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Short communication

The functional response of alginate-gelatin-nanocrystalline cellulose injectable hydrogels toward delivery of cells and bioactive molecules

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ABSTRACT

Hybrid injectable hydrogels comprising of alginate, gelatin, and nanocrystalline cellulose (NCC) were conceived and processed through adaptation of interpenetrated network of alginate-gelatin, ionic crosslinking of alginate, and supramolecular interaction approach. The design of hybrid hydrogels was based on the hypothesis that it provides an environment that is favorable for cell proliferation, exchange of nutrients via porous structure, and are characterized by mechanical properties that closely resemble the native tissue. This aspect is important for the delivery of cells or biomolecules in bone tissue engineering. The hybrid hydrogels exhibited moderate swelling behavior on formation, and the porous structure of hydrogels as imaged via SEM was envisaged to facilitate easy migration of cells and rapid transportation of biomolecules. The hybrid hydrogels exhibited desired mechanical properties and were biocompatible as confirmed though MTT assay of fibroblasts. Interestingly, osteoblasts cultured within hydrogel using bone morphogenetic protein (BMP)-2 demonstrated the capability for encapsulation of cells and induced cell differentiation. The nanocrystalline cellulose significant impacted degradation and interaction between hydrogels and cells.

Statement of Significance

The study fundamentally explores a hypothesis driven novel hybrid hydrogel that provides an environment for favorable growth and proliferation of cells, exchange of nutrients and mechanical properties that closely match the native tissue.

1. Introduction

Naturally derived polymers, including proteins and polysaccharides, have been extensively used as biomaterials. For instance, as hydrogels for wound dressing, tissue engineering scaffolds, and drug delivery vehicles [1–5]. Among these materials, alginate is a block copolymer with consecutive and alternately arranged β-D-mannuronic acid and α-L-guluronic acid residues and can be ionically crosslinked with divalent cations (e.g. Ca^{2+}, Zn^{2+}) [1,2]. The unique properties of alginate helps in the synthesis of standalone alginate hydrogel or in combination with other materials. However, because of the hydrophobic nature, alginate is not appropriate for cell adhesion unless incorporated with other hydrophilic materials [2,3]. The cell adherent components including collagen, gelatin, RGD (arginine-glycine-aspartate) or RGD derived peptide sequences, and asialoglycoprotein receptors (ASGPR) can be integrated with alginate to enhance biological and physicochemical properties [1,4]. Although bioreagent grade alginate is perceived as biocompatible, a higher mannuronic acid content or impurities in alginate has potential to be immunogenic or induce foreign body reactions upon injection or implantation [2,3].

In recent years, the alginate-based hybrid hydrogels have received significant attention because of superior mechanical properties, chemical properties, and biological activity [5]. Essentially, two or more constituents are chemically bonded via two or more crosslinked networks, such that double or multiple network polymers can be formed to obtain superior mechanical properties. For instance, alginate-polyacrylamide double network hydrogels were characterized with ~29 kPa Young's modulus and over 1700% elongation [6]. Although the mechanical properties were superior to some natural polymers, standalone alginate was characterized by low tensile modulus and elongation [7]. Thus, the

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reinforcement of alginate-based hydrogel is important to render them suitable for load-bearing tissue regeneration applications.

The nanocrystalline cellulose (NCC) is derived from plant or bacteria cellulose, and has good biocompatibility and is suitable for tissue integration [8,9]. NCC has been used in composite hydrogels of polyacrylamide, polyvinyl alcohol, and cyclodextrin to enhance mechanical properties [10–13]. NCC is not only a reinforcement material for hydrogels but also facilitates gelation in a supramolecular way through hydrophobic interaction and hydrogen bonds. Marine source NCC introduced oriented growth of myoblast in a radial pattern [14]. In another instance, alginate was combined with NCC to prepare a nanocomposite film for wound dressing [15]. Gelatin, a hydrolyzed product from collagen with high hydrophilicity, enhances cell adhesion and provides functional groups for chemical crosslinking. It can be blended with alginate to form spongy film, scaffolds and hydrogels, which provides a benign environment for cell adhesion and growth together with desirable mechanical properties [16,17]. The combination of alginate, gelatin and NCC is expected to improve mechanical and biological properties of NCC reinforced alginate hydrogel.

In the study described here, novel hybrid hydrogels were synthesized involving chemical bonding of gelatin and alginate, ionic crosslinking of alginate with zinc ions, and supramolecular interaction with nanocrystalline cellulose. The interactions led to desired properties appropriate for regeneration of bone tissue, where the components of hydrogel precursors allow ease of injection, biocompatibility, effectiveness in nutrients and cell transportation, and cell adhesion and proliferation. BMP-2, a growth factor used extensively to enhance osteogenesis and osteoinduction enabled cell proliferation and differentiation of osteoblast within the hybrid hydrogels. The studied hybrid injectable hydrogels are potentially attractive as scaffold materials for bone regeneration.

2. Materials and methods

2.1. Materials

Alginate (sodium salt from brown algae, 4-12 cP, bioreagent), zinc sulfate heptahydrate, N-hydroxy-succinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma–Aldrich USA. Gelatin (type A, from porcine skin, bioreagent) was obtained from MP Biomedicals USA.

2.2. Preparation of nanocrystalline cellulose

NCC was prepared by acid hydrolysis of cellulose from filter paper (Whatman #1). After grinding, the cellulose powder was hydrolyzed, while stirring at 45 °C for 45 min using sulfuric acid (64 wt%) at 17.5 mL/g acid-to-pulp ratio [18]. To stop the hydrolysis reaction, deionized (DI) water (10-times the volume) was added. The suspension was centrifuged at 5000 rpm for 30 min and the acid residue was decanted. After washing the precipitate and dispersing in DI water, the suspension of NCC was dialyzed against DI water using a dialysis tube with 12,000 Da molecular weight cut-off (MWCO). The dialysis was continued until the pH of the NCC suspension was greater than 5, followed by dehydration of suspension in a desiccator. Finally, NCC was sonicated for 10 min using a QSonica 750 W probe sonicator to disperse in DI water.

2.3. Synthesis of hybrid injectable hydrogels

NCC was dispersed in 2% alginate aqueous solution via sonication and then mixed with 2% gelatin aqueous solution in a 24-well tissue culture plate at varying volume ratio. 20 mg EDC and 10 mg NHS were added to each well and reacted at room temperature for 24 h. Next, 1 mL 0.05 M $ZnSO_4$ aqueous solution was added to each well for 30 min to complete ionic crosslinking, and the hydrogels were rinsed by 1X phosphate buffer saline (PBS). The details of ration of alginate, NCC, and gelatin are listed in Table 1.

In the synthesis of hydrogel precursors the ratio of gelatin/alginate/EDC/NHS/NCC used was identical to that of groups 1 and 4. The reaction was carried out in a 100 mL round bottom flask and stirred at room temperature for 24 h. Next, the hydrogel precursor was withdrawn via 10 mL medical syringes with18-gauge needle.1 mL hydrogel precursor was injected into a covered plastic mold (15.6 mm diameter, 10 mm height, modified from 24-well plate) and then 0.5 mL 0.05 M $ZnSO_4$ was injected to complete ionic crosslinking.

2.4. Characterization of hydrogel

Hybrid hydrogels were immersed in 1X PBS at 37 °C and weighed periodically after removing excessive water from the hydrogel surface though filter paper. Then, hydrogels were frozen at −20 °C and lyophilized at −50 °C for 24 h, and weighed again. Swelling ratio of hydrogels was determined using Eq. (1):

$$\text{Swelling Ratio} = \frac{W_s - W_d}{W_d} \times 100\% \quad (1)$$

where W_s is the weight of the swollen hydrogels and W_d is the weight of the hydrogels after freeze-drying.

The mechanical properties of hybrid hydrogels were tested with a Dynamic Mechanical Analyzer (RSA3, TA Instrument). The compression test (compression modulus) was carried out unconfined. Six samples prepared in 24-well plate (18 mm diameter and 5 mm thickness) from each group were tested. Compression tests were carried out at room temperature at a speed of 0.1 mm/min and the compression modulus determined by using the stress value at 10% strain.

Hydrogels were characterized using FT-IR spectrometer (JASCO FT/IR-4600). The freeze-dried hydrogel was mixed with KBr in the ratio of 1:100, and the pellet was pressed in 7 mm diameter mold. The wave number range was set between 4000 and 500 cm^{-1} and the resolution was 4 cm^{-1}.

The crystallinity of NCC and NCC-containing hydrogels was studied by X-ray diffraction (XRD) using a powder diffractometer (D8 Discover, Bruker) operating at 40 kV and 40 mA. The lyophilized NCC-free and NCC-containing hydrogels were stored in a desiccator and placed on a glass slide in the form of powder and exposed to Cu K_α X-ray.

The freeze-dried hydrogels were observed by Hitachi S-4600 field emission scanning electron microscope (SEM) operated at 20 kV. Prior to examination, the hydrogels were sputter coated with gold. The average pore size of hydrogels was determined from micrographs using 10 randomly measured pores.

Table 1
The content of alginate, gelatin and nanocrystalline cellulose used for the synthesis of hydrogels, corresponding average pore diameter and compression modulus of hydrogels.

Group	Alginate (mL)	Gelatin (mL)	NCC (mg)	Average pores (um)	Compression modulus (kPa)
1	1	1	0	35 ± 12	50 ± 10
2	1	0.5	0	54 ± 9	39 ± 17
3	0.5	1	0	48 ± 10	37 ± 19
4	1	1	5	36 ± 9	75 ± 15
5	1	1	10	47 ± 10	84 ± 22
6	1	1	20	50 ± 11	92 ± 28