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Impact of alginate concentration on the viability, cryostorage, and angiogenic activity of encapsulated fibroblasts

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ABSTRACT

Cryopreservation or cryostorage of tissue engineered constructs can enhance the off-the shelf availability of these products and thus can potentially facilitate the commercialization or clinical translation of tissue engineered products. Encapsulation of cells within hydrogel matrices, in particular alginate, is widely used for fabrication of tissue engineered constructs. While previous studies have explored the cryopreservation response of cells encapsulated within alginate matrices, systematic investigation of the impact of alginate concentration on the metabolic activity and functionality of cryopreserved cells is lacking. The objective of the present work is to determine the metabolic and angiogenic activity of cryopreserved human dermal fibroblasts encapsulated within 1.0%, 1.5% and 2.0% (w/v) alginate matrices. In addition, the goal is to compare the efficacy of dimethyl sulfoxide (DMSO) and trehalose as cryoprotectant. Our study revealed that the concentration of alginate plays a significant role in the cryopreservation response of encapsulated cells. The lowest metabolic activity of the cryopreserved cells was observed in 1% alginate microspheres. When higher concentration of alginate was utilized for cell encapsulation, the metabolic and angiogenic activity of the cells frozen in the absence of cryoprotectants was comparable to that observed in the presence of DMSO or trehalose.

1. Introduction

Research efforts have been directed towards bridging the huge gap between the demand and availability of organs for replacing those damaged due to traumatic injuries, diseases, or birth defects via fabrication of tissue engineered constructs. However, a major obstacle for the commercialization or clinical translation of such constructs is the long-term storage of these tissue-engineered products. Cryopreservation of tissue engineered products will not only enhance the off-the shelf availability of these constructs but will facilitate their transportation from manufacturing sites to the point of care. However, this requires optimization of cryopreservation protocols to maintain the viability, stability and functionality of the tissue engineered constructs.

The two principal approaches for cryopreservation involve slow freezing (1–10 °C/min) of cell suspensions in the presence of 5–10% dimethyl sulfoxide (DMSO), as cryoprotectant (CPA) and ice-free cryopreservation or vitrification. CPAs minimize the damages caused by ice formation and encourage the formation of either an amorphous state or a eutectic solid (crystalline) state in cells during the cooling–cryostorage–thawing cycle [1–2]. However, due to cytotoxicity of DMSO [3], other CPAs such as ethylene glycol, glycerol, non-reducing sugars such as trehalose and sucrose have been used for improving the cryopreservation of cells/tissue [4–9]. While cryogenic storage of cell suspensions is routinely performed around the world, the cryopreservation of complex biologics such as three-dimensional (3D) cellular aggregates or multicellular tissues/organs is still a challenge [10]. Diffusion restriction as well as uneven distribution of CPAs within the tissues often lead to the exposure of the surface cells to high and potentially toxic concentration of CPAs in order to satisfy the minimal necessary CPA concentration in the interior [11,12]. The difficulty in complete removal of CPAs from complex biologics may account for the loss of cellular functionality and metabolic activity. Thus, the commonly applied cryopreservation protocols are not optimal for the long-term storage of sensitive and complex biologics. Cryopreservation methodology based on trace or low levels of CPA or CPA-free cryopreservation strategy can immensely benefit the field of regenerative medicine, clinical medicine, and pharmacy.

Cell encapsulation technology is widely used for fabrication of tissue-engineered constructs. It is aimed at immobilizing viable cells inside a semi permeable matrix especially hydrogels. Hydrogels are cross-linked hydrophilic polymers that swell in the presence of aqueous solution but are insoluble in water [13]. Hydrogels are used in tissue engineering as they consist of a cross-linked 3D microstructure that resembles the elastic properties of a soft tissue [14–15]. Several biomaterials have been utilized for microencapsulation of cells including alginate, gelatin, poly (lactic-co-glycolic acid)/poly (L-lactic acid), agarose,

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chitosan, and hyaluronic acid [16–21]. Alginate is most commonly used because of its biocompatibility, mild gelation process (e.g. physiological temperature and pH) and non-toxicity [16,22]. Extracted from the cell walls of brown algae, alginate is a block-wise copolymer of α-L-guluronic acid (G-block) and 1, 4-linked β-D-mannuronic acid (M-block) [23], and offers an affinity to various bi-cations including Ca^{2+}, Ba^{2+} or Sr^{2+} [14]. The potential of alginate microcapsules in cell-based therapies e.g. transplantation of Langerhans' islets [24] and differentiation of mesenchymal stem cells to chondrocytes [25] has been already demonstrated.

Earlier studies have indicated encapsulating cells in alginate hydrogel itself may have a protective effect on cells during cryopreservation [26–27]. Cryo-microscopic studies revealed that the alginate microencapsulation protected the cells from physical injury caused by the growth of extracellular ice crystals [27–28]. However, whether or not alginate concentration plays a role in cryoprotective capability of the biomaterial is not known. The present study indicates that the along with encapsulation, the concentration of alginate used in encapsulation also influences post-thaw metabolic activity and functionality of cryopreserved cells. Human dermal fibroblasts were chosen as model cell line for this study. By virtue of secreting pro-angiogenic molecules and various extracellular matrix proteins including collagen and fibronectin, fibroblasts play important role in wound healing [29]. The cells were encapsulated in 1%, 1.5%, and 2% alginate gels and cryopreserved in the presence and absence of CPAs (DMSO and trehalose). The cell viability, metabolic activity, and angiogenic capability of the encapsulated cells were studied post-thawing.

2. Materials and methods

2.1. Characterization of the alginate microspheres

2.1.1. Preparation of alginate matrices

Alginic acid sodium salt from Sigma Aldrich (Catalogue # 180947, M_w = 120.000–190,000, M: G (mannuronic:guluronic) ratio 1.56, and viscosity = 15–20 cP for 1% solution) was dissolved in distilled water to prepare 1%, 1.5% and 2% (w/v) alginate solutions. The alginate microspheres were generated using 3 ml syringe with BD PrecisionGlide needle of 30G coupled to a syringe pump (Legato 270, KD Scientific, USA).The solution was pumped at a flow rate of 1 ml/min into 5 ml of 1 M $CaCl_2$ (Sigma Aldrich) solution. The alginate microspheres were incubated under stirred condition (60 rpm) at 37 °C for 10 min, following which they were washed using Dulbecco's Phosphate Buffered Saline (DPBS) and transferred to 48 well plates for further experiments.

2.1.2. Pore size

Scanning electron microscope (Hitachi S-2600N Variable Pressure SEM) was used to analyze the architecture of the porous alginate gels. 1%, 1.5%, and 2% alginate gels. For freeze drying, the samples were initially placed at −80 °C for 24 h. Following which they were dried under vacuum at a pressure of 4×10^{-4} mbar for 36 h. Towards this goal, the samples were placed in a small vacuum seal container which was placed in dry ice and connected to a vacuum pump. The freeze dried samples were then sputtered with a layer of gold under high vacuum and then the images of the cross-section were obtained. The pore size was calculated manually from the SEM images using the Quartz PCI 8.0 software. Following contrast, brightness and smoothing adjustments, the diameter across the pores was measured to assess the pore size.

2.1.3. Porosity

Porosity was determined by the liquid displacement method. Briefly, the 1%, 1.5%, and 2% alginate samples were dried at 60 °C for 24 h as described elsewhere [30]. Following which the dry weights of the samples were measured. Then the dried samples were immersed in graduated cylinders containing known volume (V_1) of distilled water for 2 h. After 2 h, the total volume of water and water impregnated gels was recorded as V_2. The volume of the gels was calculated from the volume difference (V_2-V_1). Volume of water which corresponds to the void volume within the gels was calculated from the difference the weight of the hydrated sample and its initial weight. The porosity of the gels was obtained from the ratio of volume of water inside the gels to the volume of the samples.

$$\text{Porosity percentage} = \frac{M_{wet} - M_{dry}}{[\rho(V_2 - V_1) + (M_{wet} - M_{dry})]} \times 100$$

M_{wet} weight of the wet sample
M_{dry} weight of the dried sample
ρ density of water.

2.1.4. Degradation

To measure the degradation of the alginate gels, the weights of the hydrogels were measured following fabrication (day 0). Then the hydrogels were incubated in PBS in a 37 °C shaker (60 rpm). The weights of 1%, 1.5% and 2% alginate hydrogels were measured every day for a period of 7 days. Before weighing, excess PBS was removed from the hydrogels by wiping the gels with Kimwipes. The degradation was calculated from the ratio of weights of the samples at different time points to the weights on day 0.

2.1.5. Diffusion of macromolecules

The diffusion of macromolecules into the alginate microspheres was measured as described elsewhere [31]. Briefly, microspheres of varying alginate concentrations were incubated in 200 μg/ml of fluorescein isothiocyanate (FITC)-dextran 70 kDa. The penetration of macromolecules within the spheres was monitored for 60 min by capturing time series images at every 5 min interval using Zeiss Axio ObserverA1 microscope. NIH Image J software was used to analyze the fluorescent intensities from the microspheres as well as the background. The fluorescent signal from each microsphere was then calculated as the ratio of the fluorescent intensity from the microspheres to that of the background. For comparing the influence of freezing, the frozen microspheres were thawed and the diffusion of macromolecules was monitored.

2.2. Cell culture

Normal human dermal fibroblasts and human umbilical vein endothelial cells (HUVECs) were procured from American Type Cell Culture (ATCC, USA). Fibroblasts were maintained in fibroblast basal media (ATCC, USA) supplemented with 1% (v/v) penicillin streptomycin and fibroblast growth kit containing L-glutamine, rh FGF beta, rh Insulin, hydrocortisone, ascorbic acid and fetal bovine serum. HUVECs were maintained in vascular cell basal media supplemented with 1% (v/v) penicillin streptomycin and endothelial cell growth kit containing L-glutamine, rh FGF, hydrocortisone, ascorbic acid, heparin sulfate, fetal bovine serum and bovine brain extract. The media was changed every day for optimum growth. Cells up to passage 6 were used for the experiments.

2.3. Encapsulation of fibroblast in alginate

Once confluent, fibroblasts were trypsinized and resuspended in cell culture media. Then 200 μl of cell solution at a density of 3.2×10^6 cells/ml was added to 1.5 ml sterile alginate solution. The concentration of alginate solutions were varied from 1%, 1.5% to 2% (w/v). Using 3 ml syringe (30 G × 1 BD PrecisionGlide needle), the solution was pumped at a flow rate of 1 ml/min into 5 ml of 1 M of