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Fundamental biomaterial properties of tough glycosaminoglycan-containing double network hydrogels newly developed using the molecular stent method

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ABSTRACT

The purpose of this study was to clarify fundamental mechanical properties and biological responses of the sodium hyaluronate-containing double network (HA-DN) gel and chondroitin sulfate-containing double network (CS-DN) gel, which were newly developed using the molecular stent method. This study discovered the following facts. First, these hydrogels had high mechanical performance comparable to the native cartilage tissue, and the mechanical properties were not affected by immersion in the saline solution for 12 weeks. Secondly, the mechanical properties of the CS-DN gel were not significantly reduced at 12 weeks in vivo, while the mechanical properties of the HA-DN gel were significantly deteriorated at 6 weeks. Thirdly, the degree of inflammation around the HA-DN gel was the same as that around the negative control. The CS-DN gel showed a mild but significant foreign body reaction, which was significantly greater than the negative control and less than the positive control at 1 week, while the inflammation was reduced to the same level as the negative control at 4 and 6 weeks. Fourthly, these gels induced differentiation of the ATDC5 cells into chondrocytes in the culture with the insulin-free maintenance medium. These findings suggest that these tough hydrogels are potential biomaterials for future application to therapeutic implants such as artificial cartilage.

Statement of Significance

The present study reported fundamental biomaterial properties of the sodium hyaluronate-containing double network (HA-DN) gel and chondroitin sulfate-containing double network (CS-DN) gel, which were newly developed using the molecular stent method. Both the HA- and CS-DN gels had high mechanical properties comparable to the cartilage tissue and showed the ability to induce chondrogenic differentiation of ATDC5 cells in vitro. They are potential biomaterials that may meet the requirements of artificial cartilage concerning the material properties. Further, these DN gels can be also applied to the implantable inducer for cell-free cartilage regeneration therapy.

1. Introduction

Glycosaminoglycans (GAG), such as chondroitin sulfate (CS) and sodium hyaluronate (HA), are components of the natural cartilage tissue, and play an important role in the complex functions of the cartilage tissue. In addition, CS and HA are known to enhance chondrogenesis in the field of tissue engineering for cartilage regeneration [1–6]. Therefore, there is a possibility that GAG-containing hydrogels can be potential biomaterials to be applied to development of therapeutic implants in the future, such as artificial cartilage [7] and a novel implant that can induce regeneration of the cartilage tissue in an osteochondral defect [8]. Nevertheless, GAG-containing hydrogels have not been applied to create such implants, because the previously developed GAG hydrogels are

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too weak to be applied to develop such therapeutic implants: for example, the ultimate stress is reported to be approximately 100 kPa [9]. However, if GAG-containing hydrogels with sufficient mechanical properties can be developed, such gels will greatly broaden the application of these materials to various therapeutic implants. Therefore, the authors have challenged to develop GAG-containing hydrogels with sufficient mechanical properties.

The double-network (DN) principle reported by Gong et al. [10] provides a general approach to develop robust hydrogels with high water content. The DN gels consist of two interpenetrating networks that have contrasting structures: A densely cross-linked and brittle network of low concentration and a sparsely cross-linked ductile network of high concentration. For example, a DN hydrogel composed of poly(2-acrylamido-2-methylpropanesulfonic acid) as the first network and poly(acrylamide) as the second network has tensile fracture stress as high as 2 MPa, comparable to or even superior to cartilage [11]. Recently, Nakajima et al. [12] developed a molecular stent method to synthesize the contrasting DN structure consisting of neutral polymers as both the first and the second networks. In this method, the first neutral network was pre-stretched into the extended state as like the polyelectrolyte network does by entrapping linear polyelectrolytes as the molecular stent in the neutral network. Using this molecular stent method, Zhao et al. [13] developed tough GAG-containing DN hydrogels, HA-containing DN hydrogel (abbreviated as HA-DN gel) and CS-containing DN hydrogel (abbreviated as CS-DN gel). In these gels, poly(*N,N*-dimethylacrylamide) (PDMAAm), a neutral biocompatible polymer, was used as the first and the second networks, and the biopolymers of HA and CS were used as the molecular stent (Supplementary Fig. S1). These hydrogels exhibited mechanical properties comparable to standard DN gels [11]. However, mechanical features or tissue reactions in vivo of these GAG-containing DN gels have not been clarified yet. It is necessary to know such properties in order to apply these materials for therapeutic implants.

The purpose of this study was to evaluate mechanical properties and tissue/cell reactions of the HA-DN gel and CS-DN gel. The specific aims asked in this study are as follows: (1) Are the mechanical properties and the water content of the HA-DN gel and the CS-DN gel stable for autoclave sterilization and storage in the phosphate buffered saline solution (PBS)? (2) Do these properties of the GAG-containing DN gels change after implantation into the living body for 12 weeks? (3) What kind of biological reaction is induced in the muscle by implantation of these GAG-containing gels? (4) Can the GAG-containing gels induce differentiation of murine ATDC5 cells, representing a chondroprogenitor clone, into chondrocytes in the culture on these gels with the insulin-free maintenance medium?

2. Materials and methods

2.1. Gel preparation

To synthesize the first network, *N,N*-dimethyl acrylamide (DMAAm) (Kojin Co., Ltd., Tokyo), as a monomer, was purified by distillation under reduced pressure before usage. *N,N*-methylenebisacrylamide (MBAA) (Tokyo Kasei Co., Ltd., Tokyo), as a cross-linker, was recrystallized twice from pure ethanol before usage. 2-Oxoglutaric acid (Wako Pure Chemical Industries, Ltd., Tokyo), as a UV initiator, was used as received. HA, made from *Streptococcus zooepidemicus* by fermentation (molecular weight 1.8×10^6–2×10^6 Da, Kibun Food Chemifa Co., Ltd., Noda) was used as purchased. E-Type chondroitin sulfate peptides (CS-E), made from squid cartilage (molecular weight 7×10^4–3×10^5 Da, Yantai Changsen Chemicals Co., Ltd., China) were used as-received. HA (3 wt%) or CS (5 wt%) was added to the precursor monomer solution, including 1-M DMAAm, 4-mol% MBAA, and 0.1-mol% UV initiator. Each biopolymer used as a molecular stent was incorporated into the precursor solution of the first network using a planetary centrifugal vacuum mixer. Then, the solutions were poured into a simple reaction cell we created, consisting of a pair of glass plates kept apart with silicone elastomer spacers 1 mm or 5 mm thick. The first network hydrogel containing HA or CS was synthesized by irradiation of the reaction cell with 365 nm UV radiation for 8 h in an argon environment. In the first network gel, the HA or CS trapped in the PDMAAm network exerts extra ionic osmotic pressure on the PDMAAm network to substantially swell the network [12,13]. Then, the first network gel was immersed in an aqueous solution of 4 M DMAAm, containing 0.1 mol% MBAA, and 0.1 mol% UV initiator for 1 day until reaching the equilibrium to synthesize the second network. The second network (PDMAAm) was polymerized in the presence of the 1st network gel for 8 h by irradiation of the reaction cell with 365 nm UV radiation in an argon environment. After polymerization, the HA- or CS-PDMAAm DN gel was immersed in pure water for 1 week and the water was changed twice daily to remove any unreacted materials. From the 5-mm HA-DN or CS-DN gel sheet, we created disks having a diameter of 10 mm for subcutaneous implantation test and $1 \times 1 \times 10$-mm^3 rectangular parallelepiped shape rods for intramuscular implantation test. From the 1-mm HA-DN or CS-DN gel sheet, we created disks having a diameter of 15 mm for cell culture.

2.2. Study design

The present study was composed of 4 sub-studies. In the second and third sub-studies, a total of 31 mature female Japanese white rabbits, weighing 3.5 ± 0.2 kg, were used. Animal experiments were carried out in the Institute of Animal Experimentation, under the Rules and Regulation of the University's Animal Care and Use Committee. An operation for each animal was performed under intravenous anesthesia (pentobarbital, 25 mg/kg) and sterile conditions.

2.2.1. The first sub-study

The first sub-study was performed to clarify whether the mechanical properties and the water content of the GAG-containing DN gels change due to autoclave sterilization, and then, due to immersion in the PBS for 12 weeks. The HA-DN and CS-DN gel disks having a diameter of 10 mm and a height of 5 mm were soaked in the PBS and autoclaved (120 °C) for 20 min. The, sterilized gel disks were immersed in the PBS, and put in an incubator (37 °C under 5% CO_2). The mechanical properties and the water content of each disk were measured before and after the autoclave sterilization at 0 (immediately after sterilization), 6, and 12 weeks (n = 10 at each period).

2.2.2. The second sub-study

The second sub-study was carried out to determine changes of the material properties of the HA-DN and CS-DN gels due to in vivo implantation, according to the international guideline for biological evaluation of the safety of biomaterials [14]. For the implantation test, a total of 10 mature female Japanese white rabbits were used. Four longitudinal skin incisions of 3 cm were made at the back of each rabbit. Two incisions were made on the right side, and the other two were made located on the left side. The distance between the 2 spaces was 2 cm or more. Then, two sterilized HA-DN gel disks and two sterilized CS-DN gel disks, having a diameter of 10 mm and a height of 5 mm, were separately implanted into the four subcutaneous spaces, respectively. At 6 and 12 weeks after surgery, each animal was sacrificed by a lethal