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Enhanced angiogenesis of growth factor-free porous biodegradable adhesive made with hexanoyl group-modified gelatin

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ABSTRACT

The bonding behavior of hexanoyl (Hx: C_6) group-modified alkaline-treated gelatin (HxAlGltn) porous films ((P)HxAlGltn) on the porcine intestine was evaluated. (P)HxAlGltns with various porosities were prepared by the salt-leaching method for various solid–liquid ratios. (P)HxAlGltns bonded more strongly to porcine intestine surfaces than did porous AlGltn films ((P)AlGltns). L929 cells cultured on (P)HxAlGltns showed adhesivity than cells cultured on (P)AlGltns. Faster tissue infiltration and a shorter degradation time of highly porous (P)HxAlGltns were observed after implantation in rat subcutaneous tissues. The angiogenic markers CD34 and α-SMA were highly expressed around (P)HxAlGltns that had high porosity. These results indicated that highly porous (P)HxAlGltns have advantages with respect to not only bonding strength on wet soft tissues, but also angiogenesis.

1. Introduction

Fast and effective wound care requires the promotion of processes involved in the acute inflammatory and growth phases, including angiogenesis and tissue migration. Biomaterials for wound care should demonstrate strong tissue adhesion under wet conditions to close the wound and tissue regeneration properties, including cell adhesion, angiogenic activity, and appropriate porosity.

Biomaterials that are developed for strong adhesion to soft tissues under wet conditions are classified into three types: nanomaterials, biomimetic materials, and naturally derived polymer-based materials. Nanomaterials include nanoparticles [1] and nanosheets [2] and are driven mainly by van der Waals' forces. Biomimetic materials are typically prepared by introducing a catechol moiety [3], which is the major component of the marine mussel adhesion protein, into biocompatible polymers under wet conditions. Gecko feet [4] have been reported to adhere to tissue/organ surfaces with fibrous structures exhibiting strong bonding ability on solid substrates. Naturally derived polymers including gelatin [5], alginate [6], and albumin [7,8] have been employed as basic components of tissue adhesive materials.

Highly porous materials [9] have been proposed for faster tissue regeneration and for their ability to release angiogenic factors [10–14]; however, angiogenic factors such as basic fibroblast growth factor (bFGF) [15–18] and vascular endothelial growth factor (VEGF) [19,20] are expensive cytokines and are not stable in physiological environments [21–24].

Hexanoyl (Hx: C_6) group-modified heparin has a high binding constant with bFGF [25], indicating that Hx-modified heparin can be a reservoir for bFGF. Furthermore, we recently reported that a liquid tissue adhesive containing hydrophobically-modified gelatin (hm-Gltn) shows higher interfacial bonding strength to soft tissues under wet conditions than an adhesive containing non-modified, original Gltn [26–28]. Moreover, film-type adhesives composed of hm-Gltn, especially Hx-Gltn, bond strongly to soft tissue under wet conditions [29,30]. These results suggest that biopolymers combined with the Hx group will show high affinity with angiogenic factors such as bFGF and VEGF.

Hx group-modified alkali-treated Gltn (HxAlGltn) films with porous structures were fabricated using NaCl as a porogen to

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evaluate soft tissue bonding under wet conditions. Furthermore, the angiogenic properties and mechanisms of the resulting films were clarified.

2. Materials and methods

2.1. Synthesis and characterization of HxAlGltns

Following previously reported methods [29,30], Hx group-modified AlGltn (HxAlGltn) was prepared by the reaction between Hx chloride and primary amino groups of AlGltn (Fig. 1A). First, alkaline-treated gelatin (AlGltn: BeMatrix®, Nitta Gelatin Inc., Osaka, Japan) (10 g) was fully dissolved into 99 mL of dehydrated dimethyl sulfoxide (DMSO: Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 80 °C. Then, 1 mL of hexanoyl (Hx: C_6) chloride (Wako Pure Chemical Industries, Ltd.) was added to the AlGltn/DMSO solution to obtain 100 mL of 10 w/v% AlGltn/DMSO solution under a dry N_2 atmosphere at room temperature. One milliliter of triethylamine (TEA: Wako Pure Chemical Industries, Ltd.) was subsequently added to the AlGltn solution and stirred for 17 h at room temperature. The resulting HxAlGltn/DMSO solution was poured into 300 mL of cold ethanol (EtOH: Wako Pure Chemical Industries, Ltd.) and stirred for 1 h. Subsequently, the HxAlGltn precipitate was washed twice with 300 mL of cold EtOH followed by evaporation under a vacuum to leave a white cake, and the yields were calculated.

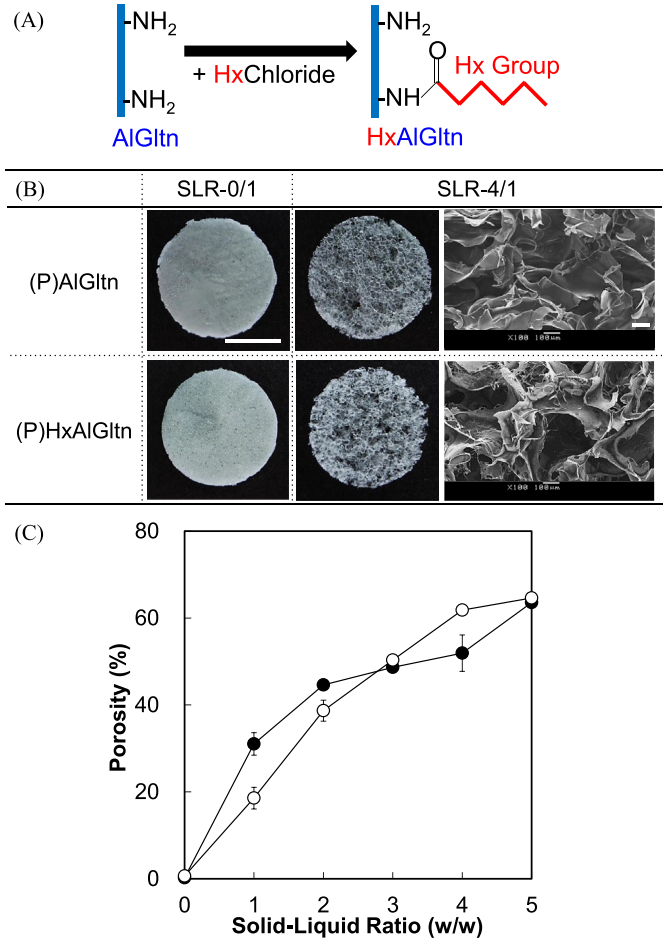

Fig. 1. Preparation of the porous films. (A) HxAlGltn synthesis after the reaction between AlGltn and Hx chloride. (B) Macro/microscopic images of tissue-adhesive porous bodies fabricated without/with NaCl using AlGltn/HxAlGltn. Scale bars: 5 mm in macroscopic and 100 μm in microscopic. (C) Porosity of (P)AlGltns or (P)HxAlGltns with various amounts of NaCl (●(P)AlGltns and ○(P)HxAlGltns). Data represent the means ± SD of three samples (n = 3).

The modification ratio for Hx groups in HxAlGltn was quantified using a previously reported method with 2,4,6-trinitrobenzenesulfonic acid (TNBS: Wako Pure Chemical Industries, Ltd.) [26,27,31–33]. Briefly, HxAlGltns or the original AlGltn was dissolved into DMSO to obtain 0.05 w/v% solutions. Then, 100 μL of 0.1 v/v% TEA/DMSO, 50 μL of 0.1 w/v% sodium dodecyl sulfide (SDS: Wako Pure Chemical Industries, Ltd.)/DMSO, and 100 μL of 0.1 w/v% TNBS/DMSO were added to 100 μL of the HxAlGltn or AlGltn/DMSO solution followed by incubation at 37 °C for 2 h under light-shielding conditions. Then, 50 μL of 2 N hydrochloric acid (HCl: Wako Pure Chemical Industries, Ltd.)/DMSO was added to stop the reaction. Finally, the intensity of light absorbance was measured spectrophotometrically at 340 nm using a microplate reader (GENios A-5082, Tecan Japan, Kanagawa, Japan). The percentage of amino groups substituted with Hx chloride was then calculated based on the relative intensities of HxAlGltn compared with the original AlGltn. Also, modification of Hx group in AlGltn was confirmed using fourier transform infrared spectrophotometry (FTIR) (FTIR-8400S, Shimadzu Co., Ltd., Japan).

2.2. Preparation and characterization of (P)HxAlGltns and (P)AlGltns

The porous AlGltn or HxAlGltn films ((P)AlGltns and (P)HxAlGltns, respectively) were fabricated using the salt-leaching method with NaCl particles as a porogen. To crosslink AlGltn or HxAlGltn, trisuccinimidyl citrate (TSC) was employed [35] (Fig. 1B). HxAlGltn was first dissolved in 10% L-lactic acid (LA: Wako Pure Chemical Industries, Ltd.)-DMSO solvent to prepare 25 w/v% HxAlGltn solutions, and trisuccinimidyl citrate (TSC) synthesized as previously reported [34] was added to 10 mL of the HxAlGltn/LA-DMSO solution to match the amount of *N*-hydroxysuccinimide (NHS) group in TSC and the amount of amino group residues of the HxAlGltn molecule, and the TSC-HxAlGltn/DMSO was stirred quickly. The required amount of sodium chloride (NaCl: Wako Pure Chemical Industries, Ltd.) (for liquid–solid ratios of 1/1, 2/1, 3/1, 4/1, or 5/1 (w/w)) was added to the solution and mixed to disperse the NaCl uniformly. The solutions were packed into zipper bags (Unipack, Seisannipponsha Ltd., Tokyo, Japan) and the compounds were put between glass plates with 1-mm thick silicone spacers. After overnight crosslinking, the obtained NaCl-HxAlGltn gels were immersed in 4 °C ultra-pure water for 3 days with frequent water changes to remove LA-DMSO, NaCl, NHS, and unreacted TSC. The hydrogels were frozen at −80 °C and were lyophilized using VirTis lyophilizer (Advantage, The VirTis Company, Gardiner, NY, USA) for 3 days to obtain the (P)HxAlGltn film. The (P)AlGltns were fabricated following the same method. The image was taken with stereoscopic microscope (Stemi 2000-C, Carl Zeiss, Jena, Germany) with a ×10 objective and a camera (Canon Power Shot A6, Cannon, Tokyo, Japan).

The porosity of each porous film was determined by a water content calculation considering the weight of water absorbed for porous films with cold water filled-pores (W_f) and the weight of water absorbed for porous films with empty pores (W_e). The porosity was calculated using the following formula:

$$\text{Porosity}(\%) = \left(W_f - W_e\right) \Big/ W_f \times 100 \quad (1)$$

By changing solid–liquid ratios (SLR) of NaCl and AlGltn or HxAlGltn/10%LA-DMSO ranged from 0/1 to 5/1, and the porosity of each porous film was regulated with an accuracy of 1%–65% (Fig. 1C).