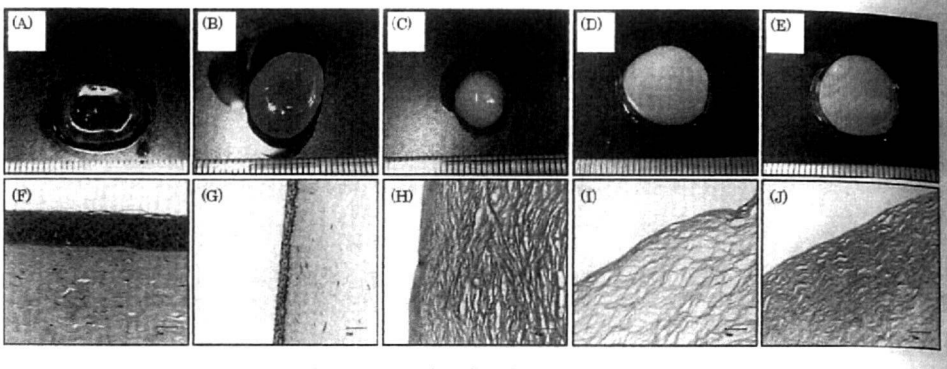

図6　種々の脱細胞化角膜の肉眼所見と組織切片像

(A), (F) 未処理／(B), (G) TritonX-100による脱細胞化角膜／(C), (H) SDSによる脱細胞化角膜／(D), (I) 超高圧処理による脱細胞化角膜（30℃）／(E), (J) 超高圧処理による脱細胞化角膜（10℃）。Scale bar 50 μm。

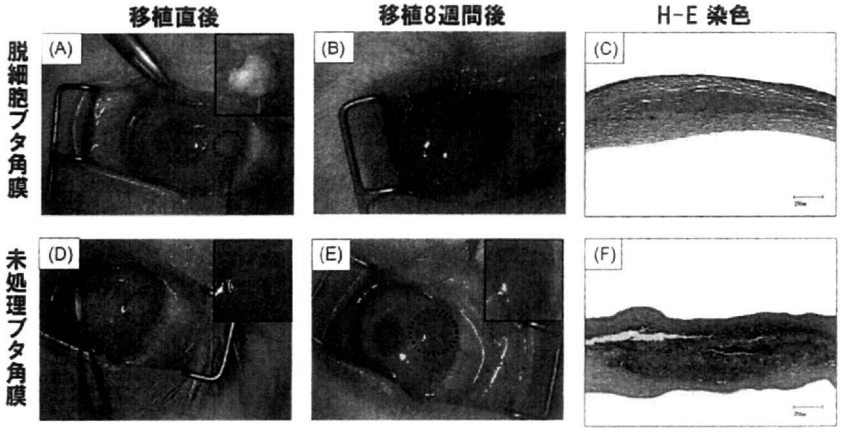

図7　脱細胞化ブタ角膜のウサギ眼移植実験の経時変化

5　将来展望

未だ長期間に渡って満足できる性能を発揮する人工臓器や組織は開発されておらず，また，患者の成長に伴って人工臓器に成長性を与えることはほとんど不可能である。再生医療は，皮膚，角膜，心臓弁など比較的簡単な構造機能型の「組織」から応用が開始され，今後は，脳，肝臓，心臓，膵臓など複雑な機能を有する「臓器」へと発展していくことが期待されている。これを実現するためには，足場材料，細胞，成長因子，バイオリアクターに加えて，任意の細胞を足場材料の任意の位置に配置できるような新しい技術が必要である。今後も再生医療の発展には，医学，生物学，工学，薬学の研究の融合が必要である。本稿がその一助になれば幸いである。

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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 1min 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-

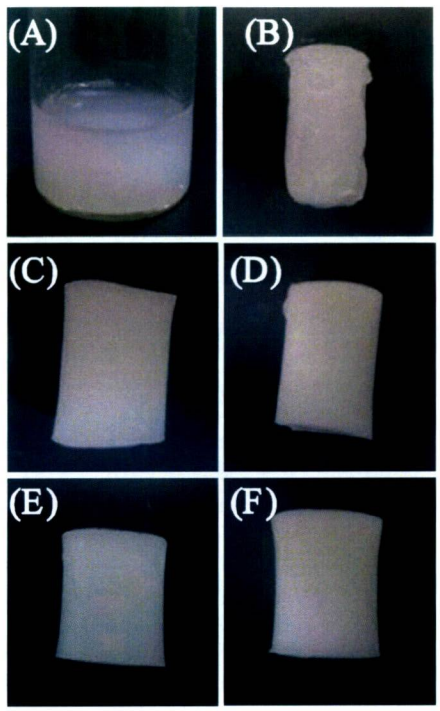

Fig. 1. Photographs of poly(vinyl alcohol) (PVA) hydrogels (**A–D**) and PVA/DNA (**E,F**) hydrogels at concentrations of **A** 3% w/v, **B** 4% w/v, **C,E** 5% w/v, and **D,F** 10% w/v obtained by ultra-high pressure treatment

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,

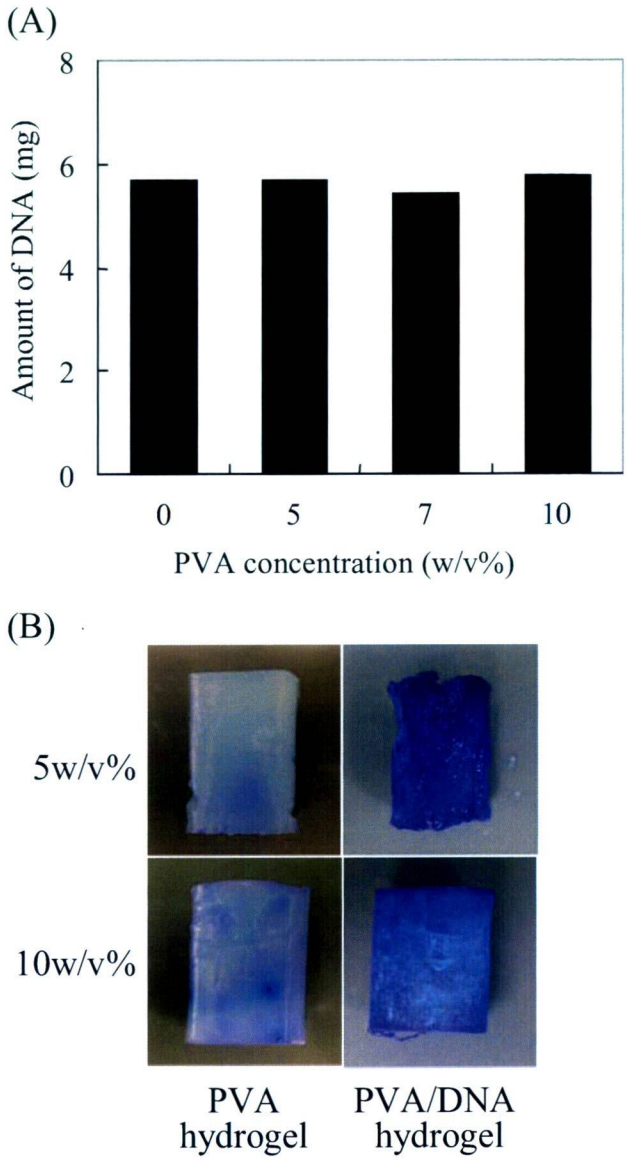

Fig. 2A,B. Presence of DNA in PVA/DNA hydrogels. **A** Amount of DNA in solution obtained by melting PVA/DNA hydrogels prepared using ultra-high pressure processing. **B** Photographs of PVA hydrogels and PVA/DNA hydrogels stained with nucleic acid dye

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

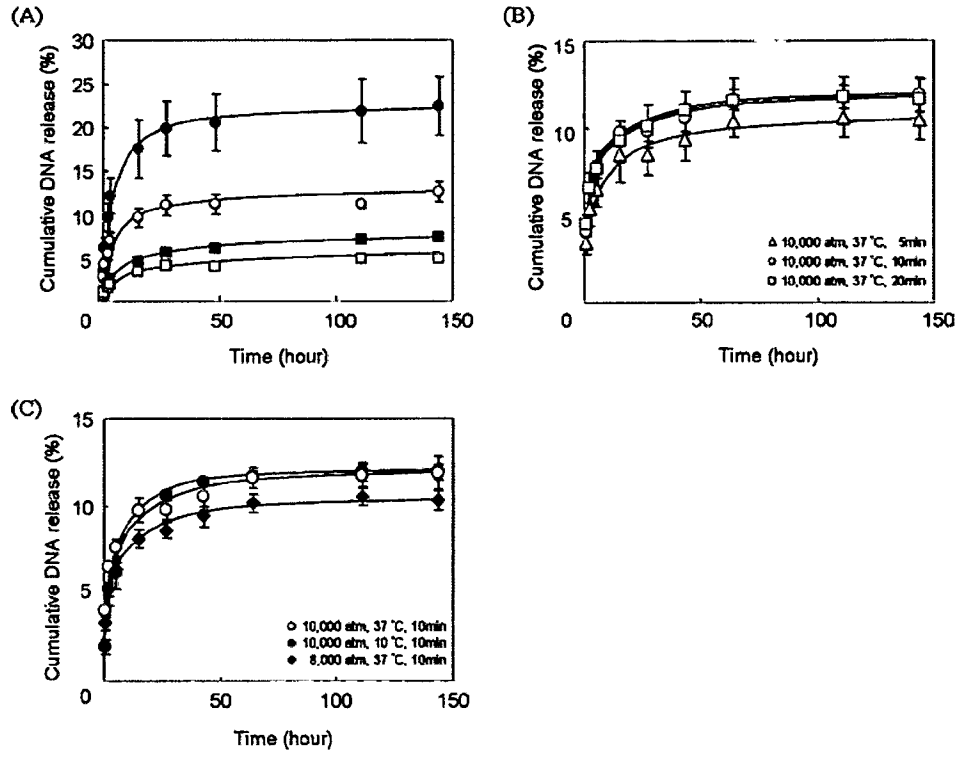

Fig. 3A–C. DNA release test from PVA/DNA hydrogels produced by pressurization under various conditions or by the freeze–thaw method. **A** Release profiles of DNA from hydrogels at PVA concentrations of 5% w/v (○, ●) and 10% w/v (□, ■) PVA concentration. *Open* and *solid* symbols indicate DNA from hydrogels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

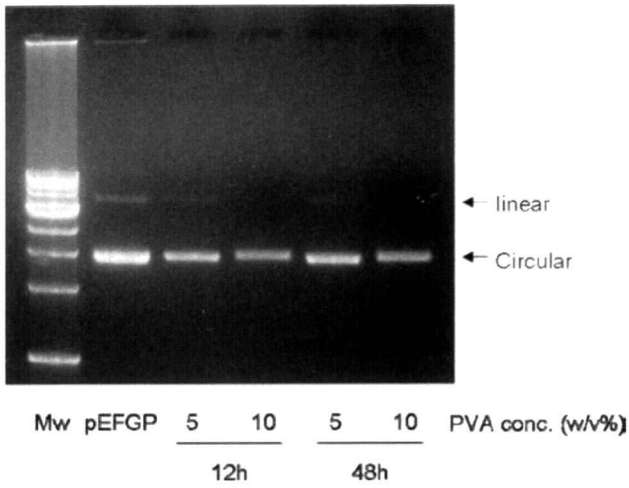

Fig. 4. Agarose gel electrophoresis of plasmid DNA (pDNA) released from PVA/pDNA hydrogels with PVA concentrations of 5% and 10% w/v produced by pressurization at 10000 atm and 37°C for 10 min after immersion in phosphate-buffered saline for 12 and 48 h

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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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

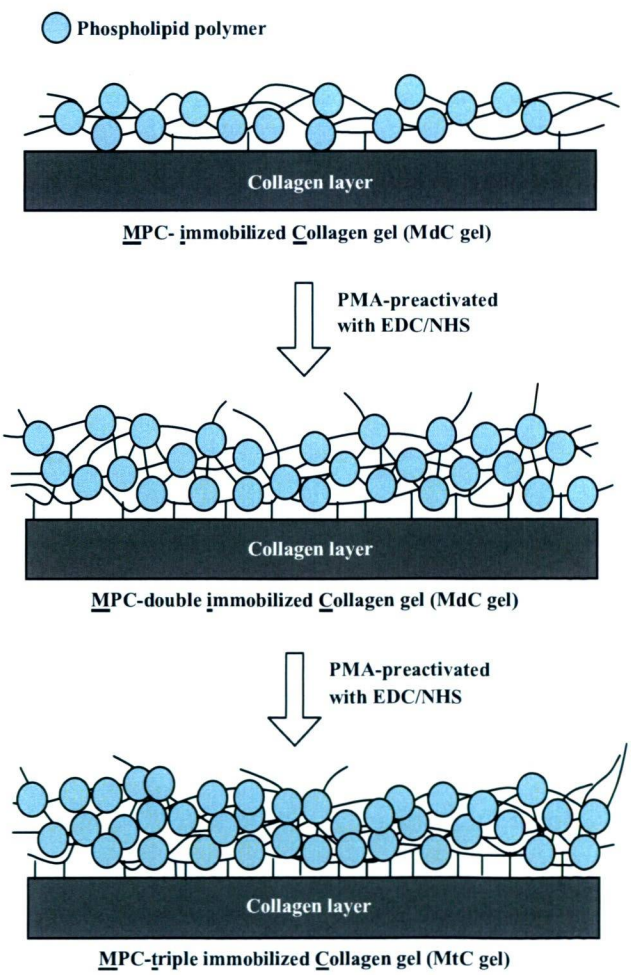


Fig. 1. Schematic diagram of the immobilization process of PMA on collagen.

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.

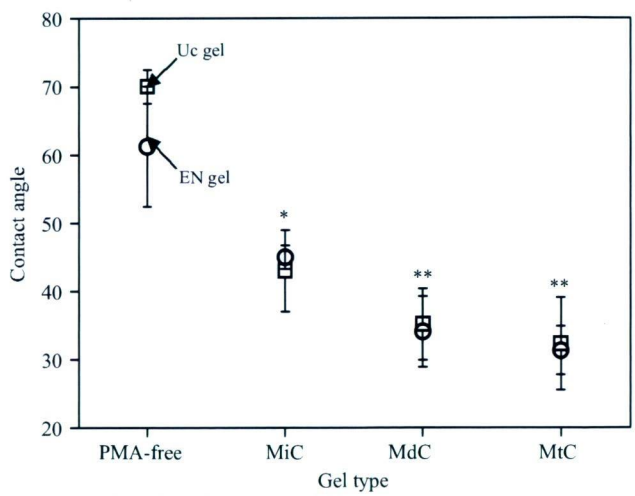
Fig. 3 illustrates the results of the reacted amine group content of the respective collagen gels. It decreases from approximately 60–30% as the PMA is immobilized compared to Uc gel. The lowest reacted amine group content was observed for G-gel, which was approximately 15% of Uc gel.

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

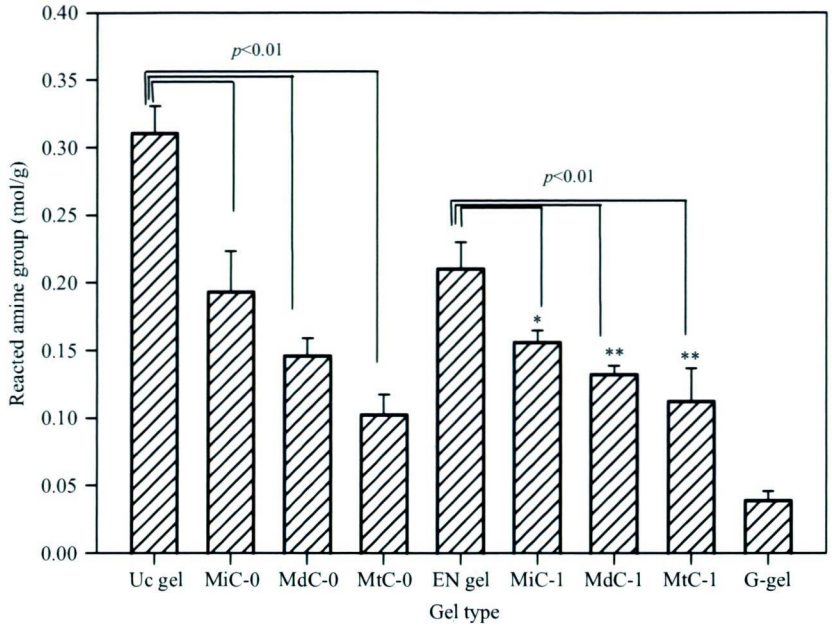


Uc gel and EN gel $p < 0.01$ vs. * and **
* $p < 0.01$ vs. **

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

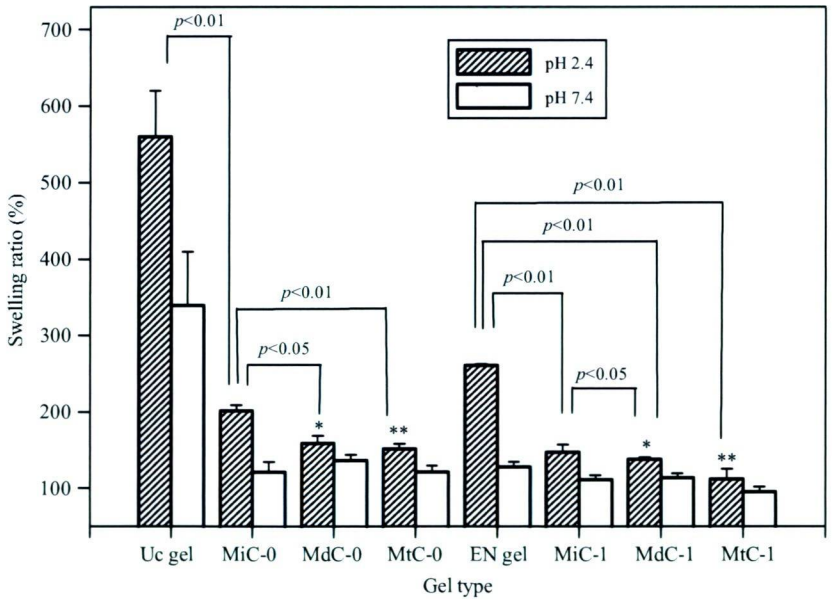
Table 2
Atomic phosphorus concentration of respective collagen gels

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.

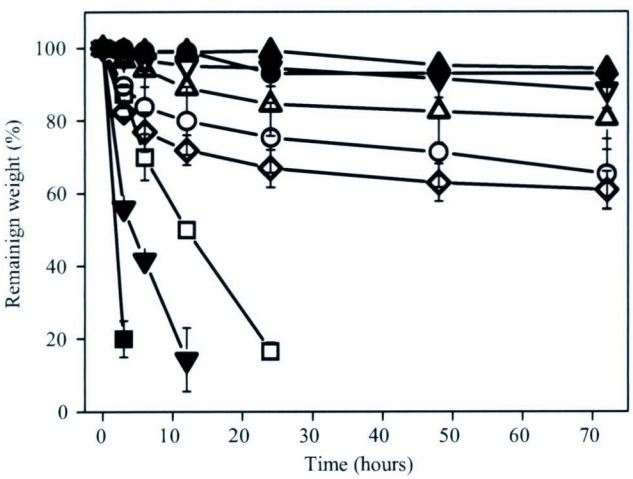


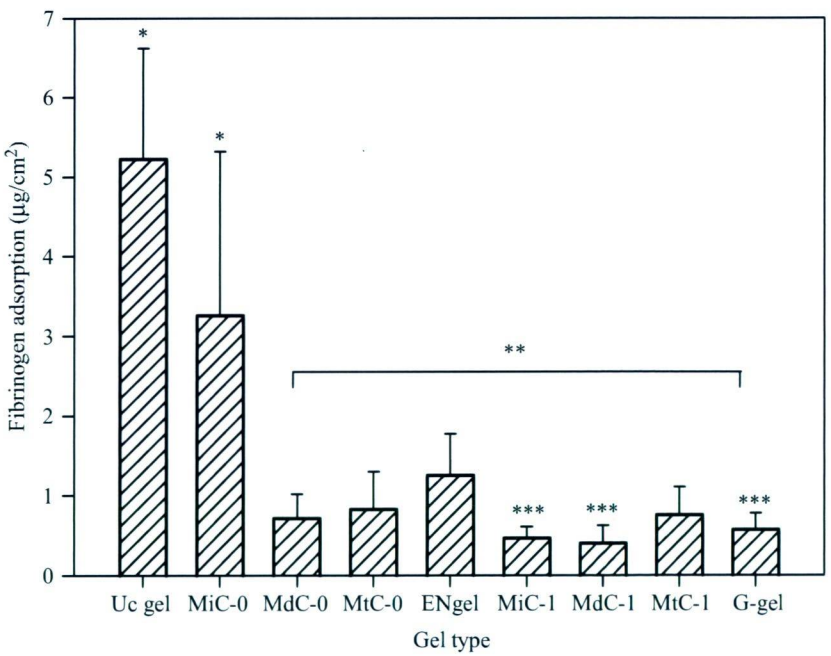
Fig. 5. Degradation of collagen gels by collagenase in Tris–HCl buffer (pH 7.4) at 37 °C. (■) Uc gel, (●) MiC-0 gel, (▲) MdC-0 gel, (▼) MtC-0 gel, (□) EN gel, (○) MiC-1 gel, (△) MdC-1 gel, (▽) MtC-1 gel, and (◇) G-gel. Closed symbols indicate gels without interhelical cross-links while open symbols indicate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

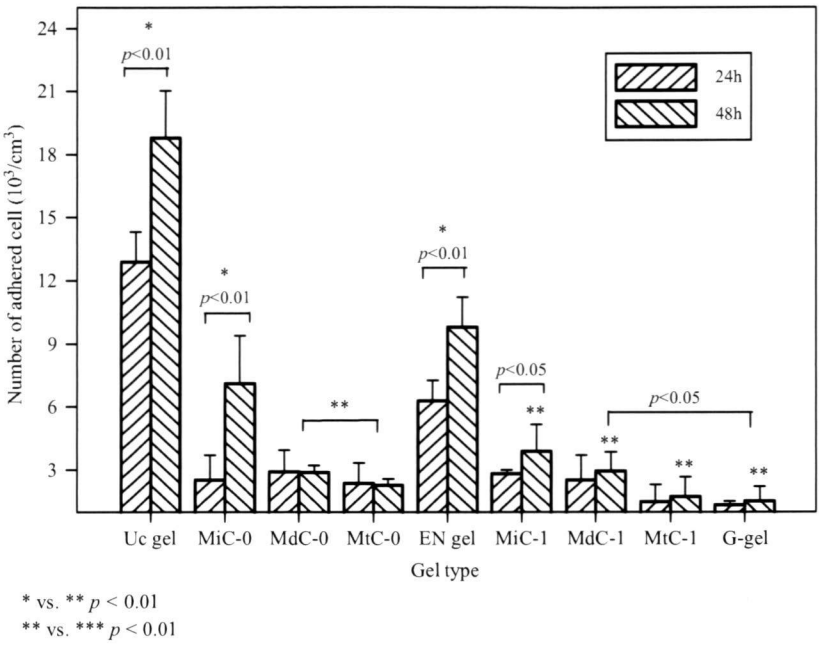


Fig. 7. Cell adhesion property of the respective collagen gels at a seeding density of 5000 cells/cm^2. Each value represents the mean ± SD ($n = 5$).

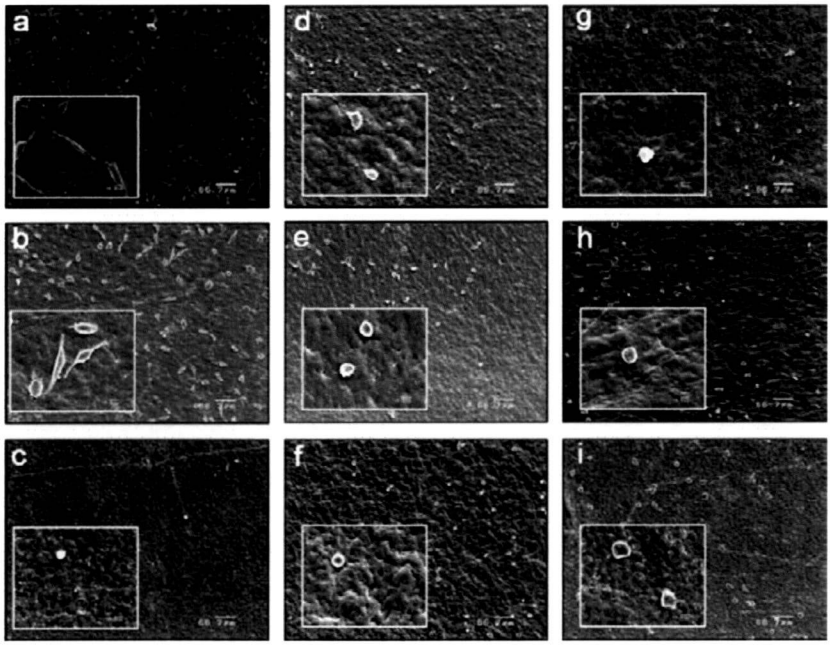


Fig. 8. SEM images of adhered fibroblast cells on respective collagen gels after 48 h of incubation. (a) Uc gel, (b) EN gel, (c) G-gel, (d) MiC-0, (e) MdC-0, (f) MtC-0, (g) MiC-1, (h) MdC-1, and (i) MtC-1 gel. SEM images in the large frame are shown at a magnification of × 150 and the small frame at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

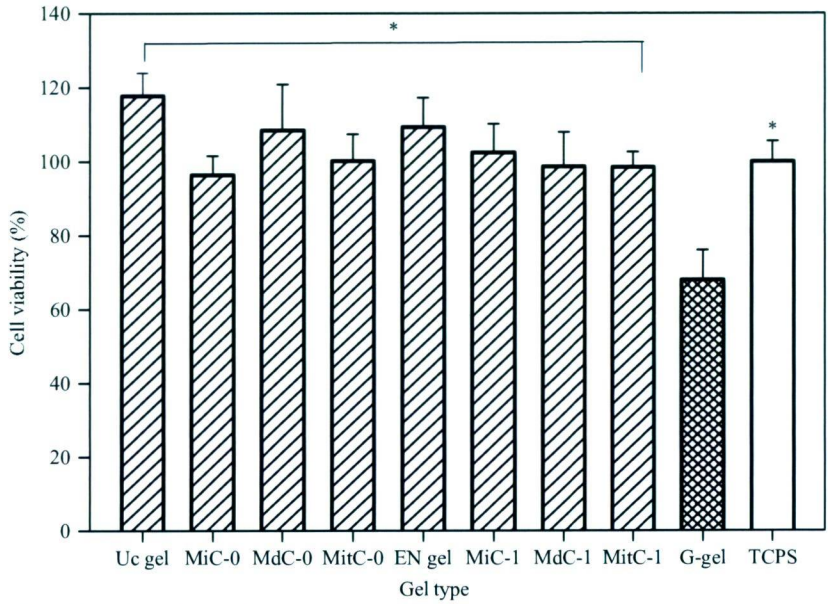


Fig. 9. Cell viability of L929 at 48 h of incubation with at a seeding density of 5000 cells/cm^2 on the respective collagen gels. Each value represents the mean ± SD ($n = 3$).

(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*.

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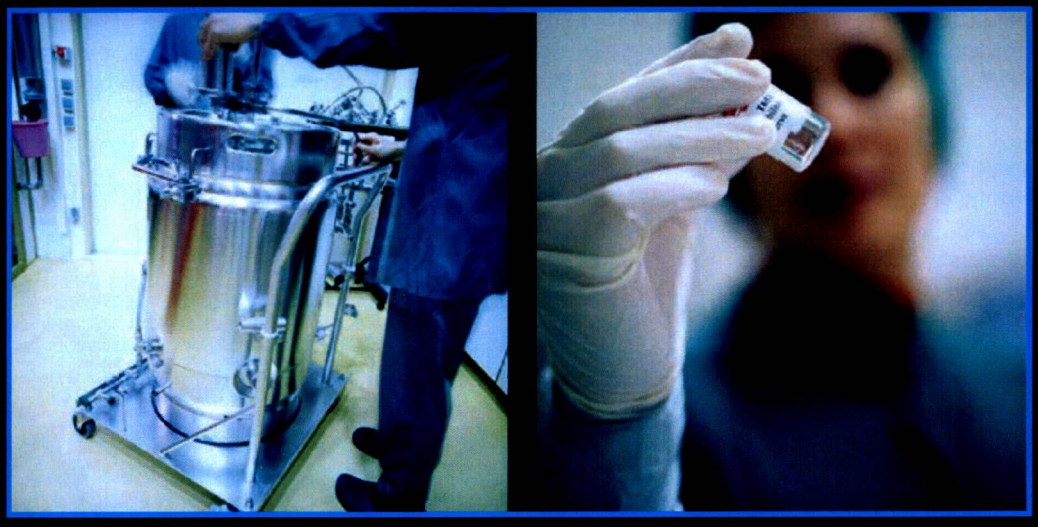

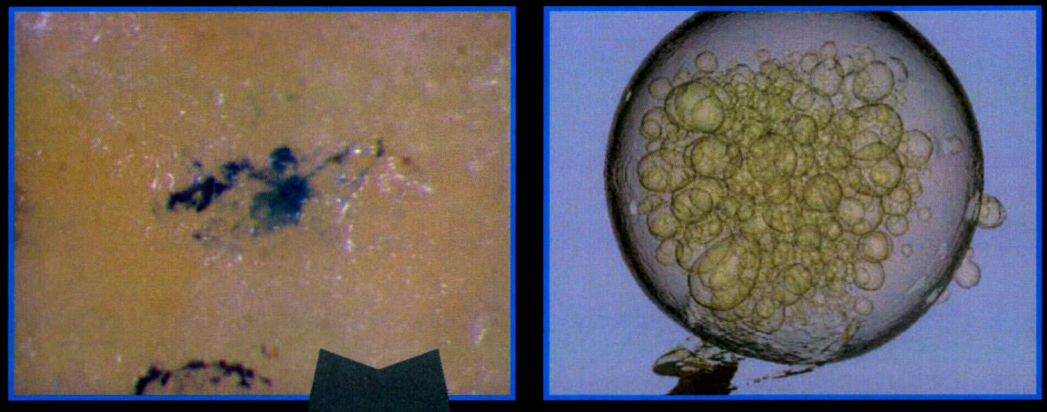

Scientifically Speaking

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).

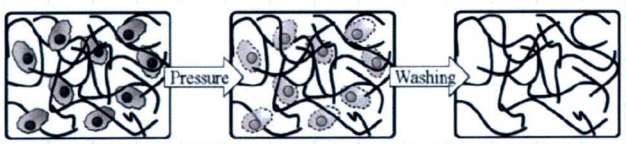

Figure 1. Preparation procedure for decellularizing tissue using ultra-high pressure treatment.

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

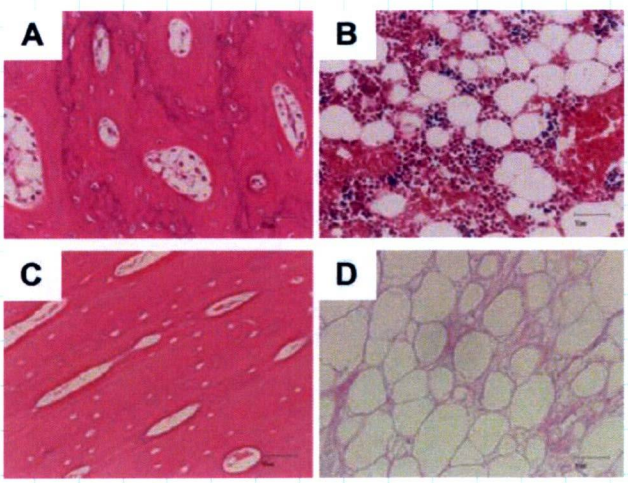

Figure 2. Hematoxylin eosin staining of (A) native cortical bone, (B) native bone marrow, (C) decellularized cortical bone, and (D) decellularized bone marrow by pressurization at 10,000 atm (980 MPa) for 10 min.

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.

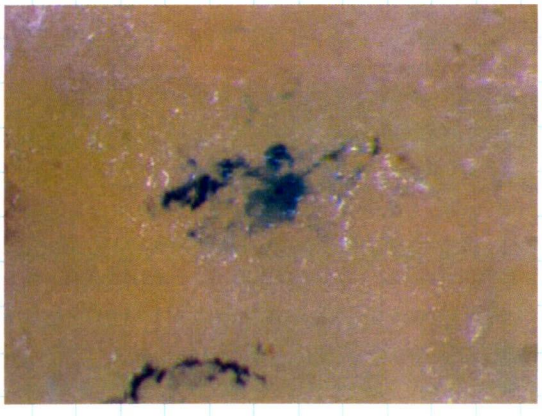

Figure 3. X-gal staining of cells reseeded on decellularized costa with calcium phosphate/DNA co-precipitate.

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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