Journal of Materials Chemistry B

PAPER

View Article Online
View Journal | View Issue

Cite this: J. Mater. Chem. B, 2014, 2, 8346

Fabrication of modified dextran–gelatin *in situ* forming hydrogel and application in cartilage tissue engineering

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Received 24th July 2014
Accepted 28th September 2014

DOI: 10.1039/c4tb01221f

www.rsc.org/MaterialsB

Hydrogels play a very important role in cartilage tissue engineering. Here, we oxidized dextran (Odex) and modified gelatin (Mgel) to fabricate a fast forming hydrogel without the addition of a chemical crosslinking agent. The dynamic gelling process was measured through rheological measurements. The microstructure was examined by lyophilizing to get porous scaffolds. Biological assessment was performed through CCK-8 assays by using synovium-derived mesenchymal cells (SMSCs) at 1, 3, 7 and 14 days. *In vivo* evaluation for application in cartilage tissue engineering was performed 8 weeks after subcutaneous injection of SMSC-loaded Odex/Mgel hydrogels combined with TGF-β3 in the dorsa of nude mice. According to the results, a fast forming hydrogel was obtained by simply modifying dextran and gelatin. Moreover, the Odex/Mgel hydrogel exhibited good biocompatibility in cultures of SMSCs and a homogeneous distribution of live cells was achieved inside the hydrogels. After 8 weeks, newly formed cartilage was achieved in the dorsa of nude mice; no inflammatory reaction was observed and high production of GAGs was shown. The method provides a strategy for the design and fabrication of fast *in situ* forming hydrogels. The Odex/Mgel hydrogel could be used for the regeneration of cartilage in tissue engineering.

Introduction

Cartilage is a type of load-bearing connective tissue, and chondrocytes are embedded in a highly hydrated, but stiff extracellular matrix. It has a low metabolic rate and limited self-repair capacity due to its avascular supply.[1] The loss or failure of cartilage tissue is a frequent, devastating, and costly problem in health care. Every year a large number of patients undergo a variety of medical procedures for the repair of articular cartilage defects caused by sports injuries, trauma or degenerative diseases. Clinically, the treatment protocols for cartilage defect repair include subchondral drilling, autologous chondrocyte implantation and osteochondral allograft transplantation.[2–5] However, these approaches have several shortcomings, including secondary donor site injury, immune rejection and incomplete integration.[6] Tissue engineering represents a promising method to solve this problem. It uses a set of tools at the interface of the biomedical and engineering sciences such as stem cells, bioactive agents and appropriate materials to aid tissue formation or regeneration. Thus, it is essential to develop cartilage tissue engineering strategies that can restore cartilage tissue defects to a normal state.

Several scaffolds have been investigated including the varieties of natural materials and composites. Hydrogels have received significant attention because of their unique properties that make them desirable candidates in biomedical applications. The extracellular matrix of cartilage is composed of long-chain polymers of glycosaminoglycans (GAGs) and collagen, which are swollen in a significant amount of water and essentially make cartilage a hydrogel. Collagen forms up to two-thirds of the dry weight of cartilage and is mainly classified as type II collagen.[7] Glycosaminoglycans play important roles in the water retention properties of cartilage, and water constitutes about 65–80% of the entire wet weight of articular cartilage.[8] Natural materials such as collagen,[9] alginate,[10] glycosaminoglycans,[11] and agarose[12] have been used to construct hydrogels. Dextran, which is analogous to glycosaminoglycans, is a naturally colloidal, hydrophilic, biocompatible, and nontoxic polysaccharide. It has been widely researched as a macromolecular carrier for the delivery of drugs or proteins for biomedical applications.[13] Gelatin is a collagen-hydrolyzed protein with unique gelation behavior and is the one most frequently used materials in food and pharmaceutical industries.[14] While these hydrogels composed of natural materials promote chondrogenesis, their structures are difficult to control and they are too

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weak to support the loads that are typically placed on articular cartilage. Thus, it is promising to modify natural materials and allow considerably greater control over the composition, structure and crosslinking density. In this case, their properties can be tailored to mimic those of healthy cartilage.

In the present study, we have modified gelatin with ethylenediamine to obtain the amino groups (Scheme 1) and oxidized dextran to obtain aldehyde groups (Scheme 2). In the molecular chain of gelatin there are amino groups and carboxyl groups. The carboxyl group can be converted into an amino group by reaction with ethylenediamine (ED) in the presence of EDC. The neighboring hydroxyl groups in the glucose ring of dextran can be oxidized by sodium periodate into dialdehyde groups to introduce aldehyde groups.[15] Thus, the amino groups of modified gelatin (Mgel) are increased and react with the aldehyde groups of oxidized dextran (Odex) to rapidly form hydrogels. The formation process and stability of Odex/Mgel hydrogels was investigated. The internal microstructures were observed using scanning electron microscopy of lyophilized hydrogels. The synovium-derived mesenchymal cells (SMSCs) are multipotent progenitor cells and have the capacity to differentiate into chondrocytes both *in vitro* and *in vivo*. We further examined the cytotoxicity and biocompatibility of the Odex/Mgel hydrogel in cultures of SMSCs. Specifically, the distribution and viability of SMSCs encapsulated in the hydrogel were studied through the Live/Dead Viability Assay by using laser scanning confocal microscopy. We also evaluated the application of a SMSC-loaded Odex/Mgel hydrogel combined with TGF-β3 for cartilage tissue engineering, which was subcutaneously injected in the dorsa of athymic nude mice. The results show that the Odex/Mgel hydrogel is suitable to support the survival of SMSCs and provides space for SMSCs to proliferate and differentiate in the presence of TGF-β3 *in vivo* for cartilage regeneration.

Materials and methods

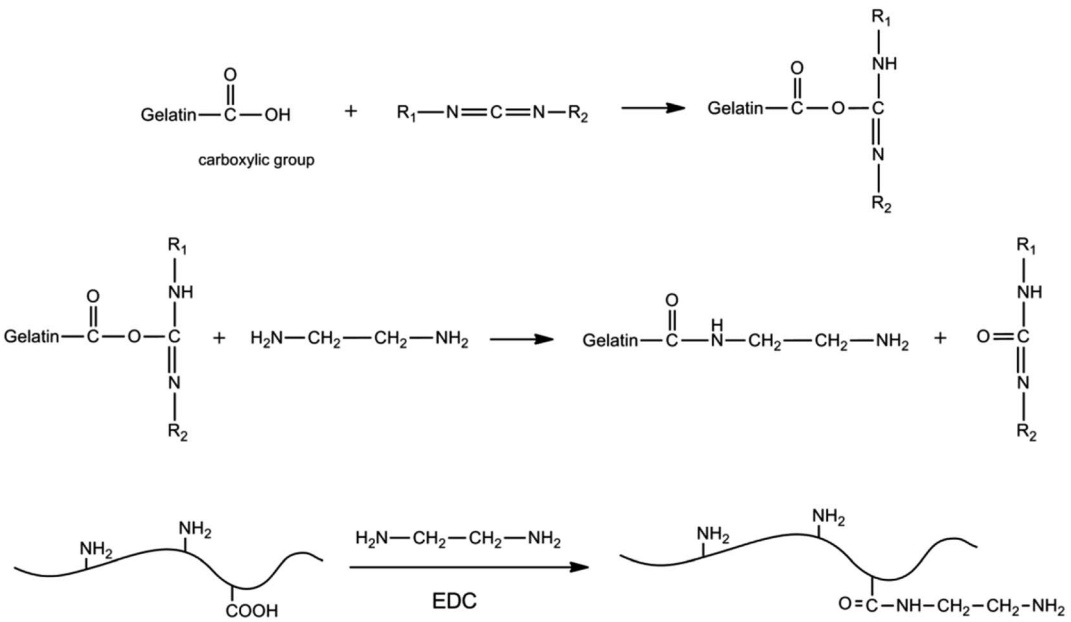
1. Materials

Dextran, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride crystalline (EDC), gelatin, sodium periodate, ethylenediamine (ED), 2,4,6-trinitrobenzene sulfonic acid (TNBS) and hydroxylamine hydrochloride were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), the Live/Dead Viability Assay Kit, collagenase II, penicillin–streptomycin solution, trypsin–EDTA and other culture media and reagents were purchased from Gibco Life Technologies Corporation (Carlsbad, CA, USA). CCK-8 was purchased from Dojindo Corporation (Kumamoto, Japan). Tissue culture flasks were obtained from BD Biosciences Corporation (San Jose, CA, USA). Athymic nude mice (BALB/c mice) were purchased from Fudan University (Shanghai, China).

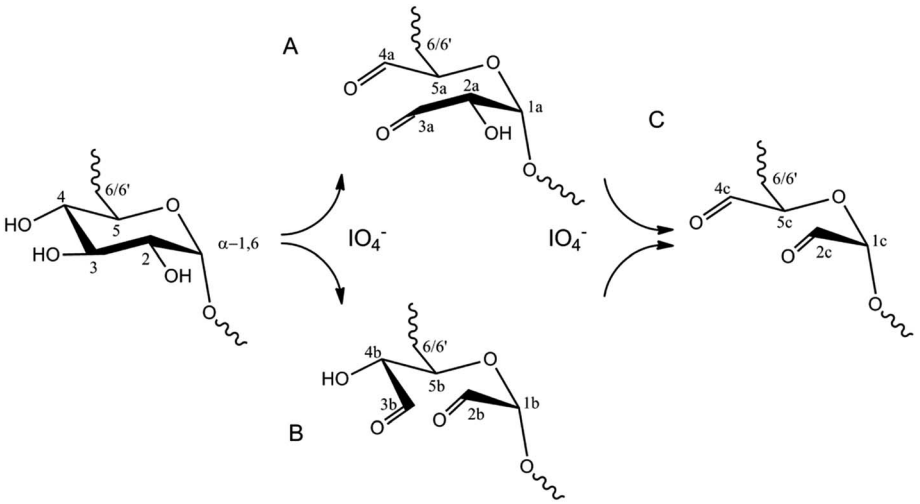
2. Synthesis of oxidized dextran and modified gelatin

Oxidized dextran (Odex) was synthesized from dextran by reaction with sodium periodate as reported.[16] In brief, dextran solution was prepared by dissolving 10 g of dextran in 100 ml of distilled water. Then, 6.34 g of $NaIO_4$ (dissolved in 100 ml of distilled water) was added dropwise to the dextran solution. The solution was stirred at room temperature for 6 h and shielded from light. Then, 2 ml of ethylene glycol was added to terminate the oxidation reaction. The resulting solution was dialyzed exhaustively for 3 days against water and lyophilized to obtain the final Odex.

The carboxyl groups in gelatin were converted into amino groups by reaction with ED in the presence of EDC. Gelatin was dissolved in 100 ml of phosphate buffered solution (PBS) to a final concentration of 5 wt% at room temperature and 16 ml of



Scheme 1 Synthesis of modified gelatin carrying more amino groups.



Scheme 2 Preparation of oxidized dextran.

ethylenediamine was added. Immediately after that, the pH of solution was adjusted to 5.0 by adding hydrochloric acid (HCl). After that, 2.3 g of EDC was added into the gelatin solution. The molar ratio of the carboxyl groups on gelatin chains, EDC and ED was 1 : 2 : 40. The reaction mixture was stirred at room temperature overnight, and then dialyzed against distilled water for 48 h to remove the excess ED and EDC. The dialyzed solution was freeze-dried at −80 °C to obtain a modified gelatin (Mgel).

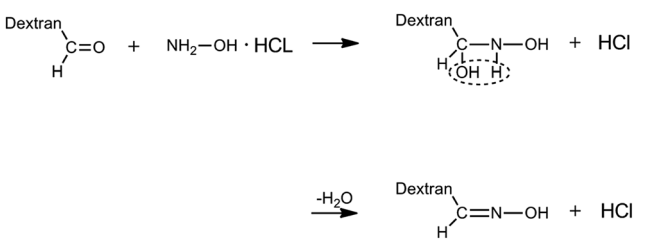
3. Determination of oxidation degree of Odex and amino content of Mgel

The oxidized degree (OD) of Odex was determined with a quantitative reaction between hydroxylamine hydrochloride and aldehyde groups, while producing a Schiff base and releasing hydrochloric acid (HCL) (Scheme 3). Through a titration method to calculate the amount of HCL, the concentration of aldehyde groups can be calculated. Odex sample (0.1 g) was mixed with 5 ml of hydroxylamine hydrochloride solution. A blank test was performed with Odex dissolved in 5 ml of ethanol solution. The OD of Odex was calculated by:

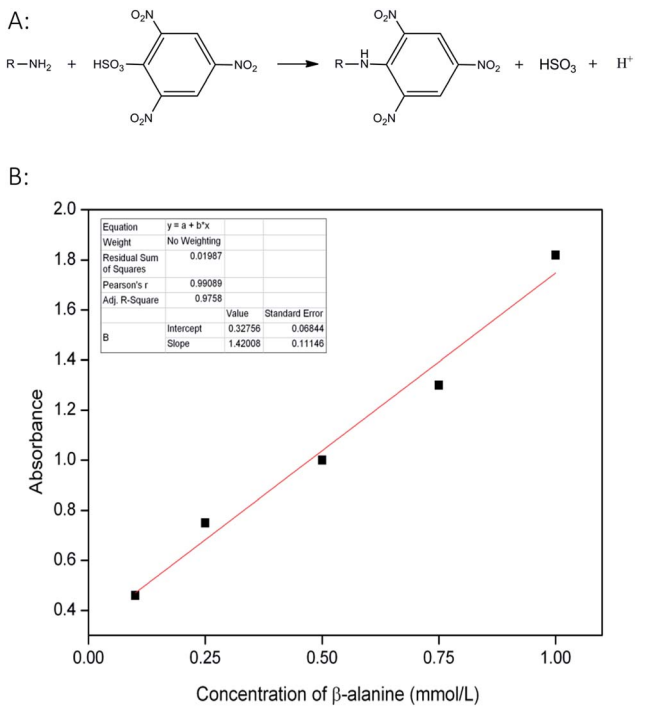
$$\mathrm{OD}\ (\%) = (V_{\mathrm{blank}} - V_{\mathrm{sample}}) \times C_{\mathrm{HCL}} \times M_{\mathrm{dextran}} \times 100/W_{\mathrm{sample}}$$

where C_{HCL} is the HCL solution concentration (mol L^{-1}), V_{blank} is the volume of HCL used for the blank (L), V_{sample} is the volume of HCL used for the sample (L), M_{dextran} is the molecular weight of Odex, and W_{sample} is the weight of the oxidation product added in the hydroxylamine hydrochloride solution.

TNBS assay was performed to determine the amino content of Mgel. The amino groups react with sulfonic acid groups of TNBS to form equimolar amounts of trinitrophenylated (TNP) amino groups and bisulfite ions (Scheme 4A). The trinitrophenylation reaction mixture has an absorption peak at 415 nm and the amino content is directly related to the absorbance. To determine the absorbance values of different amino contents, a standard curve is constructed from the known



Scheme 3 Determination of oxidation degree of Odex by hydroxylamine hydrochloride method.



Scheme 4 Determination of amino content of Mgel by TNBS method.

concentrations of β-alanine (Scheme 4B). Then, the absorbance value of Mgel reacting with TNBS was measured and the amino content was determined.

4. Hydrogel preparation and the effect of Odex/Mgex ratio on hydrogel properties

First, 10 wt% Odex (100 mg ml^{-1}) and 20 wt% Mgel (200 mg ml^{-1}) was separately prepared in PBS for physicochemical characterization. Odex and Mgel were mixed in the tube at the ratio of 3/7, 4/6, 5/5, 6/4 and 7/3 at 37 °C. The gelation time was determined using the tube tilting method. When no fluidity was visually observed upon inverting the tube, the time cost was defined as the gelation time. The weight loss because of hydrolytic degradation was examined for time-control degradation at different points (day 0, day 3, day 7, day 14, day 21, day 28). The initial dry weight of the sample was recorded, and the hydrogels were completely submerged in PBS with 0.02% NaN_3. The solution was changed each day. At different time points, the samples were thoroughly rinsed with PBS and lyophilized before weighing the dry residuals. The weight loss was calculated as the ratio of original dry weight and the dry residuals weight. All the samples were tested in triplicate. The mechanical strength of Odex/Mgex hydrogels at different ratios was also characterized by compression stress–strain measurements using a Dejie DXLL-20000 materials testing instrument at 25 °C. All the samples were incubated in PBS at 37 °C for 24 h before testing, in order to reach complete swelling equilibrium. After measuring the diameter and thickness of the specimens, they were placed on the lower plate and compressed by the upper plate at a strain rate of 1 mm min^{-1}. All compression testing groups had a sample quantity of $n = 3$. Then, the cytotoxicity of Odex/Mgex hydrogels at different ratios was characterized by seeding MC3T3-E1 cells on the surface of the hydrogels at a density of 5×10^4 cells per well. Next, 1 ml of complete medium was added to each well, and the cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. The cells were tested for viability using a Live/Dead Viability/Cytotoxicity Assay Kit. By these studies, the optimized hydrogel formulation was selected for the following investigation:

5. Rheological measurements

All the rheological experiments were performed at 37 ± 0.1 °C in the strain-controlled mode using a Physica MCR 301 rheometer (Anton Paar GmbH, Austria) and a parallel plate geometry (diameter = 25 mm). To minimize evaporation, a solvent trap was employed and a low viscosity mineral oil was applied around the sample. Time sweep experiments were conducted *via* dynamic oscillatory shear at 1% strain and 1 Hz. Dynamic frequency sweep experiments were performed in the range of 0.1–10 Hz at 1% strain. To study the stability of the hydrogels, strain sweeps in the range of 0.1–1000% were run at 1 Hz.

6. Swelling measurement and *in vitro* degradation

To measure the equilibrium swelling ratio of the Odex/gelatin hydrogels, the samples were lyophilized in a vacuum oven. The dry hydrogel samples were weighed (W_d), and then immersed in PBS at 37 °C. After 24 h of incubation, the samples were removed from the PBS and the water on the surface was wiped off with a filter paper. The weight of the swollen mass was measured (W_s). The swelling ratio (SR) was calculated according to the following equation: $SR = W_s/W_d$.

To assess the degradation properties of the hydrogels, samples were divided into four groups (day 3, day 7, day 14, day 21) for a time-dependent degradation study. The samples ($n = 3$) were prepared as described above and pre-swollen in PBS overnight. Subsequently, the samples were weighed (W_0) and submerged in PBS at 37 °C. At different time intervals, the samples were removed from the solution and placed on a filter paper. After the removal of the excess superficial water, the weight of the residual mass was measured (W_1). The PBS was replaced every 3 days. The residual mass fraction (%) was calculated using the following formula: residual fraction $= W_1/W_0 \times 100\%$.

7. SEM observations

To characterize the internal microstructures of the Odex/Mgel hydrogels, the samples were rinsed three times with distilled water at room temperature, and then lyophilized in liquid nitrogen to obtain porous scaffolds. After that, the dried hydrogel samples were cut to expose the cross-sections and coated with gold for 120 seconds. The samples were observed using a scanning electron microscope (JSM-5600, Japan) under an accelerating voltage of 10 kV.

8. Synovium-derived mesenchymal cell culture

Synovium-derived mesenchymal cells (SMSCs) were isolated and expanded as reported by De Bari.[17] In brief, synovial tissue was aseptically harvested from the knee joint of New Zealand white rabbits in accordance with the guidelines approved by the animal committee of Fudan University, China. The synovial tissue collected was rinsed three times with PBS solution containing antibiotics (100 units ml^{-1} penicillin, 100 units ml^{-1} streptomycin) and finely minced. Subsequently, the tissue was digested with trypsin–EDTA (0.1% trypsin, 0.4 mM EDTA) at 37 °C for 30 min and digested using 0.1% collagenase II in complete medium (low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics) at 37 °C for 2 h. After that, cells were collected from the digestion solution with a filter, centrifuged at 1500 rpm for 5 min to obtain a cell pellet and resuspended in complete medium at a suitable concentration. The cells were seeded in culture flasks and allowed to proliferate in the complete medium at 37 °C in a humidified atmosphere of 5% CO_2. The complete medium was replaced every 3 days. When the attached cells reached 90% confluence after 9–12 days in primary culture, they were washed twice with sterilized PBS solution, collected by treatment with trypsin–EDTA (0.25% trypsin, 1 mM EDTA) and seeded in new culture flasks at a dilution rate of 1 : 4 for the subculture.

9. Viability and long-term proliferation of SMSCs cultured with hydrogel

The cell viability of cells cultured with hydrogels was quantified using the CCK-8 assay on 1, 3, 7 and 14 days *via* a Transwell system (Costar 3422). In brief, the synovium-derived mesenchymal cell suspension with a cell density of 1×10^6 cells per ml was injected in 24-well culture plates. Odex and Mgel were premixed and placed within the upper chambers at 37 °C for gel formation. Then, the upper chambers were inserted into 24-well culture plates and complete medium was added in the hydrogel group. SMSCs seeded in a 24-well culture plate without an upper chamber served as the control group. The culture plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. After 1, 3, 7 and 14 days culture, the culture medium was removed, and 400 μl medium containing 40 μl CCK-8 reaction solution was added to each well and incubated for 4 h at 37 °C and 5% CO_2. Then, the medium with CCK-8 was transferred to a 96-well tissue culture plate, and the absorbance was read at 450 nm using a multidetection microplate reader (MK3, Thermo, USA). All the experiments were carried out in triplicate. After 14 days in culture, SMSCs in the control group and hydrogel group were also stained with Live/Dead staining solution and incubated at 37 °C for 30 min according to kit instructions. After that, SMSCs were washed twice using PBS and observed through the microscope to estimate the viability and proliferation.

10. Distribution and long-term viability of encapsulated cells in 3D culture

For cell distribution, SMSCs were premixed with Odex and Mgel solutions at a density of 5×10^6 cells per ml. The mixture was injected in culture dishes and incubated at 37 °C for gel formation. After that, the cells were encapsulated in hydrogels by the crosslinking process between aldehyde groups and amino groups. Then, the complete medium was added in culture dishes, followed by incubation in a 5% CO_2 humidified atmosphere at 37 °C. SMSCs encapsulated in hydrogels were stained using the Live/Dead assay at 12 and 24 h. A laser scanning confocal microscope (FV1000, Olympus Corporation, Japan) was used for cell observation. Live/Dead fluorescence staining was performed according to the instructions provided by the Live/Dead Viability Assay Kit. In brief, the SMSC-loaded hydrogel samples were immersed in Live/Dead staining solution containing calcein acetoxymethyl (AM) and ethidium homodimer-1 (EthD-1) and incubated at 37 °C for 30 min. After this step, the samples were washed twice using PBS before laser scanning confocal microscopy viewing. The live and dead cells exhibit green-fluorescent and red-fluorescent, respectively, under confocal microscopy. In order to obtain the 3D distribution and Live/Dead condition of the encapsulated cells, stacks of images were acquired in the *z* dimension, consisting of successive slices with a thickness of 10 μm each.

For investigating the long-term effects of the hydrogels on cell viability, MC3T3-E1 cells inside the hydrogel were also stained at day 1, day 3, and day 7 using Live/Dead Viability/Cytotoxicity Assay Kit. In brief, the cell-laden hydrogels were rinsed twice with PBS and stained with Live/Dead staining solution at 37 °C for 30 min. Then, the samples were rinsed twice with PBS and observed using a QCapture 5 Imaging System (Surrey, Canada) through a fluorescent microscope (UL100HG Olympus Corporation, Japan).

11. *In vivo* evaluation of SMSC-loaded Odex/Mgel hydrogel combined with TGF-β3

For *in vivo* evaluation, SMSCs were premixed with Odex and Mgel solutions at a density of 5×10^6 cells per ml. In the TGF-β3 group, growth factor (TGF-β3) was incorporated in the SMSC-loaded Odex/Mgel hydrogel at a concentration of 10 ng ml^{-1}, while the control group was free of TGF-β3. The mixture was subcutaneously injected in the dorsa of athymic nude mice. After 8 weeks of *in vivo* chondrogenesis, the mice were sacrificed and the samples were harvested. The animal experiment was conducted in accordance with guidelines approved by the animal committee of Fudan University, China.

12. Histological analysis

For histological staining, the SMSC-loaded hydrogel samples were harvested at 8 weeks after injection and fixed in neutral buffered paraformaldehyde. Then, the samples were sequentially dehydrated in a series of increasing ethanol concentration solutions and cleared by exposure to xylene solutions with increasing concentration. Next, the samples were embedded in paraffin after an overnight incubation with paraffin saturated xylene, and xylene was evaporated by placing the samples in an oven. The samples were sectioned at a thickness of 8.0 μm and laid overnight to adhere to glass slides. The sections were stained with hematoxylin and eosin (H&E) for histological observation and subsequently stained with toluidine blue for glycosaminoglycan (GAG) observation. All incubations were at room temperature.

13. Immunohistochemistry analysis

The deparaffinized sections were incubated with proteinase K enzyme in Tris-buffered saline (1 : 50 working dilution, pH 7.4, TBS) for 5 min for antigen retrieval, followed by incubation with 0.3% hydrogen peroxide in water for 20 min at room temperature. After washing with PBS, sections were blocked with 10% goat serum solution for 30 min. Then, the sections were incubated with primary antibodies: anti-type I collagen or anti-type II collagen (1 : 150 working dilution) overnight at 4 °C. After washing with PBS three times, the biotinylated secondary antibody (anti-mouse immunoglobulins) was applied for 30 min. Then, the sections were washed with PBS and incubated with horseradish peroxidase-conjugated streptavidin for 30 min. Sections were washed and incubated with 3,3-diaminobenzidiine-tetrahydrochloride as a color substrate for 3 min to visualize the bound antibodies.

14. Statistical analysis

Tests were performed on three replicates, unless otherwise stated. All quantitative data were recorded and statistically analyzed by SPSS 19.0. Values were expressed as the mean of

three replicates and standard deviation (SD). Experimental results were also analyzed by one-way ANOVA. For all statistical tests, the level of significance was set at $p < 0.05$.

Results

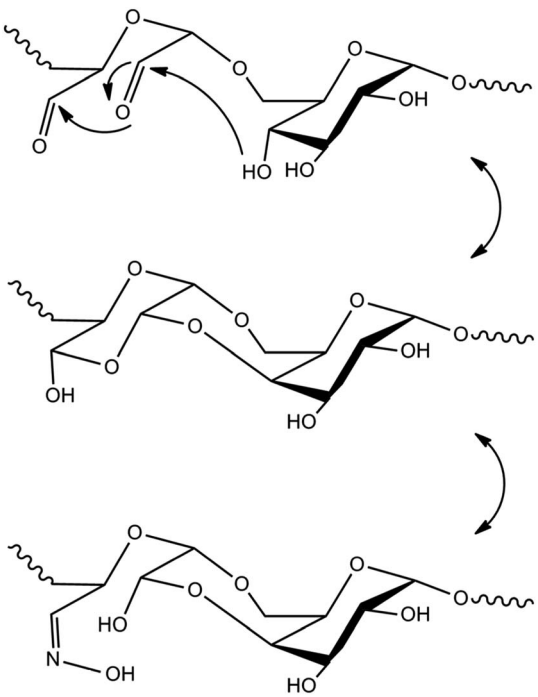
1. Preparation and characterization of Odex and Mgel

Dextran, composed of linear α-1,6-linked anhydroglucose units with a low fraction of α-1,2-, α-1,3- and α-1,4-linked side chains, has a structure of a glucopyranose ring and adjacent hydroxyl groups located in C2, C3, C4. The periodate oxidation of dextran is a complicated two-step reaction as shown in Scheme 2. First, the adjacent hydroxyl groups (C2, C3 or C3, C4) of an α-1,6-linked anhydroglucose unit are oxidized to form the α-hydroxy aldehyde groups. In the second step, the α-hydroxyl aldehyde group (C3) can be further oxidized to generate formic acid and a dialdehyde group. As a result, the periodate ion attacks one of the vicinal hydroxyl groups in dextran and yields two aldehyde groups. In our study, dextran was oxidized by sodium periodate at the theoretical oxidation degree of 49%. However, the measured oxidation degree was only 43% as quantified by reaction with hydroxylamine hydrochloride. This is possibly because to some extent two aldehyde groups of Odex only react with one amino group of hydroxylamine hydrochloride. Sodium periodate oxidizes adjacent hydroxyl groups of dextran and yields two vicinal aldehyde groups (Scheme 5). The intramolecular hydrogen atom is transferred from the closest hydroxyl group in the adjacent glucopyranose ring to the aldehyde group, which forms the transition state of a six-membered-ring hemiacetal. Then, the amino groups of hydroxylamine hydrochloride attack the hemiacetal group, which actually consumes one amino group. FTIR-ATR was carried out to confirm the appearance of aldehyde groups on oxidized dextran. Fig. 1A shows the changes in the FTIR spectra of oxidized dextran synthesized by the process of oxidation. As illustrated, an absorbance peak located at around 1733 cm^{-1} corresponds to the aldehyde groups.

Generally, gelatin maintains a gel state under room temperature. In this study, through reaction with ethylenediamine, carboxyl groups were chemically modified to amino groups and the intermolecular forces between amino and carboxyl groups were weakened, which is mainly attributed to the formation of solid gelatin. As a consequence, gelatin at a concentration of 20 wt% could stay a fluid at room temperature (Fig. 1B). The degree of chemical modification of gelatin was measured to be approximately 0.802 mmol g^{-1} by the TNBS method. While Odex and Mgel solutions were mixed together, the hydrogels were rapidly formed through a Schiff-base reaction between aldehyde groups and amino groups.

2. The effect of Odex/Mgex ratio on hydrogel properties

When changing the blending ratio of oxidized dextran and amino gelatin, a conspicuous difference was found in hydrogel properties (Fig. 2). With the increase in dextran's content, the gelation time dramatically decreased and slowly increased. The Odex/Mgel hydrogel at ratio of 5/5 has a shorter gelation time than other formulated hydrogels. As for dry weight degradation, the 3/7 Odex/Mgel hydrogel exhibited the fastest degradation



Scheme 5 Intramolecular hydrogen atom transfer in oxidized dextran.

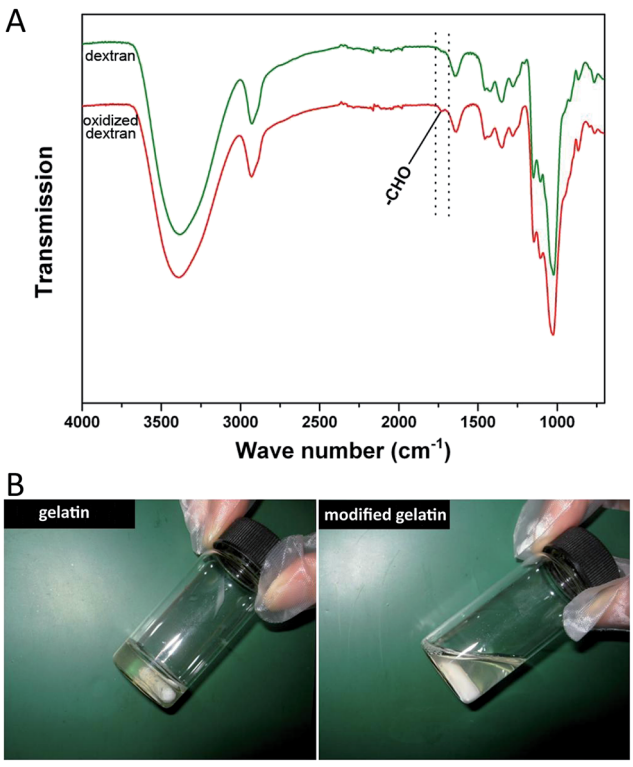


Fig. 1 Preparation and characterization of Odex and Mgel. (A) FTIR-ATR characterization of dextran and oxidized dextran; (B) gelatin and modified gelatin at room temperature.

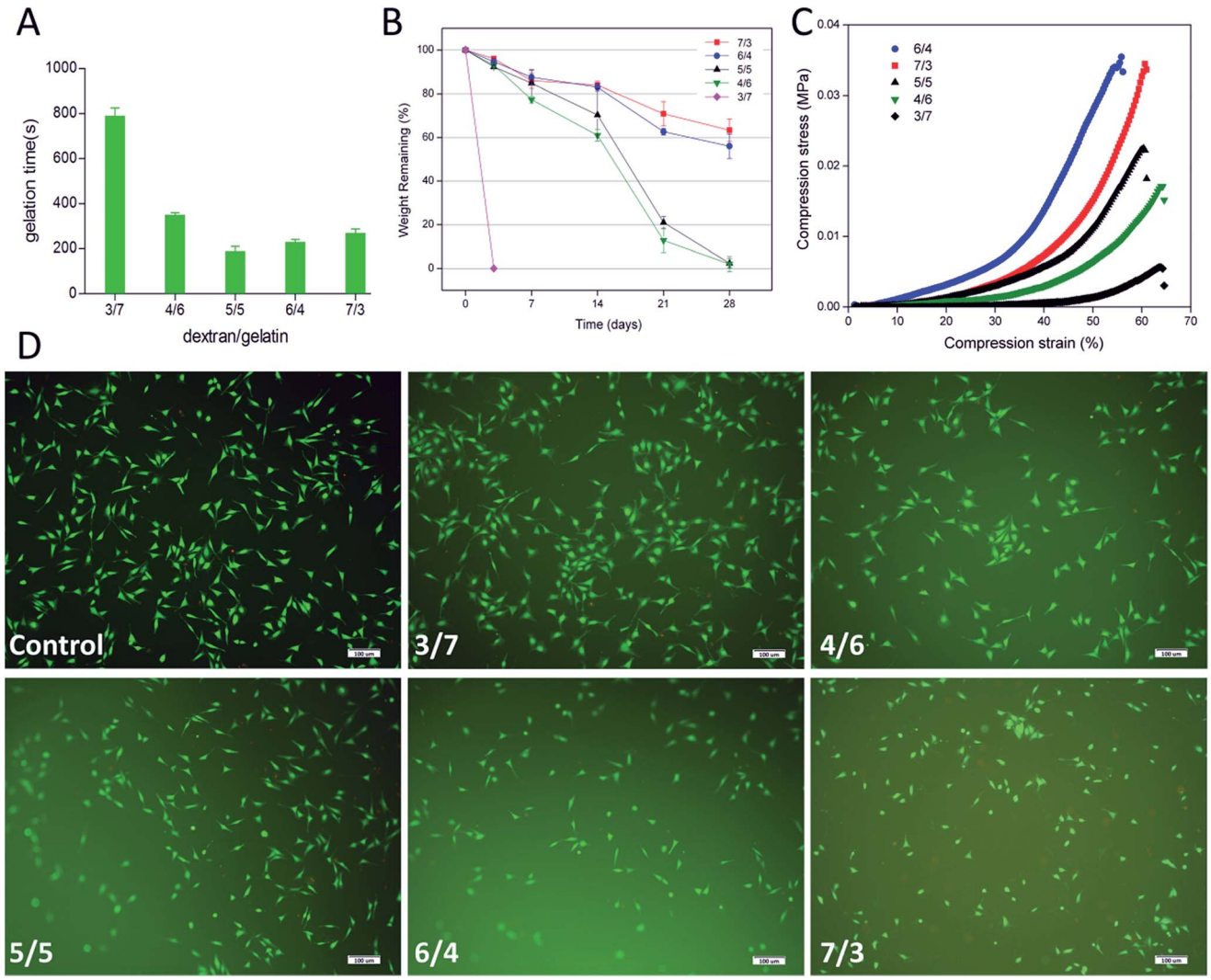


Fig. 2 The effect of Odex/Mgex ratio on hydrogel properties. (A) Gelation time of hydrogels at different ratios; (B) *in vitro* degradation of the hydrogel at day 3, day 7, day 14, day 21, and day 28 through the measurement of dry weight; (C) mechanical strength of the different formulated hydrogels; and (D) cell attachment and spreading on tissue culture plate (control) and hydrogels (3/7, 4/6, 5/5, 6/4 and 7/3). Scale bar represents 100 μm.

rate and was totally degraded at day 3. By contrast, the 4/6 and 5/5 hydrogels showed lower degradation rates because of higher crosslinking densities. Odex/Mgel hydrogels at ratios of 6/4 and 7/3 have the strongest resistance to degradation in that they could still hold approximately 60% of the initial dry weight after 28 days. Fig. 2C shows the typical compressive stress–strain curves of the five formulated hydrogels. Corresponding to the same compressive strain, compressive stress value showed an increasing trend with an increase in the Odex amount from 3/7 to 5/5. In addition, Odex/Mgel hydrogels at ratios of 6/4 and 7/3 exhibited better mechanical properties than other hydrogels. However, with the ratio of Odex/Mgel changing from 3/7 to 7/3, the hydrogel became adverse to cell responses such as attachment, spreading and survival. Poor attachment properties were exhibited in the 6/4 and 7/3 groups. One possible explanation is that the toxicity caused by the aldehyde groups in oxidized dextran hampered cell responses. Following the characterization of five different hydrogels, the 5/5 Odex/Mgel hydrogel was shown to display favorable physical properties and cell–material interactions.

3. Rheological measurements

Fig. 3 shows the time sweep profiles of storage modulus (G') and loss modulus (G'') for the Odex/Mgel hydrogel formation process. The system behaved as a viscous fluid (sol) at the beginning, where G'' was higher than G'. Both G' and G'' increased with gelation time, along with the Schiff based reaction. However, G' increased faster than G''. Afterwards, there was a crossover point where G' became higher than G''. The time required for this crossover to occur was referred to as the gelation time, and this point was referred to as the gelation point.[18] For Odex/Mgel hydrogel, the gelation time was approximately 107 s. Finally, both moduli leveled off and a well-defined three-dimensional network formed.

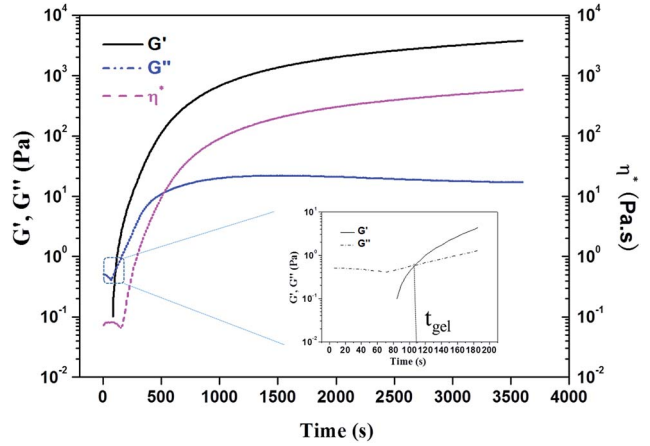


Fig. 3 The time sweep profiles of the Odex/Mgel hydrogel formation process.

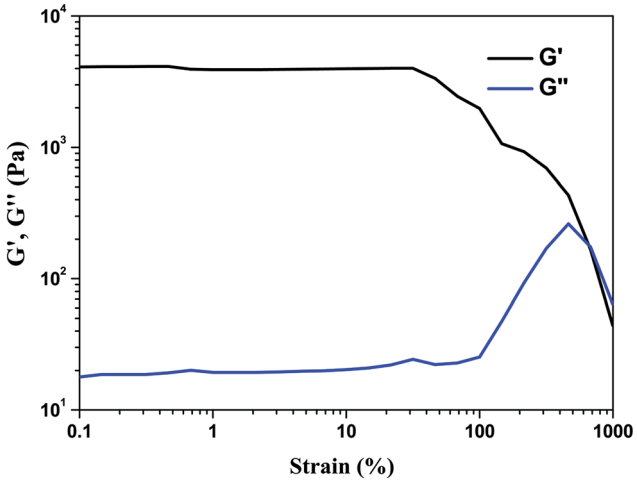


Fig. 5 The strain of Odex/Mgel hydrogel.

The dynamic moduli of Odex/Mgel hydrogels were measured as a function of frequency at 1 h after mixing, as is shown in Fig. 4. G' was considerably larger than G'' and presented nearly frequency-independent behavior in the entire frequency range tested, which was a characteristic of solid (gel)-like behavior.[19]

Fig. 5 shows the stability of Odex/Mgel hydrogels under shear stress, which is crucial for cartilage.[20] A strain sweep from 0.1% to 1000% was performed on the hydrogel. Both G' and G'' changed a little below the critical strain γ_L (about 35%), which represented the linear viscoelastic range and the deformation limit.[21] In the gel state, G' was larger than G''. Above γ_L, G' rapidly decreased, and at flow strain γ_f (about 650%), G' became lower than G'', indicating a collapse of the gel to produce a liquid-like system.

4. Swelling and degradation measurements

The equilibrium swelling behavior is an important property of a hydrogel because it relates to the diffusion of nutrients and cellular waste. Fig. 6A indicates the swelling results for lyophilized hydrogels in PBS. The dry Odex/Mgel hydrogel could absorb a large quantity of water from 18.73 to 19.42 times its original dry weight, suggesting that these gels could maintain significant amounts of tissue fluid and nutrients *in vivo*, as reflected by their equilibrium swelling ratios.

The degradation of Odex/Mgel hydrogels was monitored by incubation in PBS at 37 °C, as shown in Fig. 6B. Apparently, with the increase in time, the Odex/Mgel hydrogel gradually lost mass. However, after 2 weeks because of hydrolytic degradation, the Odex/Mgel hydrogel still held more than 50% of the initial weight. Concretely, at day 3, day 7, day 14, and day 21 the residual mass fraction (%) is 89.94 ± 0.83, 79.09 ± 2.09, 62.64 ± 3.07, and 27.84 ± 0.96, respectively.

5. Morphology of hydrogels

In this study, the samples were observed by using scanning electron microscopy to confirm the internal microstructures. All hydrogel samples were flash-frozen at −80 °C in liquid nitrogen and lyophilized (Fig. 7A1). After that, the corresponding cross-section of the dry samples was observed (Fig. 7A2). Fig. 7B shows the SEM micrographs of freeze-dried hydrogels. All hydrogels had good pore interconnectivity as indicated by the red arrows in Fig. 7B1. The pore interconnection throat size inside the lyophilized hydrogels was in the range of 40–80 μm,

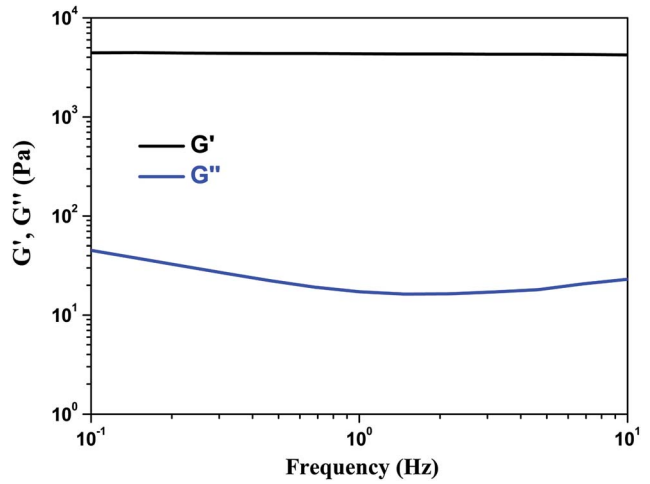


Fig. 4 Dynamic mechanical analysis of Odex/Mgel hydrogel: storage modulus (G') and loss modulus (G'') *versus* frequency at 37 °C.

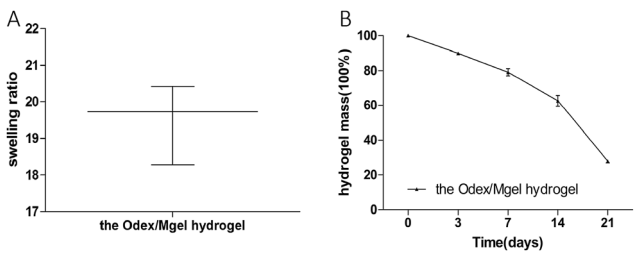


Fig. 6 Swelling and degradation measurements of the Odex/Mgel hydrogel.

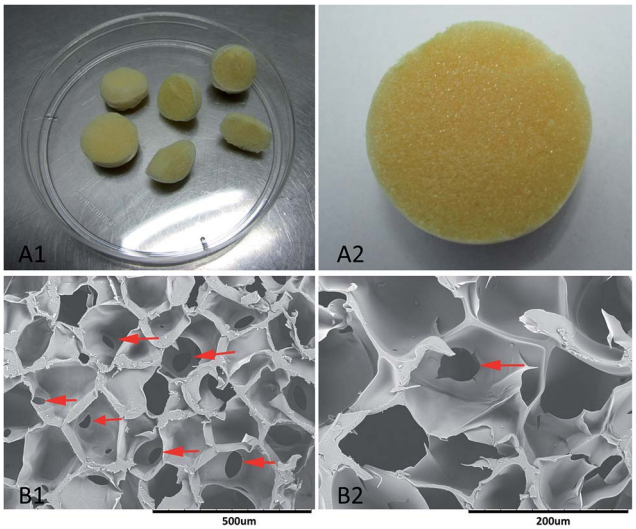


Fig. 7 Images of gross view (A1, A2) and SEM micrographs of lyophilized Odex/Mgel hydrogel (B1, B2).

which facilitates oxygen and nutrient supply and waste removal. The diffusion of nutrients and gas is a prerequisite for the regeneration of cartilage tissue, which is avascular. Compared to an isolated pore structure, an interconnected pore network enhances the diffusion rates to and from the center of the scaffold. The pore size of the Odex/Mgel lyophilized hydrogel is about 200 μm (Fig. 7B2). The porosity of Odex/Mgel hydrogel makes an indispensable contribution to its performance. The porous structure provides two critical functions. First, pore channels are ports of entry for cells migrating from adjacent tissues and for interstitial fluid permeating from the surrounding *via* capillary suction. Second, pores endow a material with an enormous specific surface that is made available for adhesion and spreading of invading cells. Therefore, the interconnected porous structure is necessary for tissue engineering scaffolds to allow cellular ingrowth and retain nutrient solution.

6. Viability and long-term proliferation of SMSCs cocultured with hydrogel

SMSCs were isolated from synovial membrane tissue and exhibited a high expandability in the culture flasks. In the first subculture, SMSCs were plastic adherent with an elongated or spindle-shaped appearance, which is typical of fibroblastic morphology (Fig. 8A). Round nuclei were observed by DAPI staining (Fig. 8B). To quantitatively assess the cell proliferation of SMSCs, CCK-8 tests were performed on days 1, 3, 7 and 14. As shown in Fig. 9A, there was no statistically significant difference between the hydrogel group and control group ($p < 0.05$), indicating that the Odex/Mgel hydrogel has no effect on cell viability. The statistical analysis revealed that SMSCs had an increased metabolic activity on the hydrogels with the increase of culture time. After 14 days of *in vitro* culture, there was no statistically significant difference compared with 7 days culture ($p < 0.05$). We attribute this to the condition that the attached cells reached greater than 95% confluence in culture plate after 7 days. Throughout the course of the CCK-8 experiment, SMSCs cultured with hydrogel remained viable and proliferative. After 14 days in culture, SMSCs cultured with or without hydrogel were also stained with Live/Dead staining solution. According to fluorescence microscopy there was no visual difference between the control group and the hydrogel group (Fig. 9B and C). The cells in the two groups reached complete confluence and had an organized arrangement. All of them exhibited a green-fluorescent, spindle-shaped morphology. These results indicate that the degradation products derived from Odex/Mgel hydrogels did not inhibit key metabolic pathways of cells and kill SMSCs.

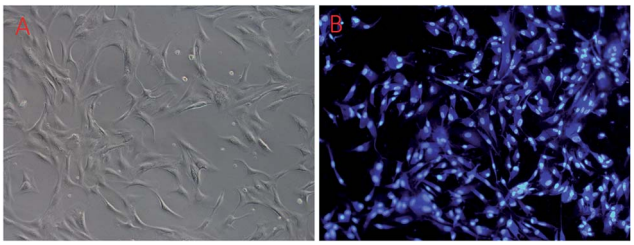


Fig. 8 Image of first passage SMSCs cultured in growth medium (A). SMSCs were stained by DAPI (B).

7. Distribution and long-term viability of encapsulated cells in 3D culture

The 3D structure of Odex/Mgel hydrogels appears to mimic the environment *in vivo*, which provides an opportunity to study their performance as an *in vitro* matrix for tissue engineering. In our experiment, the cells were premixed with the Odex and

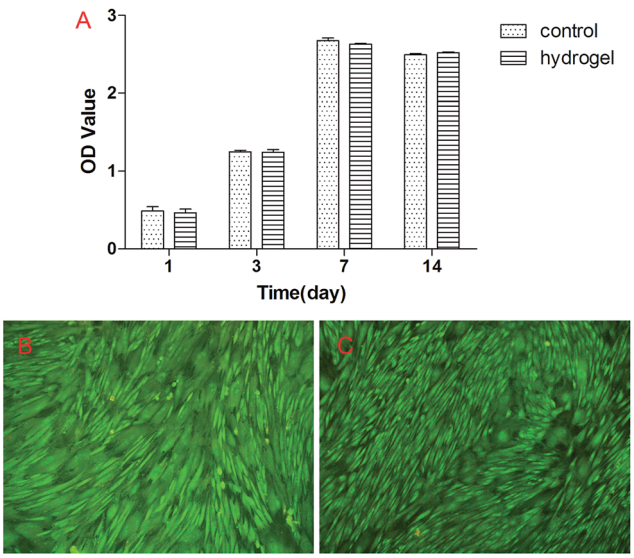


Fig. 9 CCK-8 assay of SMSCs cultured with or without hydrogel (A). SMSCs were stained with Live/Dead staining solution (B: control group; C: hydrogel group).

Mgel solutions for encapsulation. Further, the Dead/Live test was used to confirm the viability of SMSCs after encapsulation in hydrogels (Fig. 10). As shown in Fig. 10A, after 12 h in culture, most encapsulated cells were alive, while only a few were dead based on the simultaneous staining of live (green-fluorescent) and dead (red-fluorescent) cells. At 24 h (Fig. 10B), the overwhelming majority of encapsulated cells remain alive. Most of the live cells were round in shape. The large preponderance of live cells revealed that Odex/Mgel hydrogels exert no cytotoxicity on SMSCs and allow their proliferation, which is in accordance with the results of the CCK-8 assay. Additionally, SMSC distribution inside the hydrogel samples was investigated by laser scanning confocal microscopy (Fig. 10A3 and B3). The cell distribution inside the samples was observed to be homogenous, suggesting that a uniform and ordered cell distribution is able to allow SMSCs to proliferate in high numbers and to differentiate into chondrocytes.

With respect to the long-term effect of the hydrogels on cell viability, MC3T3-E1 cells in hydrogels were stained with the Live/Dead Viability Assay Kit and are shown in Fig. 11A–C. After 1 day and 3 days in culture, the overwhelming majority of encapsulated cells was alive and stained as green. At day 7, there were also rarely dead cells stained as red, indicating that the Odex/Mgel hydrogels possess good biocompatibility. Moreover, the number of viable cells in samples increased with culture time. Most of the living MC3T3-E1 cells exhibited a round shape and could be homogeneously encapsulated within the hydrogels after culture.

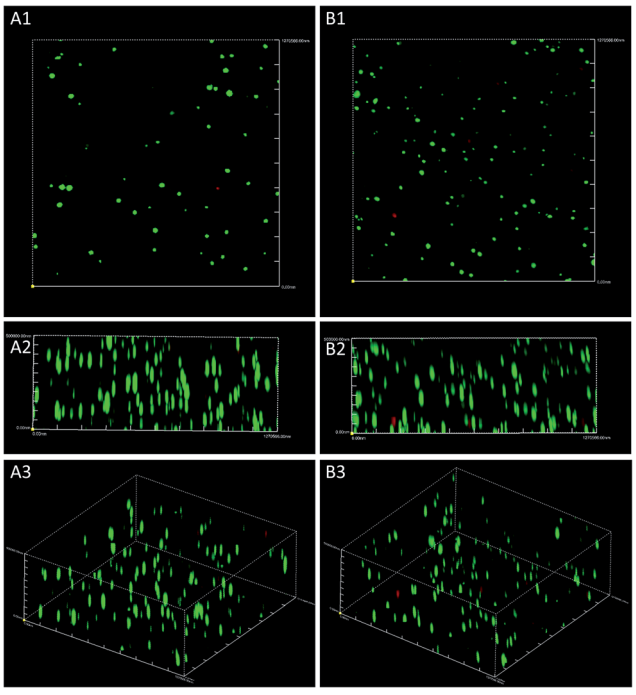


Fig. 10 Confocal images of encapsulated cell distribution in 3D culture. SMSCs were stained with Live/Dead staining solution after 12 hours (A) and 24 hours (B) in culture.

8. *In vivo* study

The SMSC-loaded Odex/Mgel hydrogels with or without TGF-β3 were subcutaneously injected in the dorsa of athymic nude mice (Fig. 12A1 and A2). After 8 weeks *in vivo* for chondrogenesis, the samples were harvested. No evidence of superficial inflammatory reaction such as tissue necrosis or abscess formation was observed in both the hydrogels and the adjacent sites (Fig. 12B1). The hydrogels subcutaneously injected in the dorsa of nude mice were very easily dissected from the surrounding soft connective tissue. In the TGF-β3 group, a gross examination of the hydrogels showed that the hydrogels retained the shapes for the chondrogenic period (Fig. 12B2), while the samples in the control group disappeared at 8 weeks after injection and only a thin layer of membrane-like tissue remained. Compared with the control group, more cartilage-like tissue was formed in the TGF-β3 group. The color of the samples became white, and the quality became strong and felt like cartilage tissue.

Images of the histological and immunohistological staining of the overall transverse-sections of the hydrogel samples are shown in Fig. 13. The histological images of the specimens in the control group showed weakly positive toluidine blue stain and cells were scattered throughout the amorphous basophilic matrix stained by hematoxylin eosin (Fig. 13A1 and A2). In contrast, histological examination in the TGF-β3 group indicates that SMSCs in the TGF-β3 incorporated hydrogels continued to proliferate in a high number and to secrete extracellular matrix components to form homogeneously compact cartilage tissues. A uniform cell distribution throughout the hydrogels was achieved after 8 weeks according to hematoxylin and eosin staining (Fig. 13B1). The bright toluidine blue positive stain indicates that glycosaminoglycans were abundant and homogeneously distributed throughout the hydrogels (Fig. 13B2), which illustrates that SMSCs have great chondrogenic differentiation ability. Moreover, the cells in the TGF-β3 incorporated hydrogels showed a round morphology (Fig. 13B1 and B2), which indicates that SMSCs are capable of differentiating into chondrocytes and secreting extracellular matrix.[22] Immunohistological staining showed a minimal detection of collagen type I in the control group and the TGF-β3 group (Fig. 13A3 and B3). However, there was increased extracellular staining for type II collagen in the TGF-β3 group (Fig. 13B4). These results showed that during *in vivo* implantation with TGF-β3, more new cartilage matrices were regenerated while the scaffolds gradually degraded and were substituted.

Discussion

The aim of biomaterial scaffolds used in a wide variety of applications is to achieve a suitable combination of functional properties to match those of the replaced tissue and facilitate tissue regeneration. Hydrogels have been intensively studied and used as tissue engineering scaffolds, because they can provide a highly swollen three-dimensional environment similar to soft tissues and allow diffusion of nutrients and cellular waste through the elastic networks.[23–26] Due to their

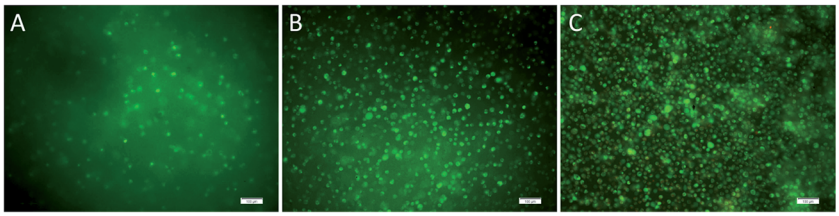


Fig. 11 Phase contrast microscopy images of MC3T3-E1 cells encapsulated in Odex/Mgel hydrogels for 1 day (A), 3 days (B) and 7 days (C). Scale bar represents 100 μm.

exceptional promise in biomedical applications, hydrogels have received significant attention in cartilage tissue engineering. In this study, the Odex/Mgel hydrogels were produced by cross-linking of two comonomer units (oxidized dextran, modified gelatin), which are hydrophilic to render them swellable. Dextran and gelatin offer the advantage of being very similar to macromolecular substances that the biological environment is prepared to recognize and to deal with metabolically. An intriguing characteristic of them is their ability to be completely degraded by enzymes *in vivo*, which guarantees that the Odex/Mgel hydrogels will be eventually metabolized and disposed of by normal metabolic processes. Furthermore, the abundant hydroxyl groups on the dextran enable it to be oxidized with sodium periodate to obtain aldehyde groups. The existence of aldehyde groups after the process of oxidation was confirmed through FTIR spectroscopy. The characteristic absorbance peak of the aldehyde group was detected at 1733 cm^{-1} as previously reported.[27] Normally, gelatin maintains a gel state under room temperature, which is unfavourable for injection into cartilage defects. On the basis of our study, carboxyl groups on the gelatin main-chain were chemically modified to amino groups through making gelatin react with ethylenediamine. The intermolecular forces between amino and carboxyl groups, which is mainly attributed to the gel state of gelatin, would be weakened, and the Mgel solution could maintain its liquid state at room temperature. As a result, Odex and Mgel solutions could be injected into the site of cartilage defects to form a hydrogel by Schiff-base reaction between aldehyde groups and amino groups. This does not require surgical implantation and provides a minimally invasive strategy for the repair of cartilage defects through arthroscopic surgery. Moreover, due to its viscous behavior, the Odex/Mgel hydrogel could easily fit cartilage defects and circumvent incomplete integration between the graft and host tissue. Various potential therapeutic agents, such as drugs, cells and growth factors, could also be incorporated into the hydrogel by pre-mixing.

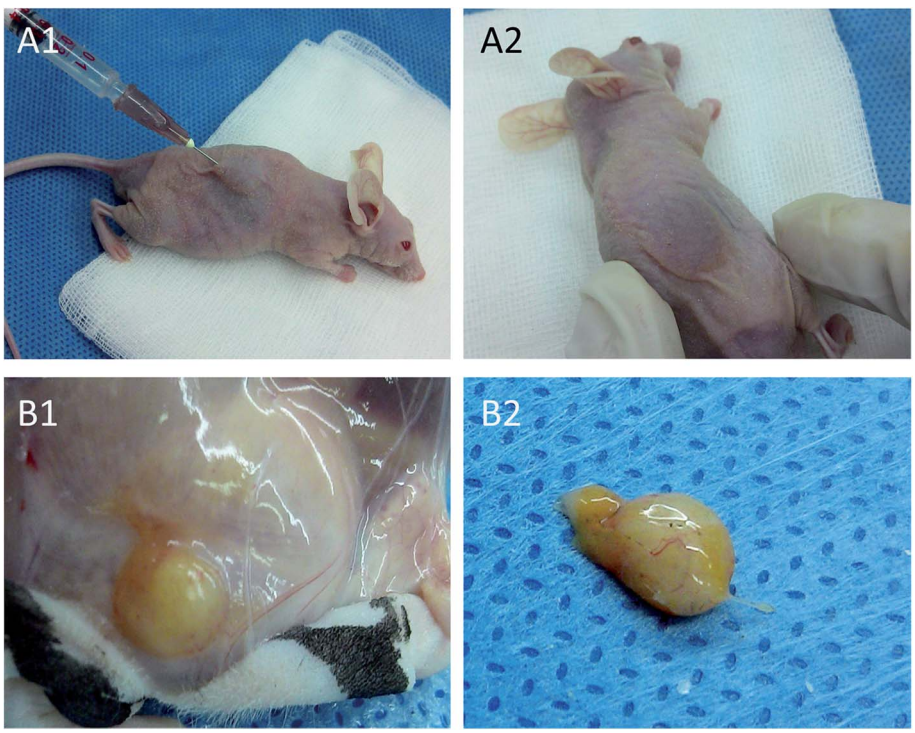


Fig. 12 The mixed solution was injected subcutaneously in the dorsa of athymic nude mice (A1) and Odex/Mgel hydrogel was formed (A2). Gross images of typical sample after 8 weeks *in vivo* (B1 and B2).

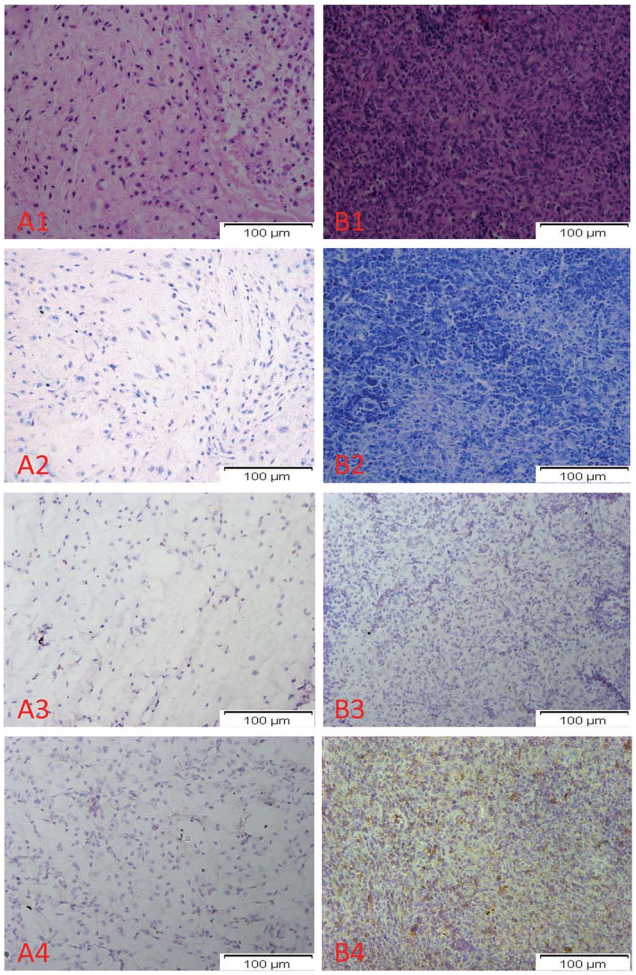


Fig. 13 *In vivo* evaluation of SMSC-loaded Odex/Mgel hydrogel combined with or without TGF-β3 (A1: hematoxylin eosin staining of SMSC-loaded Odex/Mgel hydrogel without TGF-β3; A2: toluidine blue staining of SMSC-loaded Odex/Mgel hydrogel without TGF-β3; A3: collagen type I immunohistological staining of SMSC-loaded Odex/Mgel hydrogel without TGF-β3; A4: collagen type II immunohistological staining of SMSC-loaded Odex/Mgel hydrogel without TGF-β3; B1: hematoxylin eosin staining of SMSC-loaded Odex/Mgel hydrogel with TGF-β3; B2: toluidine blue staining of SMSC-loaded Odex/Mgel hydrogel with TGF-β3; B3: collagen type I immunohistological staining of SMSC-loaded Odex/Mgel hydrogel with TGF-β3; B4: collagen type II immunohistological staining of SMSC-loaded Odex/Mgel hydrogel with TGF-β3).

For hydrolytically degradable hydrogels, the synchronization of the degradation rate with cellular growth and extracellular matrix accumulation is critical to match tissue formation. Through the chemical cross-linking, it is possible to alter the degradation rate and control the lifetime of the Odex/Mgel hydrogels, since the degradation behavior of hydrogels is dependent on the crosslinking density.[28] In general, gelatin and dextran can be easily solubilized in aqueous solutions. On the basis of this study, we increased the number of amino groups on the gelatin main-chain by changing the carboxyl groups into amino groups. Thus, the Odex/Mgel hydrogels had stronger resistance to degradation due to the higher crosslinking density. To examine the weight loss rate by hydrolytic degradation, we incubated the Odex/Mgel hydrogels in PBS at 37 °C for a time-dependent degradation assay. Our work found that the Odex/Mgel hydrogels could still hold approximately 60% of the initial weight after two weeks. In the process of neo-cartilage formation, it takes at least 14 days for the stem cells encapsulated in hydrogels to generate extracellular matrix. Therefore, the Odex/Mgel hydrogels meet this criterion and could generate adequate space to allow cells to generate extracellular matrix as the gel network degrades.

Moreover, the porosity is crucial to enable sufficient nutrient diffusion in the scaffold, as well as to provide a suitable environment for cell seeding, attachment, growth and ECM production. In order to serve as a suitable scaffold for guiding cell growth and differentiation, sufficient porosity and an interconnected pore structure need to be achieved to allow efficient mass transport. To characterize the internal microstructures of the Odex/Mgel hydrogels, the hydrogel samples were lyophilized and observed by using SEM. Previous experiments have demonstrated that for ingrowth of cartilage tissue, the recommended pore size ranges from 200 to 300 μm.[29,30] In our study, the pore size of the Odex/Mgel lyophilized hydrogel is about 200 μm, which is suitable to facilitate the formation of cartilage tissue. Therefore, these porous scaffolds with large void volume and large surface-area-to-volume ratio are desirable for cell attachment, migration and proliferation.

Stem cells, with advantages of self-renewal and multipotency, are thought to hold great promise for the repair of articular cartilage defects. Recently, synovium-derived mesenchymal stem cells have emerged as a new cell source for musculoskeletal regeneration.[22] Because they were first identified and isolated as a new member of MSC families in 2001, they have been intensively regarded as a promising cell source for the reconstruction of cartilage.[17] Some studies have revealed that SMSCs maintain a greater proliferative ability and chondrogenic potential than other sources of MSCs.[31,32] Therefore, we isolated and expanded SMSCs according to the method reported by De Bari. In this study, SMSCs showed great performance as a cell source for musculoskeletal regeneration. A minimal amount of synovial membrane tissue was harvested for SMSC isolation, and adequate yields per number of nucleated cells were achieved due to its high expandability *in vitro*. With regard to the availability of SMSCs for clinical use in the future, the synovial membrane can be obtained through arthroscopic surgery without causing complications at the donor site.

To evaluate the biocompatibility of Odex/Mgel hydrogels, the metabolic activity of SMSCs cultured with the hydrogel were analyzed by CCK-8 assay. Since a degradable hydrogel does not need to be removed surgically, unlike non-degradable scaffolds, degradable materials that require temporary presence to support cartilage regeneration are of value in cartilage tissue engineering. In addition, the use of degradable materials can also circumvent some of the problems related to the long-term safety of permanently implanted scaffolds. However, a potential concern relating to the use of degradable hydrogels is the toxicity of the scaffold's degradation products. Since all of the

scaffold's degradation products are released into the body of the patient, the design of a degradable hydrogel requires careful attention to testing the potential toxicity of the degradation products. Three primary assays are used for evaluating biocompatibility: direct or indirect contact, extracts of materials and filter diffusion.[33] In this experiment, we accomplished the filter diffusion assay through a Transwell system and found that there was no statistically significant difference between the hydrogel group and control group after 14 days in culture. This indicates that the Odex/Mgel hydrogels, which are judged to be nontoxic and compatible, will function in a biologically appropriate manner.

For the distribution of encapsulated cells in the Odex/Mgel hydrogels, whether cells could scatter in a homogenous order may directly affect the deposition of extracellular matrix and the formation of cartilage. Therefore, confocal microscopy was used to observe the distribution of synovium-derived mesenchymal stem cells. The results in Fig. 10 showed that the SMSC distribution inside the hydrogels was homogeneous and uniform. In addition, after 24 hours of culture, most encapsulated cells were alive based on the Live/Dead Viability Assay. Most of the live cells were round in shape, and a rounded cell shape is required for cell-specific functions such as extracellular matrix secretion. The homogeneous distribution of live SMSCs in the Odex/Mgel hydrogels is an important prerequisite for the successful formation of cartilage tissue. Thus, the Odex/Mgel hydrogels conform to this requirement for medical application in the area of healing cartilage defects.

Strategies for influencing MSC proliferation and differentiation have been well investigated. One of the most widely applied strategies for the enhancement of chondrogenesis *in vitro* is the addition of growth factors in the medium.[34] Microenvironmental factors, such as TGF-β3, FGF-2, BMP-6 and IGF-1, largely contribute to the regulation of these MSCs.[35–38] TGF-β3 could enhance MSC proliferation and differentiation potential during *in vitro* expansion. This classic growth factor supplementation for chondrogenic medium is 10 ng ml^{-1} of TGF-β3. TGFβ3 is the only well-established full inducer of chondrogenesis that leads to deposition of proteoglycan and collagen type II when added in the medium as a single factor.[39] To take advantage of this, we hypothesized that the incorporation of TGF-β3 in the Odex/Mgel hydrogel would produce a biomimetic microenvironment suitable for cartilage regeneration applications. Chondrogenesis of stem cells combined with TGF-β3 was also investigated in the Odex/Mgel hydrogel. In this study, we accomplished this test by adding TGF-β3 in the Odex/Mgel prepolymer solutions at a concentration of 10 ng ml^{-1} and then injecting SMSC-loaded Odex/Mgel hydrogel subcutaneously in the dorsa of athymic nude mice. After 8 weeks, cartilage-like tissues were formed and all the samples appeared ivory white without visible blood vessel infiltration. In the control group without TGF-β3, cartilage formation was very little and the samples disappeared due to absorption *in vivo*. In the process of differentiation of stem cells toward the chondrogenic lineage, extracellular matrix is a critical product and was stained basophilic by hematoxylin eosin. Glycosaminoglycans, stained bright blue by toluidine blue, are responsible for maintaining the viscoelastic compressive properties of cartilage tissue.[40] In this animal experiment, chondrogenesis in the SMSC-loaded Odex/Mgel hydrogel combined with TGF-β3 was confirmed *via* hematoxylin-eosin and toluidine-blue staining.

In summary, the Odex/Mgel hydrogel is a promising scaffold in cartilage tissue engineering. In the first place, the two natural materials of the Odex/Mgel hydrogel offer the advantage of being very similar, even identical, to macromolecular substances that the biological environment is prepared to recognize and to deal with metabolically. The stimulation of a chronic inflammatory reaction, which is often provoked by many synthetic materials, may thereby be suppressed. The immunogenicity of gelatin is typically far lower than that of most proteins. Dextran is also a weak immunogen relative to the majority of proteins. An additional advantage is that the use of Odex/Mgel hydrogel provides a minimally invasive way to repair cartilage defects.

Conclusion

In the present study, we prepared a biodegradable, fast *in situ* forming hydrogel based on oxidized dextran and amino gelatin without the addition of a chemical crosslinking agent and evaluated its application in cartilage tissue engineering through injection of SMSC-loaded Odex/Mgel hydrogel combined with TGF-β3 subcutaneously in the dorsa of athymic nude mice. The results demonstrated that the Odex/Mgel hydrogel was fabricated through oxidizing dextran and modifying gelatin. A homogeneous cell distribution inside the hydrogels was achieved and the cells cultured with hydrogel were capable of proliferating over long-term culture. Upon injection of SMSC-loaded Odex/Mgel hydrogel combined with TGF-β3 subcutaneously in the dorsa of athymic nude mice, no inflammatory reaction was observed. High production of GAGs and large cell accommodation were shown and newly formed cartilage was achieved. Our method provides a strategy for the design and fabrication of fast *in situ* forming hydrogels in tissue engineering. Our findings suggest that the lyophilized Odex/Mgel hydrogel with a porous structure is nontoxic as well as biocompatible and can be used for the regeneration of articular cartilage in tissue engineering.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Contract Grant no. 81171671, no. 81071468, no. 31470941, no. 31271035).

References

1 B. A. Jones and M. Pei, Synovium-derived stem cells: a tissue-specific stem cell for cartilage engineering and regeneration [J], *Tissue Eng., Part B*, 2012, **18**(4), 301–311.
2 J. R. Steadman, W. G. Rodkey, S. B. Singleton, *et al.* Microfracture technique for full-thickness chondral defects: Technique and clinical results[J], *Operat. Tech. Orthop.*, 1997, 7(4), 300–304.

3 W. Richter, Mesenchymal stem cells and cartilage in situ regeneration[J], *J. Intern. Med.*, 2009, **266**(4), 390–405.
4 B. Weber, M. Y. Emmert, R. Schoenauer, *et al.* Tissue engineering on matrix: future of autologous tissue replacement[C], *Semin. Immunopathol.*, 2011, **33**(3), 307–315.
5 E. B. Hunziker, Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects[J], *Osteoarthritis Cartilage*, 2002, **10**(6), 432–463.
6 P. H. Hernigou, A. Poignard, F. Beaujean, *et al.* Percutaneous autologous bone-marrow grafting for nonunions Influence of the number and concentration of progenitor cells[J], *J. Bone Jt. Surg.*, 2005, **87**(7), 1430–1437.
7 D. Eyre, Collagen of articular cartilage[J], *Arthritis Res.*, 2002, **4**(1), 30–35.
8 J. Martel-Pelletier, S. Kwan Tat and J. P. Pelletier, Effects of chondroitin sulfate in the pathophysiology of the osteoarthritic joint: a narrative review[J], *Osteoarthritis Cartilage*, 2010, **18**, S7–S11.
9 D. Guenther, A. Oks, M. Ettinger, *et al.* Enhanced migration of human bone marrow stromal cells in modified collagen hydrogels[J], *Int. Orthop.*, 2013, 1–7.
10 W. Liu, M. Griffith and L. I. Fengfu, Alginate microsphere-collagen composite hydrogel for ocular drug delivery and implantation[J], *J. Mater. Sci.: Mater. Med.*, 2008, **19**(11), 3365–3371.
11 Y. Liu, S. Cai, X. Z. Shu, *et al.* Release of basic fibroblast growth factor from a crosslinked glycosaminoglycan hydrogel promotes wound healing[J], *Wound Repair Regen.*, 2007, **15**(2), 245–251.
12 T. Gros, J. S. Sakamoto, A. Blesch, *et al.* Regeneration of long-tract axons through sites of spinal cord injury using templated agarose scaffolds[J], *Biomaterials*, 2010, **31**(26), 6719–6729.
13 A. C. Lima, W. Song, B. Blanco-Fernandez, *et al.* Synthesis of temperature-responsive dextran-MA/PNIPAAm particles for controlled drug delivery using superhydrophobic surfaces [J], *Pharm. Res.*, 2011, **28**(6), 1294–1305.
14 K. B. Djagny, Z. Wang and S. Xu, Gelatin: a valuable protein for food and pharmaceutical industries: review[J], *Crit. Rev. Food Sci. Nutr.*, 2001, **41**(6), 481–492.
15 X. Mo, H. Iwata and Y. Ikada, A tissue adhesives evaluated in vitro and in vivo analysis[J], *J. Biomed. Mater. Res., Part A*, 2010, **94**(1), 326–332.
16 X. Mo, H. Iwata, S. Matsuda, *et al.* Soft tissue adhesive composed of modified gelatin and polysaccharides[J], *J. Biomater. Sci., Polym. Ed.*, 2000, **11**(4), 341–351.
17 C. De Bari, F. Dell'Accio, P. Tylzanowski, *et al.* Multipotent mesenchymal stem cells from adult human synovial membrane[J], *Arthritis Rheum.*, 2001, **44**(8), 1928–1942.
18 G. M. Kavanagh and S. B. Ross-Murphy, Rheological characterisation of polymer gels[J], *Prog. Polym. Sci.*, 1998, **23**(3), 533–562.
19 H. H. Winter and M. Mours, Rheology of polymers near liquid-solid transitions[M], *Neutron spin echo spectroscopy viscoelasticity rheology*, Springer, Berlin Heidelberg, 1997, pp. 165–234.
20 J. P. St-Pierre, L. Gan, J. Wang, *et al.* The incorporation of a zone of calcified cartilage improves the interfacial shear strength between in vitro-formed cartilage and the underlying substrate[J], *Acta Biomater.*, 2012, **8**(4), 1603–1615.
21 H. H. Winter and F. Chambon, Analysis of linear viscoelasticity of a crosslinking polymer at the gel point[J], *J. Rheol.*, 1986, **30**(2), 367–382.
22 J. Fan, R. R. Varshney, L. Ren, *et al.* Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration[J], *Tissue Eng., Part B*, 2009, **15**(1), 75–86.
23 A. A. Amini and L. S. Nair, Injectable hydrogels for bone and cartilage repair[J], *Biomed. Mater.*, 2012, **7**(2), 024105.
24 J. Zhu, Bioactive modification of poly (ethylene glycol) hydrogels for tissue engineering[J], *Biomaterials*, 2010, **31**(17), 4639–4656.
25 K. Y. Lee and D. J. Mooney, Hydrogels for tissue engineering [J], *Chem. Rev.*, 2001, **101**(7), 1869–1880.
26 J. L. Drury and D. J. Mooney, Hydrogels for tissue engineering: scaffold design variables and applications[J], *Biomaterials*, 2003, **24**(24), 4337–4351.
27 H. Tan, C. R. Chu, K. A. Payne, *et al.* Injectable in situ forming biodegradable chitosan–hyaluronic acid based hydrogels for cartilage tissue engineering[J], *Biomaterials*, 2009, **30**(13), 2499–2506.
28 S. J. De Jong, B. Van Eerdenbrugh, C. F. van Nostrum, *et al.* Physically crosslinked dextran hydrogels by stereocomplex formation of lactic acid oligomers: degradation and protein release behavior[J], *J. Controlled Release*, 2001, **71**(3), 261–275.
29 H. Y. Cheung, K. T. Lau, T. P. Lu, *et al.* A critical review on polymer-based bio-engineered materials for scaffold development[J], *Composites, Part B*, 2007, **38**(3), 291–300.
30 B. D. Ratner, A. S. Hoffman and F. I. Schoen, *et al.*, *Biomaterials science: an introduction to materials in medicine [J]*, San Diego, California, 2004, pp. 162–164.
31 Y. Sakaguchi, I. Sekiya, K. Yagishita, *et al.* Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source[J], *Arthritis Rheum.*, 2005, **52**(8), 2521–2529.
32 H. Yoshimura, T. Muneta, A. Nimura, *et al.* Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle[J], *Cell Tissue Res.*, 2007, **327**(3), 449–462.
33 A. Wennberg, G. Hasselgren and L. Tronstad, A method for toxicity screening of biomaterials using cells cultured on millipore filters[J], *J. Biomed. Mater. Res.*, 1979, **13**(1), 109–120.
34 S. Boeuf and W. Richter, Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors[J], *Stem Cell Res. Ther.*, 2010, **1**(4), 31.
35 L. Bian, D. Y. Zhai, E. Tous, *et al.* Enhanced MSC chondrogenesis following delivery of TGF-β3 from alginate microspheres within hyaluronic acid hydrogels in vitro and in vivo [J], *Biomaterials*, 2011, **32**(27), 6425–6434.

36 S. Tsutsumi, A. Shimazu, K. Miyazaki, *et al.* Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF[J], *Biochem. Biophys. Res. Commun.*, 2001, **288**(2), 413–419.
37 S. Vukicevic and L. Grgurevic, BMP-6 and mesenchymal stem cell differentiation[J], *Cytokine Growth Factor Rev.*, 2009, **20**(5), 441–448.
38 N. Indrawattana, G. Chen, M. Tadokoro, *et al.* Growth factor combination for chondrogenic induction from human mesenchymal stem cell[J], *Biochem. Biophys. Res. Commun.*, 2004, **320**(3), 914–919.
39 A. M. Mackay, S. C. Beck, J. M. Murphy, *et al.* Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow[J], *Tissue Eng.*, 1998, **4**(4), 415–428.
40 V. P. Willard, A. J. Reimer, K. A. Athanasiou, *et al.* The regional contribution of glycosaminoglycans to temporomandibular joint disc compressive properties[J], *J. Biomech. Eng.*, 2012, **134**(1), 011011.