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Cryopreservation of cell/hydrogel constructs based on a new cell-assembling technique

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Abstract

Organ manufacturing products hold the promise to be used widely in the future for complex organ failings. The cryopreservation of the product is a very important step in the commercialization activities. In this article, a new cell cryopreservation technique, whereby, cryoprotectants were directly incorporated into the cell/hydrogel constructs, prototyped according to the predesigned structure and then subjected to a special freezing/thawing process. The rheological and hydration properties of the cryopreservation systems indicated that the hydratabilities of the gelatin/alginate hydrogels were greatly increased while the eutectic temperatures were greatly decreased by the addition of glycerol. Dextran-40 was found to be effective to improve the cell survival when incorporated with glycerol. The optimal volume concentration of the

cryopretectans was 2.5% (v/v) glycerol and 5% (g/mL) dextran-40 in the gelatin/alginate hydrogel. Under these conditions, the cell viability in the construct was 96.3 ± 3.4%. Microscopic, thiazolyl blue (MTT) and hematoxylin and eosin (HE) staining results all indicated that the cells began to proliferate after thawing. The cells in the gelatin/alginate hydrogel with both glycerol and dextran-40 had greater proliferations than with only glycerol or dextran-40. This approach holds promise for a variety of applications in cell assembly for high-throughput drug screening and complex organ manufacturing areas.

Keywords: Cell-assembly technique; rapid prototyping; tissue Engineering; cryopreservation; adipose-derived stem cells (ADSCs)

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Introduction

It is anticipated that there will be a demand for a broad range of organ manufacturing products, such as “ready-to-use” and patient-specific products. The manufacturing process must accommodate a large number of cells in the matrix, particularly different cell types [1]. However, the maintenance of these living constructs is costly and labor-intensive. However, well-preserved viable cells contained in constructs can eliminate lengthy awaiting periods for needy individuals, and bring down the medical costs significantly as the preparation for solutions can take place at scheduled and moderate timing. It is foreseeable that future application of organ manufacturing products will dependant largely on the establishment of an effective distribution method for the patients in need and the cryopreservation techniques will play a central role in the manufacturing of complex organs [2].

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Cryopreservation of cells was developed by scientists more than 300 years ago and is now routinely used to store semen, embryos, as well as tissues from animals and humans [3]. During the cryopreservation procedure, the cells are suspended in certain cryoprotectant contained solutions and are stored in a deep cryogenic temperature (ordinarily below -70°C). Cryoprotectants are used with the purpose to replace water in cells/tissues to minimize the damage caused by ice formation during the cooling-cryostorage-warming cycle [4]. Cryopreservation can be considered as an alterative for the long-term maintenance and large-volume distribution of the cell containing products. The development of an effective, safe, and sterile cryopreservation protocol is a prerequisite for the long-term storage of the cell-assembly and organ manufacturing products with respect to the serious donor shortage of organs and labor-intensive cultures.

One of the vital issues is to develop an effective and suitable cooling/warming procedure for the cell containing constructs [5]. In our preliminary study, a 78.7% cell viability was obtained with the incorporation of DMSO in the gelatin/alginate/fibrinogen hydrogel [2]. During the warming stage, the 3D construct was first incubated at 37 until the surface of the construct began to thaw. Then, 5% $CaCl_2$ was added to the container at 4 for 2 min to crosslink the alginate. The construct was finally put into a thrombin solution at 37 ^{o}C for 20 min to polymerize the fibrinogen. However, without fibrinogen in the hydrogel, the cell containing gelatin/alginate was easily broken during the warming period.

Cryoprotectants can be divided into two classes; penetrating (such as dimenthysulfoxide (DMSO) and glyercol) and nonpenetrating (such as dextran and hydroxcellulose) that can protect cells and their membranes from damage during the freezing/thawing processes. Protection

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against intracellular ice formation has been attributed to the colligative effects of cryoprotectant chemicals [6]. Careful component selection is another vital issue to avoid toxic effects of the cryoprotectant, as well as the prevention of ice formation during cryopreservation [7]. Glycerol was the first penetrating agent used 60 years ago [8,9] but is potentially toxic to cells. Sugars (nonpenetrating agents) are also used in cryopreservation as they form an important component in osmotic buffers. It has been suggested that some sugars are capable of preserving the structural and functional integrity of cell membranes at low water activities [10]. The addition of carbohydrate sugars to penetrating cryoprotectants aids in the dehydration of embryos [10].

In this article, penetrating cryoprotectant glycerol was used alone or in combination with non-penetrating dextran-40 as additives in the adipose-derived stem cell (ADSC)/gelatin/alginate assembly constructs. The incorporation of cryoprotectants directly into the cell/hydrogel system

is an easy and convenient way in the controlled cell assembly techniques. After assembly at 4°C, the constructs were maintained at 4°C for 30 min. Then -20 for about 1 h before cooling slowly to -80 for long-term storage. During the warming stage, 17°C was chosen as the recovery temperature to keep the integrity of the gelatin/alginate construct. The component selection and warming temperature of 17°C have promise for a variety of applications in cell assembly and organ manufacturing areas.

Results and Discussion

Rheological property analyses and recovery temperature selection of the hydrogel systems

The curves in Figure 1 indicate how viscosities (ŋ) of the hydrogel systems changes with temperature. The viscosities (ŋ) of the hydrogels change dramatically between 15 – 20°C. Similar results were found in the control P0 group without glycerol and the groups from P1-1 to P1-4 with different amount of glycerol. The viscosities remained almost the same when the temperature was higher than 18.8°C. When the temperature was higher than 18.8°C, the hydrogel systems were at

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viscous fluid states with very low viscosities. When the temperature was decreased to 16.3^{o}C in the control group P0, and 15.1 ^{o}C in the groups from P1-1 to P1-4, the viscosities of the systems increased rapidly. The viscosities of all the systems changed greatly in a narrow temperature range, showing that there is a significant thermo-sensitivity in all the systems at about 17°C.

It can be seen from Figure 2 that when the test temperature was 15°C and the test frequency was less than 100 rad/s the elastic moduli of all the hydrogel systems were larger than the viscous ones. Under these conditions, the mechanical ullage (tg)s were below 0.35 and the structural properties were relatively stronger. When the test frequency was increased the mechanical ullage and the liquidity of the hydrogel systems increased. Comparing groups from P1-1 to P1-4, with the decrease of gelatin concentrations in the groups of P1-3 and P1-4, the storage modulus became larger, the mechanical ullage (tg) became smaller and the structures became more stable. This means that the addition of glycerol can enhance the structural stability of the gelatin/alginate hydrogel system.

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Through the rheological analysis above, it is clear that each group remained in a relatively stable gel state at 15 and a mutation around 17°C in the viscosity vs temperature curve, above 17°C the system becomes a Newtonian fluid. Therefore, 17°C was chosen as the recovery temperature during the thawing stage. Under this condition, the hydrogels were maintained as gels with no obvious rheology. During the thawing stage, the constructs were directly put into a 17°C water bath and observed visually. When the color of the constructs turned from being opaque white to milk-white with transparent cells (about four minutes), the constructs were put into the 5% crosslinking $CaCl_2$ solution for one minute. The constructs were washed four times with the culture medium and then cultured in an incubator at 37°C and 5% CO_2. The constructs thawed rapidly during the recovery process and the grid structures maintained their stability

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(Figure 3). The diameters of channels in the constructs were 400 600 m (Figure 3a). After freeze-drying, the micropores, 5 100 m in diameter, were produced in the hydrogel filaments (Figure 3b).

The cryopreservation process based on the controlled cell assembly technique can be divided into three major phases; a) a pre-freezing phase in where the ADSCs are enwrapped in the glycerol contained gelatin/alginate hydrogel at 4 - 10°C, b) a critical freezing phase at -20°C, cells underwent osmotic and thermal stresses, and c) a thawing phase in which a reverse process occurs at 17°C. During the relatively slow-cooling phase, ice formed outside the cells could potentially decrease the osmotic imbalance. The formation of intracellular ice and the toxic effect of the glycerol were largely responsible for the diminished cell recovery rates. On thawing, the penetrating glycerol can be easily removed from the system through dilution. It is possible that

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the glycerol and dextran-40 altered the cooling rate required to avoid ice crystallization in the hydrogel. It is, also, logical to use the lowest possible concentration of penetrating glycerol to minimize problems of toxicity.

The warming temperature that is used to return the 3D constructs to normothermic temperatures is very important. It is necessary to avoid ice formation and coalesce while maintaining the integrity of the 3D constructs. We found that when the thawing temperature was 37°C, the grid structure was destroyed by the swelling and conglomeration of the hydrogel. To avoid this situation, a specific warming temperature of 17°C was chosen to ensure the 3D structures remain integrity after the thawing and crosslinking stages. It was technically feasible to restore the cells as well as the 3D architecture at this temperature with minimal loss of cell viability during preservation.

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Hydration properties and the melting points of the hydrogel systems

The DSC curves of the heat flow during the cooling and warming processes of the hydrogel systems are shown in Figure 4. There was a clear exothermic peak ranging from -20°C to -11°C or an endothermic peak from -10 to 10 in each curve. The exothermic peak was due to the free water which froze inside the hydrogels during the cooling process while the endothermic peak was due to the bond water which could not be frozen inside the hydrogel during the cooling process. Each of the exothermic peaks was proportional to the enthalpy of crystalline phase transition. Based on Figure 4, it could inferred that the addition of glycerol lowered the enthalpy of the gelatin/alginate hydrogel systems, which means there is an apparent decrease in free water that could be frozen during the cooling process.

The drop of the crystallization enthalpy in the curves with the addition of glycerol

represents the prevention of crystal formation during the cooling processes. Compared with the control group, some of the free water formed hydrogen bonds with the glycerol lowering the freezing temperature. The unfrozen water represents the water binding ability of the hydrogel systems.

The bonded water contents ( ) and melting points (Tm) of the hydrogel systems are shown in Table 3. The water bonding ability of the gelatin/alginate system was improved and the melting points (eutectic temperatures) were reduced by the addition of glycerol. The water bonding ability was increased in a linear relationship with the increase of the glycerol concentrations. This can be explained that there are three hydroxyls in the molecule of glycerol, and these hydroxyls can act with water and reduce the driving force needed for phase transition of water crystal formation [25]. With the increase of the water bonding ability and the decease in

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the eutectic temperature, the cryoinjury to cells during the cooling/warming stages can be reduced to the minimum.

These data highlight the importance of cryoprotectants incorporated in the cell/hydrogel constructs. In the control gelatin/alginate group, ice crystal formation outside of the cells during the cooling stage may disrupt cells and thus explains the low post-thaw recovery rates. This method is a safe and effective approach for ADSC cryopreservation and the results are highly reproducible. The method that we describe provides excellent recovery of ADSCs after thawing with high proliferation ability. In combination, these techniques could be particularly useful for the cryopreservation of the ADSC lines.

Viability of the cells in the construct containing 10% glycerol after thawing

The 3D construct in the culture medium was a milk-white color with transparent cells but

after MTT coloration, the construct in the culture medium turned purple (Figure 5a). Under a light microscope, blue-violet crystal deposition in cells in the constructs were observed (Figure 5c).

The viabilities of the five different concentrations of glycerol incorporated in the 3D gelatin/alginate construct containing ADSCs are shown in Figure 6. There was a gradual increase in the post-thaw cell changes from 64.0 ±4.5% to 72.3 ±2.9% with increasing glycerol concentration from 5% to 10% (v/v). Further increases in glycerol caused significant reduction in the post-thaw cell viability. For example, the viability of the two groups with 15% and 20% (v/v) glycercol, respectively, were below 38% (i.e. 37.8±4.1% and 31.9±1.8%), indicating toxicity due to the penetrating glycerol and the poor cryoperservation results. The cell viability of the control gelatin/alginate group

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without glycerol was 25.4±2.1%. However, the cell viability in the pure DMEM culture medium was 0 ±0.005%, and cell viability in the DMEM culture medium with 5% and 10% (v/v) glycerol was 54.2 ±5.2% and 53.7 ±5.1%, respectively. Significance statistical differences were found among groups containing different amounts of glycerol ($P<0.01$).

The cell viability in the gelatin/alginate groups with different amounts of dextran-40 are shown in Figure 7. The cryopreservation effects in group P2-1, P2-2, P2-3 and P2-4 was 33.2 ± 3.9%, 49.7 ± 5.2%, 47.4 ± 4.1% and 28.8 ± 2.6%, respectively. The cell viability in the group with 10% (g/mL) dextran-40 was 49.7±5.2%. However, when a combination of 2.5% (v/v) glycerol and 5% (g/mL) dextran-40 was used, a synergetic effect was found and a relatively higher cell survival rate of 96.3 ± 3.4% was achieved. While the cell viability in the combination

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group with 5% (v/v) glycerol and 5% (g/mL) dextran-40 was 92.1 ± 5.2%.

These results clearly indicate that the 3D constructs containing ADSCs can be stored and maintained with a high degree of viability. The gelatin/alginate hydrogels themselves have certain protective capabilities during the cooling/warming stages. Unlike the technique in preservation of cell suspensions in cryoprotectant containing solutions, the glycerol and dextran-40 were directly incorporated in the gelatin/alginate hydrogel to reduce the risk and possible damage caused by ice crystal formation during the cooling/warming stages. The ADSCs were enveloped in the cryoprotectants that were contained in the hydrogels before assembly. A relatively easily diffusion of glycerol in the cell membranes seems to occur before the gelatin/alginate turned into gel state. However, when the hydrogen turned into gel state, the penetrating glycerol might be enmeshed with the gelatin and alginate molecular chains making to

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entry into the cell membranes difficult.

When the temperature was decreased from 4°C to -70°C, there could be a loss of cell water, consequently, the electrolyte concentration inside and outside the cells would be increased and ice might form inside the cells. The cooling rate can profoundly affect the fate of the cells in the frozen 3D constructs. At the relatively slowing cooling rates, free water in the cells are able to penetrate the cell membrane and equilibrate the osmotic differences caused by the extracellular ice formation. Greater dehydration could increase the viscosity of the cytoplasm and decrease the cytoplasmic supershocks. Thus, at sufficiently low temperatures, the cell membrane becomes effectively impermeable.

Microscopic and histological observation of cells in the gelatin/alginate hydrogel with 10% glycerol after thawing

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After 2 days of post-thaw *in vitro* culture, the cells in the construct were transparent under the microscope appeared round or pellet shapes while the grid structure maintained well (Figure 8).

After 9 days of culture, the number of cells inside the construct increased significantly (Figure 9a, 9b). The 3D construct were full of cell clusters while the grid structure became little blurry with endothelial-like cells surrounding the exterior of channels.

Cell post-thaw proliferation ability in the gelatin/alginate hydrogel containing 10% glycerol or the combination of 2.5% glycerol and 5% dextran-40

The cell post-thaw proliferation states during the two weeks of *in vitro* culture is shown in Figure 10. At day of 0, 2, 5, 8, 11 and 14, the MTT value was 0±0.1%, 11.9±2.3%, 43.2±3.8%, 117.6±8.2%, 203.7±18.3% and 376.6± 26.5%, respectively. This indicates that ADSCs in the 3D

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gelatin/alginate constructs containing 10% glycerol proliferated in a progressional state and formed aggregates inside the hydrogel during the post-thaw culture.

In the gelatin/alginate hydrogel with 2.5% glycerol and 5% dextran-40, the cells proliferated to a greater extent higher progressional state (Figure 11). At day of 0, 2, 5 and 8, cell MTT value was 0 ± 0.1%, 19.8 ± 2.5%, 74.1 ± 5.3%, 202.6 ± 2.4%, respectively. The inclusion of dextran-40 within the system has lead to further improvements in cell survival rates. This indicates that dextran-40, as a nonpenetrating solute, dehydrated the cells prior to cryopreservation which reduced the degree of ice crystallization. Dehydration is considered to be a more important factor than the absolute amount of cryopretectants for successful cryopreservation [26].

The toxicity of the cryoprotectant components and the cooling/warming procedures are of great

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importance in any cell cryopreservation protocol and are particularly important when the concentrations and procedures are limited by cell assembly techniques. At present, an important goal is to design an effective protocol that is stable and nontoxic to cells even after prolonged storage stages. Shaw and coworkers indicated that polymers, such as PVP, Ficoll, and dextran, in particularily, can replace penetrating cryoprotectants with minor or no changes altering a solution's physical properties at low temeratures [27]. Consequently, sugars, such as glucose, raffinose, sucrose, fructose, trehalose [28-30], are often used in cryopreservation as they form an important component of osmotic buffers.

In this study, glycerol and detran-40 proportions in the gelatin/alginate hydrogels were selected. It is logical to use the lowest possible concentration of penetrating cryoprotectant glycerol in the cell containing hydrogels to minimize toxicity problems. The addition of the

dextran-40 to the penetrating cryoprotectant improved the cell survival and proliferation rates. This means that the toxicity of the penetrating glycerol was greatly reduced by the use of dextran-40. This may be due to the aqueous dextran-40 solution has high glass transition temperatures [31]. The selected concentrations of the cryoprotectants and gelatin/alginate hydrogel were sufficient to support dehydration during the cooling and warming stages while the cells progressed through the thawing process successfully. The efficacy of the method depends largely on the selection of the cryoprotectant types and the cooling/warming procedures.

Conclusions

Organ manufacturing technique holds the potential to eventually develop complex human organs. The current study, of the direct incorporation of cryoprotectants, such as penetrating glycerol and non-penetrating dextran-40 in the 3D construct, is an effective way to avoid

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cryoinjures during the cooling/thawing procedures. Glycerol enhances the water bonding ability and reduces the melting point of the gelatin/alginate hydrogel. The cell survival (64.0 ± 4.5% to 72.3 ± 2.9%) was obtained with 5% and 10% of glycerol, respectively. A composite of cryoprotectants (5% dextran-40 and 2.5% glycerol) and warming temperature of 17 was considered optimal for our experiments. A much higher cell viability (96.3 ± 3.4%) was obtained with these conditions. Compared with the average cell survival rate of 25.4 ± 2.1% of the control groups without glycerol and dextran-40, the incorporation of glycerol and dextran-40 in the gelatin/alginate hydrogel is an effective way to preserve the 3D constructs based on the cell assembly techniques.

Experimental protocol

Adipose-derived stem cells (ADSCs)

ADSCs were isolated from rat subcutaneous adipose tissue [11]. The epididymal adipose tissues from Sprague-Dawley rats (150 ~ 200 g), medical Center of Laboratory Animals, Beijing university were excised, washed sequentially in serial dilutions of betadine and finely minced in a phosphate-buffered saline (PBS, pH 7.2). Tissues were digested with 0.075% Type II collagenase (sigma) at 37 for 45 min. Neutralized cells were centrifuged to separate mature adipocytes and the stromal-vascular fraction. Floating adipocytes were removed and pelleted stromal cells were filtered through a 100 μm cell strainer before seeding. ADSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK), containing 10% fetal bovine serum (FBS) (Gibco, Paisley, UK) at 37°C in an atmosphere of 5% CO_2. Cells were grown to subconfluence and passaged by a standard trypsinization method.

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Sample preparation.

Gelatin, purchased from the Tianjin Green-Island Company, China, was dissolved in PBS with different concentrations (i.e. 20%, 22.5%, 25% and 30%) (w/v). Sodium alginate, purchased from the Sigma-Aldrich, USA, was dissolved in PBS with a concentration of 8% (w/v). All the solutions were sterilized three times in an oven at 80°C for 1 h. The gelatin solution, sodium alginate solution and glycerol (analytic grade, purchased from Beijing Organic Chemical Plant, Beijing China) were mixed at room temperature with different ratios (Table 1). The final volume of each group is 600 L . Cells were mixed into each group at a density of 1×10^7 cells/mL.

Dextran-40 (molecular weight 4×10^4, analytic grade, purchased from Beijing Organic Chemical Plant, Beijing China), was added to the gelatin/alginate hydrogel at

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different concentrations as Table 2.

When the dextran-40 and glycerol was used together, equal volumes of P2-2 in table 2 with P1-1 or P1-2 in table 1 containing the same density of cells was mixed. Two different cooperation concentrations of the glycerol and dextran-40 were obtained in the gelatin/alginate hydrogel.

Fabrication and freezing-thawing procedures

Grid 3D constructs without or with different amount of glycerol (i.e. 5%, 10%, 15%, 20%), dextran-40 (i.e. 5%, 10%, 15%, 20%) or the combination of the glycerol and dextran-40 were fabricated at 4°C in 20 min using a cell-assembling machine as described previously [12-21]. After assembling, the constructs were firstly stored at 4 for 15 min, then at -20°C for 30 min and finally at -80°C for a long-term storage. Ten days later, the constructs were resuscitated at 17°C in a water bath for 4 min, crosslinked in a 5% $CaCl_2$ (w/v) solution for 1 min, diluted with

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DMEM containing 10% FBS 15 times and finally cultured at 37°C in an atmosphere of 5% CO_2.

Rheological analyses of the hydrogels

Different concentrations of gelatin and sodium alginate solutions were obtained according to Table 1. MCR300 advanced extended rheometer with coaxial cylinder sensor system was used to measure the visco-elastic properties of the gelatin/alginate hydrogels with or without glycerol. The use of dynamic viscoelastic spectroscopy to study the visco-elastic properties of polymer make it possible for us to know the conformational changes of the molecular chains and the aggregation of different molecular chains. In the dynamic test, the apparatus imposes strain in a sinusoidal oscillation and then measures the response to the stress of the sample, which contains two parts, the elastic stress response and the viscous stress response. The elastic stress is in the same phase with strain, denoted as storage modulus (G'), which indicates the characterization of

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the spring back of the materials after reformed, and the viscous stress have a 90º hysteresis with the strain, denoted as loss modulus (G’’). The elastic stress and viscous stress reflect the extent of matter’s appearance as ideal elastic solid and ideal viscous fluid respectively. The mechanical ullage shown as tan means the ratio of G’’ and G’, expressing the strength of viscoelastic characterization and the liquidity of the system, which is, the liquidity appears strongest if tan >>1 and the structural property is strong when tan <<1.

When the hydrogel system is homogeneous, G < G , and as the temperature decreased, the system changes from a homogeneous liquid into elastic solids with a 3D network structure. This process is called gelling. The critical point of the liquid and solid is known as the gel point, at which the viscosity tends to be infinity and the modulus G’ equals to G’’ making up of two important characteristics of the system. With the strengthening of the gelation degree, the trend

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that G' goes over G'' becomes larger, the solid form of the system gradually takes shape, and part of the separated molecular chains get closer to the network, strengthening the gel network structure. Thus, the rigidity of the network becomes larger with strengthened network, along with increased nodes, smaller meshes, denser networks and decreased liquidity until a complete conversion of the hydrogel.

Differential scanning calorimeter (DSC) and melting point detection of the hydrogel systems

The differential scanning calorimeter (DSC) used in the experiment was the American TA Instruments company's DSC Q2000. The temperature calibration was based on the melting point (156.6^{o}C, temperature determination) of 99.99% purity metal of indium (In), the enthalpy calibration used the melting changing enthalpy of indium (In) (28.71J/g), and the rate of

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calibration was 10°C/min. Purge nitrogen (N2) (more than 99.999%) with a flux of 50 mL/min was used. The process of cooling depends on the refrigeration apparatus of the equipment, i.e. the DSC refrigerated cooling system.

Standard fluid sealing aluminum plate was used for the measurement with a volume of 2~5 mg, accurate of 0.01 mg. The temperature ranges from -80°C ~ 40 °C. At first the temperature was lifted at a rate of 5 °C/min, waited for five minutes, then decreased to -80°C at a rate of 5 °C/min, and waited for another five minutes. To eliminate the thermal history of samples, the temperature was increased rapidly to 40°C at the rate of 150°C /min and then decreased rapidly to -80°C at the same (or twice or three times) rate. The temperature was set at -80 °C for about 3~5 min until the heat was stable before data were collected. The transition temperatures and enthalpy values were collected using an analyzing software called Thermal Analysis developed by

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Amrican TA company. If the heat capacity changed little after phase transition, standard baseline was used, while S-shaped baseline was used when the heat capacity changed greatly. The temperatures of crystallization and melting were determined with the intersection of the tangent of the largest slope at the cutting-edge of the curve and the extended line of baseline, namely, the extrapolated beginning temperature. There were three samples in each group.

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The empirical formula below of the freezing enthalpy of pure water at different temperatures derives from an analysis of data in a literature [22].

$$\Delta H(T_s) = \text{-}334 \text{-} 2.05 \quad (T_s \text{-} 273) \qquad (1)$$

The formula for calculating the content of unfrozen water in the polymer solution is shown as below:

$$\alpha = 1 - \frac{\Delta H(T_s)_{DSC}}{\Delta H(T_s)} \quad \frac{1}{w} \qquad (2)$$

Among them,

the content of unfrozen water in the solution (g/g water)

w water content in the solution (%)

T_s the freezing temperature of the samples in the process of cooling ( )

$\Delta H(T_s)_{DSC}$ freezing enthalpy of the solution at the temperature of T_s measured by DSC (J/g)

$\Delta H(T_s)$ freezing enthalpy of pure water determined by equation (1) at the temperature of T_s (J/g).

Cell viability and proliferation assays

Thiazolyl blue (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrasodium bromide) (MTT, Zhongshan Company, China) was adopted to determine the viability and proliferation ability of the ADSCs within the constructs after cryopreservation [23,24]. Small pieces of $1 \times 1 \times 1$ mm^3 were cut from the constructs and weighed before put into serum free culture medium containing 0.5 mg/mL thiazolyl blue. Following 4 h of

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incubation at 37 in an air atmosphere containing 5% CO_2, the supernatant was replaced by 200 L DMSO. The plates were shaken for 10 min to ensure the complete dissolution of the formazan. 160 L of each solution sample was placed in a 96-well plate and the optical densities (OD) values at 570 nm were measured using a BIO-RAD680 immunoanalyzer (Japan). Some samples in which ADSCs were suspended in the DMEM culture medium without or with 5% and 10% (v/v) glycerol were also used as controls for the cell viability tests.

Microscopy and histological examination

Samples were washed with PBS after cryopreservation and then fixed with 2.5% glutaraldehyde in the PBS for 0.5 h. After being thoroughly washed with PBS, the cells were dehydrated through a series of concentration graded alcohol and freeze-dried.

Some of the constructs were gold sputtered in vacuum and examined using a scanning electron microscopy (SEM, Hitachi S-450, Japan). Some of the constructs were embedded in methyl methacrylate, cut into 10 m thick sections, stained with hematoxylin and eosin (HE) and visualized using a light microscopy .

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Statistical analysis

Sample values were expressed as means±standard deviation (SD). Values were analyzed using ANOVA. P-values < 0.01 were considered significant. SPSS 13.0 statistic software was used.

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Figure legends

Figure 1. Relationship between the viscosity and the temperature: P0 represents the control group without glycerol; P1-1 P1-4 represent the groups with different concentrations of glycerol.

Figure 2. Rheological (frequency scanning) curves about the different hydrogel systems: P0 represents the control group without glycerol; P1-1 P1-4 represent

the groups with different concentrations of glycerol.

Figure 3. 3D constructs containing ADSCs and 10% glycerol before cryopreservation (a); an image of the construct after thawing (b), a SEM photograph of the construct after freeze-drying (c).

Figure 4. DSC curves of the heat flow during the cooling and warming processes of the hydrogel systems: P0-1 the control group without glycerol during cooling process; P0-2 the control group without glycerol during warming process; P1-1 the gelatin/alginate hydrogel with 5% (v/v) glycerol during the cooling and warming processes; P1-2 the gelatin/alginate hydrogel with 10% (v/v) glycerol during the cooling/warming processes; P1-3 the gelatin/alginate hydrogel with 15% (v/v) glycerol during the cooling/warming processes; P1-4 the gelatin/alginate hydrogel with 20% (v/v) glycerol

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during the cooling/warming processes.

Figure 5. MTT coloration and cell state observation: (a) before coloration, the construct presented clear milk-white with transparent cells under microscope; (b) 1 h after coloration, the construct turned to be purple; (c) 1 h after coloration, microscopic blue-violet crystal deposition in cells are observable.

Figure 6. MTT results of cell viabilities in different groups of gelatin/alginate hydrogels containing different amount of glycerol (i.e. 0%, 5%, 10%, 15%, 20%).

Figure 7 MTT results of cell viabilities in different groups of gelatin/alginate hydrogels containing different amount of dextran-40 (i.e. 5%, 10%, 15%, 20%).

Figure 8. Cells inside the gelatin/alginate hydrogel with 10% glycerol after 2 days of post-thaw *in vitro* culture.

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Figure 9. Microscope and HE images of the construct after 9 days of culture: (a) a microscope image of the construct; (b) a higher magnification view of (a); (c) HE staining of the froze section showing cell aggregates in the 3D construct.

Figure 10. Cell post-thaw proliferation states in the gelatin/alginate hydrogel containing 10% (v/v) glycerol during the 14 days *in vitro* culture.

Figure 11. Cell post-thaw proliferation states in the gelatin/alginate hydrogel containing 2.5% glycerol and 5% dextran-40 during the 8 days *in vitro* culture.

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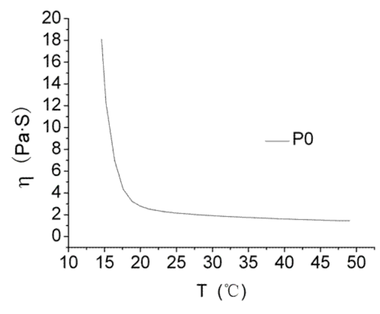

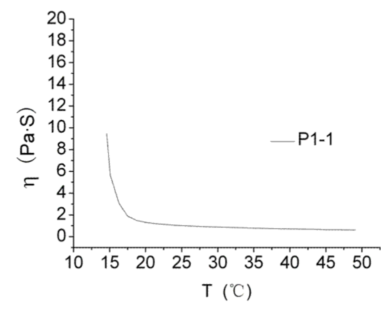

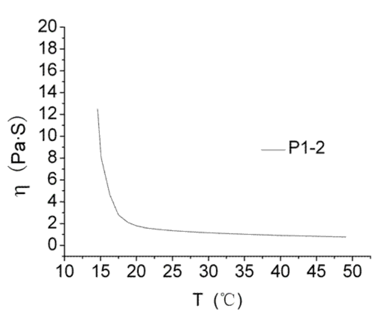

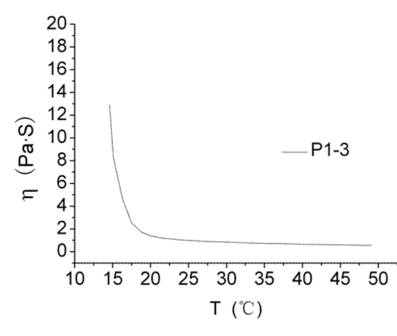

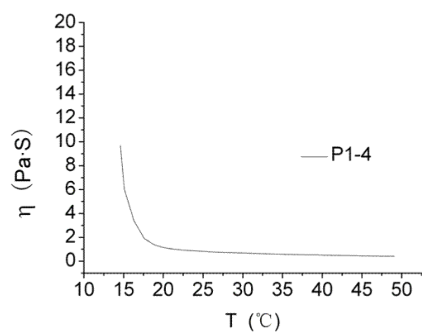

Figure 1

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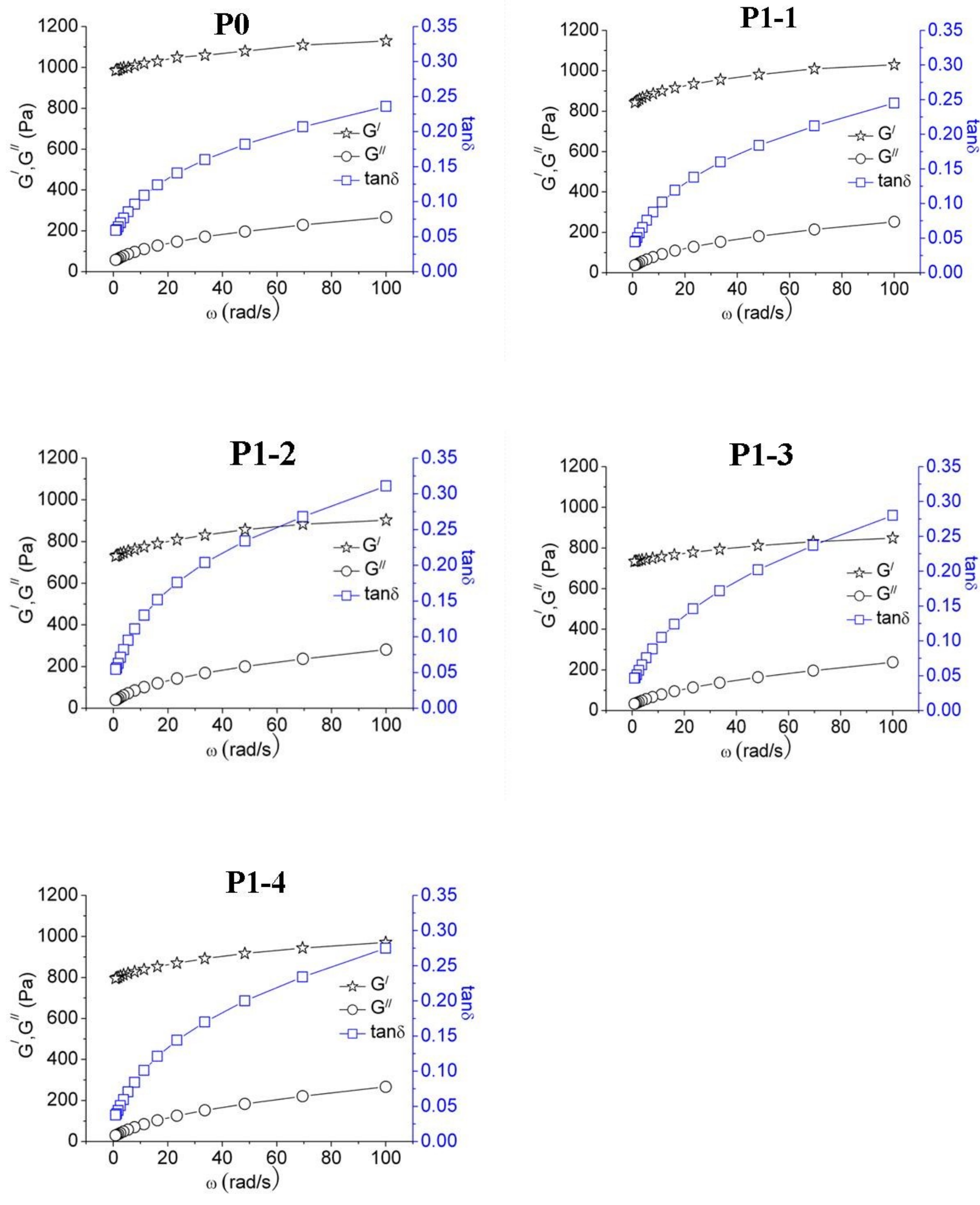

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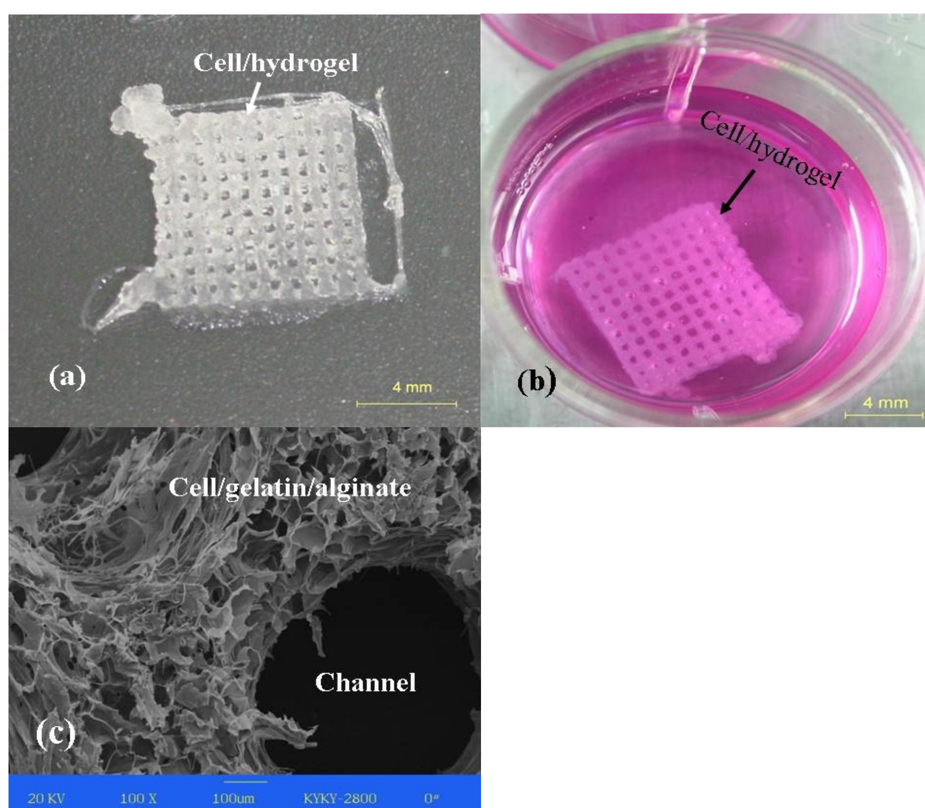

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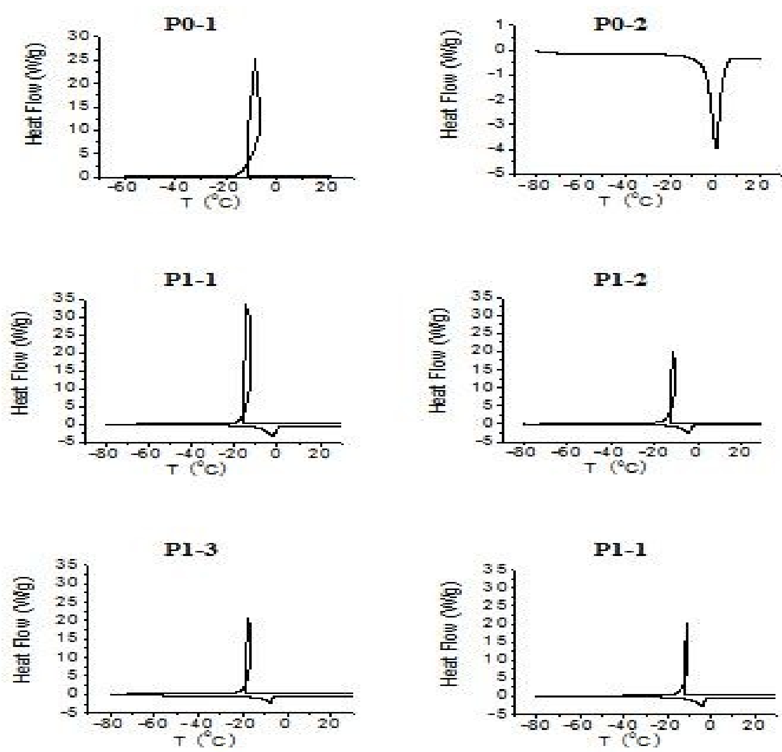

Figure 4

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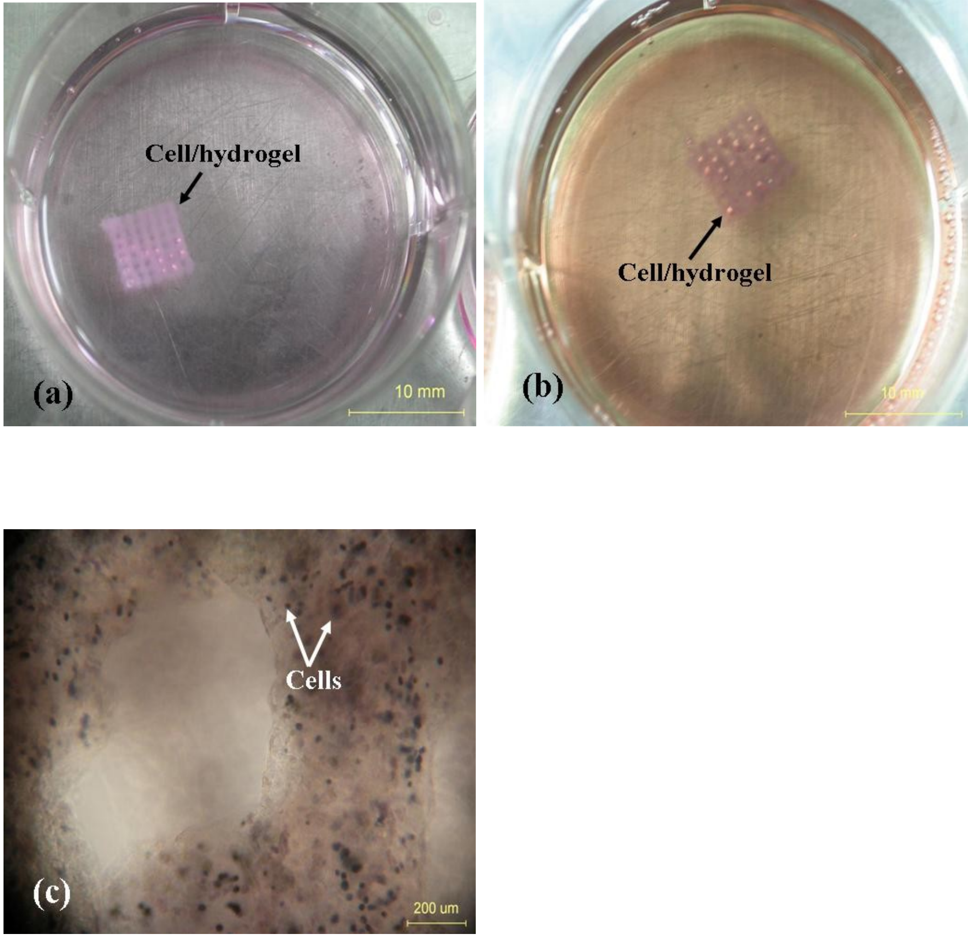

Figure 5

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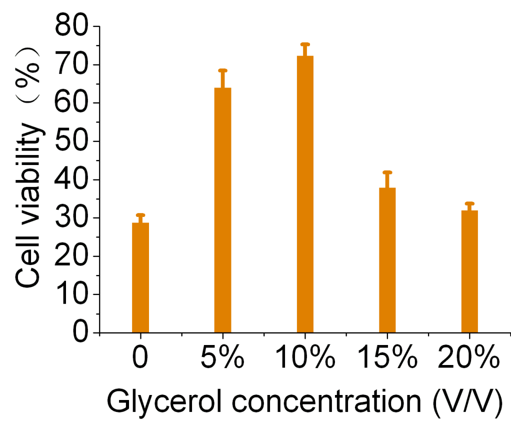

Figure 6

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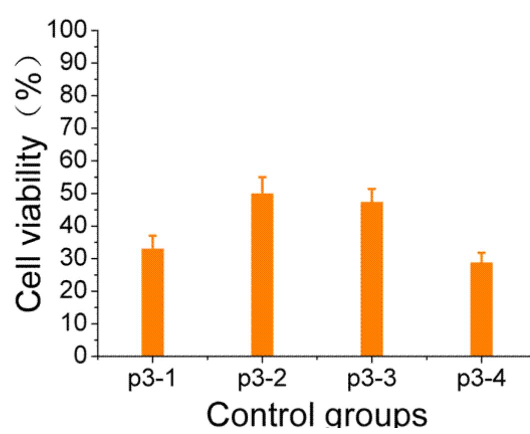

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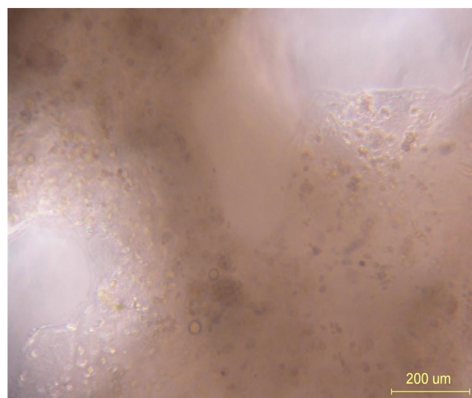

Figure 8

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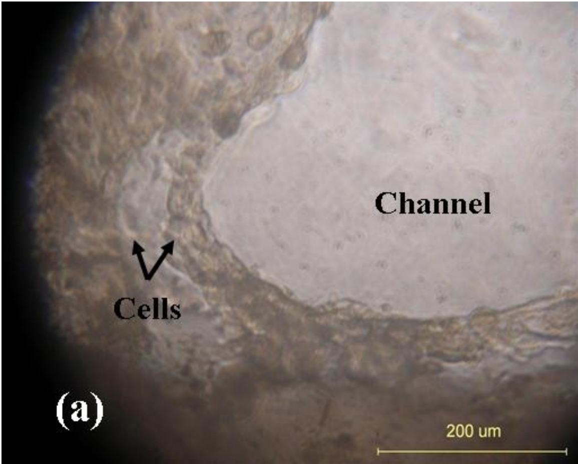

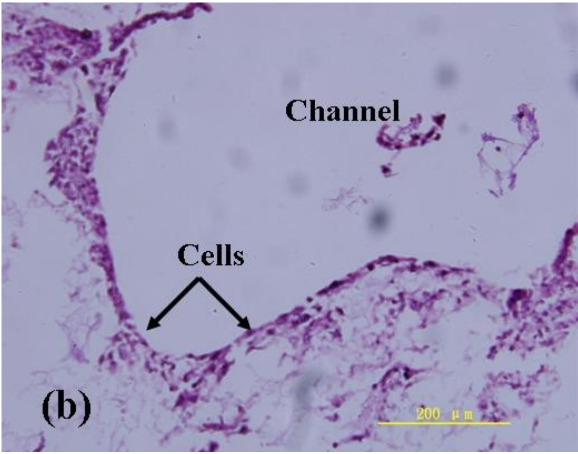

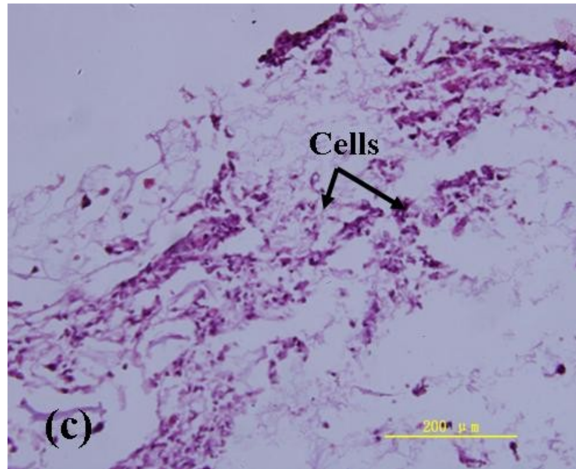

Figure 9

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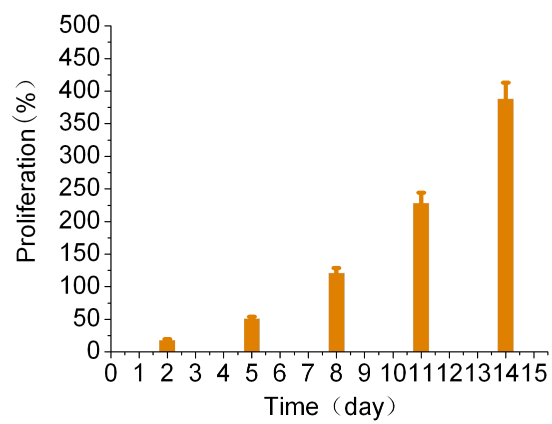

Figure 10

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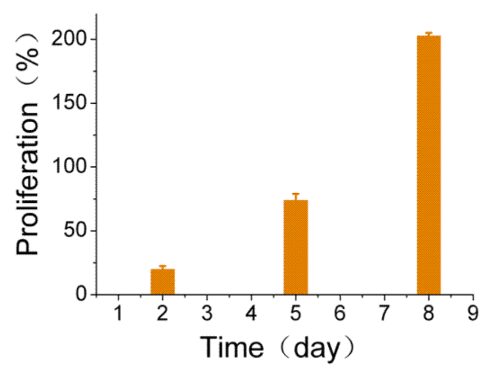

Figure 11

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Table 1. Composition of the gelatin/alginate hydrogel containing different amount of glycerol.

Sample	Glycerol (v/v %)	8% alginate/ L	20 % gelatin/ L	22.5% gelatin/ L	DMEM/ L
P0	0	205	240	0	155
P1-1	5	205	235	0	130
P1-2	10	205	235	0	100
P1-3	15	205	225	0	80
P1-4	20	205	0	195	80

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Table 2 Composition of the gelatin/alginate hydrogel containing different amount of dextran-40.

Sample	Dexrtran-40 (g/100mL)	8% alginate/ L	20% gelatin/ L	22.5% gelatin/ L	25% gelatin/ L	30% gelatin/ L	DMEM/ L
P2-1	5	205	255	0	0	0	110
P2-2	10	205	0	240	0	0	95
P2-3	15	205	0	0	225	0	80
P2-4	20	205	0	0	0	195	80

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Table 3 Binding water contents and melting points of the hydrogel systems

Sample	Glycerol (v/v)%	T_s ( )	$\Delta H(T_s)_{DSC}$ (J/g)	$\Delta H(T_s)$ (J/g)	W (g/g)	(g/g)	Tm ( )
P0	0	-11.43	249.1	255.7	0.90	0.10	-3.78
P1-1	5	-15.99	-301.2	211.1	0.85	0.176	-7.54
P1-2	10	-12.61	-308.2	168.8	0.80	0.31	-9.31
P1-3	15	-18.55	-296.0	133.9	0.75	0.39	-12.52
P1-4	20	-23.38	-286.1	108.7	0.70	0.46	-16.73

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