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Carbodiimide crosslinked collagen from porcine dermal matrix for high-strength tissue engineering scaffold

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ABSTRACT

Naturally-derived collagens for tissue engineering are limited by low mechanical strength and rapid degradation. In this study, carbodiimide is used to chemically modify the collagen derived from porcine acellular dermal matrix (PADM). The results show that the strength and resistance of PADM to enzymatic digestion can be adjusted by the reconnection of free amino and carboxyl groups of the collagen fibers. The cytocompatibility of the crosslinked PADM was evaluated by cell adhesion and proliferation assays. The cell culture studies on crosslinked and uncrosslinked PADM showed that the modification does not affect the scaffold's biocompatibility. These results demonstrate that the PADM collagen materials can be strengthened through a low-cost, non-toxic crosslinking method for potential use in biomedical applications.

1. Introduction

As the most promising strategy for regeneration medicine, tissue engineering has attracted a great deal of attention since it first appeared in the 1980s. The scaffold is one of the three most important elements (scaffold, stem cell, and growth factor) of tissue engineering, providing a matrix for cell attachment, proliferation, and differentiation during the construction of active biomaterials *in vitro*, or during cell aggregation to form cell groups and new tissues *in vivo* [1]. Therefore, the design and preparation of 3-dimensional porous tissue engineering scaffolds with biocompatibility and bioactivity, as well as controllable biodegradability have constituted the most important tasks for tissue engineering research [2]. Inspired by nature, researchers have posited that imitation of the composition and structure of the extracellular matrix (ECM) is the best way to obtain a high-performance tissue engineering scaffold, due to the fact that ECM is the natural foundation of tissue building cells [3]. Although some artificial biomimetic ECMs have demonstrated good performance for *in vitro* cell construction and *in vivo* tissue regeneration, some of their functionality is still not as good as natural ECM [4,5].

Over the past 10 years, extracting ECMs from animal tissues by a decellularization process has become a general methodology for obtaining high-performance tissue engineering scaffolds because the decellularized matrix possesses natural polymers biocompatible with cell and tissue growth, as well as native porous structures suitable for cell infiltration and tissue regeneration [6,7]. The most popular and widely applied natural ECM product is the acellular dermal matrix (ADM), which is extracted from human or animal skin (especially of porcine origin) [8].

Recently, achievements in organ regeneration by using whole organ decellularization point to a very bright future for tissue and whole organ regeneration [9,10]. An acellular matrix can be produced by removing the cellular components from tissue or whole organs. These decellularization techniques include chemical, enzymatic and mechanical means of removing cellular components, leaving a material composed essentially of extracellular matrix components [11]. During the decellularization process, the chemical, biological and physical agents affect the interface between the cells and the ECM, and often cause the cleavage of the collagen fibrils, disruption of the collagen crosslinks, and breakdown of the ultrastructure in the ECM, all of which lead to

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poorer mechanical properties and an accelerated biodegradation speed of the decellularized matrix [12–14]. In fact, the primary strength and biodegradation rate of the tissue regeneration process are both very important factors for characterizing the tissue engineering scaffold. Therefore, mechanical reinforcement and tuning of the degradation properties of the decellularized matrix have become an important task for tissue regeneration research. Although damage to the protein structure of the decellularized matrix has been noticed for many years [11,13], there is little published work on the re-crosslinking of cleaved protein fibers or disrupted collagen crosslinks in a decellularized collagen matrix.

Generally, crosslinking methods for collagen-based biomaterials can be divided into three groups: physical methods (*e.g.*, applying dehydrothermal treatment or UV light) [15–17], chemical methods (*e.g.*, using chemical agents like glutaraldehydes, carbodiimide or genipin) [18–20], and biological methods (*e.g.*, using transglutaminase as the crosslinking agent) [21,22]. Among the chemical crosslinking methods for artificial and natural macromolecules, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) (EDC/NHS crosslinking) and glutaraldehydes are the most commonly used chemical crosslinking agents. Glutaraldehydes are efficient in crosslinking proteins and other macromolecules, but the release of free glutaraldehyde molecules from the scaffold during the biodegradation process *in vivo* would be harmful to the tissue and the health of the patient. Because free glutaraldehydes can induce DNA–protein crosslinking [23]. Compared with glutaraldehydes, EDC/NHS crosslinking does not attach harmful reagents to the scaffold and EDC is a zero-length crosslinker, which does not form crosslink bonds [24]. In addition, excess EDC/NHS reagent and urea by-product are water soluble and can be easily removed by washing from the crosslinked scaffolds with dilute acid or water [25].

The aim of this study is to use the EDC/NHS crosslinking process to reinforce the decellularized tissue or organ matrix. Porcine acellular dermal matrix (PADM), a very popular collagen-based scaffold material for tissue regeneration, was taken as a sample material in order to evaluate the efficacy of reinforcement and the degree of degradation tuning. Cytocompatibility of the crosslinked PADM was also assessed.

2. Materials and methods

2.1. Preparation of PADM

PADM was prepared as described in our previous report [26]. Briefly, fresh full thickness porcine skin obtained from a local market was kept at −20 °C after removal of hair and subdermal fat tissue. Thawed skin was incubated on a vortex shaker at 150 rpm at 37 °C and decellularized by using the following solutions: 2.0 g/L NaOH with 0.8 g/L sodium dodecyl sulfate (SDS), 12 h; deionized water, 15 min, 2 times; 2.4 g/L SDS, 2 h; deionized water, 15 min, 2 times; 3.0 g/L pancreatin, 12 h; deionized water, 15 min, 2 times; 2.4 g/L SDS, 2 h; deionized water, 15 min, 2 times; 6% NaCl, 6 h; and finally deionized water, 20 min, 3 times. PADM product with a thickness of about 2 mm was finally obtained after lyophilization at −60 °C for 4 h. To facilitate experimental use, PADM samples were punched into disks of 8 mm diameter.

2.2. Crosslinking procedures

Freeze-dried PADM disks were rehydrated and incubated in different concentrations of EDC (Alfa Aesar, TianJin) solution (0, 0.5, 1, 5, 10, 20, 30 and 50 mM) for 6 h at room temperature. The disk samples were immersed in MilliQ water (1 mL/disk) containing EDC and NHS (Alfa Aesar, TianJin) with a molar ratio of 2.5:1 (EDC:NHS). Following crosslinking, the samples were rinsed twice for 2 h with a 0.1 M Na_2HPO_4 solution and four times for 4 h with MilliQ water to neutralize and remove any residual EDC or NHS. The 0 mM group was chosen as the control group.

2.3. Determination of extent of crosslinking

The extent of crosslinking was determined by measuring the number of free amino groups in the crosslinked and uncrosslinked PADM samples with the use of a ninhydrin assay [27]. Briefly, the freeze-dried samples were weighed and then rehydrated before being heated with a ninhydrin solution (170 mg ninhydrin and 30 mg hydrindantin dissolved in 20 mL ethylene glycol monomethyl ether) for 15 min in a boiling water bath. Then, the test solution was cooled by running water and diluted in 60% ethanol. The absorbance at 570 nm was determined using a microplate reader (MULTISKAN MK3, Thermo, USA). The number of free amino groups in the PADM before ($A1$, $n = 3$) and after crosslinking ($A2$, $n = 3$) is obtained using a calibration curve obtained by measuring the absorbance of glycine solutions at various concentrations. The extent of crosslinking was then calculated by using the formula $(A1 - A2)/A1 \times 100\%$.

2.4. Characterization of crosslinked and uncrosslinked PADM

The morphology of the scaffolds was imaged using a scanning electron microscope (SEM; Hitachi, S-4800) at an accelerating voltage of 10 kV. To identify amide formation, the FT-IR spectrum was recorded for crosslinked and uncrosslinked PADM scaffolds using an IR spectrophotometer (Thermo Nicolet, NEXUS 670). The spectrum was obtained with 64 scans per sample ranging from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}.

To determine the effect of crosslinking on the mechanical properties of the PADM framework, compressive testing was carried out using an automatic mechanical testing machine (Zhixing Co., China) fitted with a 10-N load cell. Samples of 8 mm diameter punched from the PADM sheets were tested separately under dry and wet conditions (prehydrated in phosphate buffered saline for 2 h prior to testing). All testing was performed at a strain rate of 10%/min. The stress–strain curve was then determined and the compression modulus was defined as the slope of a linear fit to the curve over the 2–5% strain range.

2.5. In vitro biodegradation studies

The stability to enzymatic degradation of each tested sample was evaluated by exposing the scaffolds to collagenase (from Clostridium histolyticum, Sigma). Freeze-dried scaffolds were weighed, rehydrated and then incubated in a 12.5 U mL^{-1} collagenase solution buffered with Tris–HCl buffer solution (50 mM, pH = 7.4) containing 10 mM $CaCl_2$ at 37 °C in a water bath. At 6, 12, 24, 48, 72, and 96 h, the digested suspension was centrifuged at 10,000 rpm. The supernatants were discarded and the deposits were oven dried at 37 °C for 12 h. All dried digested samples were weighed again. The biostability of EDC crosslinked and uncrosslinked PADM was characterized by calculating the percentage of mass remaining after enzyme digestion.

2.6. Cell culture

MC3T3-E1 mouse pre-osteoblast cells (Cell Bank of Chinese Academy of Sciences) were used in this study. All scaffolds (8 mm in diameter) were sterilized by immersing in 75% ethanol for 4 h, washing three times in sterile PBS (pH = 7.4), and presoaking in α-minimal essential medium (αMEM, Gibco) in 48-well-plate overnight before cell seeding. The presoaking process was aimed