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Carbodiimide cross-linking of amniotic membranes in the presence of amino acid bridges

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ABSTRACT

The purpose of this study was to investigate the carbodiimide cross-linking of amniotic membrane (AM) in the presence of amino acid bridges. The biological tissues were treated with glycine, lysine, or glutamic acid and chemically cross-linked to examine the role of amino acid types in collagenous biomaterial processing. Results of zeta potential measurements showed that the use of uncharged, positively and negatively charged amino acids dictates the charge state of membrane surface. Tensile strength and water content measurements demonstrated that the addition of lysine molecules to the cross-linking system can increase the cross-linking efficiency and dehydration degree while the introduction of glutamic acid in the AM samples decreases the number of cross-links per unit mass of chemically modified tissue collagen. The differences in the cross-linking density further determined the thermal and biological stability by differential scanning calorimetry and in vitro degradation tests. As demonstrated in matrix permeability studies, the improved formation of covalent cross-linkages imposed by lysine facilitated construction of stronger cross-linking structures. In contrast, the added glycine molecules were insufficient to enhance the resistances of the proteinaceous matrices to thermal denaturation and enzymatic degradation. The cytocompatibility of these biological tissue membranes was evaluated by using human corneal epithelial cell cultures. Results of cell viability, metabolic activity, and pro-inflammatory gene expression level showed that the AM materials cross-linked with carbodiimide in the presence of different types of amino acids are well tolerated without evidence of detrimental effect on cell growth. In addition, the amino acid treated and carbodiimide cross-linked AM implants had good biocompatibility in the anterior chamber of the rabbit eye model. Our findings suggest that amino acid type is a very important engineering parameter to mediate carbodiimide cross-linking of AM collagen.

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1. Introduction

Clinically, amniotic membrane (AM) transplantation is carried out in order to promote corneal epithelialization and prevent corneal melting. However, with the increasing severity of limbal stem cell deficiency, the transplantation of limbal epithelial cells cultivated on AM carriers has been devoted to the development of an effective treatment modality [1]. To overcome the poor stability of AM collagen, the biological tissue matrices are treated by chemical cross-linking. Over the past decades, investigators have attempted to modify the AM grafts with glutaraldehyde (i.e., a non zero-length cross-linker) because of its high efficiency of collagenous biomaterial stabilization [2,3]. More recently, our group has also used this type of cross-linking agent for studying molecular stability related to cross-link formation [4,5]. Although glutaraldehyde cross-linking is beneficial to enhance the resistance to enzymatic degradation, the remaining aldehyde groups in the proteinaceous matrices may contribute to cytotoxicity and induce apoptosis [6].

Biomaterial modification with carbodiimide is considered as an alternative in achieving good biocompatibility for many ophthalmic applications. Corneal endothelial cell sheet engineering using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) cross-linked porous gelatin hydrogel carriers may offer a new minimally invasive surgery for patients with pseudophakic bullous keratopathy and Fuch's endothelial dystrophy [7]. A nanoscale modification strategy based on carbodiimide chemistry can fabricate gelatin/chondroitin sulfate scaffolds for controlled three-dimensional corneal stromal cell culture [8]. In addition, during EDC cross-linking of gelatin molecules, the solvent composition is found to greatly affect the performance of delivery carriers for retinal sheet transplantation [9]. To effectively administer the antiglaucoma medications, the gelatin and poly(*N*-isopropylacrylamide) graft copolymers synthesized by means of EDC/*N*-hydroxysuccinimide (NHS) are proposed as biodegradable in situ forming delivery systems [10]. Although the by-products of the carbodiimide reaction are in the form of water-soluble urea derivatives,

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the treatment of AM collagen with EDC/NHS to catalyze the cross-link formation exhibits only limited cross-linking [4]. A promising way to improve this problem is the use of additional molecular bridges to increase carbodiimide cross-linking efficiency while maintaining biocompatibility of chemically modified collagenous tissues.

Amino acids are natural substances in the body and serve as the building blocks of protein molecules. Usha et al. have reported that the stabilization of collagen with EDC/NHS in the presence of lysine and found that the addition of foreign amino acid residues to the cross-linking system may have a profound influence on the self assembly process of collagen fibrils [11]. A study from Hu et al. has indicated that the hyaluronic acid strands cross-linked by glutaraldehyde and resurfaced with amino acids show good biocompatibility toward fibroblasts [12]. To eliminate the disadvantage of rapid degradation of porous collagen scaffolds, Ma et al. have also proposed a strategy for biomaterial modification with carbodiimide using different types of amino acids as cross-linking bridges between collagen molecular chains [13]. These encouraging findings have led us to develop the stable and biocompatible AM by the treatment of amino acid bridges and EDC/NHS cross-linking.

For the first time, here, we studied the carbodiimide cross-linking of AM in the presence of amino acids such as glycine, lysine, and glutamic acid. The zeta potential and cross-linking density measurements were performed to gain insights in the role of amino acid type on the surface charge and cross-linking structure of biological tissue membranes. The efficiency of collagenous biomaterial stabilization was evaluated by tensile strength measurements, water content analyses, differential scanning calorimetry studies, and in vitro degradation tests. The nutrient transport analysis of limbal epithelial cell carrier was used to assess the correlation of matrix permeability of AM with its extent of cross-link formation. After exposure of human corneal epithelial cells to these AM materials, the cytocompatibility was investigated by determinations of cell viability, metabolic activity, and pro-inflammatory gene expression level. The in vivo biocompatibility of amino acid treated and carbodiimide cross-linked AM implants was analyzed using the anterior chamber of a rabbit eye model. The biological tissue membranes were inserted in the ocular anterior chamber and were evaluated by clinical observations. The obtained information from specular microscopy and corneal thickness measurements can be used to estimate the tissue responses following implantation. To the best of our knowledge, the biocompatibility of carbodiimide cross-linked AM materials in the anterior chamber of the eye has yet to be examined.

2. Materials and methods

2.1. Materials

This study followed the tenets of the Declaration of Helsinki involving human subjects and received approval from the Institutional Review Board of our institution. Human AM tissues (i.e., the innermost layer of the placental membranes) were obtained with informed consent at the time of elective cesarean section from mothers when human immunodeficiency virus, syphilis, and hepatitis B and C had been excluded by serologic tests. The separation of AM was carried out using blunt dissection to cut the tissue samples approximately 2 cm from the placental disc. The average thickness of AM samples used in this work was 100 μm. 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), glycine, lysine, glutamic acid, collagenase (type I *Clostridium histolyticum*, EC 3.4.24.3), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-hydroxysuccinimide (NHS) was supplied by Acros Organics (Geel, Belgium). Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Deionized water used was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Biochrom (Berlin, Germany). Balanced salt solution (BSS, pH 7.4) was obtained from Alcon Laboratories (Fort Worth, TX, USA). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical (Rockford, IL, USA). FNC Coating Mix (i.e., a fibronectin/collagen mixture) was obtained from Athena ES (Baltimore, MD, USA). Dulbecco's modified Eagle's medium (DMEM), keratinocyte serum-free medium (KSFM), gentamicin, trypsin-ethylenediaminetetraacetic acid (EDTA), and TRIzol reagent were purchased from Gibco-BRL (Grand Island, NY, USA). The antibiotic/antimycotic (A/A) solution (10,000 U/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). 24-well tissue culture polystyrene (TCPS) plates (Falcon 353047) were purchased from Becton Dickinson Labware (Franklin Lakes, NJ, USA). All the other chemicals were of reagent grade and used as received without further purification.

2.2. Preparation of chemically cross-linked amniotic membranes

The AM samples were aseptically washed three times with PBS containing 1% A/A solution and 50 μg/ml of gentamicin according to the protocols reported previously [4]. The membranes were immersed with sequential concentrations of DMSO, followed by freezing and storing at −80 °C in DMEM containing 50% glycerol. After a further incubation with 0.02% EDTA at 37 °C, the AM was denuded of its amniotic epithelial cells by gentle scraping. Prior to chemical cross-linking, the AM materials were immersed in 30 ml of MES buffer containing 5 mM of different types of amino acids for 6 h. Then, the EDC and NHS were added to this buffer solution to catalyze the formation of zero-length covalent cross-links between protein molecules. The cross-linker concentration was fixed at 0.05 mmol EDC/mg AM. Additionally, a 5:1 EDC to NHS molar ratio was used for all cross-linking. The cross-linking reaction was allowed to proceed at 25 °C for 3 h. The resulting membrane samples were thoroughly washed with deionized water to remove excess EDC and urea by-product. In this study, the chemically modified biological tissue materials prepared in the presence of glycine, lysine, and glutamic acid were respectively designated as Gly-E/A, Lys-E/A, and Glu-E/A. Cross-linking of AM with EDC/NHS in the absence of amino acid bridges was conducted simultaneously for comparison (E/A groups).

2.3. Zeta potential measurements

To determine the charge of the membrane surface, the zeta potential of various test samples was quantified with the streaming potential method [14]. An electrokinetic analyzer (EKA) (BI-EKA; Anton Paar, Austria) located at the Center for Emerging Material and Advanced Devices at National Taiwan University (Taipei, Taiwan, ROC) was utilized to measure the streaming current. Prior to testing, the AM samples were equilibrated in 1 mM KCl for 2 h. Zeta potential measurements were performed using 1 mM KCl as electrolyte solution. The pH of the solution was adjusted to 7.4 by the addition of 0.1 M KOH. Results were averaged on five independent runs.

2.4. Cross-linking density measurements

The cross-linked structure of the chemically modified AM such as degree of cross-link and average molecular weight of polymer chains between two consecutive junctions was analyzed according to the method reported previously [15]. After immersion in deionized water for 12 h at 25 °C, the membrane samples (20 mm × 10 mm) were mounted between two clamps of an Instron Mini 44 universal testing machine (Canton, MA, USA). The lower clamp was then adjusted downward until the sample was just in tension and the unstressed length was noted. Maximum load was recorded as an indicator of the tensile strength of the various AM samples. Following determination of mechanical properties, the test specimens were removed from the clamps and blotted with tissue paper, and the density was determined by the specific gravity bottle method. A graph of σ against (α-α^{-2}) would be

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