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Glucosamine loaded injectable silk-in-silk integrated system modulate mechanical properties in bovine ex-vivo degenerated intervertebral disc model

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ABSTRACT

Injectable hydrogels offer a tremendous potential for treatment of degenerated intervertebral disc due to their ability to withstand complex loading, conforming precisely to the defect spaces and eliminating the need for invasive surgical procedures. We have developed an injectable hydrogel platform of N-acetyl-glucosamine (GlcNAc) loaded silk hollow spheres embedded in silk hydrogel for *in situ* therapeutic release and enhanced mechanical strength. The assembled silk hydrogel provided adequate structural support to the *ex vivo* degenerated disc model in a cyclic compression test at par with the native tissue. Spatiotemporal release of GlcNAc in a controlled manner from the silk hollow microspheres trigger enhanced proteoglycan production from ADSCs embedded in the composite system. Role of MAPK and SMAD pathways in increasing proteoglycan production have been explored by immunohistological analysis as a result of the action of GlcNAc on the cells, elucidating the potential of injectable silk microsphere-in-silk hydrogel for the regeneration of degenerated disc tissue.

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1. Introduction

Nucleus pulposus (NP) of intervertebral disc (IVD) is a highly hydrated (70–90%) tissue that contains mainly proteoglycans and collagen type II [1]. The IVD tissue provides support and dissipates the axial compressive forces in spinal joints. With aging process, disc degeneration pathophysiology initiates in the NP due to loss of cellularity, decreased water content and loss of proteoglycans from the extracellular matrix [2]. These incidents alter the swelling properties of the NP that translates into the reduction in disc height and poor ability to absorb the mechanical pressure applied on the spine [3]. To date, there is no successful treatment strategy available for degenerative disc diseases.

Hydrogels have been implanted into the degenerated discs in a minimally invasive manner; in an effort to reduce the surgery time and post-surgical trauma. Fibrin [4–6], hyaluronan [7,8], collagen [9,10] and chitosan [11–13] based hydrogels have been used as carriers of cells and for the delivery of biochemical cues for promoting the regeneration of NP tissue. Various growth factors, such as TGF-β1 [14] and BMP-2 [14,15], delivered along with cells, have been shown to enhance ECM deposition. The presence of these factors has resulted in increased deposition of ECM components; however, a major disadvantage is that it has also lead to the ossification of the adjacent AF region [16]. Gellan gum hydrogels have been found to maintain native phenotype of NP cells [17,18], but the mechanical properties of the crosslinked hydrogels differed from the native NP tissue. In both the aforementioned conditions, the hydrogel implant may fail to maintain biomechanical stability of the disc and hence promote further disc degeneration. Most of these hydrogels developed for supporting cell growth and growth factor delivery inside the degenerated tissue have not been successful in restoring the viscoelastic and mechanical properties of the NP tissue. This challenge can be addressed by designing a

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hydrogel system that can encapsulate cells and deliver biochemical cues; while being robust enough to mimic the viscoelastic properties of the native tissue.

Silk was the material of choice because of its known cellular compatibility and low immunogenicity [19,20]. Silk fibroin hydrogels have been used as injectable materials for healing confined critical size cancellous defects due to their excellent intrinsic mechanical behavior [21]. Injectable sonication-induced silk hydrogels have been used to deliver VEGF and BMP-2 for the elevation of the maxillary sinus floor [22]. Silk microspheres and nanospheres have been fabricated from silk/PVA blend films, by dissolution of silk-PVA films in water and their subsequent ultrasonication [23]. This process generated spheres of non-uniform sizes [23]. Some studies have demonstrated the use of silk hydrogels in combination with other synthetic polymers for NP tissue engineering. A silk fibroin-fibrin-hyaluronic acid composite gel was used for NP tissue engineering [24], where 1% and 2% silk hydrogels were used in conjugation with fibrin and hyaluronic acid for controlling the degradation rate of the scaffolds. The silk fibroin helped in enhancing the mechanical properties of the fibrin/hyaluronic acid hydrogels, which still lacked the viscoelastic properties and injectability that are a pre-requisite in NP tissue regeneration. Another study demonstrated that the injectable silk fibroin/polyurethane composite hydrogels augment NP tissue [25]. In context of the current literature, our study demonstrates that the use of synthetic polymers, like polyurethane, can be avoided by optimizing the weight percentages of silk hydrogel itself to make its rheological and mechanical properties at par with the native NP tissue. The reinforcement of salt leached silk scaffolds by silk particles could improve the specific modulus from 205.8 ± 93.7 kPa g^{-1} cm^3 to 7722.4 ± 812.6 kPa g^{-1} cm^3 [26]. Also, the incorporation of short staple silk fibers in silk hydrogels has previously shown to enhance the mechanical properties of silk hydrogels [27]. Hence, reinforcement of silk hydrogels by silk microspheres may improve mechanical properties of silk hydrogel. During loading, intermolecular interactions between silk fibers and silk hydrogels could provide a smooth interface for the transfer of forces, resulting in a better mechanical profile.

Glucosamine is a critical component in the biosynthetic pathway of hyaluronic acid and provides a backbone for the self-assembly of several proteoglycan molecules to form the large proteoglycan aggregates present in a healthy NP tissue [28]. It is demonstrated that N-Acetyl-D-glucosamine (GlcNAc) modulates the expression of TGF-β with a switch-like response (Hill coefficient, pH~5.4), in turn differentially regulating the ERK and SMAD signaling pathways downstream [29]. GlcNAc also upregulates TGF-β1 mRNA levels in a dose-dependent manner in both 2D and 3D hydrogels seeded with articular chondrocytes [30]. In mesangial cells, GlcNAc mediated TGF-β1 up-regulation could increase the production of specific ECM components, possibly through the hexosamine pathway that exogenously supplied glucose [31–33]. Thus a controlled delivery of glucosamine in an optimum concentration is required for enhanced GAG production that can be beneficial for treating degenerated IVDs. Further, generation of insights about the mechanism through which this GlcNAc-mediated switch like behavior is caused can help to device strategies for degenerated IVD treatment.

The implantation of autologous NP cells for IVD regeneration is not feasible, as the procedure requires puncturing the annulus fibrosus (AF) tissue. This damage may lead to further degeneration of adjacent discs [34]. Thus, human adipose derived stem cells (hADSCs) appear to be the best candidates for this purpose owing to their easy isolation from liposuctioned waste fat tissue. Moreover, the high proliferation rate of hADSCs helps in achieving a significant population of cells for implantation. ADSCs have reportedly been differentiated towards the NP phenotype either in the presence of TGF-β [35–37] or when co-cultured with NP cells [38], and are thus a potent candidate for NP tissue engineering. Recently, we reported that microgels composed of collagen type II and hyaluronan helped in the differentiation of ADSCs towards a NP-like phenotype by providing a native-like NP microenvironment to the cells [39].

Thus, in the present study we introduce a silk fibroin hydrogel-based biomaterial delivery platform, consisting of silk fibroin hollow microspheres embedded within fibroin hydrogel, offering well suited rheological features for injectability, and shape-conformability into defect sites as well as controlled delivery rate. We hypothesized that the hollow silk microspheres, developed using template sacrificial method using polystyrene templates, loaded with GlcNAc can offer a spatiotemporally controlled release of the moieties into the silk hydrogel system, providing the cells with physiologically relevant amounts of GlcNAc for enhanced GAG production.

2. Materials & methods

2.1. Preparation of silk fibroin solution

Bombyx mori silk cocoons were provided by Central Silk Technological Research Institute (Central Silk Board), Bangalore, Ministry of Textiles, Government of India. Fibroin protein was isolated as mentioned earlier [40,41]. Briefly, 5.0 gm of cocoons were cut into small pieces and degummed in boiling water containing 4.4 gm Na_2CO_3 (Sigma) for 30 min. Extracted fibroin fibres were given three consecutive rinses in deionized MilliQ water for 15 min each, air dried and then subsequently dissolved in 9.3 M LiBr (Sigma) at 60 °C for 4 h. The silk-LiBr solution was dialyzed against deionized water using Slide-A-Lyzer cassette (Thermo, molecular weight cut off 3500) to obtain a 6% (w/v) solution of fibroin protein.

2.2. Fabrication of silk hollow microspheres

Hollow silk fibroin microspheres were fabricated using the template based method [42–46]. Commercially available beads (Spherotech, USA) of 0.5 μm, 0.8 μm, 1.4 μm and 4.4 μm were first coated with a 1% w/v poly-L-Lysine solution to impart a positive charge to the polystyrene surface. Following coating, the beads were spun at 4000 rpm for 20 min and then washed with deionized (DI) water by vortexing for 5 min. The beads were then resuspended in 2% w/v silk fibroin solution overnight and then washed with DI water twice and incubated with 70% ethanol for 2 h. The beads were then resuspended in DI water and crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC)/N-hydroxysuccinimide (NHS) (12 mM EDC, 4.8 mM NHS) in an aqueous solution for 1.5 h and subsequently the reaction was stopped by adding 1 mM Na_2HPO_4. The beads were washed twice with DI water and the final wash was with 70% ethanol for 30 min. The beads were resuspended in water and the polystyrene core was dissolved by adding tetrahydrofuran (THF) to the solution (1:1 v/v) for 2 min. The silk hollow spheres were then spun down, washed twice with DI water and then with 70% ethanol. The beads were then lyophilized for 24 h at −80 °C and stored in dried condition at 4 °C for subsequent experiments.

2.3. Sample preparation for scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The samples were vacuum dried and coated with gold using a gold sputter coater (EMITECH K550X, UK) at 25 mAmp for 1 min, to form a 15–20 nm thick coating. The samples were imaged on a Hitachi S-4700 scanning electron microscope. For TEM (Hitachi H7500 TEM), the samples were taken in DI water suspension and loaded onto the TEM grid for subsequent imaging.

2.4. Analysis of polystyrene removal

To confirm complete removal of polystyrene core from the microspheres following THF treatment, an Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR) analysis of the spheres was performed after crushing them in a mortar and pestle to expose any traces of polystyrene present within the core of silk microspheres. Particular attention was given to the characteristic peaks of polystyrene and silk in the obtained spectrum. Uncoated polystyrene beads and hollow silk fibroin spheres were examined after vacuum drying on Varian 660-IR ATR-FTIR instrument.

2.5. Size and zeta potential analysis

The particle size and zeta potential of silk hollow microspheres were analyzed by using dynamic light scattering (Zeta sizer, Nano-ZS90, Malvern). The DLS measurements were performed with a detection angle of 90° at a wavelength of 630 nm at room temperature. For particle size analysis, the silk hollow microspheres were

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