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Injectable natural polymer compound for tissue engineering of intervertebral disc: In vitro study

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ABSTRACT

Intervertebral disc degeneration is recognized to be the leading cause for chronic low-back pain. Injectable hydrogel is one of the great interests for tissue engineering and cell encapsulation specially for intervertebral (IVD) affecting rate of regeneration success, in this study we assessed viscoelastic properties of a Chitosan-β glycerophosphate-hyaluronic acid, Chondroitin-6-sulfate, type 2 of Collagen, gelatin, fibroin silk (Ch-β-GP-HA-CS-Col-Ge-FS) hydrogel which was named as NP hydrogel that is natural extracellular matrix of IVD.
Chitosan-based hydrogel was made in the ratio of 1.5%: 7%: 1%:1%:1%–1.5%–1% (Ch: β-GP: HA-CS-Col-Ge-FS). Gelation time and other rheological properties were studied using amplitude sweep and frequency sweep tests. Also, the cytotoxicity of the hydrogel invitro assessed by MTT and trypan blue tests. Morphology of the hydrogel and attachment of NP cells were evaluated by SEM.
Our result showed that NP hydrogel in 4 °C is an injectable transparent solution. It started gelation in 37 °C after about 30 min. Gelation temperature of NP hydrogel was 37 °C. Storage modulus (G′) of this hydrogel at 37 °C was almost constant over a wide range of strain. MTT and trypan blue tests showed hydrogel was cytocompatible. The obtained results suggest that this hydrogel would be a natural and cytocompatible choice as an injectable scaffold for using in vivo study of IVD regeneration.

1. Introduction

Low back pain is a worldwide prevalent health care issue nowadays. 60–80% of people in United States of America have experienced low back pain [1] Herniation of intervertebral disc (IVD) and its degeneration are the major reasons of low back pain, which occur because of structural damage of disc [2]. Therapeutic strategies for disc degeneration treatment are tissue engineering and gene transferring, which have done in laboratory animals [3].

Use of appropriate scaffold is an important point in tissue engineering and especially for cartilage restoration. Scaffolds prepare a three-dimensional condition for proliferation, production and secretion of extracellular matrix and formation of normal tissue [4–7]. The purpose in tissue engineering is to find the proper substances with significant traits for restoration of tissue. These traits are biodegradable [6–8] and biocompatible, which mean don't induce inflammatory reactions and toxic production [6–8]. Having proper pores and controlled porosity, scaffold surface must be appropriate for adherence, proliferation and migration of cells [9].

Injectable hydrogel scaffolds are an important category of biomaterials in tissue engineering [9]. Softness, inherent flexibility, injectability, easily casting into different shapes and three dimensional structures of hydrogels which mimic the extra cellular matrix of natural tissues make them very useful in this field [10]. These hydrogel materials form by the changes in the environmental conditions, such as ionic charge, electrical stimulus, temperature or pH of aqueous polymer solutions [11]. Yan et al. suggested Temperature-sensitive hydrogels are more attractive than other ones because growth factors, cells and other biologically active elements can be loaded more easily inside them [12].

Chitosan as a natural, biocompatible, biodegradable and abundant polymer can form injectable thermoresponsive hydrogels [13]. This scaffold has been used in regeneration of bone and cartilage and also in tissue engineering [14,15]. Injectable thermosetting chitosan based

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hydrogels at first described by Chenite et al. [16]. They used chitosan/glycerophosphate salt combination [17]. Chitosan/glycerophosphate salt (CH–GP) hydrogels are physically cross linked and don't have toxicity of synthetic hydrogels. So many studies performed about CH-β-GP hydrogels in different concentrations [18,19]. IVDs are located between spines, which contain 3 parts. The outer part is annulus fibrosis (AF), the middle part is transitional zone (TZ) and the inner part is nucleus pulposus (NP), which produce the nucleus of disc [1,3]. AF and NP formation are mainly from extracellular matrix. Water, proteoglycans, and Collagen in the extracellular matrix (ECM) of NP tissue provide fluidity and viscoelasticity to the structure, acting as a shock absorber, and maintaining loads in IVDs Some polymers could help chitosan to improve its mechanical and biological virtues. The proportion of each component of GAG in the Human NP is contain 90% sulfated GAG and 10% hyaluronan [20]. Gelatin is one of them, which improves the biological activity of scaffold because of its specific sequence that increases cell adhesion and migration [21].

In a previous study, it was found that Chondroitin-6-sulfate in combination with other natural scaffolds increases proliferation, NP cell survival and even the strength of the composite scaffolds [22].

Injectable hydrogel is one of the great interests for tissue engineering and cell encapsulation especially for intervertebral (IVD) affecting rate of regeneration success [14,15].

The goal of this study was to prepare and assess injectable natural compound hydrogel (NP hydrogel) that is similar to ECM structure of IVD with appropriate efficiency. We assessed behavior of NP cells on NP hydrogel in vitro model. To do that, we used chitosan based hydrogel containing chondroitin-6-sulfate and collagen II. We added gelatin and fibroin silk to increase hydrophilicity, stability and strength of compound hydrogel. Compound hydrogel was characterized by rheometer. We also assessed cytotoxicity of hydrogel by cultured of NP cells on NP hydrogel.

2. Methods and materials

2.1. Preparation of injectable hydrogel

Step 1: Preparation of silk fibroin solution

Mori cocoons were prepared from Iranian silkworm research center (Guilin, Iran) and silk fibroin was extracted following the as described previously [23]. Briefly, silk sericin was removed using boiling of mori cocoons in 0.02 M Na2CO3 solution for 30 min. The silk fibers were then rinsed three times with deionised water and dried at 37 °C for 24 h. Subsequently, extracted fibroin were dissolved in 9.3 M LiBr solution for 3 h and then dialyzed against distilled water us by a cellulose dialysis membrane with 12 kDa molecular weight cut-off. Water was exchanged several times to remove LiBr salt from the fibroin protein solution .The final concentration of the fibroin silk solution was determined gravimetrically using drying the solution. The fibroin protein had a 9% (w/v) concentration.

Step 2: Preparation of Col/HA solution

Type II Collagen (Col) dissolved in 0.01 M acetic acid to yield 1% (w/v) aqueous solution. Hyaluronic acid sodium (HA) (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) separately to yield 1% (w/v) aqueous solution. Finally, collagen solution mixed in HA solution.

Step 3: Preparation of FS/Col-HA solution

Two solutions were mixed using repeated pipetting at a ratio of 50:50 (vol%) to obtain homogeneous FS/Col-HA solution (1% w/v FS and 1% w/v Col-HA).

Step 4: Preparation of Ch/CS-Ge solution

Ch (Chitosan) 1.5% (w/v) obtained using dissolved in acetic acid (0.1 M). CS (Chondroitin-6-sulfate) and Ge (Gelatin) were dissolved in distilled water separately to yield 1% (w/v) and 1.5% (w/v) aqueous solutions, respectively. Then Ch solution were mixed with CS-Ge solution at a ratio of 50:50 (vol%) (1.5% w/v Ch, 1% w/v CS and 1.5% w/v GE).

Step 5: Preparation of FS/Col-HA/Ch-CS-Ge solution:

Solutions FS, Col-HA and Ch-CS-Ge mixed with equal ratio 1:1:1

Step 6: β-GP solution

1.12 g of sterilized β-glycerol phosphate (β-GP) (Sigma, Germany) dissolved in 1.7 mL of sterile deionised water to yield 7% (w/v) aqueous solution.

Final step: Thermoresponsive hydrogel

Final mixed solution and β-GP solution kept on ice for 15 min and then cold β-GP solution added drop wise to the cold final mixed solution with continuous stirring to form a clear solution .obtained solution were thermoresponsive hydrogel that named NP hydrogel.

2.2. Rheological assessments

All rheological tests that mentioned below performed by a Physica MCR 300 rheometer (Anton-Paar, Ashland, VA, USA) with appropriate tools.

2.3. Gelation time determination

The gelation time of thermo responsive hydrogel was measured by using the rheometer equipped with a cone-cup tool (CC27). To simulate in vivo injection, samples from 4 °C transferred to 37 °C at time zero and gelation time was measured as a function of time at constant temperature of 37 °C. The elastic modulus (G′) and the viscous modulus (G″) were evaluated from the oscillatory measurements at a frequency of 1 Hz. The gelation time was determined as the time past in which G′ and G″ curves intersect each other.

2.4. Amplitude sweep

Amplitude sweep was done using cone-plate tool (CP25–2) at 37 °C. The distance between the plates was 0.05 mm. Samples were placed directly between the parallel plates. Beneath plate was heated up to 37 °C prior to measurement. Mineral oil ₀ was used to prevent dehydration. Amplitude sweep records the linear viscoelastic region (LVER) of a material. Storage (elastic) modulus (G′) is used to define it. The limit of the LVER is where the structure of the material starts to break down. Thus LVER is a direct measurement of structure and indicates the stability of sample. We performed amplitude sweep at a frequency of $\omega =$ 10 rad/s with a strain range from 0.1% to 100% [19].

2.5. Frequency sweep

Frequency sweep such as amplitude sweep was done using cone-plate tool (CP25–2) at 37 °C. Based on this output; material can be classified into 3 general categories: viscoelastic solid, viscoelastic liquid and gel materials. Storage (elastic or solid like) modulus (G′), loss (viscous or liquid like) modulus (G″) and complex modulus were drawn versus frequency. LVER should be recorded using amplitude sweep prior to frequency sweep. This test was done in strain (γ) = 5% (in LVER region), and frequency range from 0.1 to 100 rad/s.