Tamibarotene-loaded citric acid-crosslinked alkali-treated collagen matrix as a coating material for a drug-eluting stent

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Received 11 July 2012
Accepted for publication 3 October 2012
Published 23 November 2012
Online at stacks.iop.org/STAM/13/064208

Abstract
Tamibarotene-loaded biodegradable matrices with antithrombogenic and drug-releasing properties were prepared in a crosslinking reaction between amino groups of alkali-treated collagen (AlCol) and active ester groups of trisuccinimidyl citrate. The resulting matrices were characterized by their residual amino group concentrations, swelling ratios and thermal, antithrombogenic and drug-releasing properties. It was clarified that the addition of tamibarotene does not inhibit matrix formation. After immersion in water, the swelling ratio of a matrix became lower than that prior to immersion. Thermal analysis indicated that AlCol interacted with tamibarotene. The addition of tamibarotene to the matrix did not influence the antithrombogenic property of the resulting matrix. A matrix with a high crosslinking density had a prolonged tamibarotene elution time. These results demonstrate that tamibarotene-loaded matrices have great potential as a coating material for drug-eluting stents.

Keywords: alkali-treated collagen, citric acid, tamibarotene, chemical crosslinking, drug-eluting stent (DES)

1. Introduction
Drug-eluting stents (DES) are powerful biomedical devices used to prevent restenosis after a percutaneous coronary intervention. In general, biodegradable or synthetic polymeric matrices such as poly(lactic acid), phosphorylcholine, poly(butyl methacrylate) and styrene–isobuthylene–styrene triblock copolymer are coated to act as drug reservoirs and elute drugs for several time periods [1–3]. However, after drug elution, matrices with slow biodegradation lead to complications such as exaggerated inflammatory response, the formation of thrombus and prevention of endothelialization [4–6]. Therefore, it is necessary to develop a novel biodegradable matrix with an antithrombogenic property that can promote endothelial cell adhesion after drug elution [7–11]. Recently, we have developed novel crosslinkers, organic acids with active ester groups, for use in biocompatible medical devices [12, 13]. These crosslinkers, made of citric acid, maleic acid and tartaric acids, were reported to form biopolymer matrices as a result of the reaction between amino groups of the biopolymer and active ester groups of crosslinkers [14–16]. The resulting matrices exhibit excellent cytocompatibility compared with commercially available crosslinkers such
as glutaraldehyde [17, 18]. In addition, it was clarified that alkali-treated collagen (AlCol) crosslinked with trisuccinimidyl citrate (TSC) matrices (AlCol–TSC) showed not only excellent adhesion to human umbilical vein endothelial cells (HUVECs) but also good antithrombogenic properties [19].

At present, immunosuppressive and anticancer compounds are used as DES drugs [20, 21]. As these drugs non-specifically inhibit cell proliferation and suppress neointimal hyperplasia and re-endothelialization [22]. Therefore, a wound in a DES-implanted region takes a long time to heal [23]. In addition, it has been established that tibamabarotene (Am80) specifically inhibits smooth muscle cell (SMC) proliferation without affecting the proliferation of HUVECs [24, 25]. If Am80 could be impregnated into AlCol–TSC matrices, the resulting Am80-loaded matrices would be useful as a coating material for drug-eluting stents.

In this paper, we report on the preparation of novel Am80-loaded AlCol–TSC matrices. The resulting matrices were characterized by the determination of residual amino groups, swelling ratios, thermal analysis, antithrombogenic properties and drug-releasing behaviours.

2. Materials and methods

2.1. Materials

AlCol derived from porcine skin was provided by Nitta Gelatin Inc. (Osaka, Japan). AlCol, whose isoelectric point is 5, has a carboxyl group generated by the hydrolysis of residual amide groups that exist in asparagine and glutamine of collagen. Am80 was donated by the Research Foundation Itsuu Laboratory (Tokyo, Japan). Citric acid, N-hydroxysuccinimide, dimethyl sulphoxide (DMSO) and lactic acid were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). All chemical reagents were used without further purification. TSC was prepared according to the previously reported methods [26].

2.2. Preparation of Am80-loaded AlCol–TSC matrices

AlCol was dissolved in 10% (w/w) lactic acid-containing DMSO to obtain a 15% (w/v) solution. TSC solutions of various concentrations were prepared in 10% (w/w) lactic acid-containing DMSO. The TSC solutions were added to the AlCol solutions, then Am80 was dissolved so that the final concentration was 35 mM. The resulting solution was stirred, poured into a mould with a 1 mm silicone rubber spacer between two glass plates and held for 24 h at 25 °C. The obtained matrices were subsequently immersed in water for 60 h at 4 °C to remove DMSO, lactic acid and N-hydroxysuccinimide. Then, disc-shaped samples (diameter 10 mm) were punched out from the matrices for further experiments.

2.3. Characterization of Am80-loaded AlCol–TSC matrices

The determination of the residual amino groups in AlCol–TSC was carried out using a spectrophotometric method employing 2,4,6-trinitrobenzene sulphonic acid (TNBS) [27]. Briefly, 1 ml of a 4% sodium hydrogen carbonate solution and 1 ml of 0.1% TNBS were added to AlCol–TSC followed by incubation for 2 h at 37 °C. Subsequently, 3 ml of 6 M hydrochloric acid was added, followed by autoclaving for 1 h at 120 °C to hydrolyse the matrices. The absorbance values of the mixed solutions were measured at 340 nm using a plate reader (GENios-A-5082, Tecan Group Ltd, Männedorf, Switzerland). The swelling ratio of Am80-loaded AlCol–TSC was determined using the following equation:

\[
\text{Swelling ratio} = \frac{W_D - W_W}{W_D},
\]

where \(W_D\) and \(W_W\) are the weights of the matrix swollen with DMSO and water at 4 °C, respectively. The thermal analysis of the mixture of AlCol and Am80 was carried out using a differential scanning calorimeter (DSC-8230, Rigaku, Tokyo, Japan). Ten microgram of various molar ratios of AlCol and Am80 was placed in an aluminium sampling pan and heated from 40 to 60 °C at a rate of 5 °C min\(^{-1}\) in a nitrogen atmosphere.

2.4. Antithrombogenic properties of Am80-loaded AlCol–TSC matrices

The antithrombogenic properties of the matrices were evaluated according to established methods [28, 29]. Each matrix was pre-incubated in 0.1 M phosphate buffered saline (PBS) for 24 h, then placed in a tube and immersed in 1 ml of arterial porcine whole blood for 30 min at 37 °C. After immersion in blood, the matrix was removed and gently rinsed three times with 1 ml of 0.1 M PBS. Then, the matrix was fixed with 10% neutral buffered formalin, and subjected to critical point drying using tert-butyl alcohol. Scanning electron microscopy (SEM) observations were carried out using a JSM-5600 microscope (JEOL, Tokyo, Japan). The observed samples were coated with platinum using an ESC-101 SEM sample coating system (Elionix, Tokyo, Japan).

2.5. Release profile of Am80 from Am80-loaded AlCol–TSC matrices

Am80-loaded AlCol–TSCs were immersed in 200 ml of sterilized 0.1 M PBS (pH 7.4) under sterile conditions. Tubes of the solution were stored at 37 °C, and 20% of the incubated PBS was collected and replaced with fresh PBS each time. The quantification of the released Am80 in the collected PBS was performed using a high-performance liquid chromatography (HPLC) system equipped with a C18 analytical column (4.6 id × 150 mm, 4.4 μm, Cosmosil 5C18-Ar-II, Kyoto, Japan). The flow rate of the mobile phase (65% (v/v) acetonitrile, 1.75% (v/v) acetic acid and 32.25% (v/v) water) was 1 ml min\(^{-1}\) at 40 °C; the flow was delivered by an HPLC pump (PU-2080, Jasco, Tokyo, Japan) equipped with column oven (CO-2060, Jasco). The eluent was monitored with an UV detector (UV-2070, Jasco) at 286 nm.
Figure 1. Percentages of residual amino groups in AlCol–TSC with (□) and without Am80 (●) at different TSC concentrations. Error bars represent standard deviations (n = 3).

2.6. Statistical analysis

The results are expressed as a mean ± standard deviation. Statistical comparisons were made using a two-tailed Student’s t-test on Microsoft Office Excel 2010.

3. Results

3.1. Effect of Am80 addition on crosslinking reaction between AlCol and TSC

The effects of the addition of Am80 on the crosslinking of AlCol with TSC were investigated by determining the amino groups in the resulting matrices with the TNBS method. Figure 1 shows the percentage of residual amino groups in AlCol–TSC prepared with or without Am80. The percentage of residual amino groups in AlCol–TSC decreased with increasing TSC concentration up to 60 mM, saturating at higher values. As can be seen in figure 1, there was no significant difference between AlCol–TSCs with or without Am80. From this result, we conclude that the addition of Am80 does not influence the matrix formation.

3.2. Swelling of matrices

Figure 2 shows the swelling behaviour of AlCol–TSC crosslinked with 20 mM of TSC in the presence of Am80 after immersion in water. Due to the low solubility of Am80 in water at 4 °C (0.157 mg ml⁻¹), we immersed the resulting matrices in cold water for the substitution of DMSO to minimize the dissolution of Am80 in water. As shown in figure 2(a), the transparent matrices immediately become opaque and swell after immersion in cold water, presumably because white crystals derived from Am80 were precipitated by the substitution of DMSO with cold water. As shown in figure 2(b), the precipitation of Am80 was observed inside as well as on the surfaces of the matrices. We investigated the relative weights of AlCol–TSC prepared in the presence of Am80 after immersion in cold water (figure 2(c)) and verified that the swelling ratio of AlCol–TSC increased with a soaking time up to 1 h and decreased at longer times. The matrix shrank after 24 h immersion in water.

3.3. Thermal analysis of Am80-loaded AlCol

Thermal analysis was performed to evaluate the interaction between AlCol and Am80. Figure 3(a) shows typical DSC curves of AlCol (molar ratio of Am80 to AlCol Xa = 0) and Am80-loaded AlCol (Xa = 0.6). AlCol exhibits an endothermic peak centred at approximately 50 °C, associated with the helix-coil transition of AlCol with denaturation. When Xa = 0.6, the thermal stability of AlCol increases, as shown by the shift in the denaturation temperature to higher values (56 °C). The transition temperatures (Tm) obtained from the DSC curves of the matrices with various molar ratios of Am80 are plotted in figure 3(b); Tm values increase with Xa. The increased thermal stability of AlCol is due to the fact that AlCol interacts with Am80.
3.4. Antithrombogenic property of Am80-loaded AlCol–TSC

The effect of the presence of Am80 on the antithrombogenic property was evaluated. Figure 4 shows the morphology and SEM images of AlCol crosslinked with 20 mM TSC with or without Am80 after immersion in arterial porcine whole blood for 30 min. The concentration of Am80 was fixed at 35 mM. As can be seen from figure 4(a), no significant thrombus formation was observed on the surface of AlCol–TSC with or without Am80. Figure 4(b) shows SEM images of the surface of AlCol–TSC after immersion in blood. No platelets or fibrin network formation were observed on the surface of the matrices, with or without Am80.

3.5. Effect of TSC concentrations of matrices on releasing behaviour of Am80

To investigate the effect of TSC concentration on the release behaviour of Am80 from matrices, AlCol was crosslinked at TSC concentrations of 5, 20 and 100 mM in the presence of 35 mM Am80. Figure 5 shows the release profiles of Am80 from matrices prepared at various TSC concentrations. In the case of TSC concentration of 5 and 100 mM, Am80 was sustainably released for approximately 80% within 6 h. When the TSC concentration was 20 mM, approximately 50% of Am80 remained in the matrix.

4. Discussion

A DES consists of three components: a metal, a polymeric matrix and a drug. Although, there is no significant difference in the metal components used in a bare-metal stent (BMS) and DES, it has been reported that the incidence of late stent thrombosis (LST) is higher for DES than BMS [30, 31]. Therefore, we hypothesized that LST was caused by drug and polymeric matrices. In this paper, we report the fundamental properties of AlCol-based matrix as a coating material for an Am80-eluting stent.

Matrices were formed by the reaction between amino groups of AlCol and active ester groups of TSC. To examine reactive efficiency by the addition of Am80, we quantified residual amino groups in a matrix after the preparation reaction. Regardless of the presence of Am80, the density of residual amino groups in AlCol–TSC decreased with an increase in TSC concentration. At same TSC concentrations, there are no significant differences in the amino group in the matrices with or without Am80. This result indicates that the addition of Am80 does not influence the matrix formation.

To remove DMSO, lactic acid and \(N\)-hydroxysuccinimide, the resulting matrices were immersed in cold water. Upon immersion in cold water the matrices immediately turned white, presumably due to the precipitation of Am80 in the matrix. As Am80 was barely detected in the soaking solution, this observation suggests that most of the Am80 was impregnated into the matrix. During the initial period, the matrix swelled remarkably in water because of electrostatic repulsion between the negative charge of matrix and the eluted lactic acid nearby. After DMSO was substituted with water the shrinkage of the matrix was likely caused by the formation of a helix in water.

The drug–matrix interaction is a key factor in controlling drug release from the matrix. Therefore, we analysed the interaction between AlCol and Am80 using DSC. AlCol exhibits an endothermic peak centred at approximately 50 °C, associated with the helix–coil transition of AlCol with denaturation. The thermal stability of AlCol increased with increasing Am80 content, indicating that the stability of AlCol with aging is related to the interaction between AlCol and Am80. These results suggest that Am80 interacts with the hydrophobic amino acids of AlCol via hydrophobic interactions and \(\pi–\pi\) stacking.

The antithrombogenic property of biomaterials has been introduced into hydrophilicity, and negatively charged groups...
on the surface of an Am80-loaded AlCol–TSC matrix, indicating that the addition of Am80 does not inhibit the antithrombogenic property of matrices. Since serum proteins were absorbed in the matrices and were fixed with formalin through the sample preparation process, the surface of the matrix become rough.

SMCs are one of the most important targets of therapies aimed at preventing restenosis. SMCs are proliferated and migrated in the healing process after stent implantation in the short term. It has been reported that Am80 specifically inhibits SMCs [24]. While Am80 inhibited the proliferation and migration of SMC, it had no effect on proliferation of HUVECs after 16 h. We also reported that the surface of this matrix shows excellent endothelialization property after 1 day [19]. Considering that Am80 has been eluting from Am80-loaded matrices for a day, we hypothesized that the re-endothelialization did proceed. We therefore monitored the Am80 release for 24 h in this study. To investigate the effects of the crosslinking density of matrices on the release behaviour of Am80, Am80-loaded matrices were prepared with 5, 20 and 100 mM of TSC. It has been revealed that AlCol–TSC with 20 mM of TSC had the highest crosslinking density compared with matrices prepared at other TSC concentrations [19]. Figure 5 shows the slowest release profile obtained when the TSC concentration was 20 mM, because the Am80 interacted with the AlCol in the matrices. In this matrix, 50% of Am80 remained even after immersion for 24 h. Therefore, if this Am80-loaded matrix-coated metallic stent were implanted into a coronary, it would be exposed to matrix metalloproteinase through the wound-healing process and release residual Am80 from the matrix. Because the organic acid-crosslinked AlCol is enzymatically degradable [34], the

Figure 4. Optical (left) and SEM images (right) of AlCol–TSC with (a) and without (b) Am80 after immersion in arterial porcine whole blood. Black bar: 2 mm, white bars: 50 µm.

Figure 5. Am80 release profiles from AlCol–TSC in PBS (0.1 M, pH 7.4) at 37 °C. The concentration of TSC is 5 mM (●), 20 mM (△), 100 mM (■). Error bars represent standard deviations (n = 3).
Am80 released into the matrix will inhibit SMC growth and inflammation. Am80-loaded AlCol–TSC matrices with highest crosslinking density show excellent Am80 eluting property.

5. Conclusions

We reported the preparation and characterization of Am80-loaded AlCol–TSC. Am80-loaded AlCol–TSC samples with different crosslinking densities were obtained as a result of the reaction between the amino groups of AlCol and the active ester group of TSC in the presence of Am80. DSC results clarified the interaction between AlCol and Am80. Matrices prepared with 20 mM of TSC exhibited sustained release of Am80. Am80-loaded AlCol–TSC matrices may find applications in Am80-eluting stents that prevent in-stent restenosis and thrombosis. Further research on Am80-eluting stents and in vivo experiments using AlCol–TSC are currently in progress.

Acknowledgments

This research was supported by the Japan Society for the Promotion of Science (JSPS) through its Funding Program for World-Leading Innovation R&D on Science and Technology (FIRST Program). This research was also financially supported in part by a Grant-in-Aid from the National Institute of Biomedical Innovation of Japan and the World Premier International Research Center (WPI) Initiative on Materials Nanoarchitectonics, MEXT, Japan.

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