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Tuning mechanical performance of poly(ethylene glycol) and agarose interpenetrating network hydrogels for cartilage tissue engineering

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ABSTRACT

Hydrogels are attractive for tissue engineering applications due to their incredible versatility, but they can be limited in cartilage tissue engineering applications due to inadequate mechanical performance. In an effort to address this limitation, our team previously reported the drastic improvement in the mechanical performance of interpenetrating networks (IPNs) of poly(ethylene glycol) diacrylate (PEG-DA) and agarose relative to pure PEG-DA and agarose networks. The goal of the current study was specifically to determine the relative importance of PEG-DA concentration, agarose concentration, and PEG-DA molecular weight in controlling mechanical performance, swelling characteristics, and network parameters. IPNs consistently had compressive and shear moduli greater than the additive sum of either single network when compared to pure PEG-DA gels with a similar PEG-DA content. IPNs withstood a maximum stress of up to 4.0 MPa in unconfined compression, with increased *PEG-DA molecular weight* being the greatest contributing factor to improved failure properties. However, aside from failure properties, *PEG-DA concentration* was the most influential factor for the large majority of properties. Increasing the agarose and PEG-DA concentrations as well as the PEG-DA molecular weight of agarose/PEG-DA IPNs and pure PEG-DA gels improved moduli and maximum stresses by as much as an order of magnitude or greater compared to pure PEG-DA gels in our previous studies. Although the viability of encapsulated chondrocytes was not significantly affected by IPN formulation, glycosaminoglycan (GAG) content was significantly influenced, with a 12-fold increase over a three-week period in gels with a lower PEG-DA concentration. These results suggest that mechanical performance of IPNs may be tuned with partial but not complete independence from biological performance of encapsulated cells.

1. Introduction

Mechanical integrity is an important property of hydrogels used for cartilage regeneration and is often a major barrier in usability. Progress in improving hydrogel mechanical strength was significantly advanced with the development of a special class of interpenetrating network (IPN) hydrogels with superior mechanical properties (termed “double-network hydrogels”) by the research team of Gong et al. at Hokkaido University (Japan) [1,2]. An IPN is synthesized by constructing a single-network hydrogel (first network), soaking the gel in a monomer solution of the second component long enough to equilibrate, and then photopolymerizing the second network that interlocks with the first. While IPN formation typically enhances the performance of hydrogels in biomedical applications, conventional IPNs do not show more than additive improvement in mechanical integrity over either individual network [1,3]. However, double-network (DN) hydrogels exhibit mechanical properties sometimes orders of magnitude greater than their individual components [2,4].

Since the development of the DN hydrogel in 2003, Gong et al. have recognized the comparability of DN properties to native cartilage and have consequentially studied their potential for cartilage replacement [5,6], with other research groups following suit [7,8]. More recently, they developed a method using a DN hydrogel to attempt *in vivo*

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articular cartilage regeneration without the use of encapsulated cells [9]. Other studies have led to extremely strong DN hydrogels [10,11], though without the specific aim of cartilage repair or regeneration. Most methods for synthesizing DN hydrogels are not compatible for cell encapsulation, and there have been very few attempts to produce cell-laden DN gels. Recently, Tang and coworkers [12] reported the encapsulation of chondrocytes into a DN hydrogel made of gellan gum and carboxymethyl chitosan to evaluate the material's biocompatibility. Mechanical testing was also done on acellular gels, with their stiffest gel showing a compressive modulus of ~600 kPa, which is relatively high for a cellularly-viable gel. However, this gel permanently deformed upon compression, raising the concern for recovery for this particular formulation. Shin and coworkers [13] recently encapsulated fibroblasts into gelatin/gellan gum DN hydrogels to evaluate their cytocompatibility and mechanical properties. They reported an acellular IPN that could withstand a maximum compression of ~7 MPa and had a compressive modulus of ~110 kPa. While both studies showed cells could survive the encapsulation process, viability was not tested beyond 1 week for Tang and coworkers and 3 days for Shin and coworkers.

Network parameters such as swelling degree and crosslinking density, which are important in determining whether the DN hydrogels provide a microenvironment conducive to waste/nutrient transport, have been reported for single-network hydrogels, particularly to determine their relationship with proteoglycan distribution [14]. However, the equations for calculating these parameters have not yet been applied to hydrogels with more than one network for cartilage regeneration. Furthermore, because the role of cell encapsulation is to stimulate regrowth of the patient's own cartilage, it is also important to evaluate not only the viability of encapsulated cells over a longer period of time but also whether the cells are healthy and producing ECM. Additionally, reporting the fracture properties of hydrogels meant for load-bearing tissue applications is just as essential as reporting mechanical stiffness. Finally, encapsulated cells that produce ECM while degrading or metabolizing the gel could potentially affect the gel's mechanical properties, so mechanical testing on cell-laden hydrogels is also crucial in determining whether the material is truly appropriate in providing mechanical compensation *in vivo*.

Our group has recently shown that chondrocytes can survive the encapsulation process in an IPN consisting of 2% *w*/*v* agarose and 15% *w*/*v* poly(ethylene glycol) diacrylate (PEG-DA, MW = 2000 Da) [15,16]. Many agarose studies have shown a high degree of success for cell viability and GAG production, though these constructs are severely limited by their inferior mechanical performance. PEG-DA, a synthetic, hydrophilic polymer, has also been widely studied for cartilage regeneration and has mechanical properties far exceeding those of agarose. Nguyen and coworkers [17] recently released an extensive overview of the use of PEG-DA for cartilage tissue engineering, including the concentrations, molecular weights, methods, mechanical properties tested, and results of several research groups who have studied PEG-based hydrogels.

While our previous studies of agarose/PEG-DA IPNs showed a large improvement in mechanical performance compared to both single networks, we hypothesized that further mechanical improvements would be observed by increasing the concentrations of both networks as well as the molecular weight of PEG-DA while still maintaining cell viability. Three PEG-DA concentrations (10%, 15%, and 20% *w*/*v*), three agarose concentrations ("0%" for pure PEG-DA hydrogels, 2% and 5% *w*/*v* for IPNs), and three PEG-DA molecular weights (2000, 3400, and 6000 Da) were studied in a full-factorial design for a total of 27 formulations. Our objective was to determine which of these three factors most significantly contributed to differences in mechanical properties, network parameters, and swelling characteristics. Of these 27 formulations, nine representative groups were selected for encapsulation to examine viability as well as the mechanical properties of cell-laden scaffolds compared to their acellular counterparts, and three were further selected for biochemical analysis.

2. Materials and methods

2.1. Materials

High-purity PEG-DA (molecular weights 2000, 3400, and 6000 Da; purity >99%) was purchased from SunBio (Anyang City, South Korea). 2-Hydroxyethyl agarose (Type VII) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The photoinitiator Irgacure 2959 (I-2959) was acquired from Ciba (Basel, Switzerland).

2.2. Agarose network synthesis

Agarose powder was added to phosphate-buffered saline (PBS, 0.01 M) to yield 2% and 5% *w*/*v* solutions and autoclaved for 30 min. Solutions were then pipetted into cylindrical silicon rubber molds (~5 mm diameter, ~2 mm height), pressed between glass plates, and cooled at 4 °C for at least 10 min. Gels were then placed in PBS to equilibrate for at least 24 h before use.

2.3. PEG-DA network synthesis

Irgacure 2959 was added to a 70/30 mixture of ethanol and deionized (DI) water to create a 10% *w*/*v* photoinitiator solution. 10%, 15%, and 20% *w*/*v* solutions of PEG-DA (molecular weight 2000, 3400, and 6000 Da each) in PBS were prepared, and 10 μL of photoinitiator solution was added to each mL of PEG-DA solutions. The solutions were then placed in rectangular silicon rubber molds (~2 mm height), pressed between optical glass microscope slides, and exposed to 312 nm light (3.0 mW/m^2, XL-1000, Spectronics Corp.; Lincoln, NE) for 5 min on each side. Gel samples were cut using a 3 mm biopsy punch, and gels were transferred to PBS to equilibrate for at least 24 h before use.

2.4. IPN formation

Four cylindrical agarose gels were added for each mL of PEG-DA/photoinitiator solution and soaked under constant agitation. The length of the soaking times needed for adequate diffusion was calculated based on literature data [18] and were dependent on the PEG-DA molecular weight (2, 3.5, and 6 h for molecular weights of 2000, 3400, and 6000, respectively). The agarose gels were then placed in rectangular molds (~2 mm height) between two optical glass microscope slides and exposed to 312 nm light, 3.0 mW/m^2 (XL-1000, Spectronics Corp.) for 5 min on each side. Samples were then cut using a 3 mm biopsy punch and added to PBS to equilibrate for at least 24 h before use. The formulations are reported according to the following structure: agarose concentration/PEG-DA concentration (PEG-DA molecular weight). As an example, the formulation 2%/15% (2k) is an IPN with 2% *w*/*v* agarose soaked in a 15% *w*/*v* PEG-DA (2000 Da molecular weight) monomer solution prior to photopolymerization.

2.5. Chondrocyte isolation

10 ankles from five juvenile Chester White hogs (female, ~3 months old, ~120 lbs) were obtained from Winchester Meat Processing, Inc. (Winchester, KS). Cells were harvested within 24 h after slaughter following methods described in our previous publications [19,20]. The articular cartilage was removed manually with a scalpel and minced under sterile conditions. The cartilage was then digested in a 2 mg/mL sterile-filtered solution of type II collagenase (300 U/mg, Worthington Biochemical; Lakewood, NJ) for 24 h at 37 °C. Cells were then filtered from their ECM, centrifuged, and resuspended in culture medium consisting of Dulbecco's modified Eagle medium with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% sodium pyruvate, and 1% penicillin–streptomycin fungicide. Cells were fed every other day with the supplemented medium until ~90% confluent. The medium and supplements were obtained from Invitrogen (Grand Island, NY). Cells were not pooled until after the first passage, during the encapsulation process.

2.6. Cell encapsulation

2.6.1. Encapsulation into IPN gels

Two solutions of agarose (3% and 7.5% *w*/*v*) in PBS were prepared and autoclaved for 30 min. Meanwhile, cells were detached from their flasks with trypsin–ethylenediaminetetra acetic acid and labeled as passage 1 (P1). At this point, P1 cells were pooled and resuspended in PBS at 30 million cells/mL to begin the encapsulation procedure. Once cooled to 39 °C, the cell suspension was added to each solution of molten agarose in a 1:2 ratio to produce 2% and 5% agarose solutions with a seeding density of 10 million cells/mL. Each solution was pipetted into sterilized silicon rubber molds (~5 mm diameter, ~2 mm height), pressed between two glass plates,