117–2202
6. Kimura T, Yamaoka T, Iwase R, Murakami A. Effect of physicochemical properties of polyplexes composed of chemically modified PL derivatives on transfection efficiency in vitro. Macromol Biosci 2002;2:437–446
7. Futaki S. Membrane-permeable arginine-rich peptides and the translocation mechanisms. Adv Drug Deliv Rev 2005;57:547–558
8. Reschel T, Koňák Č, Oupický D, Seymour LW, Ulbrich K. Physical properties and in vitro transfection efficiency of gene delivery vectors based on complexes of DNA with synthetic polycations. J Control Release 2002;81:201–217
9. Elouahabi A, Ruysschaert JM. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther 2005:11:336–347
10. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterals 2006;24:1121–1131
11. Choksakulnimitr S, Matsuda S, Tokuda H, Takakura Y, Hashida M. In vitro cytotoxicity of macromolecules in different cell culture systems. J Control Release 1995;34:233–241
12. Sakurai K, Mizu M, Shinkai S. Polysaccharide–polynucleotide complexes. 2. Complementary polynucleotide mimic behavior of the natural polysaccharide schizophyllan in the macromolecular complex with single-stranded RNA and DNA. Biomacromolecules 2001;2:641–650
13. Kimura T, Okuno A, Miyazaki K, Furuzono T, Ohya Y, Ouchi T, Mutsuo S, Yoshizawa H, Kitamura Y, Fujisato T, Kishida A. Novel PVA-DNA nonoparticles prepared by ultra high pressure technology for gene delivery. Mater Sci Eng C 2004;24:797–801
14. Doi E, Shimizu A, Kitabatake N. Gel-sol transition of ovalbumin by high pressure. In: Hayashi R (ed) High pressure bioscience and food science. Kyoto: Sanei Press, 1993;171–177
15. Shea LD, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue engineering. Nat Biotech 1999;17: 551–554
16. Chun KW, Lee JB, Kim SH, Rark TG. Controlled release of plasmid DNA from photo-cross-linked pluronic hydrogels. Biomaterials 2005;26:3319–3326
17. Ochiya T, Takahama Y, Nagahara S, Sumita Y, Hisada A, Itoh H, Nagai Y, Terada M. New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: the Minipellet. Nat Med 1999;5:707–710
18. Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, Tabata Y. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. Biomaterials 2005;26:3319–3326
19. Kushibiki T, Tomoshige R, Fukunaka Y, Kakemi M, Tabata Y. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. J Control Release 2003;90:207–216
20. Miyashita H, Shimmura S, Kobayashi H, Taguchi T, Asano-Kato K, Uchino Y, Kato M, Shimazaki J, Tanaka J, Tsubota K. Collagen-immobilized poly (vinyl alcohol) as an artificial cornea scaffold that supports a stratified corneal epithelium. J Biomed Mater Res Part B: Appl Biomater 2006;76B:56–63
21. Hyon SH, Cha WI, Ikada Y. Preparation of transparent poly (vinyl alcohol) hydrogel. Polymer Bull 1989;22:119–122
22. Yamamoto K, Furuzono T, Kishida A, Mustuo S, Yoshizawa H, Kitamura Y. Formation of a supramolecular assembly of poly (vinyl alcohol) by ultrahigh pressure. Meeting Report of the Poval Committee 2002;121:25–26
23. Walter E, Moelling K, Pavolvich HP. Microencapsulation of DNA using poly (D,L-lactide-co-glycolide): stability issues and release characteristics. J Control Release 1999;61:361–374

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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

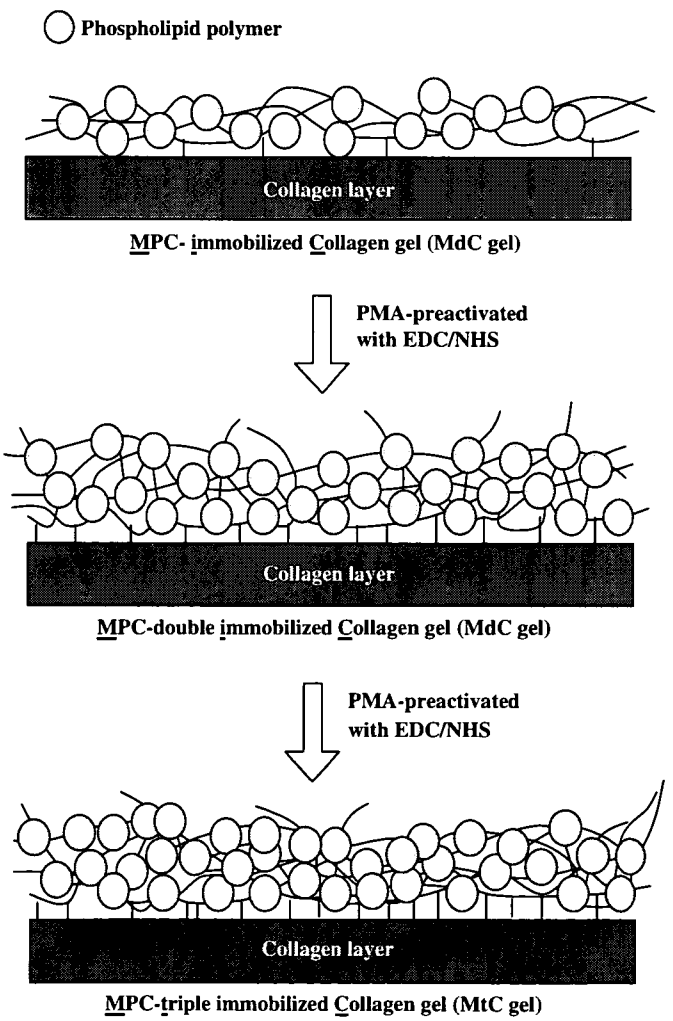


Fig. 1. Schematic diagram of the immobilization process of PMA on collagen.

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.

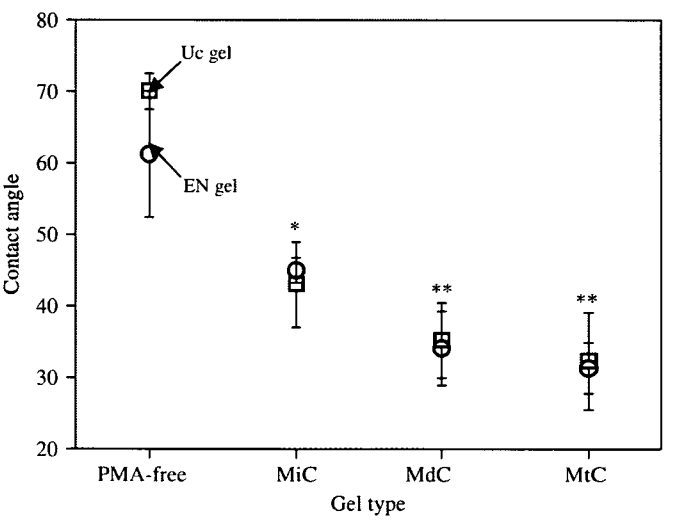
Fig. 3 illustrates the results of the reacted amine group content of the respective collagen gels. It decreases from approximately 60–30% as the PMA is immobilized compared to Uc gel. The lowest reacted amine group content was observed for G-gel, which was approximately 15% of Uc gel.

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

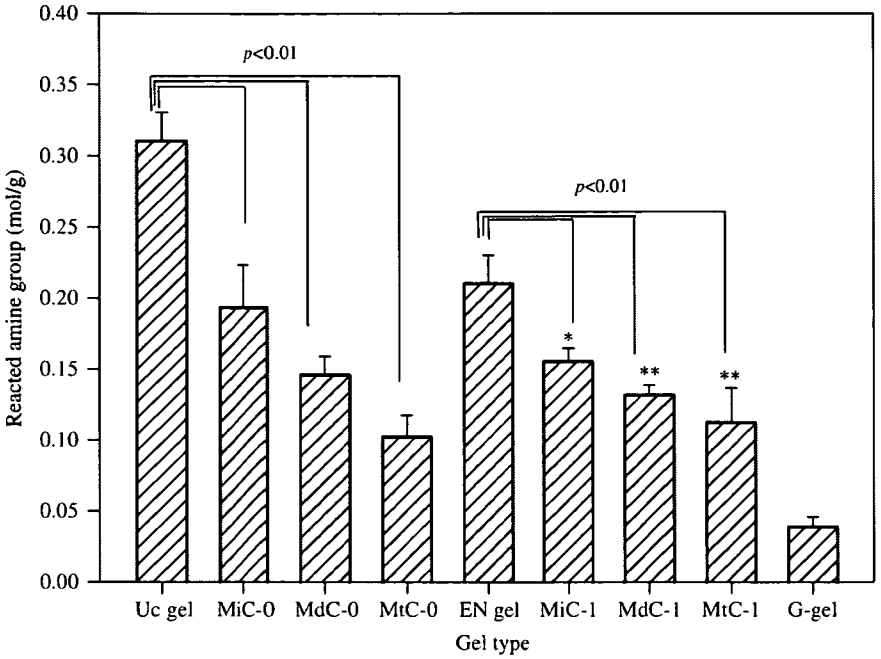


Uc gel and EN gel $p < 0.01$ vs. * and **
* $p < 0.01$ vs. **

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

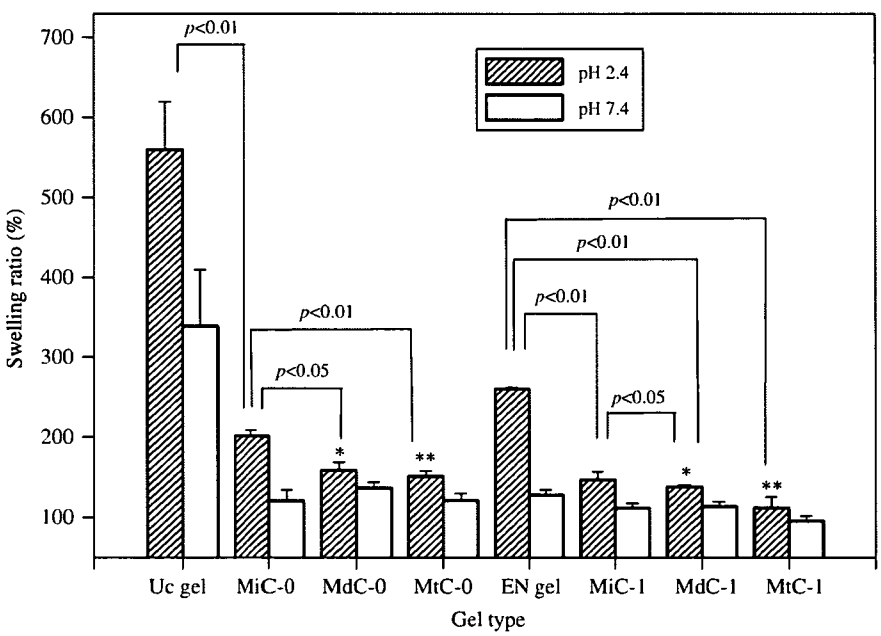
Table 2
Atomic phosphorus concentration of respective collagen gels

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.

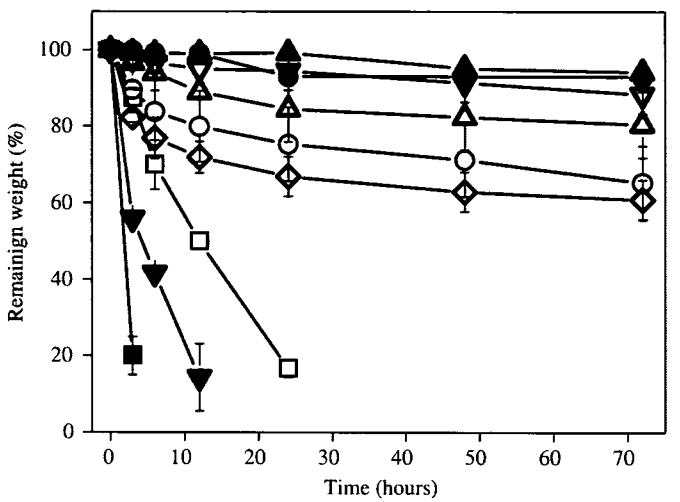


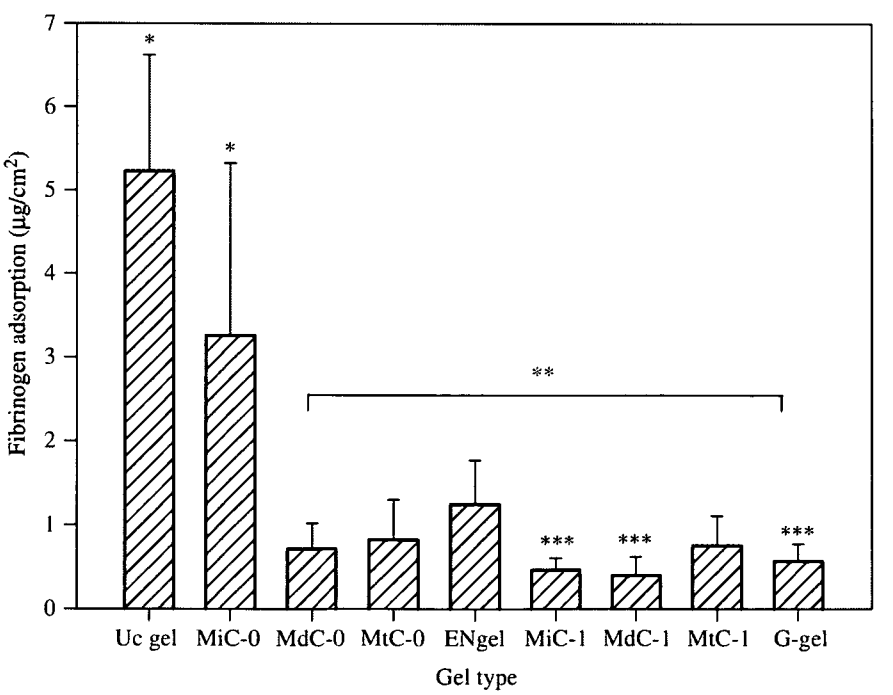
Fig. 5. Degradation of collagen gels by collagenase in Tris–HCl buffer (pH 7.4) at 37 °C. (■) Uc gel, (●) MiC-0 gel, (▲) MdC-0 gel, (▼) MtC-0 gel, (□) EN gel, (○) MiC-1 gel, (△) MdC-1 gel, (▽) MtC-1 gel, and (◇) G-gel. Closed symbols indicate gels without interhelical cross-links while open symbols indicate 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 ($\approx 50\,\mu m$), 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 ($n = 5$).

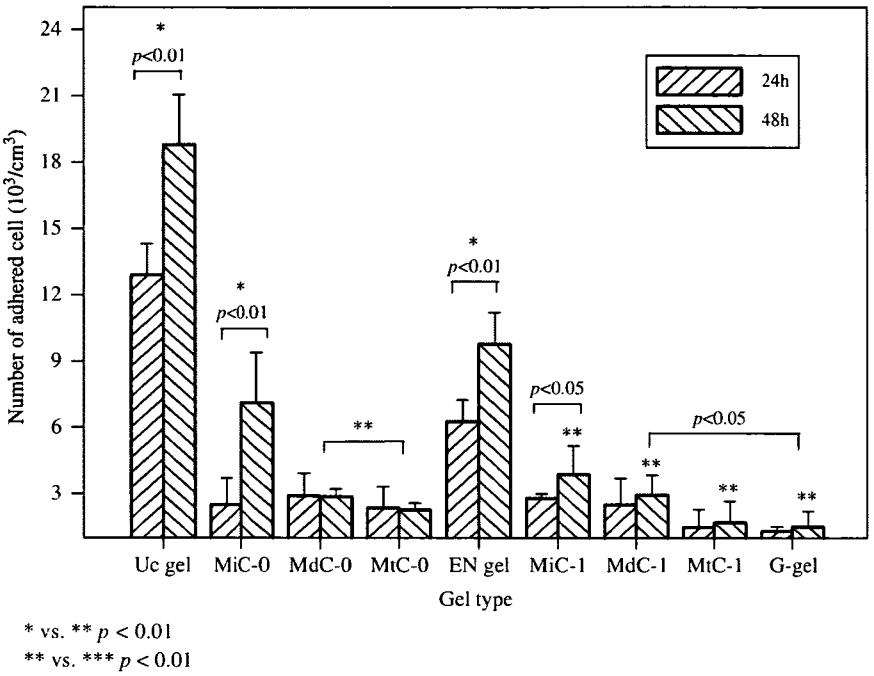


Fig. 7. Cell adhesion property of the respective collagen gels at a seeding density of 5000 cells/cm^2. Each value represents the mean ± SD ($n = 5$).

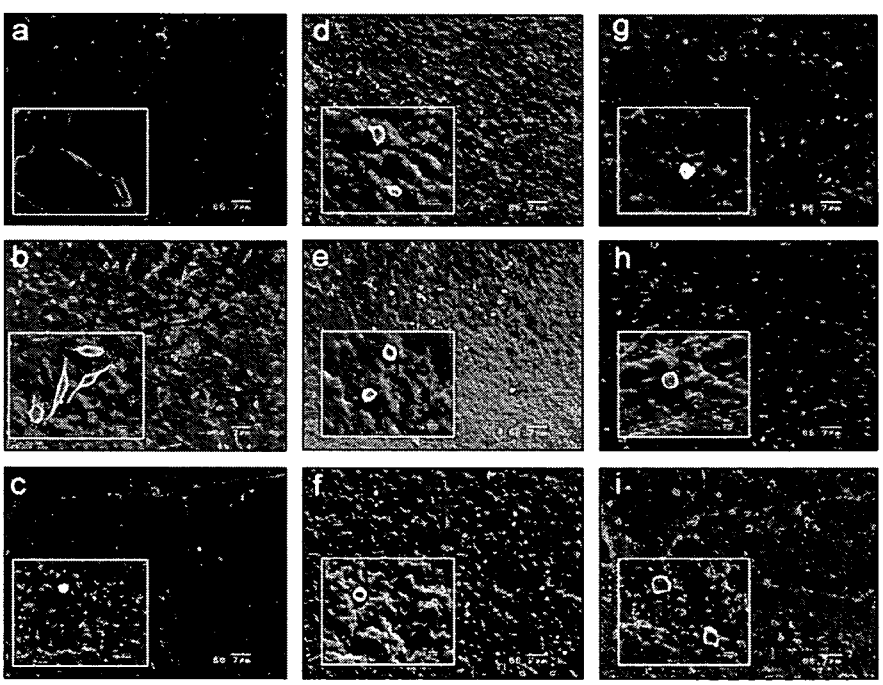


Fig. 8. SEM images of adhered fibroblast cells on respective collagen gels after 48 h of incubation. (a) Uc gel, (b) EN gel, (c) G-gel, (d) MiC-0, (e) MdC-0, (f) MtC-0, (g) MiC-1, (h) MdC-1, and (i) MtC-1 gel. SEM images in the large frame are shown at a magnification of × 150 and the small frame at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