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Preparation of a collagen/polymer hybrid gel designed for tissue membranes. Part I: Controlling the polymer–collagen cross-linking process using an ethanol/water co-solvent

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ABSTRACT

The drawback with collagen/2-methacryloyloxyethyl phosphorylcholine (MPC) polymer hybrid gels (collagen/phospholipid polymer hybrid gels) prepared in alkaline morpholinoethane sulfonic acid (MES) aqueous solution is that the cross-linking rate between the polymer and the collagen is low. To solve this problem, ethanol has been adopted as the reaction solvent, to prevent 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) hydrolysis. Alterations in the ethanol mole concentration changed the cross-linking rate between the MPC polymer and the collagen gel. Prevention of EDC hydrolysis is clearly observed; protonation of carboxyl groups implies that the ratio of ethanol to water should be controlled. The polymer shows signs of penetration into the collagen gel layer, thus forming a totally homogeneous phase gel. This affects the mechanical strength of the collagen gel, making the gel much stiffer and brittle with an increase in the swelling ratio, as compared with that prepared in MES buffer. However, it is possible to obtain a collagen/phospholipid polymer hybrid gel with a high polymer portion and the cross-linking rate can be successfully controlled.

1. Introduction

Collagen is the major constituent of connective tissues and is widely used in biomaterial applications [1–4]. The use of collagen as a biomaterial offers advantages such as biocompatibility, low toxicity and natural abundance, in addition to well-documented structural, physical, chemical and immunological properties [5]. However, purified collagen possesses weak mechanical properties, which are inadequate for application as a biomaterial. The formation of covalent intermolecular cross-links between collagen molecules in macromolecular fibrils with appropriate biocompatible molecules is an effective method of improving mechanical integrity and stability [6–9].

Covalent cross-linking using 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) is a widely used method. The coupling reaction produces "zero length" amide cross-links between carboxylic acid groups and amine groups; this reduces side-effects that may be induced by a cross-linking agent [10]. In addition, adoption of an additional polymer that contains a carboxyl group enables the cross-linking of collagen and the polymer using EDC and NHS to reinforce the mechanical strength of the collagen gel.

By coupling to anti-coagulant substances, such as heparin, it is possible to provide collagen with properties such as hemocompatibility, which collagen alone does not possess [11]. However, the conditions under which the coupling reaction cross-links the collagen and the polymer to control a particular property remain unclear. We adopted 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer, which has good hemocompatibility. We succeeded in preparing an MPC polymer immobilized collagen gel using [poly(MPC-co-methacrylic acid)] (PMA) and EDC/NHS in 0.05 M 2-morpholinoethane sulfonic acid (MES) buffer (pH 9.0), as explained in our previous report [12]. We expected the immobilized MPC polymer to express good hemocompatibility and non-cell adhesive properties, but the immobilized fraction of MPC head groups was low, implying that cross-linking did not occur efficiently and gel could not be applied as a tissue membrane.

To solve the above mentioned problem we adopted ethanol as the reaction solvent, because hydrolysis of EDC in water occurs over a very short timespan [13,14] and it has been predicted that ethanol may prevent this hydrolysis. The use of ethanol has been reported by many researchers, but its reactivity has not yet been focused upon [15–17]. In a previous study, we adopted an ethanol/water co-solvent to prepare a collagen gel using EDC and NHS. We found that the ethanol/water co-solvent did not affect the collagen triple helix

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until the ethanol mole concentration (N_A) reached ~0.42 (70 vol.% ethanol) [18]. The reactivity of EDC with the carboxylate anion groups of collagen helices could be enhanced by the ethanol/water co-solvent, but the concentration ranged from $N_A \sim 0.07$–0.17 (~10–40 vol.% ethanol) because a balance between hydrolysis of EDC and protonation of carboxylate anions had to be maintained.

To apply this method to polymer–collagen cross-linking we first characterized the physical behavior of the MPC polymers and poly(methacrylate) in the ethanol/water co-solvent in order to investigate the reactivities of EDC and NHS with the polymers. For MPC polymers we selected two types of PMA: PMA30 [30 mol.% MPC, 70 mol.% methacrylic acid (MA)] and PMA90 (90 mol.% MPC, 10 mol.% MA). Subsequently we cross-linked the polymer with collagen to characterize network formation by the collagen gel in the ethanol/water co-solvent. In this manner we attempted to establish a theory of the polymer–collagen gel cross-linking system for preparation of a tissue membrane based on collagen. The second phase of the study, which involved characterization of the biological properties, will be reported in part II.

2. Materials and methods

2.1. Preparation of the collagen/phospholipid polymer hybrid gels (MiC gels)

PMA was synthesized according to a previously published method [12,19]. In brief, MPC and MA were co-polymerized in ethanol solution for 16 h at 60 °C using 2,2-azobisisobutyronitrile as initiator. The molar ratios of PMA were MPC:MA 3:7 (PMA30) and 9:1 (PMA90) and the average molecular weights were 3.2×10^5 and 4.5×10^5.

Cross-linked collagen gel was prepared using a previously reported method [12,18,19]. Instead of 0.5 wt.% collagen type I solution (pH 3) (KOKEN, Tokyo, Japan), 2 wt.% collagen type I aqueous solution was prepared and used for film preparation (thickness 50 ± 3 μm). The collagen/phospholipid polymer hybrid gel (MiC gel) was prepared using collagen film. PMA30 and PMA90 were added to the ethanol/water co-solvent series (ethanol mole concentration N_A = ~0–0.32), along with EDC and NHS. The polymer was activated for 10 min before the collagen film was immersed in the solvent. The molar ratios of each chemical were fixed – EDC:NHS:collagen carboxylic acid groups 10:10:1. Immobilization of PMA to collagen was allowed to continue for 24 h at 4 °C to form a MiC30 (cross-linked with PMA30) gel and MiC90 (cross-linked with PMA90) gel. To evaluate the physical properties, a collagen film stabilized in MES buffer, pH 9.0, was prepared (Uc gel). The basic preparation scheme and an image of the MiC30 gel are shown in Fig. 1. The abbreviations for the collagen gels used in this study are listed in Table 1.

2.2. Characterization of the polymers

The molecular sizes of PMA00, PMA30 and PM90 were measured using a dynamic scattering method using a Zetasizer light scattering system (Malvern Instruments Ltd., Malvern, UK) equipped with a He–Ne laser (λ = 633 nm, 4.0 mW) at 25 °C. The samples were prepared in an ethanol/water co-solvent series with a concentration of 1 mg ml^{-1}. The viscosity and transparency of the ethanol/water co-solvent series were calculated for precise measurement of molecule size. The ζ potential of the polymers measured using the same instrument was executed after mathematical calculation of the permittivity of the co-solvents [20]. All samples were filtered through a 0.45 μm Millex filter (Millipore, Bedford, MA) before measurement.

2.3. Characterization of the MiC gels

2.3.1. Surface analysis

Surface analysis was performed using X-ray photoelectron spectroscopy (XPS) (AXIS-HSi, Shimadzu/KRATOS, Kyoto, Japan). Samples that had been cut into small pieces (2 × 2 cm) were lyophilized overnight (FDU-2000, EYELA, Tokyo, Japan). The chemical composition of the gel surface was determined from the release angle of photoelectrons fixed at 30°.

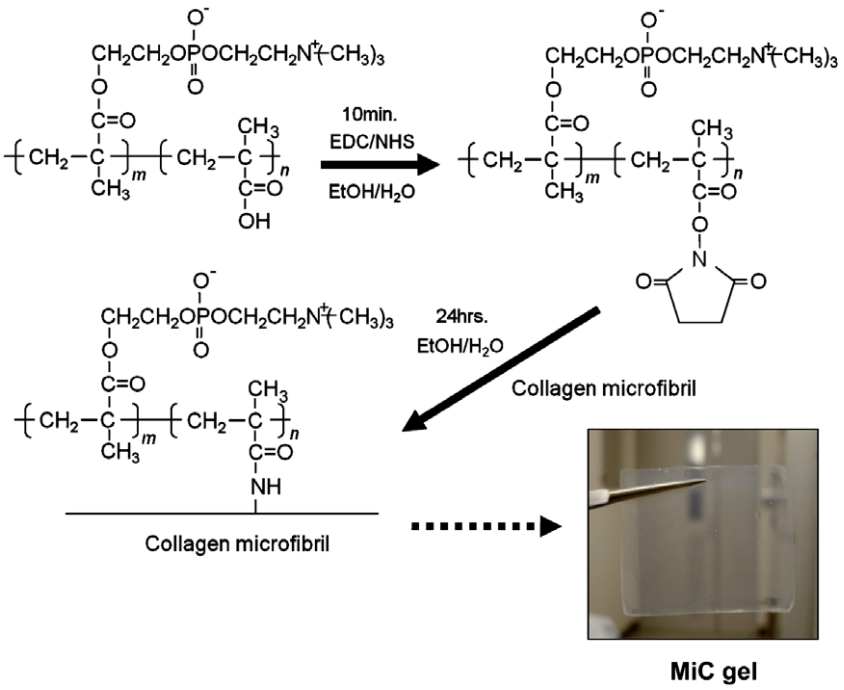

Fig. 1. Basic preparation scheme and image of the MiC gel.

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