Thermo-sensitive injectable glycol chitosan-based hydrogel for treatment of degenerative disc disease

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\begin{abstract}
The use of injectable hydrogel formulations have been suggested as a promising strategy for the treatment of degenerative disc disease to both restore the biomechanical function and reduce low back pain. In this work, a new thermo-sensitive injectable hydrogels with tunable thermo-sensitivity and enhanced stability were developed with $N$-hexanoylation of glycol chitosan (GC) for treatment of degenerative disc disease, and their physico-chemical and biological properties were evaluated. The sol-gel transition temperature of the hydrogels was controlled in a range of 23–56 °C, depending on the degree of hexanoylation and the polymer concentration. In vitro and in vivo tests showed no cytotoxicity and no adverse effects in a rat model. The hydrogel filling of the defective IVD site in an ex vivo porcine model maintained its stability for longer than 28 days. These results suggest that the hydrogel can be used as an alternative material for treatment of disc herniation.
\end{abstract}

1. Introduction

Disc herniation is one of the most common causes of low back pain, which is an age-related complication most commonly caused by intervertebral disc (IVD) degeneration, a condition that has significantly increased recently (Anderson & Tannoury, 2005; Luoma et al., 2000; Urban & Roberts, 2003). Numerous therapeutic options have been developed, including fusion, disc replacement and discectomy (Larson, Levicoff, Gilbertson, & Kang, 2006; Yabuki, 1999). Despite the development of a vast array of therapeutics, the currently available treatment for disc herniation is not able to completely restore or repair the biomechanical function of the degenerated disc, and many patients continuously suffer from low back pain even after treatment (An et al., 2003; Lee, 1988). Therefore, advanced therapeutic strategies are needed for improved disc herniation healing to overcome the existing barriers via a convenient treatment procedure. Recent progress in biomaterials-based three-dimensional (3D) hydrogel scaffold research for IVD tissue engineering has suggested a promising strategy that can both restore biomechanical function and reduce low back pain (Ahrrens et al., 2009; Iatridis, Nicoll, Michalek, Walter, & Gupta, 2013; Li et al., 2008; Stagni et al., 2012; Wei, Brisby, Chung, & Diwan, 2008; Whatley & Wen, 2012). Accordingly, design and development of a biocompatible and thermo-sensitive hydrogel that shows high stability and imitates the IVD has attracted much attention for the treatment of degenerative disc disease.

Various biomaterials have been used to mimic the natural IVD extracellular environment. Cloyd et al. (Cloyd et al., 2007) and Revel et al. (Revel et al., 2007) showed that hyaluronic acid (HA)-based hydrogels have the ability to mimic the natural IVD extracellular environment, including characteristics such as elastic modulus, Poisson’s ratio, and stress relaxation properties of the nucleus pulposus (NP). The synthetic HA-based hydrogels approximated the NP well and the injected discs had a central NP-like region which had a close similarity to the normal biconvex structure and contained viable chondrocytes forming matrix like that of normal disc. Leone et al. (Leone, Torricelli, Chiumento, Facchini, & Barbucci, 2008) reported that amide alginate hydrogels exhibited the thixotropic behavior of native nucleus tissue. From a rheological point of view, the amide alginate hydrogel showed a behavior, in terms of complex modulus and phase shift angle, strictly comparable with that of nondegenerated human NP, and so can be...
considered as an attractive material for the NP replacement. Although those chemically cross-linked hydrogel systems have been generally studied, these methods often suffer from limitations in controlling their stability and rely on the use of chemical cross-linkers that might be toxic to cells or tissues. On the other hand, physically cross-linkable hydrogels have been recently developed to fabricate injectable systems that can create 3-dimensional macromolecular structures without the use of any additional toxic cross-linkers or solvents (Drury & Mooney, 2003; Ruel-Gariepy & Leroux, 2004; Ta, Dass, & Dunstan, 2008). In particular, thermo-sensitive hydrogels have been considered as a useful system for various biomedical applications because they can maintain a flowing liquid state at room temperature but instantaneously form a non-flowing gel state at body temperature (Berger et al., 2005; Chenite et al., 2000; Roughley et al., 2006). Thermo-sensitive hydrogels are easily prepared without application of severe conditions such as pH adjustments, oxidants or UV light and also support patient compliance with non-invasive surgical procedures for treating irregularly shaped tissue sites (Cavalcanti, Zeitlin, & Noer, 2013; Galler, Hartgerink, Cavender, Schmalz, & D’Souza, 2012; Hatefi & Amsden, 2002; Yu & Ding, 2008; Zhan et al., 2016). To date, experimental approaches have largely focused on the fabrication of chemically cross-linked hydrogels to treat degenerated IVD, whereas only a few studies have attempted to engineer physically cross-linkable thermo-sensitive hydrogels to treat degenerated IVD using minimally invasive procedure without non-toxic cross-linker.

Chitosan is a naturally derived non-sulfated glycosaminoglycan that is widely used in various biomedical applications due to its biodegradability, low toxicity, non-immunogenic and mucoadhesive properties (Li, Maciel, Rodrigues, Shi, & Tomas, 2015; Nagpal, Singh, & Mishra, 2010). Chitosan is a linear and partly acetylated (1→4)-2-amino-2-deoxy-β-D-glucan isolated from chitin via alkaline hydrolysis (Muzzarelli, Ilari, Tarsi, Dubini, & Xia, 1994). The presence of different functional groups such as amino groups and hydroxy groups in the polymer chains makes chitosan chemically active, facilitating various chemical modifications and efficient introduction of diverse biofunctional groups. Chitosan-based hydrogels have been prepared through chemical or physical cross-linking of the functional groups in the polymer chains. Chitosan-based thermogelling systems have been prepared using various formulations, such as combination of chitosan and glycerol-phosphate, poly(ethylene glycol)-grafted chitosan, poly(vinyl alcohol)/chitosan blends and other materials (Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005; Cao et al., 2007; Chenite et al., 2000; Tang, Du, Hu, Shi, & Kennedy, 2007). Although these hydrogels demonstrate thermogelling properties in a physiological temperature range, the use of excess glycerol phosphate, complicated multi-step reactions with organic solvents or chemical agents, and an irreversible gelation mechanism limit their practical application in biomedical fields. Moreover, the poor water solubility of chitosan under physiological conditions limits its practical use in hydrogel materials (Dash, Chiellini, Ottenbrite, & Chiellini, 2011). A water-soluble chitosan derivative with glycol residues and glycol chitosan (GC) retains the physico-chemical and biological properties of chitosan and is more suitable for pharmaceutical and biomedical applications (Knight, Shapka, & Amsden, 2007).

The objective of this study was to develop a thermo-sensitive injectable N-hexanoyl glycol chitosan (HGC) hydrogel system for percutaneous treatment of disc herniation to both restore the biomechanical function and reduce low back pain. A series of HGCs were synthesized via a simple N-hexanoylation reaction of glycol chitosan using hexanoyl anhydride, and the physico-chemical and thermo-sensitive properties of this material were thoroughly characterized using various analytical methods, including 1H NMR, FT-IR, rheometry and SEM. HGC hydrogel showed thermo-reversible sol-gel transition at a low concentration (3–7 wt%). The biocompatibility of the thermo-sensitive HGC hydrogels was examined by comparing the serum biochemistry and histology of HGC-treated rats to those of saline-treated rats. Finally, the long-term stability of injectable HGC hydrogel for percutaneous treatment of disc herniation was monitored in ex vivo studies.

2. Materials and methods

2.1. Materials

Glycol chitosan (GC, DP ≥ 200, degree of acetylation = 9.34 ± 2.5% as determined by 1H NMR) was purchased from Wako (Japan). Hexanoic anhydride (97%), neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride), chondroitinase ABC and titanium (Ti) were purchased from Sigma-Aldrich (St Louis, MO, USA). Lysozyme (63,564 units per mg) was purchased from Sigma-Aldrich (Canada). Acetone, methanol, ethanol and sodium hydroxide solution (1 M) were supplied by Samchun Chemical (Korea). All chemicals were of analytical grade and used as received without further purification.

2.2. Synthesis of N-hexanoyl glycol chitosans

N-hexanoyl glycol chitosans (HGC) were synthesized by reaction of the amine groups of GC with hexanoyl anhydride under mild conditions. In brief, 1 g of GC was dissolved in 125 mL distilled water and diluted with 125 mL methanol. Hexanoic anhydride (0.25–0.43 mL) was added into the GC solution under magnetic stirring. After continuous stirring at room temperature for 48 h, HGC was precipitated in acetone (3 L) and collected by centrifugation, followed by dialysis against distilled water for 3 days using a dialysis membrane (MWCO = 2000 Da) and lyophilization.

2.3. Characterization of HGC polymer

1H NMR spectra of GC and HGCs were obtained on a JNM-AL400 spectrometer (Jeol Ltd, Akishima, Japan) operating at 400 MHz. GC and HGCs were dissolved in D2O at a concentration of 1.0 wt% in each tube. The D2O peak at 8 4.65 was used as a reference peak. FT-IR analysis was also performed to confirm the composition of HGC. FT-IR spectra of GC and HGCs were recorded using potassium bromide pellets on a MAGNA 560 spectrometer (Nicolet, USA). Each sample was scanned 32 times at a resolution of 4 cm⁻¹ over a frequency range of 4000–400 cm⁻¹.

2.4. Characterization of HGC hydrogels

2.4.1. Thermo-sensitive sol-gel transition behavior study

The thermo-sensitive sol-gel transition behavior of HGC solutions was investigated using the tube inversion method with a heating rate of 0.2 °C/min. The polymer solutions were prepared with concentrations of 3–7 wt% by dissolving HGC in PBS solution (pH 7.4, 0.01 M) at 4 °C. The sol-gel transition temperature was determined by a flow or non-flow criterion with the tube inverted for 30 s. Each data value represents the average with standard deviation (SD) obtained from three measurements. The sol-gel transition phase diagram obtained using this method is known to have a precision of ± 1 °C (Loh, Goh, & Li, 2007).

2.4.2. Rheological analysis

Rheological analysis of HGC solutions was performed using a rotating rheometer (Bohlin Advanced Rheometer, Malvern Instruments, UK) with a temperature-controlled system. The temperature was controlled from 10 to 50 °C. The elastic modulus G’ and viscous modulus G” of the HGC solutions were recorded as a function of temperature using parallel plate geometry (diameter = 20 mm) at a frequency of 1 Hz and constant stress (25 Pa). Viscosity analysis was also performed using a viscometer (RVDV-III +; Brookfield Instruments, USA). The temperature range of the measurements was 5 to 50 °C with a heating rate of 0.34 °C/min at a shear rate of 0.1/s.
2.4.3. In vitro gel stability and injectability

HGC solution (DH = 36.5%, conc. = 4 wt%, 1 mL) containing Neutral Red dye (40 μg/mL) was prepared in a 5 mL vial. After increasing the temperature to 37 °C (at which the HGC solution changed to a gel state), an excess amount of PBS solution was added on the top of the hydrogel and kept in a shaking water bath at 37 °C for 24 h to investigate gel stability. Additionally, the HGC solution was injected into excess PBS at 37 °C through a 22-gauge syringe needle to evaluate the practical feasibility of HGC for use as an injectable hydrogel.

2.4.4. Hydrogel morphology

The morphology of HGC hydrogels was observed under field emission scanning electron microscopy (FE-SEM; JSM-7000F; JEOL, Japan) at 15 kV. Freeze-dried hydrogels were prepared by quenching HGC hydrogels (polymer conc. = 3 wt%, 4 wt% and 5 wt%) in liquid nitrogen followed by lyophilization. The freeze-dried HGC hydrogels were coated with platinum by sputtering for 40 s at 20 mA before observation of the hydrogel morphology.

2.4.5. In vitro enzymatic biodegradation

In vitro biodegradation of HGC hydrogels was examined by measuring the weight change of the HGC hydrogels during incubation with or without lysozyme. Enzymatic degradation experiments were performed at 37 °C. HGC solutions (DH 36.5%, 4 wt% and 6 wt%, 1 mL) were prepared in 5 mL vials and allowed to incubate in a water bath (Series BS-21; Lab companion, Korea) at 37 °C for 10 min to form a gel. Lysozyme was dissolved in DI water at 55 μg/mL, and 3 mL of DI water containing lysozyme was added on the top of the hydrogels. At a specific time point, the solution was removed, and the residual hydrogel was lyophilized to obtain the dry weight (W_d). The weight% of hydrogels was determined by comparing the W_d after degradation with

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**Table 1** Synthetic results of HGCs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hexanoic anhydride /glucosamine residue</th>
<th>DH (%)</th>
<th>T_gel (°C)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGC1</td>
<td>0.2</td>
<td>19.0 ± 1.6</td>
<td>–</td>
<td>76.4</td>
</tr>
<tr>
<td>HGC2</td>
<td>0.3</td>
<td>28.2 ± 2.0</td>
<td>51</td>
<td>78.8</td>
</tr>
<tr>
<td>HGC3</td>
<td>0.4</td>
<td>36.5 ± 2.0</td>
<td>23</td>
<td>82.3</td>
</tr>
<tr>
<td>HGC4</td>
<td>0.5</td>
<td>–</td>
<td>Water-insoluble</td>
<td>70.2</td>
</tr>
</tbody>
</table>

*a Feed molar ratio of hexanoic anhydride to glucosamine residue.

*b Degree of hexanoylation determined by the peak integration of 1H NMR.

*c Sol-gel transition temperature measured by the tube inversion method (Polymer Conc. = 5 wt%).

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**Fig. 1.** Synthesis scheme of HGC (a), and 1H NMR (b) and FT-IR (c) spectra of GC and HGCs.
the initial weight (W_i). The resulting data are representative of triplicate experiments and presented as the mean values with standard deviation (mean ± SD).

2.5. In vitro and in vivo toxicity evaluation

2.5.1. Cytotoxicity assay

Normal human dermal fibroblast (NHDF) cells were purchased from the American Type Cell Culture Collection (CCL-2, ATCC, Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotics (Gibco, USA) under 5% CO_2 in a humidified atmosphere. The cytotoxicity assay was performed in a 96-well tissue culture plate (Costar, Corning, NY, USA) at a density of 1 × 10^4 cells/well incubated in 100 μL of DMEM/well overnight. NHDF cells were fed with 100 μL culture media containing a serial dilution of GC and HGCs (concentration: 0.3–5 mg/mL) and were cultured for 24 h and 72 h. At each time interval, the culture media was removed and 100 μL Neutral Red solution with a final concentration of 5 μg/mL in DMEM was added to each well of the plate, followed by incubation at 37 °C for 2 h. The culture media containing Neutral Red solution was removed, and the plate was gently washed twice with PBS. An amount of 150 μL Neutral Red destaining solution prepared via a given protocol was added to each well, and the absorbance was measured using a microplate reader (Spectramax 250, Molecular Device Inc., Sunnyvale, CA) at a wavelength of 540 nm. The experimental results were obtained from the average values measured from eight independent experiments.

2.5.2. Live/dead assay

Final volume of 100 μL HGC solution (DH 36.5%, final concentration of 4 wt%) mixed with NHDF cells (1 × 10^5 cells/ml) was added into each well of a 12-well plate and incubated at 37 °C for 10 min under 5% CO_2 incubator for gelation. An amount of 1.5 mL cell culture media was added to each well and was changed every other day. The viability of
cells loaded in the HGC hydrogels was verified by Live/Dead staining assay. In brief, cell-seeded HGC hydrogels were washed with PBS solution and stained with 200 \( \mu \text{L} \) of a “Live/Dead” dye solution (2 \( \mu \text{M} \) calcein and 4 \( \mu \text{M} \) ethidium homodimer) for 30 min. After staining, the HGC hydrogels were gently washed twice with PBS solution and observed under a fluorescence microscope (Leica DMI4000B, Germany). Live cells exhibited green fluorescence due to their intracellular esterase activity with green fluorescent calcein-AM, and dead cells fluoresced with red color because their compromised membranes were permeable to nucleic acids stained by the red fluorescent ethidium homodimer.

2.5.3. In vivo toxicity analysis

SD rats (six-week-old males, Orient Bio, Seoul, Korea) were randomized into PBS, fibrin gel (Greenplasts, Greencross PD Co., YongIn, Korea), and HGC hydrogel treatment groups (n = 5 per group). PBS solution was used as a control, and fibrin gel, which is a known biocompatible hydrogel, was used as a comparison. Each rat received 0.2 mL PBS, 0.2 mL fibrin gel or HGC hydrogel (4 wt% in PBS) delivered into the hind limb muscle through injection. Blood samples (1 mL) were drawn through the tail vein at days 1, 3, and 7 after injection. Complete blood count (CBC) analyses were performed using a hematology system (ADVIA 2120i, Inc., SIEMENS, Munich, Germany). The rats were sacrificed, and hind limb muscles containing the hydrogels were fixed in 10% neutral buffered formalin for at least 3 days and embedded into paraffin. Sections with a thickness of 4 \( \mu \text{m} \) were stained with hematoxylin and eosin for histological evaluation. The slides were subsequently examined on an Aperio slide scanner, (Aperio Scanner. Inc., Leica biosystem, St Louis, USA).

2.6. Ex vivo model

2.6.1. Evaluation of ex vivo degenerative IVD culture

All animals used in the ex vivo study were adult male micro-pigs (2–3 month, 15–20 kg) obtained from the barrier unit at the Korea Animal Medical Science Institute Co., Ltd. All animals were housed in single pens under controlled conditions (the temperature was held between 18 and 22 °C, and the relative air humidity was 30–70% with 15 air circulations per hour) with a 12:12-h light/dark cycle and allowed free access to water. Principles of laboratory animal care were followed in accordance with the Guide for Animal Experimental Protocol (IACUC, 15-KE-105), and the study was approved by the Institutional Animal Care and Use Committee of Korea Animal Medical Science Institute Co., Ltd. The standard feeding regime was applied to all
animals.

The procedures for evaluation of ex vivo intervertebral disc (IVD) degenerative tissue system were conducted. Micro-pigs were sacrificed, and the complete IVD tissues including the partial vertebrate body (VB), endplates (EP), nucleus pulposus (NP) and annulus fibrous (AF) of the lumbar spine from T13 to L5 (6 IVDs/porcine) were harvested under aseptic conditions. Chondroitinase-ABC was injected into the central region of the disc with a 22-gauge spinal needle to create disc degeneration. Subsequently, IVD tissues were cultured in DMEM and incubated at 37 °C with 5% CO2 for 1 week or 2 weeks, respectively, to observe CT or MR images.

2.6.2. Macroscopic observation

After the establishment and culture of degenerative ex vivo tissues, three mixtures of PBS with 2% titanium (Ti) (n = 8), alcohol with 2% Ti (n = 8), and HGC hydrogel (4 wt%) in alcohol and with 2% Ti (n = 8) were injected into the degenerated sites. PBS with 2% Ti and alcohol with 2% Ti were used as controls. In succession, the ex vivo IVD organs were incubated in DMEM at 37 °C with 5% CO2 during the designated time intervals (1 day and 28 days). After ex vivo culture of the tissue groups containing PBS, alcohol and HGC hydrogel (DH: 36.5%, PC: 4 wt%) in alcohol, the treated tissues were harvested and bi-sectioned in the transverse plane to observe the morphology of IVD, especially the nucleus pulposus (NP) and annulus fibrosus (AF).

2.6.3. Radiological image analysis using micro-CT and MRI

Micro-computed tomography image analysis of treated tissues from the experimental groups was performed using a micro-CT (Skyscan 1172, SkyScan, Belgium) with a resolution of 8.81 um (59 kv, 167 uA) at day 1 and day 28 after injection into the degenerated disc sites. The images were reconstructed using NRecon software (Skyscan, Belgium).

In parallel, specimens of treated tissues with 5 wt% barium sulfate as a radio-opaque material instead of Ti powder were used to obtain the MRI images after 2 weeks for the disc degeneration process. The images were analyzed using magnetic resonance imaging (MRI, Magnetom Essenza, Siemens Co., Germany) at designated time points before and after injection of mixtures with the following conditions: distance of 30 inches, exposure of 0.5 mAs, and penetrating power of 60 kVs. MR images were obtained with a 1.5-T magnetic field MR imaging unit. The sequence used a spin echo sequence (sagittal image TR/TE = 2300/110 msec, transverse image TR/TE = 3000/86 msec) for the MR images. The slice thickness of each section was 2.5 mm, and the scan time per slice was 2 min 9 s.

2.6.4. Histological analysis

Specimens imaged under micro-CT were processed for histological examination using H&E and alcian blue staining after fixation with 4% paraformaldehyde solution for 1 day and decalcification with decal solution (Decalifying Solution-Lite, Sigma, USA) over 2 weeks. Paraffin-embedded tissue sections cut with a rotary microtome (Leica RM 2165, Wetzlar, Germany) were stained with H&E and alcian blue and observed by light microscopy (Olympus BX51, Olympus, Center Valley, PA, USA).

2.7. Statistical analysis

Statistical analysis was performed using Origin pro version 8 software package (OriginLab Corp, MA, USA) to determine the statistical differences. The experimental data are presented as the mean ± standard deviation and were performed with one-way analysis of variance (One Way ANOVA). A value of p < 0.05 was considered statistically significant.
3. Results and discussion

3.1. Synthesis and characterization of HGCs

A series of HGCs were synthesized with N-hexanoylation of glycol chitosan using hexanoyl anhydride (Fig. 1(a)). The degree of hexanoylation (DH) was effectively controlled by varying the feed molar ratio of hexanoyl anhydride to the amino groups of glycol chitosan. The results of HGCs synthesis are summarized in Table 1 (DH: 19.0 ± 1.6 ∼ 36.5 ± 2.0%; Yields: 70.2 ∼ 82.3%). The DH of HGC was proportionally dependent on the feed molar ratio. The higher DH of HGC (36.5 ± 2.0%) was produced from a higher feed molar ratio (0.4), and the lower DH (19.0 ± 1.6%) was obtained using a smaller ratio (0.2), as shown in Table 1. The HGC synthesized from the 0.2 feed molar ratio, HGC1 (DH: 19 ± 1.6%), did not show noticeable thermosensitive gelation behavior, which was thought to be due to a low content of hydrophobic hexanoyl groups. However, when the feed molar ratio increased to 0.5, HGCs became water-insoluble due to increased hydrophobicity with the introduction of an excessive amount of hexanoyl groups. HGCs solution (5 wt%) with DH ranging from 28.2 to 36.5% showed a thermo-sensitive sol-gel transition at 23–51 °C. These thermo-sensitive sol-gel transition properties of HGC solutions result from the enhanced hydrophobic interaction among hexanoyl groups with the increasing temperature, and their transition point can be modulated by varying the content of hexanoyl groups in the glycol chitosan backbone.

The chemical structures of GC and HGCs were characterized by analysis of 1H NMR and FT-IR spectra. Fig. 1(b) presents the 1H NMR spectra of GC and HGCs, which confirm the successful introduction of hexanoyl groups into the glycol chitosan backbone. The 1H NMR spectra of GC and HGCs, which confirm the successful introduction of hexanoyl groups into the glycol chitosan backbone. The D2O peak at 4.65 ppm is used as a reference peak. The overlapped peaks at 3–4 ppm are attributed to the protons of the glucopyranosyl ring at positions 2–8.

Fig. 5. In vivo toxicity of PBS (control), fibrin gel, and HGC hydrogels evaluated in SD rats via (a) complete blood count (CBC) of 9 important cells and (b) histological analysis.
The peak at 2.6 ppm arises from the protons of the primary amine residue. The peak at 1.89 ppm is assigned to the N-acetyl protons of GC. The new proton peaks at 0.7, 1.2, 1.5 and 2.2 ppm are assigned to \(\text{eCH}_3\), \(\text{eCH}_2\text{-CH}_2\text{-CH}_3\), \(\text{eCO}\text{-CH}_2\text{-CH}_2\text{-CH}_3\), and \(\text{eCO}\text{-CH}_2\text{-CH}_2\text{-CH}_3\) of the hexanoyl groups, respectively. Based on these peak assignments, the average DH of HGC was calculated by comparing the integrated signal area of the protons of the glucopyranosyl ring with those of hexanoyl groups.

As shown in Fig. 1(c), FT-IR spectra of GC and HGC were also analyzed to confirm the conjugation of hexanoyl groups on GC by observing the amide bond formation. The broad band at 3400 cm\(^{-1}\) is assigned to the stretching vibration of hydroxyl groups, which overlaps the N–H stretching vibration in the same region. The absorption peaks at 2890 cm\(^{-1}\) are ascribed to \(\text{eCH}_2\) groups. The absorption peak at 1596 cm\(^{-1}\) is attributed to the amino-bending vibration of GC, and the appearance of absorption bands at 1655 cm\(^{-1}\) and 1555 cm\(^{-1}\) corresponds to the carbonyl stretching and the amide II bending vibration of HGCs. The disappearance of the amino vibration band at 1596 cm\(^{-1}\) and the appearance of the amide II band at 1555 cm\(^{-1}\) after hexanoylation indicate that the HGCs were successfully synthesized by introducing hexanoyl groups. These results demonstrate that HGC was successfully synthesized and that the hexanoylation of glycol chitosan was a successful process.
primarily occurred at the amino group instead of the O-position on the hydroxyl group.

3.2. Thermo-sensitive sol-gel transition of HGCs

The thermo-sensitive sol-gel transition properties of HGC solutions were investigated using the tube inversion method, viscometry and rheometry. Fig. 2(a) shows the sol-gel transition phase diagram of HGCs solutions obtained by the tube inversion method. Various HGC solutions were prepared by dissolving HGCs with DH ranging from 19.0 ± 1.6 to 36.5 ± 2.0% in PBS solution (pH 7.4, 0.01 M) at a concentration range of 3–7 wt%. Among the solutions, HGC2 (DH: 28.2%) and HGC3 (DH: 36.5%) solutions demonstrated a distinct phase transition from a flowing sol state to a non-flowing gel state as the temperature increased, whereas no thermo-sensitive gelation behavior was observed for GC and HGC1 (DH: 19%). The sol-gel transition temperature of the HGC solution could be well controlled from 23 to 51 °C by varying the DH and HGC concentrations. With increasing DH or polymer concentration, the sol-gel transition temperature was observed to decrease, probably caused by a higher physical cross-linking density formed by the enhanced hydrophobic interactions among the hexanoyl groups of HGC.

Temperature-dependent viscosities of HGC3 (DH 36.5%) solutions were observed for different HGC concentrations (2, 4, 5, 6 wt%), as shown in Fig. 2(b). Sharp increases in the viscosities of HGC3 solutions with concentrations of 4 and 5 wt% were observed at 29.80 °C and 19.90 °C, respectively. The sol-gel transition of the 6 wt% HGC3 solution occurred below room temperature, which makes this material difficult to handle. It should be noted that the critical gelation concentrations (CGCs) of thermo-sensitive HGC solutions used in this work (3–5 wt%) are much lower than those of other thermogelling polymers such as poly(ε-caprolactone)/poly(ethylene glycol) copolymers (25 wt %) and Pluronics (typically 20–50 wt%). Such high CGCs might alter lipid metabolism when injected in vivo (Dumortier, Grossiord, Agnely, & Chaumeil, 2006; Jiang, You, Gu, Hao, & Deng, 2008).

The viscoelastic properties of HGC3 (4 wt%) and GC (4 wt%) solutions with increasing temperature are shown in Fig. 2(c) and (d), respectively. This rheological analysis yielded more reproducible and quantitative results compared to the tube inversion method (Jeong, Wang, & Gutowska, 2001). Fig. 2c shows the elastic modulus (G') and viscous modulus (G") of HGC3 (DH 36.5%, 4 wt%) as a function of temperature. The G' values are smaller than the G" values of the HGC3 at the beginning, where the HGC3 exhibited the viscous fluid behavior corresponding to the sol state. With increasing temperature, both the elastic (G') and viscous (G") moduli increased rapidly as gelation proceeded, and specifically, the buildup rate of G’ was much faster than...
that of G’. The difference in the elevation rate of G’ and G” produces a crossover (defined as the gelation point), indicating the transition from a liquid phase in which a viscous property predominates a solid phase in which an elastic property dominates (Winter & Chambon, 1986). In comparison, the G’ values of the GC solution were continuously lower than the G” values without the appearance of a crossover point over the entire temperature range, which indicates that no thermo-sensitive sol-gel transition occurred in the GC solution.

To confirm the practical feasibility of HGC as a new thermogelling polymer, the in vitro gel stability and injectability of the HGC solution were investigated. With an increase in the temperature to body temperature (37 °C), HGC3 solution (4 wt%) with a red color (due to the added Neutral Red dye) changed from a liquid state to a solid-like hydrogel state, as shown in Fig. 2(e) (upper left and middle). The hydrogel maintained its three-dimensional structure without dissolution or disintegration after addition of an excess amount of PBS on the top of the hydrogel at 37 °C (Fig. 2(e) upper right and below left). In parallel, as shown in Fig. 2(e) (below middle and right), the HGC3 solution was injected through a syringe needle into PBS at 37 °C, which determines the feasibility of HGC hydrogel for use as an injectable hydrogel. As soon as the HGC3 solution was injected, HGC3 formed a hydrogel immediately in PBS at 37 °C. These in vitro gel stability and injectability results demonstrate that HGC can serve as a promising material useful for non-invasive needle injection formulations to treat degenerative disc disease or other biomedical applications such as drug delivery and tissue engineering.

3.3. Hydrogel morphology and in vitro biodegradation

To investigate the morphology of HGC hydrogels, FE-SEM images of the surfaces and cross-section morphologies of the HGC3 hydrogels were obtained, as shown in Fig. 3(a) and (b), respectively. The HGC3 hydrogels were highly macroporous, and their pores were well interconnected. The pore sizes were generally in the range of 2–20 μm. The highly macroporous and well interconnected structure resulting from the three-dimensional HGC hydrogel network is a beneficial feature that makes the HGC hydrogel more appealing for use as an injectable hydrogel scaffold to support cell growth and migration. To ascertain whether the HGC hydrogel is biodegradable by lysozyme, which is known to degrade chitosan and is a cationic protein widely present in human body fluids (e.g., serum, saliva, and tears in vitro) (Cho, Cho, Chung, Yoo, & Ko, 1999; Tomihata & Ikada, 1997), the weight losses of HGC3 hydrogels (DH 36.5%, 4–6 wt%) were monitored at designed time intervals during incubation with and without lysozyme at 37 °C. The weight percentages of the hydrogels were determined by comparing their dry weight after degradation to their initial weight.

HGC3 hydrogels (DH 36.5%, 4–6 wt%) incubated in DI water without lysozyme do not show any significant mass loss over 28 days of incubation for both 4 wt% and 6 wt%, as displayed in Fig. 3(c). However, in the presence of lysozyme, HGC3 hydrogels show an obviously noticeable weight loss of 30.8±41.7% depending on the polymer concentration. Compared with the 6 wt% HGC3 hydrogels, the 4 wt% HGC3 hydrogels exhibit a relatively faster degradation profile, probably because the 6 wt% HGC3 hydrogels have a higher physical cross-linking density. The degradation behavior of hydrogels is known to be dependent on the cross-linking density, and a larger extent of cross-linking typically produces more stable hydrogels (Lee, Bouhadir, & Mooney, 2000). As a result, thermo-sensitive HGC hydrogels are biodegradable in the presence of lysozyme, and their degradation rate is dependent on the polymer concentration.

3.4. In vitro and in vivo toxicity evaluation

The cytotoxicity of HGC was investigated using NHDF cells. Fig. 4(a) and (b) show the in vitro cytotoxic effect of GC and three HGCs, i.e., HGC1 (DH: 19 ± 1.6%), HGC2 (DH: 28.2 ± 2.0%) and HGC3 (DH: 36.5 ± 2.0%), on cultured NHDF cells in a range of polymer concentrations from 0.03 to 0.5% after 24 h (Fig. 4(a)) and 72 h (Fig. 4(b)) of incubation. Overall, the polymer solutions show almost no cytotoxicity. Even at the highest polymer concentration (0.5 wt%), GC and HGCs exhibit notably low cellular toxicity to cultured NHDF cells, displaying 90.2% and 89.5% cell viability for the HGC1 solution after incubation for 24 h (Fig. 4(a)) and 72 h (Fig. 4(b)), respectively.

Live/Dead staining assay of NHDF cells encapsulated in HGC hydrogels was performed to confirm the cytotoxicity of HGC hydrogels, and the results were observed by fluoresce microscopy. Fig. 4(c) and (d) display the stained cells (living cells stained with calcein-AM (green) and dead cells stained with ethidium homodimer (red)) residing in the HGC3 hydrogels (DH 36.5%, 4 wt%) after 24 h and 72 h incubation, respectively. The cells were uniformly distributed in the HGC 3 hydrogels. Most of the cells loaded into HGC 3 hydrogel successfully survived after 72 h incubation (Fig. 4(d)), indicating that the HGC 3 hydrogel may not exert any significant toxicity to the encapsulated cells and be useful as an injectable biomaterial for tissue engineering and other related biomedical fields.

In vivo complete blood count (CBC) analysis was conducted to evaluate the blood compatibility with foreign materials for which injected hydrogels might significantly change the CBC data. However, the total white blood cell count (WBC) was unchanged during 7 days following injection of HGC hydrogel (Fig. 5(a)). The HGC hydrogel-treated groups showed values for neutrophils and lymphocytes that were in the normal range. Furthermore, the immune responses at the injection sites remained at moderate levels for 7 days (Fig. 5(b)). No adverse effects of the HGC hydrogel were observed in the injected sites. These results suggested that HGC hydrogel could be used as a biologically safe and non-toxic material for biomedical applications.

3.5. Macroscopic observation and radiological tomography image analysis

A porcine ex vivo model was selected to mimic human lumbar segments due to their similar anatomy and physiology (Drespe, Polzhofer, Turner, & Grauer, 2005). An ex vivo degenerative intervertebral disc (IVD) tissue culture system was established (see Fig. 1(a)). Alcohol has been reported as an effective chemonucleolysis agent for treatment of disc herniation with good clinical results and no allergic complications (Riquelme, Musacchio, Mont’Alverne, & Tournade, 2001). However, the problem with pure alcohol is that the high diffusibility of the liquid leads to radicular burning pain after the procedure (Theron et al., 2010). Accordingly, in this study, HGC hydrogel (DH 36.5%, 4 wt%) dissolved in alcohol was used in treatment of disc herniation and was also compared with the use of alcohol and PBS. As shown in Fig. 6(b), the small amount of Ti (2 wt%) added into each group as a radiopaque material is observed in the form of black particles and produces stable micro-CT images because the Ti particles act as a marker for discrimination between the original tissues and injected mixtures.

The macro-morphology of the nucleus pulposus (NP) shown from the HGC hydrogel-treated group displays a more water-preserved appearance compared with those from the PBS- and alcohol-treated groups after 1 day and 28 days of culture (Fig. 6(b) above). In the micro-CT images (Fig. 6(b) below), the HGC hydrogel fills the irregular defective NP region and maintains its original gel state for 28 days, whereas PBS and alcohol showed rapid diffusion out of the injected site.

MRI images for IVD tissues obtained before and 2 weeks after disc degeneration with chondroitinase-ABC are shown in Fig. 6(c). The MRI images of the central portion of degenerative NP region, where HGC hydrogel (DH: 36.5% and PC: 4 wt%) dissolved in alcohol (30 vol%) with 5 wt% barium sulfate (a contrast for obtaining stable MRI image) was injected, were subsequently displayed. The integrity of HGC hydrogel filling the degenerated NP region was maintained for 5 weeks. This result is thought to be due to the highly differentiated tissue environment with the avascular nature of IVD without lysozyme in the NP region, as reported previously (Humzah & Soames, 1988). These results
of ex vivo studies show that HGC hydrogel has high physical stability without obvious mass loss over 5-week cultivation and suggest that the thermo-sensitive injectable HGC hydrogel could be used as an alternative material system for treatment of disc herniation using minimally invasive procedure.

3.6. Histological analysis

Histological sections of degenerative IVD tissue treated with PBS, alcohol and HGC hydrogel and incubated for 1 day and 28 days are shown in Fig. 7. H&E staining and alcin blue staining demonstrate that HGC hydrogel filled the degenerated NP region and maintained its stability for 28 days, whereas the PBS- and alcohol-treated groups showed rapid dissolution. This result was confirmed with the dispersion condition of the radio-opaque Ti particles. The particles were well dispersed in the HGC hydrogel in IVD tissue after 1 day and 28 days of incubation but were aggregated or were not found in the PBS- and alcohol-treated groups due to the rapid dissolution of PBS or alcohol. As a result, filling of thermo-sensitive injectable HGC hydrogel into defected tissue might offer great promise as a new thermogelling platform for treatment of disc herniation using minimally invasive procedure.

4. Conclusions

Thermo-sensitive injectable N-hexanoyl glycol chitosan (HGC) hydrogels were successfully synthesized and used to develop a minimally invasive needle injection formulation for treatment of disc herniation. The HGC hydrogels exhibited a thermo-sensitive sol-gel transition (25 − 56 °C) that likely resulted from hydrophobic interactions between hexanoyl groups and depended on the degree of hexanoylation (DH) and polymer concentration. The long-term gel stability, injectability, and biocompatibility were determined in vitro and in vivo studies. When injected into the NP space of the degenerated IVD in a porcine ex vivo model, HGC hydrogel filled in the irregular defective NP region and maintained its gel stability even over 28 days. According to these results, biocompatible and thermo-sensitive injectable HGC hydrogel is highly promising as a new thermogelling platform for nonsurgical treatment of disc herniation.

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Dumortier, G., Grossiord, J. L., Agney, F., & Chaumet, J. C. (2006). A review of porcine IVD tissue after 1 day and 28 days of incubation but were aggregated or were not found in the PBS- and alcohol-treated groups due to the rapid dissolution of PBS or alcohol. As a result, filling of thermo-sensitive injectable HGC hydrogel into defected tissue might offer great promise as a new thermogelling platform for treatment of disc herniation using minimally invasive procedure.

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