J Mater Sci: Mater Med (2008) 19:1297–1305
DOI 10.1007/s10856-007-3239-7

Characterization of alkali-treated collagen gels prepared by different crosslinkers

Hirofumi Saito · Shun Murabayashi · Yoshinori Mitamura · Tetsushi Taguchi

Received: 14 February 2007 / Accepted: 11 July 2007 / Published online: 13 September 2007

Abstract We have developed a naturally-derived crosslinker named malic acid derivative (MAD). In the present study, we prepared alkali-treated collagen (AlCol) gels with different crosslinkers including MAD and commercially available crosslinkers such as 1-ethyl-3-(3(′-dimethylaminopropyl) carbodiimide (EDC) and glutaraldehyde (GA). There are named as AlCol-MAD, AlCol-EDC, and AlCol-GA. We then compared their physicochemical properties. The residual amino groups in AlCol-MAD were not detected at MAD concentrations higher than 30 mM. On the other hand, the residual amino groups in AlCol-EDC and AlCol-GA were detected at crosslinker concentrations of 30 mM. The swelling ratios of AlCol-MAD, AlCol-EDC, and AlCol-GA decreased with increasing crosslinker concentration. Enzymatic degradation rate of AlCol-GA was slower than that of AlCol-MAD and AlCol-EDC. The cytotoxicity of MAD was clearly lower than that of EDC and GA. The number of adhered L929 on AlCol-MAD was higher than on AlCol-EDC and AlCol-GA after incubation for 1 day. After the culture for 3 and 7 days, excellent growth of L929 was observed on AlCol-MAD. These results suggested that MAD was excellent crosslinker for the reactivity with amino groups and cytocompatibility. Therefore, the resulting AlCol-MAD has potential for various biomedical applications like tissue engineering scaffolds and carrier for drug delivery systems.

H. Saito · S. Murabayashi · Y. Mitamura
Graduate School of Information Science and Technology, The University of Hokkaido, N-14 W-9, Kita-ku, Sapporo 060-0814, Japan

H. Saito
Furuuchi Chemical Corporation, 6-17-17 Minami-oi, Shinagawa-ku, Tokyo 140-0013, Japan

T. Taguchi (✉)
Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan
e-mail: Tetsushi@nims.go.jp

1 Introduction

Collagen-based biomaterials have been used in a variety of clinical applications such as tissue adhesive [1–3], vascular grafts [4, 5], aortic heart valves [6, 7], drug delivery matrices [8, 9], wound dressing [10, 11], and tissue engineering scaffold [12–14]. This is so, because they serve as a major component of the extracellular matrix of connective tissues. Although collagen is recognized as a promising material, concerns remain about their stability against enzymatic degradation and the low mechanical strength of untreated collagen in vivo. Therefore, collagen-based biomaterials require some chemical crosslinkers such as glutaraldehyde (GA) [15, 16], carbodiimide [17, 18], and epoxy compounds [19] in order to meet the demands for long-term clinical use. However, the use of these crosslinkers have disadvantages due to their cytotoxicity and calcification properties [20–26]. On the other hand, physical methods for crosslinking does not result in an adequate mechanical strength for tissue engineering scaffold [27–29] although they do avoid the potentially cytotoxic chemical crosslinkers. Therefore, it is necessary to develop an alternative crosslinker that has cytocompatibility.

In our previous study, we developed novel crosslinkers such as citric and malic acid derivatives (CAD and MAD) which can react with amino groups of biopolymer such as collagen and gelatin [30–32]. It was also shown that the tissue adhesive consisting of CAD and collagen, or

albumin had high bonding strength and excellent biocompatibility in vivo [30, 33, 34].

In the present study, we prepared alkali-treated collagen (AlCol) gel using MAD and commercially available crosslinkers such as 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC) and glutaraldehyde (GA), and evaluated the physicochemical properties of the resulting AlCol gels. Furthermore, the cytotoxicity tests of these crosslinkers and resulting AlCol gels were also performed for clinical application.

2 Experiments

2.1 Material

AlCol and atelocollagen (AtCol) derived from pig organs 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. Malic acid (MA), *N*-hydroxysuccinimide (HOSu), tetrahydrofuran (THF), 2,4,6-trinitrobenzenesulfonic acid (TNBS), disodium hydrogenphosphate, sodium hydrogenphosphate, dimethylsulfoxide (DMSO), GA, EDC, ethanol, and HCl were purchased from Wako Pure Chemical Industrials Ltd. (Osaka, Japan). Bacterial collagenase type I obtained from *Clostridium histolyticum* with a collagenase activity of 125 units/mg was purchased from Sigma Chemical Co., St. Louis, (MO, USA). The fetal bovine serum (FBS) minimum essential medium (MEM) and penicillin–streptomycin were purchased from Gibco Life Technologies (NY, USA). The trypsin and ethylenediamine tetraacetic acid (EDTA) were purchased from Clonetics (MD, USA). Dicyclohexylcarbodiimide (DCC) was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). All other chemicals were used without further purification.

2.2 Preparation of MAD

MAD was prepared by the method previously reported [32]. Briefly recapitulating, MA was first dissolved in THF, and then HOSu and DCC were added to it. After mixing for 30 min, the mixture was concentrated with rotary evaporator under a reduced pressure to remove THF. The resulting mixture was then recrystallized to yield pure MAD.

2.3 Preparation of AlCol-MAD, AlCol-EDC, AlCol-GA, and AtCol gel

AlCol was first dissolved in DMSO to obtain a 15 w/v% solution. MAD, EDC, and GA were added to this 15 w/v% AlCol solution so that the final crosslinker concentrations were 5–40 mM, respectively. 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 gels were subsequently immersed in excess pure water for 48 h at 37 °C to remove DMSO from AlCol gels. On the other hand, AtCol gel was prepared by mixing 0.3 w/v% AtCol, 10 M phosphate buffer solution (PBS) (pH7.4), and NaOH-HEPES buffer solution at a mixing ratio of 8:1:1 by volume. These resulting AlCol and AtCol gels were punched out in form of 10 mm diameter disks for the evaluation of physicochemical properties and cell culture.

2.4 Characterization of AlCol gels

Determination of residual amino groups in the resulting AlCol gels was performed by a spectrophotometric method using TNBS [35]. 1 mL of 4% $NaHCO_3$ and 1 mL of 0.1% TNBS were then added to AlCol gels and incubated for 2 h at 37 °C. 3 mL of 6 N HCl was subsequently added and autoclaved for 1 h at 120 °C to hydrolyze the matrices. The mixed solutions were spectrophotometrically measured at 340 nm using microplate-reader (GENios A-5082, Tecan Japan, Japan).

The storage moduli (G′) of AlCol gels were determined by a Rheometer (Rheostress RS1, Haake, Germany). The viscoelastic meter was equipped with plate-plate tools of 20 mm in diameter with a gap length of 1 mm. The temperature of the sample chamber was maintained at 25 °C. The disk shape AlCol gels were first placed onto the stage of the viscoelastic meter. Then, G′ was measured at a frequency of 1 Hz and oscillatory shear stress of 1.0 Pa.

The measurement of the swelling ratio of AlCol gels was carried out as follows: AlCol gels were weighted under an equilibrium-swollen state in pure water, and then freeze-dried at –40 °C for 24 h and –10 °C for 24 h to determine the swelling ratio 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.

2.5 Enzymatic degradation of AlCol gels

Enzymatic degradation tests of AlCol gels were performed by the method reported by Park et al. [36]. The AlCol gels were prepared with crosslinker at concentration of 20 mM. The disk shape AlCol gels were immersed in 5 mL of Tris/HCl buffer solution (pH 7.4, 2.5 mM $CaCl_2$) containing 50 μg/mL (50 units) of collagenase at 37 °C. After

different immersion times, the gels were weighted and the percentage of weight loss was calculated.

2.6 In vitro cytotoxicity test of crosslinkers

The cytotoxicities of MAD, EDC, and GA were evaluated and compared by culturing L929 fibroblasts in 10% FBS containing medium. L929 were first seeded into 96-well multiplates at a concentration of 1×10^4 cells/well. The sterilized crosslinker solutions prepared by dissolving in the medium were subsequently added to the L929 at concentrations ranging from 0.0001 to 10 mg/mL. After incubation for 3 days at 37 °C in 5% CO_2 the L929 was washed twice with 200 μL of 0.1 M PBS. The viabilities of L929 were determined by using 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 [37, 38]. The resulting data were expressed as a percentage compared to a control group that had not been treated with the medium of each crosslinker. The viabilities of L929 fibroblasts were calculated using the following equation:

$$\text{Viability } (\%) = (N_i/N_c) \times 100,$$

where, N_i and N_c are the absorbance of surviving L929 treated with and without medium of each crosslinker.

2.7 Culture of L929 on AlCol-MAD, AlCol-EDC, AlCol-GA, and AtCol gel

The experiments for the evaluation of cell culture were performed with L929 fibroblast in 10% FBS containing medium. AtCol gel was used as a control. AlCol gels at crosslinker concentration of 20 mM, and AtCol gels were placed on 24-well multiplates and fixed by a glass ring. L929 fibroblasts at a concentration of 5×10^4 cells/well were seeded on each gel, and then L929-seeded gels were incubated at 37 °C, with 5% CO_2. After 1, 3, and 7 days, L929-seeded gels were washed twice with 1 mL of PBS. The number of L929 adhered on AlCol gels were assessed by the use of WST-1. The observation of the cell morphology was performed by optical microscopy.

2.8 Statistical analysis

Statistical analysis of data was performed by Microsoft Excel's statistical function for *t*-tests. Differences were considered statistically significant at $p < 0.05$. All data were expressed as mean ± standard deviation (SD).

3 Results and discussion

3.1 Determination of residual amino groups in AlCol gels

Figure 1 shows the amounts of residual amino groups in AlCol gels as functions of crosslinker concentrations. Spectrophotometric methods were employed for the determination of the residual amino groups in AlCol gels. Residual amino groups in AlCol-MAD, AlCol-EDC, and AlCol-GA decreased with increasing crosslinker concentration. At MAD concentrations above 30 mM, no amino groups were detected in AlCol-MAD. This means that amino groups in AlCol completely reacted with active ester groups of MAD. Theoretically, 15 w/v% AlCol has 55.5 mM of amino groups derived from lysine and alginine residue. Therefore, 27.7 mM of MAD, GA, and EDC was required to completely react with all amino groups in the AlCol molecules because these crosslinkers have two reacting points in a MAD molecule as shown in Fig. 2A. In AlCol-MAD, this result is nearly in accordance with the theory. However, there was a possibility of formation of MAD-bearing AlCol which was generated by the reaction between amino group of AlCol and one active ester group of MAD. Considering this reaction a slight excess amount of MAD was required to react with all amino groups of 15 w/v% AlCol. On the other hand, the disappearance of amino groups in AlCol-EDC and AlCol-GA was not observed. It is known that DMSO will combine with some carbodiimides to produce a useful oxidising reagent [39]. We confirmed the reaction between collagen with crosslinkers by the determination of residual amino groups in resulting gels. The residual amino groups of AlCol-EDC were not disappeared, however, they decreased with increasing initial EDC concentrations. Therefore, we supposed that the reaction between collagen and EDC proceeded in our condition. Similar to the reaction in aqueous solution, condensation reaction between amino and carboxyl groups in AlCol will occur when EDC is added to the AlCol solution. In addition, it is well-known that EDC activates the carboxyl groups and then forms the amide bonds with the amino groups of AlCol. However, the formation of *N*-acylurea and carboxyl groups occurs as a result of the side reaction of *O*-acylisourea group (Fig. 2B) [40, 41]. It can be explained that these side reactions suppress the disappearance of amino groups of AlCol-EDC, whereas GA is presumed to perform inter- and intramolecular crosslinking by the formation of Schiff bases as shown in Fig. 2C [42, 43]. These Schiff bases react not only with primary amines of AlCol but also with other GA molecules resulting in the polymerization of GA molecules. These side reactions of GA also affect the

incomplete crosslinking under a sufficiently high GA concentration.

3.2 Gel strength of AlCol gels

Figure 3 shows the effect of crosslinker concentration on the behavior of G′ of AlCol gels. The G′ of AlCol-MAD, AlCol-EDC, and AlCol-GA increased with increasing crosslinker concentration. In general, the increasing of G′ is due to the increasing number of chemical junctions responsible for the formation of the crosslinking points such as amide bonds [44]. That is, the increasing of G′ indicates the increasing of crosslinking density. At MAD concentrations higher than 30 mM, the G′ of AlCol-MAD was saturated. This result suggested that crossliking density of AlCol-MAD reached a maximum level at MAD concentration of 30 mM because MAD completely reacted with all amino groups in AlCol as shown in Fig. 1. In addition, the G′ of AlCol-GA was clearly higher than that of AlCol-MAD and AlCol-EDC at crosslinker concentrations above 15 mM. This result suggested that the crosslinking density of AlCol-GA was higher than that of AlCol-MAD and AlCol-EDC due to the network formation by the aldol condensation of aldehyde groups. On the other hand, the G′ of AlCol-EDC was clearly lower than that of AlCol-MAD and AlCol-GA at any crosslinker concentration. It was thought that the crosslinking density of AlCol-EDC did not significantly increase because of the side reaction of EDC as shown in Fig. 2B.

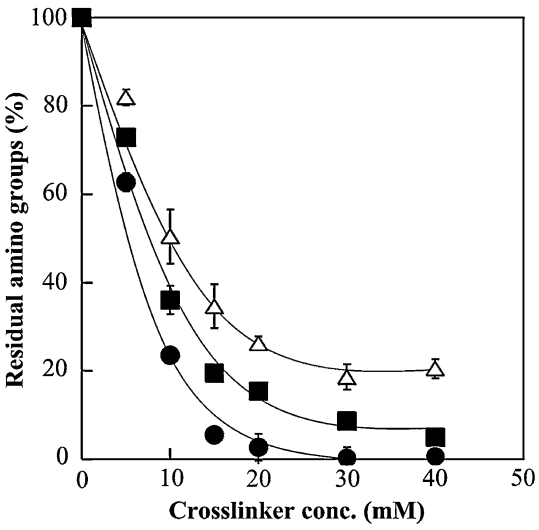

Fig. 1 Residual amino groups content in AlCol gels prepared using MAD (●), EDC (Δ), and GA (■) at different crosslinker concentrations. Error bars represent standard deviations; $n = 3$

3.3 Swelling ratio of AlCol gels

Figure 4 shows the equilibrium swelling ratio of AlCol gels at different crosslinker concentrations. It was observed that the swelling ratio of AlCol gels was saturated within 24 h. The swelling ratio of AlCol-MAD, AlCol-EDC, and AlCol-GA decreased with increasing crosslinker concentration. In general, the higher crosslinking density of a gel happens to be, the lower the swelling ratio of a gel becomes [45]. Therefore, this result indicated that the crossliking density of AlCol gels increased at elevated crosslinker concentration. In the case of AlCol-MAD, the swelling ratio showed slight increase at MAD concentrations higher than 30 mM. It was suggested that the slightly increased swelling ratio of AlCol-MAD was due to the secondary hydroxyl group of MAD residues covalently reacting with AlCol. In addition, the swelling ratio of AlCol-EDC was higher than of any other gels prepared in this study. The results of Figs. 3 and 4 indicated that the crosslinking density of AlCol-EDC was lower than that of AlCol-MAD and AlCol-GA.

3.4 Enzymatic degradation of AlCol gels

Figure 5 shows the time dependence of enzymatic degradation of AlCol-MAD, AlCol-EDC, and AlCol-GA at crosslinker concentration of 20 mM. Depending on the type of crosslinker, the degradation time of AlCol gels was found to vary. In AlCol-EDC, high degradation rate was observed. Almost half of AlCol-EDC degraded within the first 15 min and the degradation was complete in 45 min. On the other hand, AlCol-MAD and AlCol-GA completely degraded after immersion in collagenase solution in 3 and 5 h, respectively. It is well known that the degradation time depended on the crosslinking density [46]. That is, the decreasing of degradation time is indicative of the decreasing of crosslinking density. Therefore, the results from Figs. 3, 4, and 5 suggested that the high degradation rate of AlCol-EDC was due to the low crosslinking density of AlCol-EDC. In addition, it was suggested that the degradation time of AlCol-GA was slower than that of AlCol-MAD because the effect of the networks of molecular chains formed by the polymerization of GA molecules, regardless of its having nearly the same swelling ratio. The results from Figs. 1, 3, 4, and 5 showed that MAD was superior to EDC and GA in the precise design of crosslinking gel matrices. And because of the excellent crosslinker in crosslinking reactivity with amino groups it had no side reactions.

3.5 Cytotoxicity test of crosslinkers

Figure 6 shows the results of cytotoxicity test by the addition of MAD, GA, and EDC to L929 fibroblasts. The

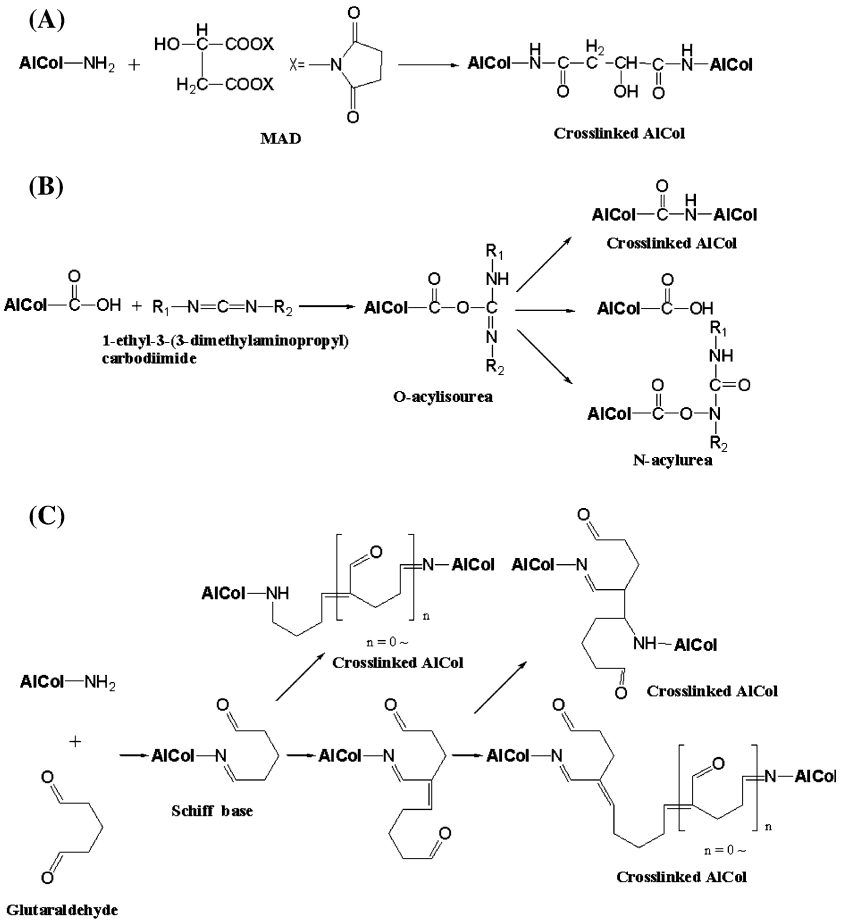

Fig. 2 The schematic illustration of crosslinking reaction mechanism of (**A**) MAD, (**B**) EDC, and (**C**) GA

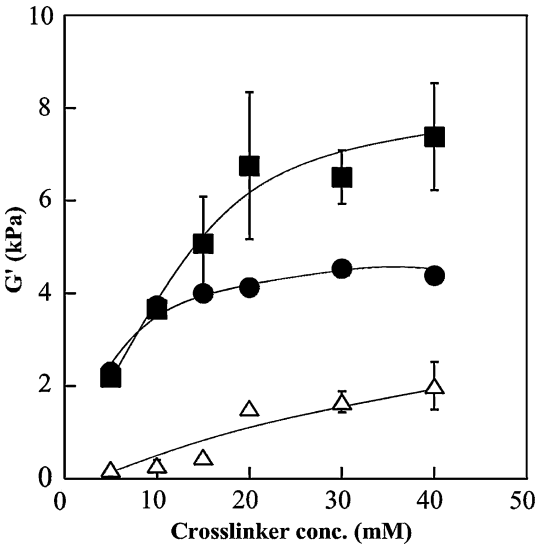

Fig. 3 Storage moduli (G′) of AlCol gels prepared using MAD (●), EDC (Δ), and GA (■) at different crosslinker concentrations. Error bars represent standard deviations; $n = 3$

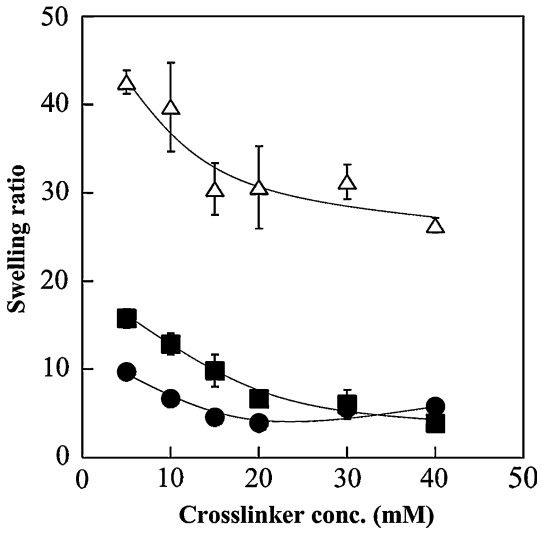

Fig. 4 The swelling ratio of AlCol gels prepared using MAD (●), EDC (Δ), and GA (■) at different crosslinker concentrations. Error bars represent standard deviations; $n = 3$

cytotoxicities of GA and EDC were not observed at low concentration of 0.0001 mg/mL, however, they increased with increasing GA and EDC concentrations. L929 could not survive the culture medium containing GA and EDC at concentrations of 0.1 and 1 mg/mL, respectively. On the other hand, the L929 cultured with medium of MAD had

little effect on viability at concentrations up to 0.01 mg/mL. At MAD concentration of 0.1 mg/mL, 80% viability of the L929 was observed. These results suggested that the cytotoxicity of MAD was clearly lower than that of GA and EDC. In addition, it was also expected that the formation of HOSu would occur by the hydrolysis of MAD in FBS-MEM. In our previous study, it was elucidated that the cytotoxicity of HOSu had lower value than that of CAD or citric acid [31]. Therefore, we expected that HOSu would show little cytotoxicity to L929 fibroblasts.

3.6 Cell culture on AlCol gels

Figure 7 shows L929 fibroblasts number on AlCol gels with crosslinker concentration of 20 mM after incubation for 1, 3, and 7 days. AtCol gel, with its commercially available cell culture matrices, was used as a control. Fig. 8A and B shows L929 fibroblasts morphologies after incubation for 1 and 7 days. The adhesion number of L929 on AlCol-MAD was nearly the same as on AtCol gel, and was higher than that of AlCol-EDC and AlCol-GA ($p < 0.05$) after incubation for 1 day (Fig. 8A). In AlCol-EDC, a few adhesion numbers of the L929 fibroblasts were there. In the literature, EDC is known as a low cytotoxicity crosslinker because it is not incorporated into the cross-linked structure, however, urea derivatives such as *N*-acylurea and *O*-acylisourea groups derived from the crosslinking reaction process shows the cytotoxicity [47–49]. Therefore, it was expected that the L929 adhesion on AlCol-EDC was inhibited due to the cytotoxicity of these urea derivatives in AlCol-EDC. On the other hand, in the case of AlCol-GA, it was thought that unreacted aldehyde

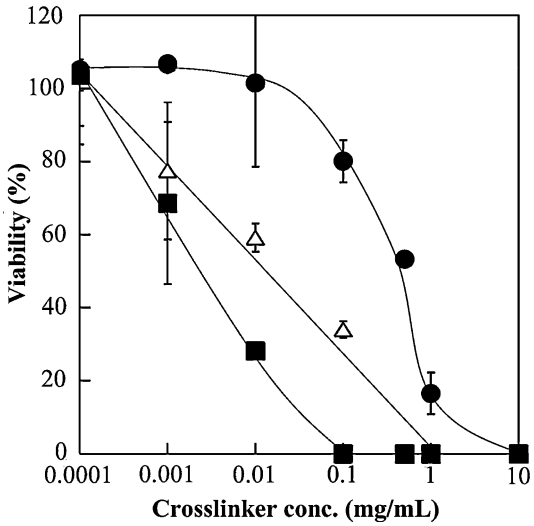

Fig. 6 Viability of L929 fibroblasts exposed to various crosslinker at different concentrations: MAD (●), EDC (Δ), and GA (■). Error bars represent standard deviations; $n = 3$

groups-bearing AlCol caused cytotoxicity. Moreover, it was also expected that MAD-bearing AlCol would show little cytotoxicity even if it remained in AlCol-MAD, as shown in Fig. 6.

After the culture for 3 and 7 days, the growth of L929 on AlCol-MAD, AlCol-EDC and AtCol were observed, whereas no significant increase in L929 number on AlCol-GA was observed as shown in Figs. 7 and 8B. In AlCol-GA, it was suggested that the release of GA-related molecules or hydrolyzed cytotoxic monomers from AlCol-GA caused the inhibition of cell growth at 3 and 7 days. In addition, it is well known that the increase of swelling ratio inhibits cell adhesion due to minimal adsorption of cell adhesion proteins on

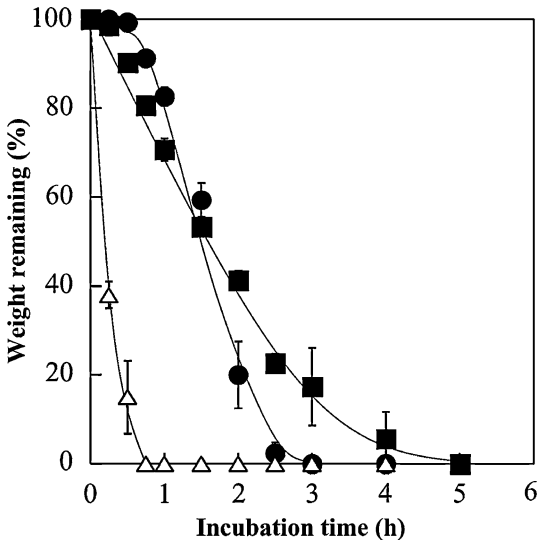

Fig. 5 Dependence of the enzymatic degradation time of MAD (●), EDC (Δ), and GA (■). Error bars represent standard deviations; $n = 3$

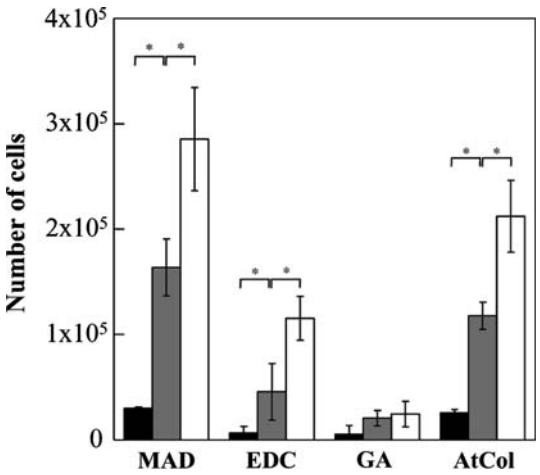

Fig. 7 Cell growth analysis results on AlCol gels prepared using MAD, EDC, and GA after incubation for 1 day (■), 3 day (▩), and 7 day (□). Error bars represent standard deviations; $n = 3$. Intersubstrate differences were deemed significant for * $p < 0.05$

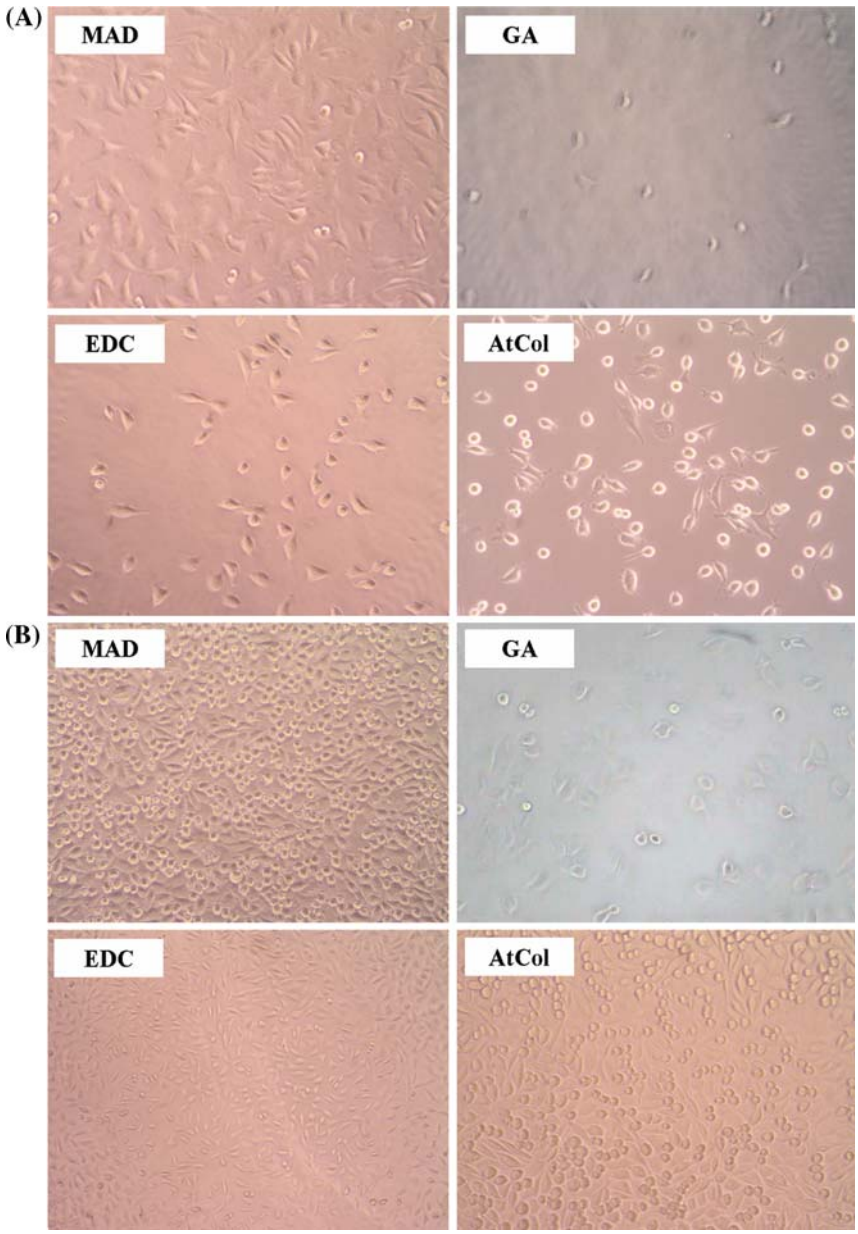

Fig. 8 Photomicrographs of L929 fibroblasts on AlCol gels prepared using MAD, EDC, and GA after incubation for (**A**) 1 and (**B**) 7 days

the gel matrices surface [50, 51]. Figure 4 shows the swelling ratio of AlCol-EDC was higher than that of AlCol-MAD. Therefore, it was suggested that the cell growth on AlCol-EDC was lower than that on AlCol-MAD. Furthermore, as shown in Fig. 7, L929 growth on AlCol-MAD was higher than AtCol gel ($p < 0.05$). This result suggested that the amounts of cell adhesion peptides such as RGD (arginine–glycine–aspartic acid) of the AlCol-MAD was much higher than on AtCol gel. Therefore, it was observed that the cell growth on AlCol-MAD was higher than on AtCol gel.

Results from Figs. 6 and 7 indicated that AlCol-MAD has excellent cytocompatibility as compared to what AlCol-EDC and AlCol-GA exhibit. Therefore, this malic acid derivative crosslinker can be successfully applied for collagen crosslinking to produce more cytocompatible collagen gel with the potential for use in tissue-engineering scaffolds and for carriers drug delivery system application.

4 Conclusion

We compared the physicochemical properties and cytocompatibility of AlCol gel prepared using MAD and commercially available crosslinkers such as EDC and GA, named as AlCol-MAD, AlCol-EDC, and AlCol-GA. At crosslinker concentrations higher than 20 mM, no amino groups were detected in AlCol-MAD, whereas the residual amino groups in AlCol-EDC and AlCol-GA were detected.

The G′ of AlCol-MAD, AlCol-EDC, and AlCol-GA increased with increasing crosslinker concentration. In AlCol-MAD, AlCol-EDC, and AlCol-GA, the increasing of swelling ratio showed at elevated crosslinker concentration. Enzymatic degradation of AlCol-GA was slower than that of AlCol-MAD and AlCol-EDC. MAD clearly showed lower cytotoxicities than shown by EDC and GA. The adhesion numbers of L929 on AlCol-MAD were higher than those of AlCol-EDC and AlCol-GA after incubation for 1 day. After the culture for 3 and 7 days, L929 growth on AlCol-MAD was superior to that of AlCol-EDC and AlCol-GA.

Acknowledgments This work was financially supported in part by Innovative Technology Research Grant from Japan Science and Technology Agency (JST), Japan, and the Coordination Found for Prompting Science and Technology for Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (Development of artificial organs utilizing with Nanotechnology and materials science) and Industrial Technology Research Grant Program in '04 from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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