Unusual Cell Adhesion and Antithrombogenic Behavior of Citric Acid-Cross-Linked Collagen Matrices

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We have developed a novel biodegradable matrix that has unusual cell adhesion and antithrombogenic properties. The prepared biodegradable matrix was alkali-treated collagen (AlCol) cross-linked with citric acid derivative (CAD), named as AlCol−CAD. The swelling ratio of AlCol−CAD decreased with increasing CAD concentration, but with a further increase of the CAD concentration, the swelling ratio of AlCol−CAD also began to increase; this behavior showed a point where the swelling ratio reached a minimum value before increasing. The highest shrinkage states of 7.5%, 15%, and 30% (w/v) in AlCol−CAD were observed at CAD concentrations of 10, 20, and 40 mM, respectively, and moreover, the residual amino groups in AlCol−CAD were found to decrease with increasing CAD concentration. On the other hand, increases in carboxyl groups of 7.5% and 15% (w/v) in AlCol−CAD were found at CAD concentrations higher than 10 and 20 mM, respectively, whereas, at CAD concentrations from 10 to 40 mM, no significant change of the carboxyl groups was observed in 30% (w/v) AlCol−CAD. Human umbilical vein endothelial cell (HUVEC) adhesion on 15% (w/v) AlCol−CAD increased with increasing CAD concentration up to 20 mM and then slightly decreased. In the case of 30% (w/v) AlCol−CAD, the number of adhered HUVECs on AlCol−CAD increased with increasing CAD concentration. Furthermore, it was observed that HUVECs had excellent cell proliferation on 15% (w/v) AlCol−CAD at CAD concentrations of 20 and 40 mM, after incubation for 7 days. No thrombus formation was observed on 15% (w/v) AlCol−CAD at CAD concentrations above 20 mM. These results suggested that the 15% (w/v) AlCol−CAD at CAD concentrations above 20 mM has both HUVEC adhesion and antithrombogenic properties.

1. Introduction

Recently, drug-eluting stents with biodegradable or nonbiodegradable matrixes such as poly(lactide-*co*-ϵ-caprolactone),[1,2] phosphorylcholine,[3] poly(*n*-butyl methacrylate),[4] and poly(dimethylsiloxane),[5] which act as drug reservoirs and elute drugs over a period of several weeks or months, have been developed and applied in the biomedical field. These stents support vessels against elastic recoil, which helps in the release of drugs to prevent thrombus formation, inflammation, or endothelial cell adhesion.[6,7] However, after the drug elutes from the matrixes, the residual matrices lead to complications such as exaggerated inflammatory response, thrombus formation, and prevention of endothelialization at the implant site.[8,9] Therefore, it is necessary to develop a novel biodegradable matrix with an antithrombogenic property that can promote endothelial cell adhesion after drug elution.

In our previous study, we developed a novel cross-linker, citric acid derivative (CAD), with three active ester groups to prepare biopolymer gel matrices.[10−13] It was shown that CAD can cross-link biopolymers such as collagen and gelatin and exhibits excellent cytocompatibility as compared to commercially available cross-linkers such as glutaraldehyde.[11,14] Furthermore, a tissue adhesive consisting of CAD and collagen/albumin exhibited high bonding strength and excellent biocompatibility in vivo.[10,11,14,15]

In the present study, we focused on the development of a novel biodegradable matrix that has antithrombogenic and cell adhesion properties; the study was part of our ongoing research on matrixes for drug-eluting stents. Using alkali-treated collagen (AlCol) and CAD (AlCol−CAD), several matrices were prepared with various cross-linking densities. We then evaluated the physicochemical properties such as the swelling ratio, residual amino groups, and carboxyl groups of the resulting AlCol−CAD to clarify the influence of thrombus formation and cell adhesion. Furthermore, cell adhesion and proliferation and antithrombogenic properties of AlCol−CAD were investigated and compared with those of atelocollagen (AtCol) gel and glutaraldehyde (GA)-treated AlCol (AlCol−GA).

2. Experimental Section

2.1. Materials. AlCol and AtCol derived from pig's tissues were provided by Nitta Gelatin Inc. (Osaka, Japan). AlCol, whose isoelectoric point is 5, has carboxyl groups generated by the hydrolysis of residual amide groups that exist in asparagine and glutamine of AtCol. Citric acid, *N*-hydroxysuccinimide (HOSu), tetrahydrofuran (THF), 2,4,6-trinitrobenzenesulfonic acid (TNBS), disodium hydrogen phosphate, sodium hydrogen phosphate, toluidine blue-O, dimethyl sulfoxide (DMSO), ethanol, *tert*-butyl alcohol, HCl, acetic acid, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 10% formalin solution, and NaOH were purchased from Wako Pure Chemical Industrials Ltd. (Osaka, Japan). Dicyclohexylcarbodiimide (DCC) was purchased

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from Kokusan Chemical Co., Ltd. (Tokyo, Japan). All other chemicals were used without further purification.

2.2. Preparation of CAD. CAD was prepared by methods previously reported.[11−13] Briefly, citric acid was first dissolved in THF, and then HOSu and DCC were added. The resulting mixture was stirred for 30 min; it was then concentrated with rotary evaporation under a reduced pressure to remove THF. The final mixture was recrystallized to yield pure CAD.

2.3. Preparation of AlCol−CAD, AtCol Gel, and AlCol−GA. AlCol was first dissolved in DMSO to obtain 7.5%, 15%, and 30% (w/v) solutions. CAD solutions were added to these AlCol solutions so that the final CAD concentration varied from 5 to 40 mM. These AlCol mixture solutions were then stirred and put into a mold with a 1 mm thick silicone rubber spacer between two glass plates for 24 h at 37 °C. The AlCol−CAD was subsequently immersed in excess pure water for 48 h at 37 °C to remove DMSO from the AlCol−CAD matrices. On the other hand, AtCol gel was prepared by mixing 0.3% (w/v) AtCol, 10 M phosphate-buffered saline (PBS) (pH 7.4), and NaOH−HEPES buffer solution. Furthermore, GA was added to 15% (w/v) AlCol solution so that the final GA concentration was 20 mM. The resulting AlCol−CAD, AtCol gels, and AlCol−GA were punched out 10 mm in diameter for the evaluation of antithrombogenic and cell adhesion and proliferation properties.

2.4. Characterization of AlCol−CAD. The equilibrium-swollen state of AlCol−CAD was weighed and freeze-dried at −40 °C for 24 h and then at −10 °C for 24 h. The swelling ratio of AlCol−CAD was determined using the following equation:

$$\text{swelling ratio} = (W_0 - W_d)/W_d$$

where W_0 and W_d are the weights of the immersed and dried matrices.

Determination of residual amino groups in AlCol−CAD was carried out by a spectrophotometric method using TNBS.[16] A 1 mL sample of 4% $NaHCO_3$ and 1 mL of 0.1% TNBS were then added to AlCol−CAD followed by incubation for 2 h at 37 °C. A 3 mL sample of 6 N HCl was subsequently added followed by autoclaving for 1 h at 120 °C to hydrolyze the matrices. The mixed solutions were spectrophotometrically measured at 340 nm using a microplate reader (GENios A-5082, Tecan Japan, Japan).

On the other hand, carboxyl groups in AlCol−CAD at different CAD concentrations were estimated by staining them with toluidine blue-O.[17] The AlCol−CAD was then stained with 5×10^{-4} M toluidine blue-O in $NaHCO_3/Na_2CO_3$ buffer solution (pH 10) for 3 h. The stained AlCol−CAD was subsequently rinsed three times with $NaHCO_3/Na_2CO_3$ buffer solution (pH 10). The toluidine blue-O in the matrixes was extracted with 50% (v/v) acetic acid solution. The spectrophotometric measurement of the extracts was carried out at 633 nm using the microplate reader.

2.5. Culture of HUVECs on AlCol−CAD, AtCol Gel, and AlCol−GA. Using AlCol−CAD, AtCol gel, and AlCol−GA, HUVEC adhesion and proliferation behaviors were evaluated. HUVECs were cultured in endothelial cell basal medium containing 2% fetal bovine serum, hydrocortisone, fibroblast growth factor-B, heparin, vascular endothelial growth factor, insulin-like growth factor-1, ascorbic acid, human epidermal growth factor, and gentamicin following the vendor's protocol (Cambrex). AlCol−CAD, AtCol gel, and AlCol−GA were preincubated in the medium for 24 h and placed on 24-well plates. HUVECs (5×10^4 cells) were subsequently seeded on each matrix followed by incubation at 37 °C with 5% CO_2. After 1 and 7 days, the HUVEC-seeded matrices were washed twice with 1 mL of PBS. The number of HUVECs adhered to the matrices was assessed by the use of a WST-1 (Dojindo Co., Ltd., Kumamoto, Japan). WST-1 is a colorimetric assay of cellular dehydrogenase activity where absorbance at 450 nm is proportional to the amount of dehydrogenase activity in the cell.[18,19] For scanning electron microscopy (SEM) observation, the HUVEC-seeded matrices were fixed with 10% formalin solution for 7 days. These matrices were subsequently dehydrated in a graded series of ethanol solutions and then critical point dried with *tert*-butyl alcohol. The observation of the HUVECs on each matrix was performed with SEM (JSM-5600LV, Jeol, Japan).

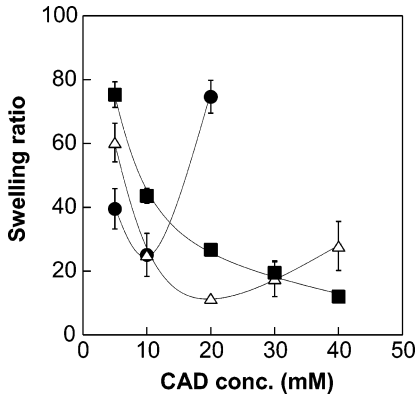

Figure 1. Swelling ratio of 7.5% (w/v) (●), 15% (w/v) (△), and 30% (w/v) (■) AlCol−CAD at different CAD concentrations. Error bars represent standard deviations ($n = 3$).

2.6. Antithrombogenic Activity Test in Vitro. The antithrombogenic activity test was carried out by methods reported elsewhere:[20,21] AlCol−CAD, AtCol gel, and AlCol−GA (all 15% (w/v)) were preincubated in 0.1 M PBS for 24 h. The AlCol−CAD, AtCol gel, and AlCol−GA were placed in 5 mL sample tubes and contacted with 1.0 mL of rat arterial blood. After incubation for 30 min, these matrices were rinsed three times with 0.1 M PBS (pH 7.4) and fixed with 10% formalin solution for 7 days. These matrices were fixed with 10% formalin solution and subjected to a critical point drying procedure for SEM observation.

2.7. Statistical Analysis. The results are expressed as the mean ± standard deviation (SD). The data were analyzed using Microsoft Excel's statistical function for one-way ANOVA. All p values were compared to an α value of 0.05 to determine significance.

3. Results

3.1. Effect of the Cross-Linker Concentration on the Swelling Ratio of AlCol−CAD. Figure 1 shows the swelling ratio of 7.5%, 15%, and 30% (w/v) AlCol−CADs at different CAD concentrations. The swelling ratio of AlCol−CAD was found to decrease with CAD concentration increasing up to 10, 20, and 40 mM, respectively. In the case of 7.5% and 15% (w/v) AlCol−CAD, the swelling ratio began to increase significantly when the CAD concentration continued to increase beyond the points 10 and 20 mM, respectively. No AlCol−CAD formation occurred at CAD concentrations above 20 mM when the AlCol concentration was 7.5% (w/v).

3.2. Determination of Residual Amino Groups and Carboxyl Groups in AlCol−CAD. Figure 2 shows the amounts of residual amino groups in AlCol−CAD as functions of the CAD and AlCol concentrations. Spectrophotometric methods were employed for the determination of the residual amino groups in AlCol−CAD. Residual amino groups in 7.5%, 15%, and 30% (w/v) AlCol−CAD decreased with increasing CAD concentration up to 10, 20, and 40 mM, respectively. At higher CAD concentrations, no amino groups were detected.

Figure 3 shows the amounts of carboxyl groups remaining in AlCol−CAD at different CAD concentrations. In the case of 7.5% and 15% (w/v) AlCol−CAD, the carboxyl groups in the AlCol−CAD increased with an increase in the CAD concentration above 10 and 20 mM, respectively, whereas no significant change of carboxyl groups was observed in 30% (w/v) AlCol−CAD.

3.3. Effect of the CAD Concentration on HUVEC Proliferation. Figure 4 shows the adhesion and proliferation numbers

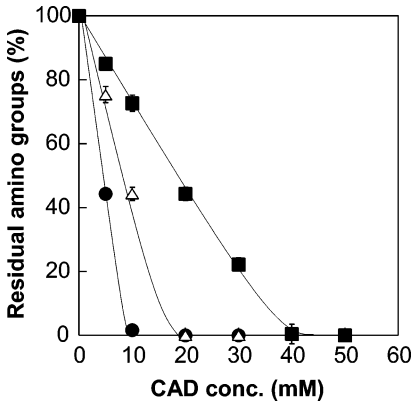

Figure 2. Residual amino group content in 7.5% (w/v) (●), 15% (w/v) (△), and 30% (w/v) (■) AlCol−CAD at different CAD concentrations. Error bars represent standard deviations ($n = 3$).

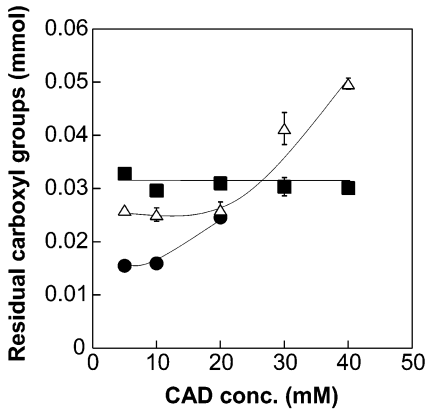

Figure 3. Carboxyl group content in 7.5% (w/v) (●), 15% (w/v) (△), and 30% (w/v) (■) AlCol−CAD at different CAD concentrations. Error bars represent standard deviations ($n = 3$).

of HUVECs on 7.5%, 15%, and 30% (w/v) AlCol−CAD with different CAD concentrations. AtCol gel and AlCol−GA were used as control materials. WST-1 assay was employed for the determination of the adhesion and proliferation numbers of HUVECs on each matrix. As shown in Figure 4A, the adhesion number of HUVECs on 7.5% (w/v) AlCol−CAD increased with an increase in the CAD concentration up to 10 mM and then began to decrease with a further increase in the CAD concentration ($p < 0.01$). Similar to the case of 7.5% (w/v) AlCol−CAD, the number of HUVECs proliferating on the 15% (w/v) AlCol−CAD increased with increasing CAD concentration up to 20 mM and then slightly decreased when the CAD concentration increased further up to 40 mM ($p < 0.01$). On the other hand, 30% (w/v) AlCol−CAD showed an increase in the adhesion number of HUVECs at elevated CAD concentration. In addition, the number of HUVECs on 15% and 30% (w/v) AlCol−CAD at CAD concentrations above 20 mM was higher than that on AtCol gel and AlCol−GA ($p < 0.001$). Figure 4B shows the proliferation number of HUVECs grown on AlCol−CAD, on AtCol gel, and on AlCol−GA after incubation for 7 days. An increase in the number of HUVECs was observed on all AlCol−CADs and AtCol gels. The 15% (w/v) AlCol−CAD at a CAD concentration of 20 mM showed almost the same HUVEC number as 30% (w/v) AlCol−CAD at CAD concentrations of 20 and 40 mM. On the other hand, no significant increase of HUVECs on AlCol−GA was observed.

Figure 5 shows SEM images of the morphology of HUVECs on 15% (w/v) AlCol−CAD, AtCol gel, and AlCol−GA after

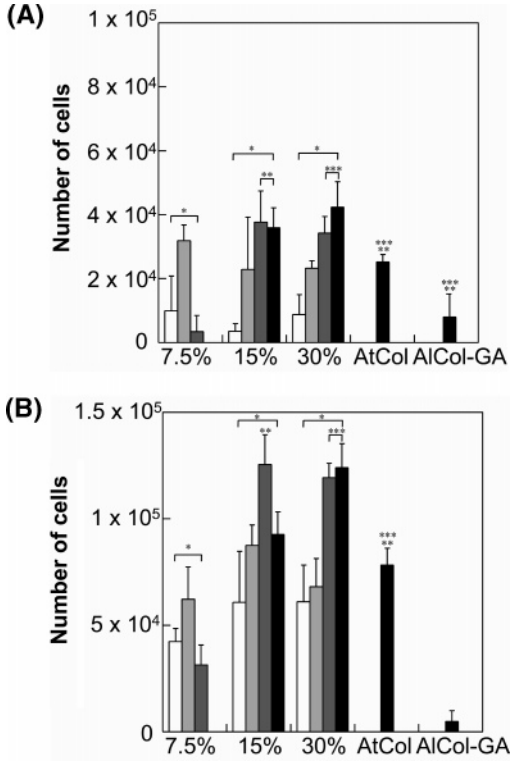

Figure 4. Number of HUVECs on AtCol gel, 15% (w/v) AlCol−CAD at different CAD concentrations, (white bar) 5 mM, (light gray bar) 10 mM, and (dark gray bar) 20 mM, (black bar) 40 mM, and AlCol−GA after incubation for (A, top) 1 and (B, bottom) 7 days. Error bars represent standard deviations ($n = 5$). Bars with the same number of asterisks are statistically significantly different for (*) $p < 0.01$ and (**, ***) $p < 0.001$.

incubation for 1 and 7 days. As shown in Figure 5a−e, cobblestone-like HUVECs were well adhered to AlCol−CAD, AtCol gel, and AlCol−GA after incubation for 1 day. After 7 days of culture, a complete monolayer was observed on AlCol−CAD at a CAD concentration of 20 mM as shown in Figure 5g. On AlCol−CAD at a concentration of 40 mM and on AtCol gel (Figure 5h,i), most areas of the gels were covered with HUVECs. A small number of HUVECs were found on AlCol−CAD at a concentration of 5 mM (Figure 5f) and AlCol−GA (Figure 5j) compared to the other AlCol−CADs.

3.4. Effect of the CAD Concentration on Thrombus Formation. Figure 6 shows the influence of the CAD concentration on thrombus formation of 15% (w/v) AlCol−CAD. As shown in Figure 6A, significant thrombus formation was observed on AtCol gel, AlCol−GA, and AlCol−CAD (5 mM) after immersion in rat arterial blood (a, b, f). On the other hand, a small amount of fibrin clot was formed on the surface of 15% (w/v) AlCol−CAD at a CAD concentration of 10 mM (c). At CAD concentrations above 20 mM (d, e), no thrombus formation was observed. Figure 6B shows the SEM images of the surface of AtCol gel, 15% (w/v) AlCol−CAD, and AlCol−GA after immersion in blood. The surfaces of AtCol gel (a), of AlCol gel at a CAD concentration of 5 mM (b), and of AlCol−GA (f) were covered by an accumulation of red blood cells and an aggregation of platelets and fibrin. In AlCol−CAD at a concentration of 10 mM, small amounts of platelets and fibrin were adhered (c). On the other hand, no fibrin clot formation and platelet adhesion were observed on AlCol−CAD at concentrations above 20 mM (d, e).

4. Discussion

Drug-eluting stents have attracted much attention in the treatment of severe coronary diseases such as myocardial infarction. These stents consist of metal and low-biodegradable or nonbiodegradable matrices containing the drugs that inhibit thrombus formation, inflammation, and cell adhesion. However, the residual matrices after drug elution show inflammatory response, thrombus formation, and prevention of endothelial cell adhesion.[22] Therefore, it is necessary to develop a biodegradable matrix with antithrombogenic activity that can promote endothelial cell adhesion. In the present study, AlCol−CAD was prepared and characterized by the determination of the swelling ratio, residual amino groups, and carboxyl groups. Then, cell adhesion and antithrombogenic properties of AlCol−CAD were investigated.

The swelling ratios of 7.5%, 15%, and 30% (w/v) AlCol−CADs with different CAD concentrations were evaluated. In general, the higher the cross-linking density of a polymer matrix is, the lower the swelling ratio of the polymer matrix becomes.[23] That is, the swelling ratio decreases with increasing cross-linker concentration. However, unusual behavior was observed in the case of 7.5% and 15% (w/v) AlCol−CAD as shown in Figure 1. The swelling ratio of 7.5% and 15% (w/v) AlCol−CAD decreased with increasing CAD concentration up to 10 and 20 mM; beyond this point, with a further increase in concentration, the swelling ratio began to increase significantly. At CAD concentrations higher than 20 mM, 7.5% (w/v) AlCol−CAD was not formed. These results suggested that the cross-linking densities of 7.5% and 15% (w/v) AlCol−CAD decreased when the CAD concentration was above 10 and 20 mM, respectively. In the case of 30% (w/v) AlCol−CAD, the swelling ratio also exhibited a significant increase when the CAD concentration was above 40 mM; this behavior was attributed to the decrease of the cross-linking density (data not shown).

To confirm the unusual phenomena, residual amino groups and carboxyl groups in AlCol−CAD were determined as shown in Figures 2 and 3. The residual amino groups of 7.5%, 15%, and 30% (w/v) AlCol−CAD were not detected at CAD concentrations higher than 10, 20, and 40 mM, respectively. This means that the amino groups in AlCol completely reacted with the active ester groups of CAD. Theoretically, a 15% (w/v) AlCol solution contains 55.5 mM amino groups derived from lysine and alginine residues. Therefore, 18.5 mM CAD is required to completely react with all amino groups in the AlCol solution because CAD has three active ester groups in a molecule. This result is in accordance with theory. However, there are two kinds of active ester groups in CAD; those two kinds show different reactivities of active ester groups and tend to cause the slight formation of CAD-bearing AlCol. Therefore, only a slight excess of CAD is required to react with all amino groups of 15% (w/v) AlCol. In the case of 7.5% and 30% (w/v) AlCol−CAD, the experimental value of the amino groups also showed coincidence with theoretical values.

On the other hand, the carboxyl groups in 7.5% and 15% (w/v) AlCol−CAD began to increase after the CAD concentration increased to higher than 10 and 20 mM, respectively, whereas no significant change of the carboxyl groups was observed in 30% (w/v) AlCol−CAD with the CAD concentration increasing up to 40 mM (Figure 3). However, at CAD concentrations higher than 40 mM, we also observed an increase in the carboxyl groups in 30% (w/v) AlCol−CAD (data not shown). In our previous study,[12] we clarified that CA-bearing AlCol generated from the hydrolysis of CAD-bearing AlCol was formed at elevated CAD concentration (Figure 7). Therefore, the swelling ratio of AlCol−CAD had a minimum value even though the CAD concentration increased because of the electric repulsion of the carboxylic groups of CA-bearing AlCol generated from the hydrolysis of CAD-bearing AlCol at elevated CAD concentrations.

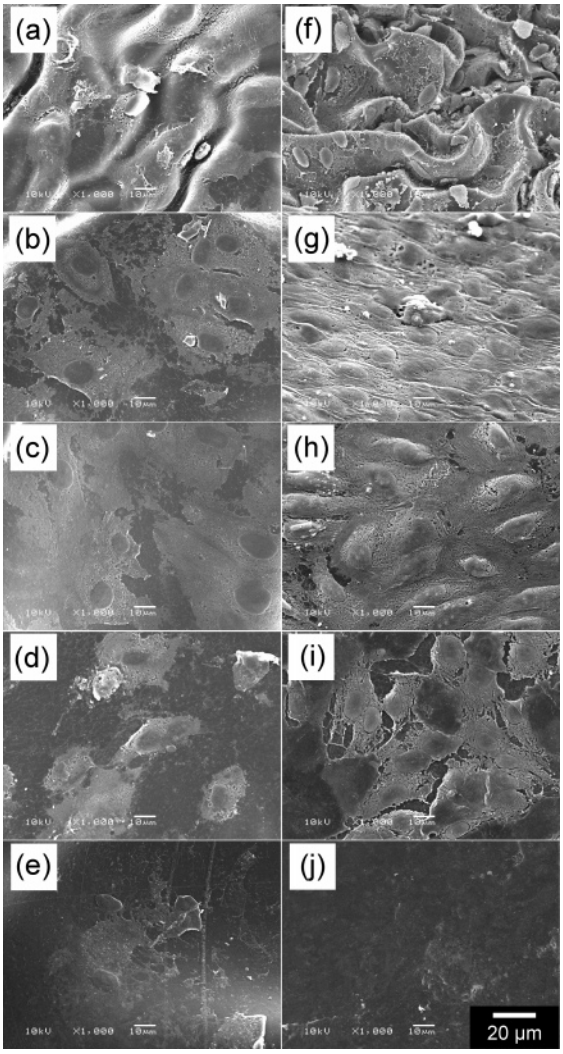

Figure 5. SEM images of HUVECs after 1 day of culture on AlCol−CAD at different CAD concentrations, (a) 5 mM, (b) 20 mM, and (c) 40 mM, (d) AtCol gel, and (e) AlCol−GA and after 7 days of culture on AlCol−CAD at different CAD concentrations, (f) 5 mM, (g) 20 mM, and (h) 40 mM, (i) AtCol gel, and (j) AlCol−GA.

HUVEC adhesion/proliferation and thrombus formation were also evaluated using AlCol−CAD with different CAD concentrations. The AlCol concentration was fixed at 15% (w/v). In general, the hydrophilic surface inhibits cell adhesion due to the minimal adsorption of cell adhesion proteins on the gel matrix surface.[24,25] From Figure 4A, the adhesion number of HUVECs on 7.5% and 15% (w/v) AlCol−CAD increased with CAD concentration increasing up to 10 and 20 mM, respectively; beyond 10 and 20 mM, the adhesion number of HUVECs decreased ($p < 0.01$). This can be explained by the increased hydrophilicity of AlCol−CAD as shown in Figure 1. In the case of 30% (w/v) AlCol−CAD, adhered HUVECs increased with increasing CAD concentration, because the swelling ratio of 30% (w/v) AlCol−CAD decreased at elevated CAD concentration. Moreover, the HUVEC number on 15% (w/v) AlCol−CAD at a CAD concentration of 20 mM and on 30% (w/v) AlCol−CAD at CAD concentrations of 20 and 40 mM showed higher values than that on AtCol gel ($p < 0.001$) as shown in

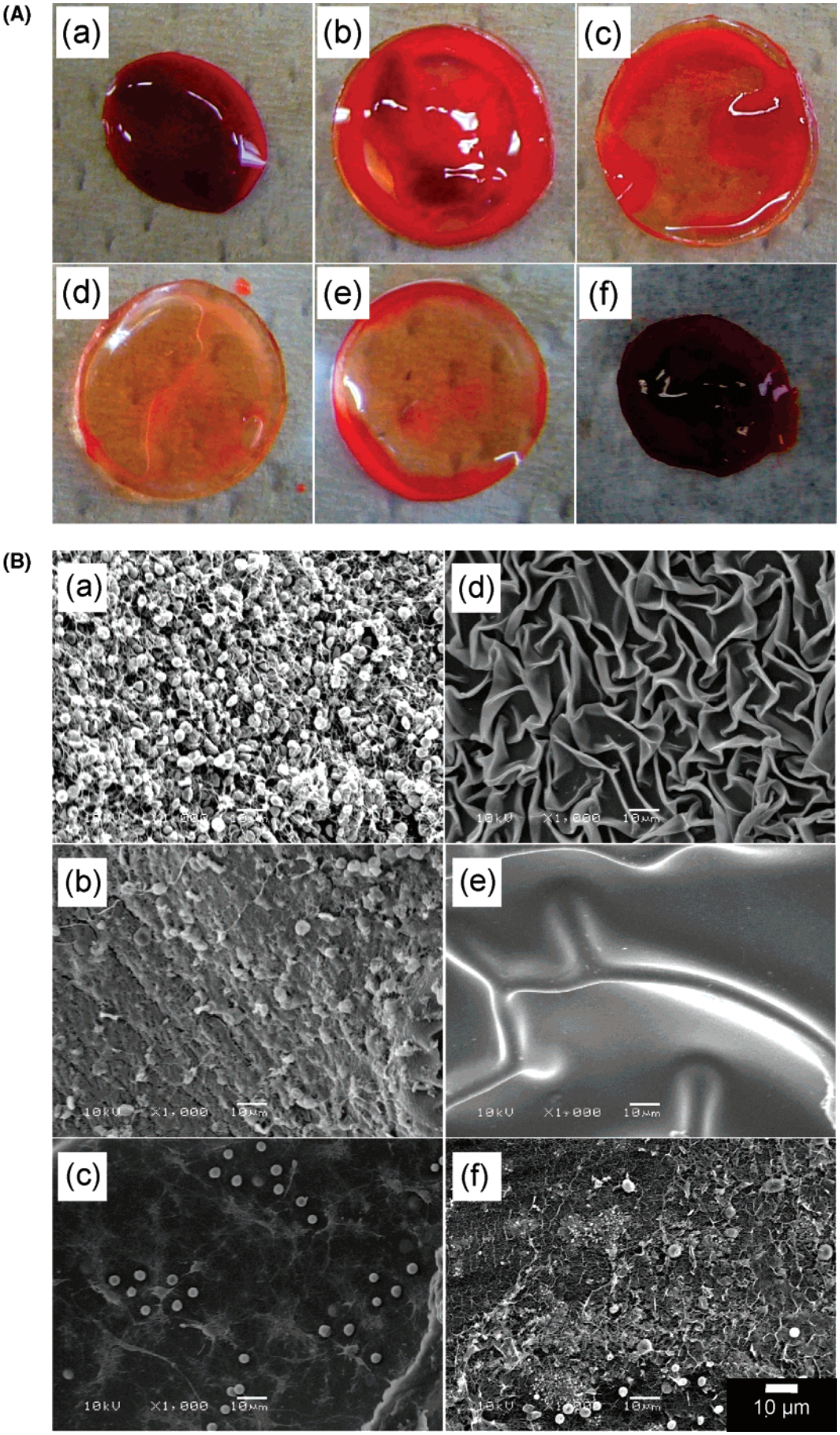

Figure 6. (A, top) Photographs of (a) AtCol gel, AlCol−CAD at different CAD concentrations after immersion of gels in rat arterial blood, (b) 5 mM, (c) 10 mM, (d) 20 mM, and (e) 40 mM, and (f) AlCol−GA. (B, bottom) SEM images of gels after immersion in rat arterial blood: (a) AtCol gel, AlCol−CAD at different CAD concentrations, (b) 5 mM, (c) 10 mM, (d) 20 mM, and (e) 40 mM, (f) AlCol−GA.

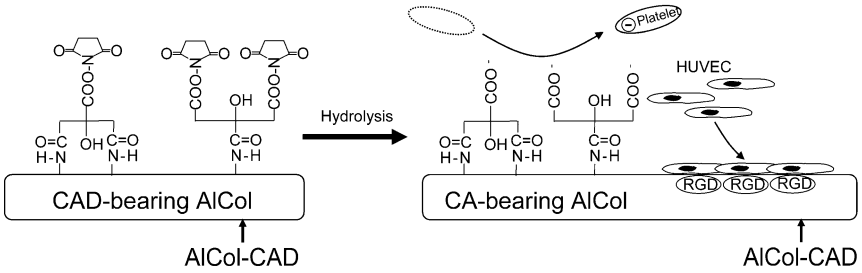

Figure 7. Schematic illustration of the expression of antithrombogenic and cell adhesion properties of 15% (w/v) AlCol−CAD (concentration >20 mM).

Figure 4B. We observed that the swelling ratio of 15% (w/v) AlCol−CAD at a CAD concentration of 20 mM and 30% (w/v) AlCol−CAD at concentrations of 20 and 40 mM was lower than that of AtCol gel. These results suggested that a cell adhesion peptide such as RGD (arginine−glycine−aspartic acid) was concentrated on the surface of AlCol−CAD. That is, the amount of RGD in AlCol−CAD was much higher than that in AtCol. Therefore, the proliferation number of HUVECs on AlCol−CAD was larger than that on AtCol gel. On AlCol−GA, only a small number of HUVECs were found after incubation for 1 and 7 days. It was thought that the unreacted aldehyde group-bearing AlCol and release of GA-related molecules or hydrolyzed cytotoxic monomers from AlCol−GA caused the inhibition of cell adhesion and proliferation. Furthermore, the elasticity of 15% (w/v) AlCol−CAD increased with increasing CAD concentration up to 20 mM and then slightly decreased. Therefore, it was suggested that the increased elasticity of AlCol−CAD promoted cell proliferation.

As shown in Figure 5g,h, AlCol−CAD at concentrations of 20 and 40 mM appeared in an almost complete HUVEC monolayer formation. The complete HUVEC monolayer is essential for expression of the antithrombogenic property.[26,27] Therefore, the 15% (w/v) AlCol−CAD at CAD concentrations above 20 mM was expected to show the antithrombogenic property.

We subsequently evaluated the antithrombogenic activity of the resulting 15% (w/v) AlCol−CAD, where AtCol and AlCol−GA were used as control matrices. In general, high thrombus formation is known to occurr on collagen-based materials because of its cationic properties.[28] However, the antithrombogenic property was observed in 15% (w/v) AlCol−CAD at elevated CAD concentration (Figure 6). It is well-known that antithrombogenic properties of biomaterials have been introduced to the hydrophilic and negatively charged groups on the surface[29−32] and the hydrophilic surface inhibits protein adsorption.[33] In addition, the isoelectric point of AlCol was 5 (the isoelectric point of AtCol was 9.6); therefore, AlCol shows a negative charge due to dissociation of carboxyl groups in blood (pH 7.4). Therefore, the antithrombogenic property of AlCol−CAD was due to the hydrophilicity and negative charge of AlCol because thrombus formation was suppressed under the elevated CAD concentration. That is, it was suggested that the AlCol−CAD at a CAD concentration of 20 mM inhibited thrombus formation due to the concentrated negatively charged carboxyl groups on the surface with a low swelling ratio and the hydrophilic tertiary hydroxyl group of CAD covalently reacting with AlCol. Furthermore, it was thought that the antithrombogenic property of AlCol−CAD at CAD concentrations above 20 mM was due to the negatively charged carboxyl groups of CA-bearing and hydrophilic AlCol-CAD (Figure 7). It was also supposed that the inhibition of thrombus formation was due to calcium-chelating properties of blood by CA-bearing AlCol, because the calcium ion promotes thrombus formation.[34] On AlCol−GA, significant thrombus formation was observed as shown in part A-f and B-f of Figure 6. It was clarified that AlCol−GA had a lower swelling ratio (6.6 ± 0.9) than other AlCol gels prepared in this study. This low swelling ratio of AlCol−GA is supposed to induce thrombus formation.

In our previous study, we confirmed that the swelling ratio of AlCol−CAD was saturated for 24 h. In addition, we evaluated the dependency of the cell number and antithrombogenic property on the incubation time; however, relationships between the swelling ratio and these biological properties were not changed over time. Therefore, it was thought that the cell adhesion and antithrombogenic property of AlCol−CAD remained unchanged over time, because the hydrophilicity of AlCol−CAD will not change over time even in the in vivo situation.

Results from Figures 4−6 indicated that the 15% (w/v) AlCol−CAD at CAD concentrations above 20 mM showed both antithrombogenic and HUVEC adhesion properties. Therefore, this AlCol−CAD has potential for biodegradable matrices for the application of a drug-eluting stent. Further evaluation and in vivo experiments are now in progress.

5. Conclusion

We have developed a novel biodegradable matrix, AlCol−CAD, which has both antithrombogenic and HUVEC adhesion properties. The swelling ratio of 7.5% and 15% (w/v) AlCol−CAD decreased with CAD concentration increasing up to 10 and 20 mM, respectively, and with a further increase in the CAD concentration the swelling ratio began to increase. In the case of 30% (w/v) AlCol−CAD, it decreases with increasing CAD concentration. The residual amino groups in 7.5%, 15%, and 30% (w/v) AlCol−CAD gels were not detected at CAD concentrations above 10, 20, and 40 mM, respectively. In 7.5% and 15% (w/v) AlCol−CAD, the carboxyl groups increased with increasing CAD concentration, whereas no significant change of the carboxyl groups was observed in 30% (w/v) AlCol−CAD with increasing CAD concentration. The adhesion number of HUVECs on 7.5% and 15% (w/v) AlCol−CAD increased with the CAD concentration up to 10 and 20 mM, respectively, and then decreased. On the other hand, 30% (w/v) AlCol−CAD showed an increase in the HUVEC number with increasing CAD concentration. Thrombus formation on 15% (w/v) AlCol−CAD decreased with increasing CAD concentration.

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References and Notes

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