

Preparation and Characterization of a Novel Injectable Hydrogel

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Abstract

Injectable hydrogels have emerged as a great candidate in tissue engineering for they can be delivered via a minimally invasive manner. Here, we report an *in situ* forming hydrogel composed of oxidized dextran (Odex) and modified gelatin. The dynamic gelling process was measured through rheological measurements. The effect of the ratio of Odex and gelatin on gelling time, microstructure, swelling ratio and *in vitro* degradation of the composite hydrogels were examined. Biological assess was performed through WST-1 Assay by using Synovium-derived Mesenchymal Cells (SMSCs). According to the results, adjustable physicochemical properties can be obtained through simply altering the ratio of Odex and gelatin. Moreover, with the increase of incorporated gelatin, better biocompatibility was shown in the composite hydrogels, which exhibited its potentially high application prospect in the field of cartilage tissue engineering.

Keywords: Oxidized dextran (Odex); Gelatin; Synovium-derived mesenchymal cells (SMSCs); In situ; Hydrogel; Tissue engineering

Introduction

Articular cartilage plays an important role in withstanding enormous mechanical load to protect the underlying bones. As the average human life expectancy increases, a significant number of patients undergo a variety of medical procedures for the repair of articular cartilage defects caused by sports injury or degenerative diseases such as osteoarthritis (OA) and Rheumatoid Arthritis (RA). Cartilage cannot undergo spontaneous repair because of a lack of access to the blood supply, so cartilage defects can further damage other articular tissues and result in pain, swelling and hopping.

Tissue engineering is a comprehensive field that involves the application of engineering principles and life sciences for the creation of substitutes to improve or replace biological functions. It involves the use of specific cells, suitable scaffolds as cell transplantation vehicles and appropriate biochemical signaling molecules as biological cues to direct cells towards differentiation. Hydrogels, highly hydrated cross-linked polymer networks and extremely similar to the nature of the extracellular matrix, have emerged as powerful and efficacious scaffolds for 3D cell culture [1-4]. Currently, there is an increasing interest in the development of novel hydrogel systems, one of which is aqueous injectable, *in situ* gel-forming system. This injectable matrix could be directly delivered into voids or cavities through a needle or a catheter and does not require surgical implantation [5-7]. Due to its viscous behavior, the system could fit a cavity or a defect easily (e.g. cartilage defect) [8]. Moreover, various potential therapeutic agents, such as drugs [9,10], cells [11,12] and growth factors [13,14], could also be incorporated into the matrix by pre-mixing.

Dextran, composed of linear α -1,6-linked D-glucopyranose residues with a few percent of α -1,2, α -1,3 and α -1,4 linked side chains, is a naturally colloidal, hydrophilic, biocompatible, and nontoxic polysaccharide [15,16]. It has been widely researched as a macromolecular carrier for delivery of drugs or proteins for biomedical applications [17]. Architecturally, the affluent hydroxyl groups on the main-chain of dextran make it possible to be oxidized with periodate to produce an entity with multiple aldehyde groups, which serves as a cross-linker for those polymers bearing free amino groups to form hydrogels [18]. Gelatin is a collagen-derived protein with unique gelation behavior ascribe to physical crosslinking of the triple-helix conformation of native collagen [19]. However, gelatin

hydrogel has a rapid solubilization in aqueous environment and melts easily within body temperature range, thus limiting its potential in biomedical applications.

In this study, we have modified gelatin with ethylenediamine to keep the gelatin an liquid status at room temperature at a high mass fraction (20 wt%). The amino groups content of modified gelatin also increased and react with aldehyde groups of oxidized dextran to form hydrogels. The physicochemical properties of the quickly *in situ* gel-forming hydrogels were investigated in terms of gelation time, swelling ratio, degradation behavior and gel morphology. The Synovium-derived Mesenchymal Cells (SMSCs) are multipotent progenitor cells and have the capacity to differentiate into a variety of connective tissue cells including bone, cartilage, and adipose tissue both *in vitro* and *in vivo*. We further characterized the cytotoxicity and biocompatibility of our hydrogel with SMSCs. Specifically, adhesion and spreading of the SMSCs on hydrogel surface were studied as well as the viability of SMSCs was evaluated by WST-1 assay. The results show that *in situ* forming hydrogel from oxidized dextran and gelatin are suitable as scaffolds to support the survival of SMSCs and they have high potential for cartilage repair.

Materials and Methods

Materials

Dextran, sodium periodate, ethylenediamine, gelatin and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride crystalline (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's modified Eagle's medium (DMEM), collagenase

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II, penicillin-streptomycin solution and all other culture media and reagents were purchased from Gibco Life Technologies Corporation (Carlsbad, CA, USA). WST-1 was purchased from Roche Applied Science (Indianapolis, IN, USA). Tissue culture flasks were obtained from BD Biosciences Corporation (San Jose, CA, USA).

Synthesis of oxidized dextran and modified gelatin

Oxidized dextran (Odex) was synthesized by reacting with sodium periodate. Briefly, Odex was prepared by first dissolving 10 g of dextran in 100 mL of distilled water, followed by the addition of a desired amount of NaIO₄ (6.34 g dissolved in 100 ml of water). The solution was stirred at room temperature for 6 hours 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 isolated yields were 75%.

Gelatin was dissolved in Phosphate Buffered Solution (PBS) to a final concentration of 5 wt% at room temperature. Ethylenediamine and EDC were added into the gelatin solution. The molar ratio of the carboxyl groups on gelatin chains, EDC and ethylenediamine was 1:2:40. Immediately after that, the pH of solution was adjusted to 5.0 by adding hydrochloric acid (HCl). The reaction mixture was agitated at room temperature overnight, and then dialyzed against Double-Distilled Water (DDW) for 48 hours to remove the excess ED and EDC. The dialyzed solution was freeze-dried at -80°C to obtain a modified gelatin.

Hydrogel preparation

10 wt% Odex and 20 wt% gelatin was prepared in PBS for physicochemical characterization, separately. Odex and gelatin were mixed at weight ratio of 7:3, 6:4, 5:5, 4:6 and 3:7 at 37°C. The different mixture solutions were injected into round molds and then incubated at 37°C for gel forming.

Rheological measurements and gelation time

Rheological measurements were performed on a rheometer. The aqueous polymer solution was mixed and pipetted directly onto the bottom plate, and the top plate was lowered to contact the gelling solution with a 1 mm gap size. For time sweeping tests, the storage moduli G' and loss moduli G'' of hydrogels were monitored as a function of time at a frequency of 1 rad/s and a shear strain of 2% under a constant temperature of 37°C.

The time to form a gel (defined as gelation time) was determined using the tube tilting method. No flow within 15 s upon inverting the tube was regarded as the gel state. In brief, a 1.5 ml eppendorf tube containing 0.5 ml of the mixture of Odex and gelatin solution was immersed in a water bath at 37°C and gently vortexed. When no fluidity was visually observed upon inverting the tube, the gel state was determined, and the time cost was defined as the gelation time. All samples were analyzed in triplicate.

SEM analysis

To characterize the internal microstructures of the composite hydrogel, the hydrogel samples were rinsed with deionized water for five times at room temperature, frozen in liquid nitrogen at -80°C and then lyophilized to get porous scaffolds. The dried hydrogel samples were cut to expose the cross-sections and coated with gold for 120 seconds using a sputter coater. The samples were observed by using scanning electron microscopy (SEM, JSM-5600, Japan) under an accelerating voltage of 10 kV.

Swelling analysis

To study the swelling kinetics of the modified hydrogels, the Odex/gelatin hydrogels at different ratios (3:7, 4:6, 5:5, 6:4, 7:3) were frozen at -80°C and lyophilized in a vacuum oven. The dry hydrogels 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 quickly wiped out with a filter paper so that the swollen weight could be measured (W_s) accurately. The Swelling Ratio (SR) was then calculated according to the following equation: $SR = W_s / W_d$.

In vitro degradation

To determine the weight loss due to hydrolytic degradation, hydrogels were divided into four groups (day 3, day 7, day 14, day 21) for time-control degradation study. The samples (n=3) were prepared from blending 10 wt% Odex and 20 wt% gelatin aqueous solutions in different ratios (3:7, 4:6, 5:5, 6:4, 7:3) and pre-swollen in PBS overnight. Subsequently, the weight of the sample was record as W_0 and the hydrogels were completely submerged in PBS at 37°C. At different time intervals, the samples were removed from the solution, blotted dry and then weighed to determine the weight of the remaining mass (W_1). The PBS was replaced every 3 days and the experiments were performed in triplicate. The weight loss (%) was calculated as the following formula: $\text{weight loss} = (W_0 - W_1) / W_0 \times 100\%$.

Cell culture

Synovium-derived mesenchymal cells (SMSCs) were isolated and expanded as reported by De Bari et al. [20]. In Brief, synovial membrane tissue was harvested aseptically 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), minced and digested with trypsin-EDTA (0.1% trypsin, 0.4 mM EDTA) at 37°C for 30 minutes, then 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 hours. After incubation cells were collected from digested solution with filter, centrifugated at 1500 rpm for 5 minutes to obtain a cell pellet and resuspended in complete medium at suitable concentration. Then cells were seeded in culture flasks and allowed to proliferate in the complete medium at 37°C in a humidified atmosphere of 5% CO₂. The complete medium was replaced once every 3-4 days. When the attached cells reached 90% confluence after 9-12 days of 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 first subculture.

SMSCs adhesion and spreading

Mixed solution was uniformly added in the 24-well tissue culture plate and crosslinked by incubated at 37°C for 12 h. SMSCs were seeded on the hydrogel surface at a density of 5,000 cells well⁻¹. Then 500 μ l complete medium was added to each well and the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. After 3 days cell-seeding hydrogels were washed with PBS for two times to remove unattached cells. Images of cells were acquired with imaging software through microscope.

Cell viability

Cell viability was determined using a WST-1 Assay Kit. For this

assay, each 300 μ l mixed solution was injected into 24-well tissue culture plate and formed hydrogel at 37°C. The synovium-derived mesenchymal cells suspension with cell density of 1×10^6 cells/mL was injected on the surfaces of hydrogel. The cell-seeding hydrogels were incubated at 37°C in a humidified atmosphere of 5% CO₂. The culture medium was exchanged every day. After 3 days and 7 days culture, 200 μ l WST-1 solution was added to each well and incubated for 2 hours at 37°C. Then the medium with WST-1 was transferred to 96-well tissue culture plate and the absorbance was read at 450 nm. All experiments were carried out in triplicate.

Statistical analysis

Tests were done in 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 and Discussion

Preparation of oxidized dextran and modified gelatin

Dextran contains vicinal hydroxyl groups and the periodate ion attacks one of the vicinal hydroxyl groups in dextran and yields two aldehyde groups (Figure 1A). The oxidized dextran bearing aldehyde groups serves as a good macromolecular crosslinker for polymers with amino groups. In general, gelatin maintains a gel state under room temperature, which was unfavourable for injectable applications. Here, through reaction with ethylenediamine, carboxyl groups on gelatin chains were chemically modified to amino groups (Figure 1B). Therefore, the strong hydrogen bonds between amino and carboxyl groups, which mainly attribute to the gel state of gelatin, could be disrupted. As a result, gelatin at concentration of 20 wt% could maintain its liquid state at room temperature.

Rheological analysis and gelation time

When Odex and gelatin solutions were mixed together at 37°C, the hydrogels were formed rapidly through a Schiff-base reaction between aldehyde groups and amino groups. The gelation process was monitored by rheological measurements. Figure 2 shows the variation of storage modulus (G'), loss modulus (G'') as well as the complex viscosity (η^*) versus time for the Odex/gelatin hydrogel (ratio 5:5) preparation during hydrogel forming at 37°C. After mixing, G' is lower than G'' and the system exhibited the behavior of a viscous fluid as the compound solution of Odex and gelatin. Then both moduli

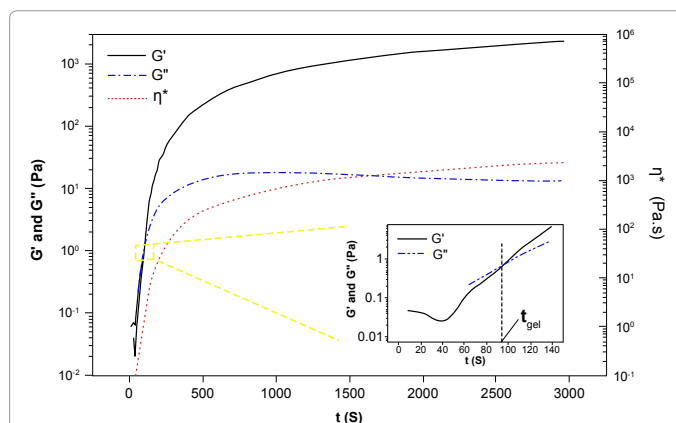
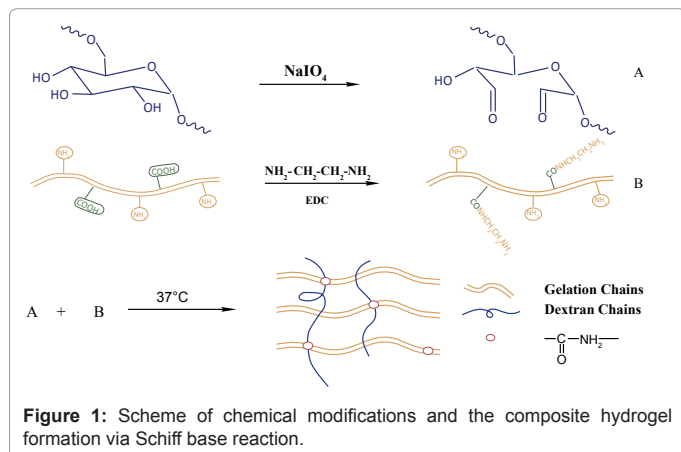


Figure 2: Rheological analysis: the relationship between storage modulus (G'), loss modulus (G'') and complex viscosity (η^*) with time after mixing at 37°C.

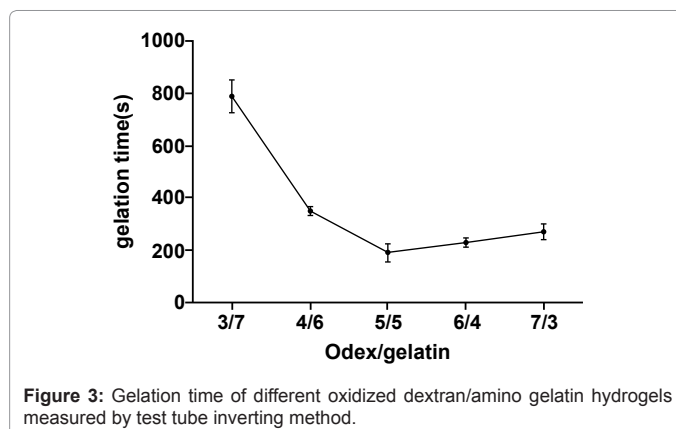


Figure 3: Gelation time of different oxidized dextran/amino gelatin hydrogels measured by test tube inverting method.

increased quickly and the solutions begin to gel. With the happening of Schiff base reaction between the aldehyde residues of Odex and the amino residues of gelatin, the growing rate of G' was much higher than that of G'' . Therefore, there was a crossover point where storage modulus is equal to loss modulus. This crossover point was normally considered as “gel point” (t_{gel}) and indicative of transition of the Odex/gelatin system from a viscous behavior dominated liquid-phase to an elastic behavior dominated solid-phase [21]. At last both moduli leveled off and a well-developed three-dimensional network formed. η^* of the system also underwent a similar transformation, a rapid increase at the beginning followed by leveling off over time. For the Odex/gelatin (ratio 5:5) system, t_{gel} was approximately 95s, which was very fast during hydrogel formation.

In the tube tilting test, the time required for solution transforming into gel was recorded as gelation time. Figure 3 shows the gelation time for different hydrogels. With the increase of Odex's content, the gel time clipped. Specifically, when the proportions of Odex/gelatin increased from 3/7 to 5/5, the gel time was shortened from 790 to 190 seconds. It is inferred that the Odex concentration has a significant impact on this *in situ* crosslinkable system and the gelation speed could be modulated by adjusting the concentrations of Odex/gelatin. This provides us an easy way to modulate the gel time.

Morphology of hydrogels

As shown in figure 4A, Odex and gelatin solutions were separately prepared in syringes and injected into culture dish. Then the gel can be formed in a short time. In this study, all hydrogel samples were

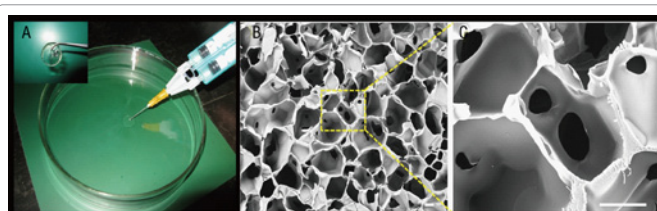


Figure 4: (A) Photograph of forming hydrogel by syringes; (B and C) SEM micrographs for lyophilized hydrogel samples. Scale bar represents 50 μm .

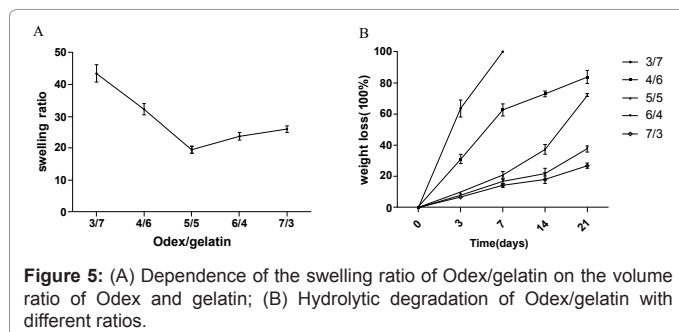


Figure 5: (A) Dependence of the swelling ratio of Odex/gelatin on the volume ratio of Odex and gelatin; (B) Hydrolytic degradation of Odex/gelatin with different ratios.

flash-frozen at -80°C in liquid nitrogen and lyophilized. After that, the corresponding cross-section of the dry samples was observed by SEM. Figures 4B and 4C showed the SEM micrographs of freeze-dried hydrogels. All hydrogels showed good interconnected porous structures with an average pore sizes ranging from 50-200 μm . The high interconnected structure is necessary for tissue engineering scaffolds to promote nutrient and gas diffusion, allow cellular ingrowth and retain high water [22]. Therefore, the hydrogels might

Swelling and degradation analyses

The swelling behavior is an important property of tissue engineering scaffold because it relates to the diffusion of signaling molecules and nutrients [23]. Figure 5A indicates the swelling results of freeze-dried composite hydrogels in PBS. The dry Odex/gelatin hydrogels could absorb large quantity of water from 19.47 to 43.45 times of their original dry weight, suggesting that they could be good scaffolds to retain tissue fluid and nutrients *in vivo*. Figure 5A also revealed the relationship between the swelling ratio of the Odex/gelatin hydrogels and the Odex content in the hydrogels. Generally, the swelling ratio of a hydrogel is related to physicochemical factors, such as the crosslinking density, gel composition, network structure, etc [24].

The swelling ratio decreased rapidly from 43.45 to 19.47 with the increase in the Odex content from 30 to 50%, which can be ascribed to the increase of crosslinking degree. Thereafter, with an elevation of Odex content from 50 to 70%, the crosslinking density in the hydrogels declined and the swelling ratio increased slightly from 19.47 to 25.94. In this regard, the variation of the swelling ratio corresponds well with the information of the gelation time depicted in figure 3. It is also worthwhile to notice that group 5/5 exhibited apparently different swelling property compared with groups of 3/7 and 4/6. We ascribe this obvious decline on swelling ratio to the decrease content of gelatin, which have a strong ability to absorb water. From the clinical aspect, over load swelling ratio of the scaffolds may cause pressure to the surrounding tissues. In contrast, under load swelling ratio of the scaffolds would result in insufficient nutrients exchanged from surrounding circumstances. Meanwhile, the scaffolds with

inadequate swelling ratio may escape from the implant point easily [25]. Therefore, the Odex/gelatin hydrogel with an adjustable swelling property can meet those requirements by changing the ratio of components.

The degradation of five composite hydrogels was monitored as by incubating in PBS at 37°C , as shown in figure 5B. Apparently, the ratio of Odex and gelatin has great impact on the weight loss of samples. Concretely, the 3:7 Odex/gelatin hydrogel exhibited the fastest degradation rate and totally degraded after 7 days. By contrast, 4:6 and 5:5 groups showed a more controllable degradation rate due to higher crosslinking density. Since gelatin can be easily solubilized in aqueous environment [26], groups of 6:4 and 7:3 with an incline of gelatin have the strongest resistance to degradation that they could still hold more than 50% of the initial weight after three weeks. Generally, it takes at least 14 days for the stem cells seeded in hydrogels to generate extracellular matrices for neo-cartilage formation [27]. The results indicate that the Odex/gelatin hydrogels may support the proliferation of stem cells to repair cartilage defect in tissue engineering.

SMSCs adhesion and spreading

In this experiment, SMSCs were used to characterize the biocompatibility of the *in situ* gelling system due to the capacity to differentiate into cartilage tissue both *in vitro* and *in vivo*. The adhesion and proliferation of SMSCs on the hydrogels are studied by culturing the cells on thin hydrogel films. SMSCs seeded in 24-well tissue culture plate served as the positive control. As demonstrated in figure 6, cell attachment and spreading varied with the changing of the Odex and gelatin ratio. In brief, with the ratio of Odex/gelatin increasing from 3/7 to 7/3 (a-e), the matrix became more and more resistant for SMSCs to adhere and spread. The explanation is based on the report by Massia that dextran limits cell adhesion and resists adsorbing protein [28]. Gelatin, like other ECM proteins such as collagen and fibrin, is capable of promoting cell adhesion [24]. Thus, with the increase of incorporated gelatin, better adhesive property was exhibited from the study.

Cell viability

The WST-1 Assay was implemented to quantitatively investigate cell viability of SMSCs on the hydrogels after 3 and 7 days. As shown in figure 7, there was a significant effect of gelatin concentration ($p < 0.05$) and culture time ($p < 0.05$) on cell viability. The statistical analysis revealed that SMSCs had an increased metabolic activity on the hydrogels with the increase of incorporated gelatin. And after 7

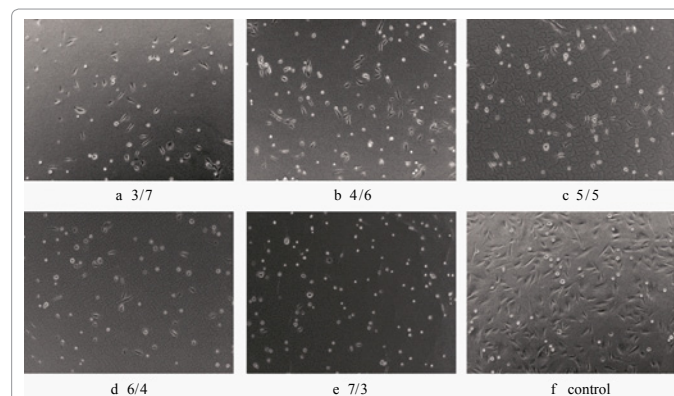
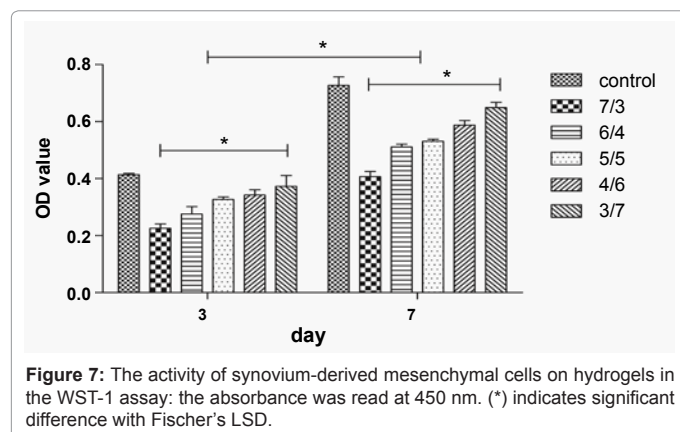


Figure 6: Micrographs of SMSCs grown on the surface of (a) 3/7, (b) 4/6, (c) 5/5, (d) 6/4, (e) 7/3 Odex/gelatin hydrogels and in (f) 24-well tissue culture plate, 3 days after seeding.



days *in vitro* culture, SMSCs on the surface of hydrogels remained viable and proliferative compared with the day 3 group, indicating the good cytocompatibility of hydrogels. These results implied that Odex/gelatin hydrogels might be good scaffold materials which have excellent biocompatibility.

Conclusion

In this study, we prepare a novel biodegradable, fast *in situ* forming hydrogel based on oxidized dextran and amino gelatin. Dextran is oxidized to generate aldehyde functional groups to react with free amino groups of modified gelatin for formulating nontoxic hydrogels with highly porous structures. The physical properties, such as rheological behavior, swelling ratio, gelling time and degradation are investigated. Results indicate that the physical properties could be modulated through simply changing the proportion of Odex and gelatin. Surface adhesion and spreading of cells have been testified by using synovium-derived mesenchymal cells, and the incorporation of gelatin into the hydrogels significantly promoted adhesion of SMSCs and supported spreading and proliferation of SMSCs *in vitro*. Furthermore, WST-1 assay demonstrated that the composite hydrogels with higher gelatin concentration possess more excellent biocompatibility and could promote cell proliferation more effectively *in vitro*. The multi advantageous features make this hydrogel an attractive material as transient cartilage tissue substitutes in tissue engineering.

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