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Gelatin-based extracellular matrix cryogels for cartilage tissue engineering

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ABSTRACT

In this study, gelatin-based cryogels were fabricated by mixing methacrylated gelatin (GelMA) with methacrylated hyaluronic acid (MeHA) or methacrylated chondroitin sulfate (MeCS) for cartilage tissue engineering. *In vitro* revealed that MeCS incorporated gelatin-based cryogel (G-MeCS) showed significant cartilaginous tissue stimulation. Furthermore, the cell-laden gelatin-based ECM cryogels were implanted into mouse subcutaneous tissue for 6 weeks and displayed uniform distribution of cells with normal phenotype maintenance. Finally, when these cryogels were implanted into osteochondral defect of New Zealand white rabbit, full integration with host tissue and increased cellularity were observed with G-MeCS cryogel.

Introduction

Articular cartilage is a highly organized and avascular tissue with limited self-repair capacity [1]. Therefore, the management of articular cartilage defects is one of the major challenging clinical problems for orthopedics surgeons [2,3]. Three-dimensional scaffolds constitute an important component of cartilage tissue engineering. Recently, biomaterials-based strategies that include zonal chondrocyte-based hydrogels or multi-layered cartilage transplantation techniques have been investigated [4,5]. Likewise, cryogel-based 3 dimensional (3D) macroporous scaffolds have been recently applied in cartilage tissue engineering [6,7]. Cryogel-based scaffolds may act as a substrate to promote repair of articular cartilage. Furthermore, cryogels have been used as cell delivering carrier due to its macroporous structure, allowing the effective mass transport of macromolecular solutes, the migration of cells by providing sufficient surface area, biocompatibility, and good mechanical properties owing to its strong association with water [7,8].

Up-to-date, various materials have been widely researched to make cryogels for cartilage regeneration purposes [9,10]. These include chitosan [11], alginate [10], type I collagen [12], poly (ethylene glycol) diacrylate (PEGDA) [7], gelatin [13,14], agarose [10], hyaluronic acids [15], and with the majority of these strategies involving optimizing crosslinking and freezing time to create optimal pore size with durable mechanical properties for physiological environment adaptation. Similarly, our group has reported the PEGDA-based cryogel with extracellular matrix components for cartilage tissue engineering [6]. In this study, we have utilized PEGDA as a base macromer and added methacrylated hyaluronic acid (MeHA) or methacrylated chondroitin sulfate (MeCS) [6]. Also, we have reported that PEG-based cryogels with MeCS can support the growth and proliferation of primary bovine chondrocytes together with the synthesis of a large amount of cartilage-specific ECMs. In addition, in a separate study, we have also demonstrated that the incorporation of integrin binding moieties and chondroitin sulfate into scaffold design can support chondrocyte phenotype maintenance [16]. Therefore, scaffold design that combines the integrin binding site along with cartilage-specific ECM scheme would mimic natural cellular microenvironment.

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Gelatin is derived from a partial breakdown of a natural triple-helical structure of collagen, and it maintains bioactive and cell binding moieties of collagen [17]. As gelatin contains cell attachable moieties such as Arg-Gly-Asp (RGD) sequence [18], it plays a crucial role in tissue engineering as a scaffold and has been widely used in several applications [19,20]. The gelatin, however, has insufficient mechanical properties to be used as a scaffold [21]. Therefore several methodologies employ a chemical modification of gelatin have been developed to remediate the mechanical problems [22–24]. These chemical modifications include the isocyanate incorporation [25], furfurylamine incorporation [26], or methacrylation to enhance the stiffness [27]. In particular, the methacrylation of gelatin for radical initiated photocrosslinking has been widely used as cost effective and simple crosslinking mechanisms.

In this study, we fabricated gelatin-based cryogel with ECM components for cartilage tissue engineering. Methacrylated gelatin (GelMA) incorporated with MeHA or MeCS was fabricated to mimic native cartilage ECM for phenotypic stability. Gelatin-based ECM cryogels were evaluated with mechanical behavior, interior structure, cell viability, and response of chondrogenic effects *in vitro* and *in vivo*. Furthermore, we evaluated the efficacy of these gelatin-based cryogels for the regeneration of subchondral cartilage defects *in vivo* using the rabbits as an experimental model. Sterile gelatin-based ECM cryogel scaffolds were implanted at the defect site developed surgically in the condyle of rabbit and repair was monitored over a period.

Materials and methods

Chondrocyte isolation and culture

Rabbit chondrocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin/streptomycin (Gibco-BRL), 1% HEPES (Gibco-BRL), 1% MEM Non-Essential Amino Acid (MEM NEAA; Gibco-BRL), 0.2% L-proline (Sigma–Aldrich, Saint Louis, MO) and 0.2% L-ascorbic acid (Sigma–Aldrich). The knees from New Zealand white rabbits (Koatech Laboratory Animal Company, Korea) were obtained and the joints were dissected and chopped tissues were digested with 0.2% (w/v) collagenase type II (Worthington, Lakewood, NJ) in DMEM for 4 h on a rotating mixer at 37 °C in a humidified 5% (v/v) CO_2 incubator. Cells were cultured at tissue culture plate at 5×10^5 cells/ml at 37 °C in a humidified 5% (v/v) CO_2 incubator. The cells on the scaffolds were then cultured for selected days and medium was changed every 2 days. Passage 1 (P1) chondrocytes were used for this study.

Synthesis of methacrylated gelatin, hyaluronic acid, and chondroitin sulfate

Methacrylated gelatins were synthesized as following. First, type A porcine skin gelatin (Sigma) was mixed at 10% (w/v) into phosphate-buffered saline (PBS; Gibco-BRL) at 60 °C for 1 h. Then, 8% (v/v) of methacrylic anhydride (Sigma) was added about 0.5 ml/min and reacted at 50 °C for 3 h to form GelMA solutions. After 5× dilution with additional warm PBS (40 °C) to stop the reaction, the mixtures was dialyzed against distilled water using 14 kDa molecular weight dialysis for 1 week at 40 °C to remove salts and residues. The solution was lyophilized and stored at −20 °C until further use. Methacrylated hyaluronic acid and chondroitin sulfate were created as described [16]. Briefly, HA (MW 64,000; Life Core Technologies) was mixed at 1% (w/v) with PBS and stirred until dissolved, after addition of glycidyl methacrylate 2% (v/v) (GMA; Sigma–Aldrich) for methacrylate group modification. The reaction solution was vigorously stirred at room temperature for 8 days. The resulting solution was dialyzed against water with 1000 MW dialysis membrane for 2 days with several changes of water during dialysis. For methacrylate chondroitin sulfate, CS (MW 20,000–40,000; Tokyo Chemical Industry) was mixed at 10% (w/v) with PBS and GMA 10% (v/v) was added. After stirring for 11 days, the product was dialyzed against water.

Fabrication of gelatin-based ECM cryogels

With ammonium persulfate (APS; Sigma–Aldrich) as the initiator agent and *N,N,N′,N′*-tetramethylethylenediamine (TEMED; Sigma–Aldrich) as catalyst the gelatin-based ECM cryogels was synthesized by free radical polymerization method. The premediated amount of GelMA, MeHA, and MeCS was dissolved in water to the final desired concentration in the presence of 4% (v/v) APS and TEMED 0.2% (v/v), respectively. To start copolymerization, this polymer solution was put into cylindrical polyethylene mold and placed in the freezer (−20 °C) for 20 h. Interconnected and macroporous cryogels (diameter, 8 mm, height, 4 mm, and total volume, 200 μl) were synthesized by thawing. Before seeding the cells, cryogels were sterilized with UV for 20 min and washed several times with PBS to remove unreacted residues. Rabbit chondrocytes were seeded onto three types of cryogels: GelMA, GelMA + MeHA (G-MeHA), GelMA + MeCS (G-MeCS). The samples were cultured in 1.5 ml of chondrocyte medium (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% HEPES, 1% MEM NEAA, 0.2% L-proline and 0.2% L-ascorbic acid) at 37 °C in 5% CO_2 environment, and the medium was changed every 2 days for up to 3 weeks *in vitro*.

Physical characterization of gelatin-based ECM cryogels

Mechanical properties of gelatin-based ECM cryogels were determined by Instron (5900S, USA). The specimens in each group were prepared as cylindrical blocks with a size of 8 mm diameter and 4 mm of height. Cylindrical samples were swollen in PBS for 24 h. The compressive modulus was calculated from the linear slope of the stress–strain curve.

Scanning electron microscopy and porosity

The morphology of freeze-dried cryogels was evaluated by (JSM-6701F; JEOL) after coated with gold. Pore sizes and porosity of each cryogels were measured from SEM using ImageJ software and MATLAB.

Swelling characterization

The water uptake ability of scaffold was determined using swelling ratios. For swelling studies, each cryogels were immersed in PBS for 24 h at room temperature. To measure the swelling ratios, the dried hydrogels were weighed, and wet weight was measured. The swelling ratios of the cryogels (n = 4) were obtained using following formula.

$$\text{Swelling ratio (Q)} = \frac{\textit{wet weight of equalibrated cryogel in PBS}}{\textit{weight of the dried cryogel}}$$

Cell viability of cryogels

Live/Dead Cell Viability/Cytotoxicity kit (Molecular Probes; L3224) was used following the manufacturer's protocol. Live cells were stained with green fluorescent calcein AM and red fluorescent Ethidium homodimer-1 (Ethd-1) stained dead cells.

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