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Influence of Cross-linking on Physicochemical and Biological Properties of Collagen-Phospholipid Polymer Hybrid Gel

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To adopt collagen as a biomaterial, collagen should be modified due to disadvantages such as poor mechanical strength and high thrombogenicity. Preparation of collagen-polymer hybrid gel for application as an artificial vascular graft was executed by immobilizing 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer, poly(MPC-*co*-methacrylic acid) (PMA), using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide and *N*-hydroxysuccinimide as cross-linkers. In order to alter the density of interchain cross-links (intermolecular bonding) between collagen fibrils and the MPC polymer chains, collagen-polymer hybrid gel was prepared by changing the mole ratio of MPC moiety of PMA. The intra- and interhelical cross-links made the gel thermodynamically stable. The interchain cross-links made the gel mechanically and dimensionally stable by supporting the network structure of the hybrid gel, which is thought to be achieved by connecting collagen fibrils. Enzymatic stability was depending on the density of interchain cross-links, because the adsorption of collagenase was prohibited. Increase in the MPC moiety made the gel cell adhesion property decrease. This implies that the interaction between cells and surface of the hybrid gel is being regulated by the MPC groups, making the hybrid gel much efficient for artificial vascular graft use.
Key words: collagen, phospholipids polymer, cross-link, gel, cell adhesion

1. INTRODUCTION

In order to use collagen for a biomaterial product, the cross-linking of collagen and/or immobilizing synthetic polymer with collagen to is indispensable. Non-treated natural collagen cannot directly be applied to the biological system due to disadvantages such as poor mechanical strength, calcium deposition, and high thrombogenicity. However, the collagen is biocompatible and non-antigenic, synergic with bioactive component, easily modifiable, and available in abundance, which makes it suitable for medical application [1-3]. While keeping the advantageous property of collagen, disadvantageous property should be eliminated or be complemented.

Cross-link method using *N*-(3-dimethyl-aminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) was chosen for this study [4,5]. Cross-linking collagen with EDC and NHS makes 'zero-length' amide cross-links between carboxylic acid groups from aspartic and glutamic acid residues, and ε-amino groups from (hydroxy-) lysine residues forming intra- and interhelical cross-link to prepare an EDC/NHS cross-link collagen gel [5]. And, 2-methacryloyloxyethyl phosphorylcholine (MPC) based copolymer, which is known for its excellent biocompatibility [6], was used to cross-link the microfibrils of collagen to produce a hybrid gel having biocompatibility and improved mechanical strength.

In this study, we investigated the network structure of the collagen-phospholipid polymer hybrid gel and the effect to the mechanical strength, thermal stability, dimensional stability, and enzymatic stability against collagenase. Furthermore, the biological property of the collagen gel was examined to evaluate the application as an artificial blood vessel.

2. EXPERIMENTAL

2.1 Preparation of EDC and NHS Cross-linked Collagen Gel

Preparation of EDC and NHS cross-linked collagen gel (E/N gel) was executed by using 0.5wt% collagen type I solution (pH 3, KOKEN, Tokyo, Japan). Collagen solution was fabricated into film. Then the collagen film was immersed into the 0.05M 2-morpholinoethane sulfonic acid (MES) buffer (pH 9) (Sigma, St Louis, USA) containing EDC (Kanto Chemicals, Tokyo, Japan) and NHS (Kanto Chemicals, Tokyo, Japan). The cross-linking procedure was executed for 4 hours at 4°C to make a cross-linked gel (E/N-al gel). After 4 hours, the reaction was stopped and the gel was then washed with 4M of Na_2HPO_4 aqueous solution for 2 hours to hydrolyze any remaining *O*-acrylisourea groups and then with distilled water for 3 day to remove salt from the gel. The molar ratio of each chemical was fixed to EDC:NHS:collagen-carboxylic acid groups=5:5:1.

2.2 Preparation of MPC-immobilized Collagen gel

Preparation of the MPC-immobilized Collagen gel (MiC gel) was executed by using E/N-al gel. poly(MPC-*co*-methacrylic acid) (mole ratio; MPC:methacrylic acid=3:7, PMA30) (Figure 1) was added with EDC and NHS in MES buffer (pH 10) and was pre-activated for 10 minutes before E/N-al gel was immersed. The molar ratio of each chemical was fixed to EDC:NHS:carboxylic acid groups of PMA =5:5:1. The immobilization of PMA to the collagen was continued for 4 hours at 4°C. Then the gel was washed with 4M of Na_2HPO_4 aqueous solution for 2 hours and then with distilled water for 1 day to remove salt from the gel to prepare a salt-free MiC30 gel. To increase the MPC moiety of the collagen-polymer hybrid gel, PMA90 (MPC:methacrylic acid=9:1) was prepared and immobilized to the collagen to make a MiC90 gel.

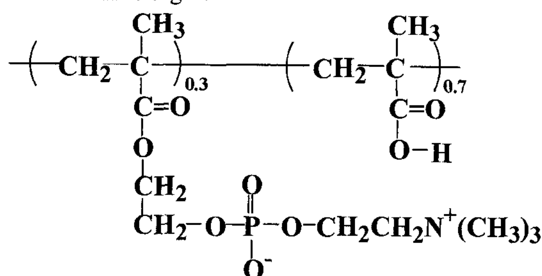

Figure 1.Chemical structure of PMA.

2.3 Surface Characterization

The 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) Samples which had been cut into small pieces were lyophilized for overnight. The chemical composition of the surfaces of the gel (upper part of the gel) was determined by releasing angle of the photoelectrons fixed at 90°. The morphologies of the gels were observed with a SEM. The razor-blade cut surfaces of respective gels were observed.

2.4 Network Characterization

The shrinkage temperature of the gels were determined using differential scanning calorimeter (DSC; DSC6000, Seiko, Chiba, Japan) in the range of 0°C to 150°C at the scanning rate of 5°C/minute.

Stress-strain curves of respective collagen gels were determined by uniaxial measurements using a tensile strength tester (STA-1150, Orientec, Tokyo, Japan). The sample for the measurement was prepared in the size of 4cm×1cm. The obtained data were changed to stress-strain curve of the samples and the elongation modulus was calculated.

The swelling test of respective samples was executed by cutting lyophilized gels into small pieces and putting into pH aqueous solution at 37°C. The pH of the aqueous solution was controlled to make 7.4. The gels were shaken gently for 24 hours and taken out to measure the changed weight of the sample. Swelling ratio was calculated in order to define the exact swelling phenomenon brought up by water absorption.

2.5 Enzymatic Degradation

The degradation test of the gel samples were executed using collagenase from Clostridiopeptidase histoluticum (EC 3.4.24.3, *Sigma*, St Louis, USA) with collagenase activity of 320 units/mg. In this experiment, collagen gels were immersed into Tris-HCl buffer solution with total concentration of collagenase 100units/mL. The weight of the gels after reaction with collagenase was measured from 1 to 72 hours.

2.6 Cell adhesion test

L-929 cells (mouse fibroblast) were used to evaluate the interaction between collagen gels and the cells. The fibroblasts were culture 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 a 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 surface. The collagen gels were sterilized by putting gels into ethanol:water 50:50 for 2 hours, than 70:30 for 2 hours, and 100:0 for an overnight before lyophilizing. The lyophilized gels were hydrolyzed with E-MEM for 5 minutes and the E-MEM was disposed just before cell seeding. After 24 hours and 48 hours, the number of adhering cells was determined using lactate dehydrogenase (LDH) assay at 560nm with UV/VIS spectrophotometer (V-560, Jasco, Tokyo, Japan).

3. RESULTS AND DICUSSION

3.1 Surface Characterization

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 400.8eV, respectively. A phosphorus peak and one nitrogen peak in $-N^+(CH_3)_3$ was observed at 134eV and 403.2eV, respectively, indicating that PMA was properly cross-linked collagen [6]. Figure 2 shows the images of the outer surface (upper part of the gel) and razor blade-cut surface (cross-section) morphology of respective collagen gels observed with SEM. All outer surfaces that are immobilized with PMA show non-porous homogenous structure. When the razor-cut surface was observed, relatively porous (or hollow) layer that is composed of many thin plates, and non-porous (or dense) layer was seen. Hollow layer is thought be the uncross-linked collagen (a collagen gel that is prepared under pH 9.0 MES buffer without any cross-linker; Uc gel) or intra- and interhelically cross-linked collagen layer. The non-porous layer representing PMA is deposited on the collagen layer and the thickness increases as more PMA is adopted. However, we are not sure yet how the deposited layer would affect the physical property of the hydrogel. We are working on this and would be reported soon.

3.2 Network Characterization

Table I shows the change of the shrinkage temperature (T_s) of each collagen gels. The cross-linking brought the increase in the T_s. And the T_s would increase further as the PMA is immobilized, but would never cross 85°C. Since the denaturization temperature is the endothermic transition of the triple helix of the collagen molecules to the random coil, it is believed that intra- and interhelical cross-link controls the T_s [7]. When the higher amount of EDC and NHS was used, the T_s would increase up to 83°C (data not shown). This implies that the increase in the T_s is not due direct connection between collagen microfibrils and polymer chain but due to complexity of the network. So, the stability of the collagen gels against temperature is dependant not only on intra- and interhelical cross-links, but also on the density of the network.

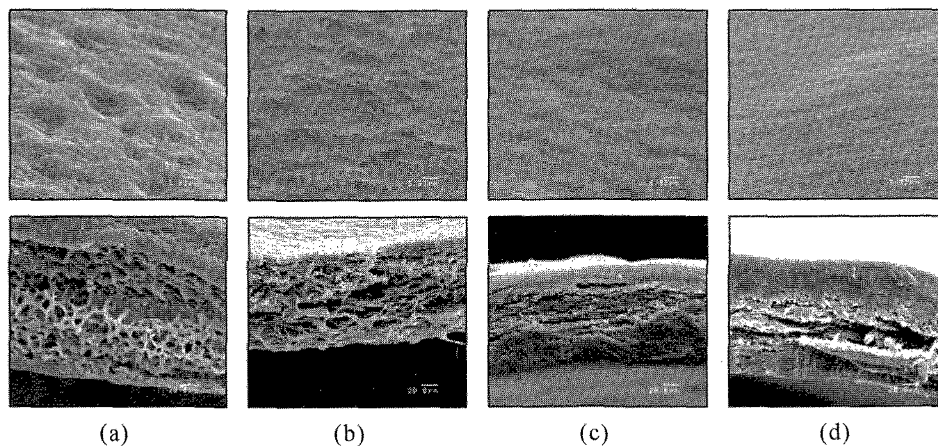

Figure 2. SEM images of collagen gels. (a) Uc gel, (b) E/N gel, (c) MiC90 gel, and (d) MiC30 gels. Upper images imply the outer surface and below images indicate the razor-cut surface of the gels.

Table I. Shrinkage temperature of respective collagen gels.

Sample	Shrinkage temperature (°C)
Uc gel	56±8
E/N gel	74±3
MiC90	76±3
MiC30	84±4

The elongational modulus increases as PMA is immobilized, indicating it is the interchain cross-link that reinforces the mechanical strength. The elongational modulus of MiC gels measured at 1% strain and 8% strain showed that approximately 10~13 times increase compared to Uc-gel while that of E/N gel showed approximately 5 times increase. This indicates that the network is much denser for MiC gels, which directly affected the mechanical strength.

All collagen gels showed 1.4~2 times increase in elongational modulus at 8% strain compared to that of at 1% strain, indicating soft tissue viscoelastic behavior can be maintained after immobilizing with PMA. So, biomaterial possessing biological property can be obtained.

Figure 3 shows the swelling of the respective gels under pH 7.4. For uncross-linked gel, the gel dissolved under pH 2.1, while swelled approximately 1400% under pH 7.4. When collagen gels absorb water, the triple helix structure is known to turns to random coil conformation, because collagen peptide chains increases accessibility to hydration. In the neutral and alkaline pH conditions, collagen film would be stabilized by forming entanglement of fibrils formed by hydrophobic and electrostatic bonds.

E/N-al gels shows swelling ratio of 320% under pH 7.4. As mentioned earlier, EDC and NHS is known to be bring inter- and intrahelical cross-links, holding the α-helices together tightly. However, its low cross-linking density due to high free amine group contents makes the gel to swell high.

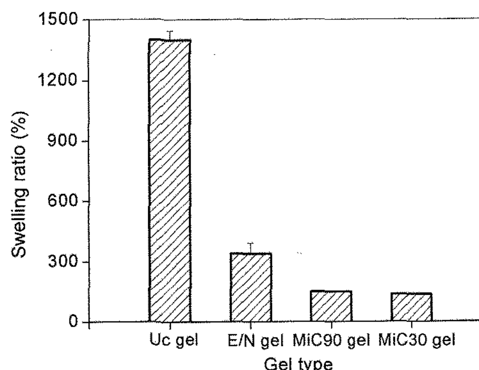

Figure 3. Swelling ratio of respective collagen gels in pH 7.4

MiC gels shows much suppressed swelling ratio. The decrease in the swelling ratio comparing to E/N gel indicates that the dense network has formed. Denser network between collagen and PMA is thought to be formed by interchain cross-links by connecting microfibrils together, increasing the toughness of the collagen gel. Furthermore, the high mechanical strength of the hybrid gel is suppressing the absorption of water, leading to low swelling ratio

3.3 Enzymatic Degradation

Figure 4 shows the degradation of collagen gels caused by the activation of collagenase in Tris-HCl buffer. Collagen gel would be degraded once it encounter with collagenase. Collagenase would cleave the helical segment, which makes the collagen gels to hydrolyze. Collagenase is known to absorb onto the collagen fibers once it penetrates into the fiber [7]. Our study shows that the collagen gel that is not cross-linked would be degraded within 2 or 3 hours. Cross-linking the collagen with EDC and NHS would maintain the helical structure firmly, extending the complete degradation time from 3 hours to 24 hours. And as mentioned previously, the E/N-al gel possesses higher intra- and intercross-link chains, making the gel to endure longer time against collagenase.

MiC gels showed higher stability against collagenase. The network of the collagen gel is thought to be denser than E/N gel, as described previously. For E/N gels, the absorption of collagenase eventually made it to be dissociated within 24 hours. On the other hand, MiC gels possess interchain cross-link, which links the microfibrils and the PMA chains, making the gel to swell much lower. And the cleavage by collagenase would be prevented by the PMA-collagen network which links fiber and polymer chain together, shielding the helices

Comparing the degradation rate between MiC gels, we can see that as MPC ratio increases, the degradation is much faster. This is because the network of the MiC90 is thought to be much sparse than MiC30, due to low mole ratio of methacrylic acid moiety. This makes the space between collagen and PMA larger, resulting in higher water absorbance.

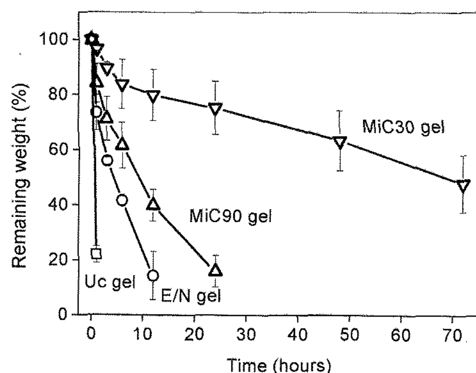

Figure 4. Degradation of collagen gels by collagenase in Tris-HCl buffer (pH 7.4) at 37°C.

3.4 Cell Adhesive property

When the number of adhered cells after 24 hours and 48 hours were compared among the collagen gels, and those with PMA immobilized on collagen gels were much lower than without PMA. This is clearly due to PMA polymer covering the surface of the collagen gel. The number of cell adhered on the surface decreased as the moiety of MPC unit increased. The difference between number of adhered cell on the surface after 24 and 48 hours was compared, the increase in the number of cells was observed for collagen gels. However, for MiC gels, increase was suppressed. Polymer immobilized on the collagen blocks the interaction between fibroblast and collagen, which is known to be the most decisive factor for cell adhesion [8].

4. CONCLUSION

The preparation of MiC gel was successfully achieved. Immobilization of MPC polymer made the gel tougher and stable. We could confirm that the stress-strain responded as generally observed for soft biological materials. Increase in the MPC unit brought the higher swelling, which lead to the faster degradation by collagenase. It is thought that the higher amount of adopted PMA have caused the formation of sparse cross-link network, which in turn make the surface of the MiC gel full of MPC head groups, reducing cell adhesion ability.

The increase in the MPC unit would bring higher biocompatilibty, while increase of MA unit would allow increment of mechanical strength. As the concentration of MPC increased, it is thought that the biocompatibility would increase but toughness decrease.

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