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Preparation and characterization of cross-linked collagen–phospholipid polymer hybrid gels

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Abstract

2-methacryloyloxyethyl phosphorylcholine (MPC)-immobilized collagen gel was developed. Using 1-ethyl-3-(3-dimethyl aminopropyl)-1-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), we cross-linked a collagen film in 2-morpholinoethane sulfonic acid (MES) buffer (EN gel). EN gel was prepared under both pH 4.5 and pH 9.0 in order to observe changes in cross-linking ability. To cross-link MPC to collagen gel, poly(MPC-*co*-methacrylic acid) (PMA) having a carboxyl group side chain was chosen. E/N gel was added to the MES buffer having pre-NHS activated PMA to make MPC-*i*mmobilized *c*ollagen gel (MiC gel). MiC gel was prepared under both acidic and alkaline conditions to observe the changes in the cross-linking ability of PMA. X-ray photoelectron spectroscopy showed that the PMA was cross-linked with collagen under both acidic and alkaline conditions. Differential scanning calorimetry (DSC) results showed that the shrinkage temperature increased for the MiC gels and that the increase would be greater for the MiC gel prepared under alkaline conditions. The data showed that swelling would be less when the MiC gel was prepared under alkaline conditions. The biodegradation caused by collagenase was suppressed for the MiC gel prepared under alkaline conditions due to stable inter- and intrahelical networks.

Keywords: Collagen; Phospholipid; Cross-linking; Surface modification

1. Introduction

Collagen is an extracellular-matrix protein that plays an important role in the formation of tissues and organs and is involved in various functional expressions of cells [1]. Collagen is non-toxic, non-antigenic, favors cell adhesion, proliferation, and differentiation to mimic the natural cell environment. However, favoring cell adhesion can be both advantageous and disadvantageous, for its strong affinity to cells and blood is uncontrollable, which may soon lead to blood coagulation and mineralization when applied for use as artificial blood vessels. Furthermore, the collagen that is prepared in a matrix form such as a gel for tissue reconstruction is mechanically insufficient [2]. Without modification, the collagen gel cannot be applied for bioprosthesis [3].

To overcome the disadvantages of collagen while maintaining its biological performance, a prosthesis-tissue complex, or bioartificial polymeric material, was developed by blending or mixing biomolecules and synthetic materials. The chief purpose for developing such a bioartificial polymer material is to overcome the poor biological performance of synthetic polymers and to enhance the mechanical characteristics of biomolecules [4].

To control cell adhesiveness and to increase mechanical strength simultaneously, collagen must be modified by cross-linking or mixing with synthetic polymers. Polymers such as poly(vinyl alcohol), poly(acrylic acid), poly(vinyl pyrrolidone), and polyethylene are used as bioartificial polymer materials because of their favorable chemical reactivity with collagen, absence of toxicity, and good mechanical performance [4–8].

However, it is very important to consider biological response in the adoption of a cross-linker or synthetic polymer because of the possibilities of severe problems

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such as toxicity, inflammatory response, or alteration of protein structure. Furthermore, some synthetic polymers that are known to be 'biocompatible' degrade in biological fluids, making the collagen structure unstable. Adoption of natural cross-linkers such as glutaraldehyde [9], genipin [10], or transglutaminase [11], and natural polymers like hyaluronic acid [12], heparin [13], or chondroitin-6-sulfate [14,15] is used as direct cross-linker or immobilizer to overcome the problems presented by the use of synthetic polymers, but cannot fully solve the problems.

To overcome these problems, we developed a biosynthetic hybrid material by cross-linking collagen with a 2-methacryloyloxyethyl phosphorylcholine (MPC) based copolymer using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as cross-linkers by activating the MPC polymer with EDC and NHS to cross-link the microfibrils and polymer chain using amide bond [3,16–18].

MPC is a blood compatible product developed in the early 1990s [19]. Design of the MPC polymer took into account the surface structure of the biomembrane. Recently, phospholipid-accumulated surfaces have been prepared by various methods, and it has been reported that the phosphorylcholine group plays an important role showing excellent blood compatibility and anti-protein adsorptivity [20–23]. The MPC units can then be introduced to conventional polymers by various methods of modification. They effectively reduce protein adsorption and denaturation and inhibit cell adhesion even when the polymer is exposed to whole blood in the absence of any anticoagulants [24]. By adopting the MPC polymer with the collagen gel, it is possible to expect a biocompatible collagen–polymer hybrid gel that is stable, has its molecular weight controlled, has no cross-linker leaking, and is mechanically tough.

In the present study, cross-linking ability between poly(MPC-*co*-methacrylic acid) (PMA) and collagen using EDC and NHS was investigated by altering several parameters, and the physical properties of PMA-immobilized matrices were characterized. In this article, the terms interchain cross-linking and immobilization are used synonymously.

2. Experimental method

2.1. Preparation of collagen–phospholipid polymer hybrid gel

2.1.1. Synthesis of PMA

PMA was synthesized by a method that has already been published [19]. In short, desired amount of MPC and MA was dissolved in ethanol in an ampoule. Then 2,2′-azoisobutyronitrile (AIBN) was added to the ethanol solution. The argon gas was bubbled into the ethanol solution to eliminate the oxygen. The ampoule was sealed and heated to 60 °C for 16 h. The solution was precipitated into diethyl ether, freeze-dried, and kept in vacuum until use. The mole ratio of PMA was controlled to MPC:MA = 3:7, and the number average molecular weight $\overline{M}_n$ of the PMA was approximately 300,000. The chemical structure of PMA is shown in Fig. 1.

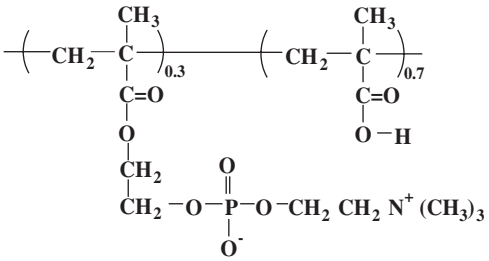

Fig. 1. Chemical structure of PMA.

Table 1
Terminology of collagen gels used in this study

Terminology	Composition
Uc-gel	Uncross-linked collagen gel (immersed in alkaline pH conditions)
EN-1	EDC/NHS-cross-linked collagen gel under acidic pH conditions
EN-2	EDC/NHS-cross-linked collagen gel under alkaline pH conditions
MiC-11 gel	PMA immobilized to EN-1 gel under acid pH conditions
MiC-12 gel	PMA immobilized to EN-1 gel under alkaline pH conditions
MiC-21 gel	PMA immobilized to EN-2 gel under acid pH conditions
MiC-22 gel	PMA immobilized to EN-2 gel under alkaline pH conditions

2.1.2. Preparation of EDC and NHS cross-linked collagen gel (EN gel)

Cross-linked collagen gel was prepared by using 0.5 wt% collagen type I solution (pH 3, KOKEN, Tokyo, Japan). Conventional film fabrication method was used for the film fabrication. The collagen solution was dropped onto the polyethylene film and dried in room temperature. The collagen film (thickness = 36 ± 2 μm) was immersed into a 0.05 M 2-morpholinoethane sulfonic acid (MES) buffer (pH 4.5) (Sigma, St. Louis, USA) containing 1-ethyl-3-(3-dimethyl aminopropyl)-1-carbodiimide hydrochloride (EDC) (Kanto Chemicals, Tokyo, Japan) and NHS (Kanto Chemicals, Tokyo, Japan). Each chemical was added at the mole ratio of EDC:NHS:collagen-carboxylic acid groups = 5:5:1 [11,13]. The cross-linking procedure was allowed to continue for 4 h at 4 °C to produce a cross-linked gel (EN-1 gel). After 4 h, the reaction was stopped by removing the gel from the solution. The gel was then washed with 4 M of Na_2HPO_4 aqueous solution for 2 h to hydrolyze any remaining *O*-acylisourea groups and then with distilled water for 3 days to remove any salts from the gel. Same preparation process was repeated under alkaline conditions (pH 9.0; adjusted with NaOH) to prepare an EN-2 gel.

2.1.3. Preparation of MPC-immobilized collagen gel (MiC gel)

Preparation of the MiC gel was done by using the EN-1 and EN-2 gels. PMA was added with EDC and NHS to the MES buffer (pH 4.5 and pH 9.0) and was pre-activated for 10 min before immersion of the EN-1 or EN-2 gel. The immobilization of PMA to the collagen was allowed to continue for 4 h at 4 °C. The gel was then washed with 4 M of Na_2HPO_4 aqueous solution for 2 h and then with distilled water for 1 day to remove any salts from the gel to prepare a salt-free MiC gel: MiC-11 gel (PMA immobilized under acidic conditions using the EN-1 gel), MiC-12 gel (PMA immobilized under alkaline conditions using the EN-1 gel), MiC-21 gel (PMA immobilized under acidic conditions using the EN-2 gel), and MiC-22 gel (PMA immobilized under alkaline conditions using the EN-2 gel). The terminology of the samples is listed in Table 1. PMA cross-linking with the collagen is shown in Fig. 2. Collagen film was immersed