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ScienceDirect
Acta Biomaterialia 3 (2007) 89–94
Acta BIOMATERIALIA
www.actamat-journals.com

pH-responsive swelling behavior of collagen gels prepared by novel crosslinkers based on naturally derived di- or tricarboxylic acids

Hirofumi Saito [a,b], Tetsushi Taguchi [c,*], Hirokatsu Aoki [b], Shun Murabayashi [a], Yoshinori Mitamura [a], Junzo Tanaka [c], Tetsuya Tateishi [c]

[a] *Graduate School of Information Science and Technology, The University of Hokkaido, N-14 W-9, Kita-ku, Sapporo 060-0814, Japan*
[b] *Furuuchi Chemical Corporation, 6-17-17 Minami-oi, Shinagawa-ku, Tokyo 140-0013, Japan*
[c] *Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan*

Received 28 April 2006; received in revised form 14 July 2006; accepted 16 August 2006

Abstract

The aim of this study was to compare the physicochemical properties of alkali-treated collagen (AlCol) gels prepared using two kinds of naturally derived crosslinkers made from citric and malic acids (CAD and MAD, respectively) that we have developed. From the crosslinking reaction between active ester groups and amino groups of AlCol, we successfully obtained AlCol gels, named AlCol-CAD and AlCol-MAD, prepared using CAD and MAD, respectively. The gelation time of the AlCol solution containing CAD initially decreased with increasing CAD concentration up to 70 mM, and then increased as the CAD concentration increased further. The gelation time reached its minimum and began to increase. On the other hand, for AlCol-MAD solution, gelation occurred within 40 s at any MAD concentration. Moreover, the residual amino groups in AlCol-CAD and AlCol-MAD were found to decrease with increasing CAD or MAD concentrations, whereas increased residual carboxyl groups were detected only in the case of AlCol-CAD. The swelling ratio of AlCol-CAD significantly increased at CAD concentrations above 50 mM. On the other hand, AlCol-MAD showed little increase in swelling ratio with increasing MAD concentration. Also, AlCol-CAD was swollen when the gels were immersed in a solution with high pH. On the other hand, no significant increase in swelling ratio was observed when AlCol-MAD was immersed in a similar solution. These results suggest that the different amounts of carboxyl groups in AlCol-CAD affected the swelling behavior of gels and that this pH-responsive AlCol-CAD has potential for drug delivery systems and tissue engineering.

Keywords: Crosslinker; Biopolymer; Citric acid derivative; Malic acid derivative; Gel

1. Introduction

pH-responsive hydrogels are polymer networks that have pendant acidic or basic functional groups which either accept or release protons [1–4]. Hydrogels of this kind that contain carboxyl or sulfonic acid groups show significant changes in their swelling behavior as a result of changing the external pH [5,6].

pH-responsive hydrogels are divided into two categories according to whether their constituent polymers are natural or synthetic. Synthetic polymers, however, are not biologically degradable by either hydrolytic or enzymatic mechanisms, and so these polymers have limited use as biomedical implant devices. Natural polymers, such as collagen and gelatin, have been used for tissue engineering and drug delivery systems [7–10]. These biodegradable polymers are often crosslinked with various compounds to obtain water-insoluble hydrogel matrices. Several crosslinkers have been used, including glutaraldehyde [11,12], carbodiimide [13] and epoxy compounds [14]. However, these crosslinkers, which remain in the resulting hydrogels, usually show high toxicity [15,16]. Therefore, it is necessary to develop an alternative, low-toxicity crosslinker. In order

* Corresponding author. Tel.: +81 29 860 4498; fax: +81 29 851 4714.
E-mail address: TAGUCHI.Tetsushi@nims.go.jp (T. Taguchi).

1742-7061/$ - see front matter
doi:10.1016/j.actbio.2006.08.003

to overcome these obstacles, we developed a novel crosslinker known as citric acid derivative (CAD) with three active ester groups [17–21]. We confirmed that CAD can crosslink biodegradable polymers, which show excellent cytocompatibility compared with commercially available crosslinkers such as glutaraldehyde [18,19]. Furthermore, a tissue adhesive consisting of CAD and collagen had high bonding strength and excellent biocompatibility in vivo [17–19].

In our current study, we developed a novel crosslinker, malic acid derivative (MAD), which contains two active ester groups, in order to prepare alkali-treated collagen (AlCol) gels. We then compared the physicochemical properties (gelation time, storage moduli (G'), swelling ratio, residual amino and carboxyl groups) of AlCol gels prepared using CAD and MAD. We also investigated the pH-responsive AlCol-CAD and AlCol-MAD.

2. Experimental

2.1. Materials

AlCol derived from pig skin was provided by Nitta Gelatin Inc. (Osaka, Japan). Citric acid, malic acid, *N*-hydroxysuccinimide (HOSu), tetrahydrofuran (THF), 2,4,6-trinitrobenzenesulfonic acid (TNBS), disodium hydrogenphosphate, sodium hydrogenphosphate, toluidine blue-O, dimethylsulfoxide (DMSO), ethanol, HCl and acetic acid were purchased from Wako Pure Chemical Industrials Ltd. (Osaka, Japan). Dicyclohexylcarbodiimide (DCC) was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). All other chemicals were used without further purification.

2.2. Preparation of CAD and MAD

CAD and MAD were prepared by the method previously reported [22]. Briefly speaking, citric or malic acid was first dissolved in THF, and then HOSu and DCC were added. After mixing for 30 min, the mixture was concentrated with rotary evaporation under a reduced pressure to remove THF. The resulting mixture was recrystallized to yield pure CAD or MAD. Characterization of CAD and MAD was performed using ^{1}H-NMR (JEOL EX-300) and elemental analysis.

The ^{1}H-NMR and elemental analysis results of CAD were follows: 1H-NMR (DMSO-d_6) $\delta = 2.8$ ppm (s, 12H, succinimidyl esters $CH_2 \times 6$), 3.4 ppm (s, 4H, $CH_2 \times 2$), 7.2 ppm (s, 1H, OH). *Analysis.* Calculated for $C_{18}H_{17}N_3O_{13}$: C, 44.73; H, 3.55; N, 8.69. Found: C, 44.83; H, 3.45; N, 8.58.

The ^{1}H-NMR and elemental analysis results of MAD were follows: 1H-NMR (DMSO-d_6) $\delta = 2.8$ ppm (s, 8H, succinimidyl esters $CH_2 \times 4$), 3.1–3.3 ppm (m, 2 H, CH_2), 4.8–4.9 ppm (m, 1H, CH), 6.73 ppm (d, 1H, OH). *Analysis.* Calculated for $C_{12}H_{12}N_2O_9$: C, 43.91; H, 3.68; N, 8.53. Found: C, 43.76; H, 3.51; N, 8.40.

2.3. Preparation of AlCol gels

AlCol, whose isoelectoric point is 5, has carboxyl groups generated by the hydrolysis of residual amide groups that exit in asparagine and glutamine of atelocollagen. AlCol was first dissolved in DMSO to obtain a 20% w/v solution. Then, 50 μl of CAD or MAD solution at crosslinker concentrations from 10 to 200 mM was added to a 200 μl of 20% w/v AlCol solution. The crosslinking reaction continued for 24 h at 37 °C. The resulting AlCol gels were subsequently immersed in excess 0.1 M phosphate buffer solution (PBS) at different pHs (5.8–8.0) for 24 h at 37 °C to remove DMSO. The AlCol gels were weighed under the equilibrium swollen state, and freeze-dried at −20 °C for 48 h to determine the swelling ratio using the following equation:

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

where W_d and W_0 are the weights of the dried and immersed AlCol gels, respectively.

2.4. Determination of residual amino and carboxyl group content in AlCol gels

Determination of residual amino groups in AlCol gels was performed by a spectrophotometric method using TNBS [23]. AlCol gels prepared using different CAD or MAD concentrations were placed in 5 ml tubes. The next stage was to add 1 ml of 4% $NaHCO_3$ and 1 ml of 0.1% TNBS to AlCol gels, which were then incubated for 2 h at 37 °C. Then, after adding 3 ml of 6 N HCl, the AlCol gels were autoclaved for 1 h at 120 °C to hydrolyze them. The mixed solutions were spectrophotometrically measured at 340 nm using a microplate-reader (GENios A-5082, Tecan Japan, Japan).

The residual carboxyl groups in AlCol gels were estimated by staining with toluidine blue-O [24]. AlCol gels, prepared using CAD or MAD at crosslinker concentrations from 10 to 200 mM, were immersed in excess 0.1 M PBS at pH 7.0 at 37 °C for 24 h. The AlCol gels were then stained with 5×10^{-4} M toluidine blue-O in $NaHCO_3$/Na_2CO_3 buffer solution (pH 10) for 3 h, and rinsed three times with $NaHCO_3$/$NaCO_3$ buffer solution (pH 10). The toluidine blue-O in AlCol gels was extracted with 50% v/v acetic acid solution. The spectrophotometric measurement of the extracts was carried out at 633 nm using ultraviolet–visible spectroscopy (Hitachi U-2001, Hitachi, Japan).

2.5. Rheological measurements

The gelation time of AlCol solution containing CAD or MAD was determined with 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 37 °C. AlCol solution (500 μl)