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Cryopreservation effects on recombinant myoblasts encapsulated in adhesive alginate hydrogels

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ABSTRACT

Cell encapsulation in hydrogels is widely used in tissue engineering applications, including encapsulation of islets or other insulin-secreting cells in pancreatic substitutes. Use of adhesive, biofunctionalized hydrogels is receiving increasing attention as cell–matrix interactions in three-dimensional (3-D) environments can be important for various cell processes. With pancreatic substitutes, studies have indicated benefits of 3-D adhesion on the viability and/or function of insulin-secreting cells. As long-term storage of microencapsulated cells is critical for their clinical translation, cryopreservation of cells in hydrogels is being actively investigated. Previous studies have examined the cryopreservation response of cells encapsulated in non-adhesive hydrogels using conventional freezing and/or vitrification (ice-free cryopreservation); however, none have systematically compared the two cryopreservation methods with cells encapsulated within an adhesive 3-D environment. The latter would be significant, as evidence suggests adhesion influences the cellular response to cryopreservation. Thus, the objective of this study was to determine the response to conventional freezing and vitrification of insulin-secreting cells encapsulated in an adhesive biomimetic hydrogel. Recombinant insulin-secreting C2C12 myoblasts were encapsulated in oxidized RGD–alginate and cultured for 1 or 4 days post-encapsulation, cryopreserved, and assessed up to 3 days post-warming for metabolic activity and insulin secretion, and 1 day post-warming for cell morphology. Besides certain transient differences in the vitrified group relative to the fresh control, both conventional freezing and vitrification maintained the metabolism, secretory activity, and morphology of the recombinant C2C12 cells. Thus, due to a simpler procedure and slightly superior results, conventional freezing is recommended over vitrification for the cryopreservation of C2C12 cells encapsulated in oxidized, RGD-modified alginate.

1. Introduction

Cell microencapsulation in hydrogels has been widely used for various tissue engineered constructs, with alginate commonly being used as the encapsulation material [1–3]. Biofunctionalized hydrogels are increasingly being studied to better control cell fate in three-dimensional (3-D) environments, with the addition of bioactive motifs to help control cellular processes such as adhesion [4,5]. Cell–matrix interactions in 3-D environments have been shown to be important for cell survival, proliferation, and differentiation, among other cell processes [6]. Specifically, hydrogels containing the RGD adhesive peptide motif have been used for the encapsulation of a variety of cell types, including myoblasts [7,8], bone marrow stromal cells [9], pre-osteoblasts [8], and human embryonic stem cells [10].

Cryopreservation is critical for the long-term storage and clinical translation of microencapsulated cell systems. The two main methods of cryopreservation are conventional freezing and ice-free cryopreservation, or vitrification. Although extracellular ice formation is generally not detrimental to single cells in suspension, ice formation during freezing may cause significant damage to multicellular systems and tissues [11,12]. Thus, vitrification has been investigated for the preservation of various natural tissues [11,13,14], as well as tissue engineered constructs [15–21]. However, vitrification may potentially lead to excessive cell osmotic excursions, as well as cytotoxicity due to the high concentration of cryoprotectants used in the procedure [11,22]. Therefore, as both methods have their potential drawbacks, it is important to investigate both vitrification and freezing in order to determine the best method of preservation for a given system.

With respect to cryopreservation of encapsulated cells, many studies have examined cellular response in non-adhesive hydrogel systems [18–20,23–32]. However, with pancreatic substitutes, cell–matrix interactions in 3-D have been shown to be

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beneficial for cell viability and/or function of encapsulated β cells or islets [33–35] and may become critical as anchorage-dependent, non-β cell types are being explored for use in an encapsulated cell therapy for diabetes. Studying the cryopreservation response of cells encapsulated in an adhesive environment is, therefore, necessary, especially as previous studies have indicated that adhesion to a substrate affects cryopreservation in different and reportedly conflicting ways [36–39]. Some studies have indicated an increased likelihood of cryoinjury due to adhesion [36,39], while others have indicated benefits of cryopreservation of attached cells compared with cells cryopreserved in suspension [38] or in non-adhesive matrices [37]. Previous studies with cells encapsulated in adhesive hydrogels derived from natural extracellular matrices showed that freezing leads to changes in cell morphology [40,41] and decreases in cell viability [40] or function [41], relative to non-preserved controls. Vitrification of similar constructs, on the other hand, resulted in viabilities similar to non-preserved controls [21]. However, no studies have systematically compared freezing and vitrification with cells encapsulated in the same adhesive hydrogel system.

In this work, we studied and compared conventional freezing and vitrification of a model pancreatic substitute consisting of murine C2C12 myoblasts, stably transfected to secrete insulin, encapsulated in partially oxidized, RGD-modified alginate hydrogels. Parental C2C12 cells have been well characterized in RGD–alginate, displaying the ability to survive, proliferate, and differentiate on two-dimensional (2-D) [42] as well as in 3-D hydrogel systems [7]. Also, as previous studies have indicated benefits of longer-term culture of encapsulated cells pre-preservation [38,41], we evaluated the effects of cryopreservation 1 and 4 days post-encapsulation. Bead integrity, cell metabolic activity and morphology, and insulin secretion after cryopreservation were quantified and compared with those of fresh controls. The implications of our findings in identifying appropriate cryopreservation procedures for cells in adhesive 3-D hydrogels are discussed.

2. Materials and methods

2.1. Alginate modification

All chemicals were obtained from Sigma (St Louis, MO), unless otherwise indicated. Alginate was partially oxidized with sodium periodate based on previously published protocols [7,43,44]. Briefly, Pronova ultrapure low viscosity high mannuronic acid (LVM) alginate (FMC Biopolymer, Philadelphia, PA) with 43% guluronic acid content and a viscosity of 24 mPa s was dissolved at a concentration of 1% (w/v) in ultrapure water. Subsequently sodium periodate (Acros Organics, Geel, Belgium) was added to the alginate solution at 21.6 mg g alginate^{-1} [7] and stirred in the dark at room temperature for 19 h. Subsequently, to ensure quenching of the oxidation reaction, a twofold molar excess of ethylene glycol, compared with the sodium periodate used, was added to the alginate solution and stirred for an additional 2 h [44]. Alginate was then placed in 2000 molecular weight cut-off (MWCO) dialysis cassettes (Thermo Fisher Scientific, Waltham, MA) and dialyzed against ultrapure water for 3 days prior to lyophilization.

Partially oxidized alginate was subsequently reconstituted and conjugated with GGGGRGDSP [7] or GGGGRGESP (Biomatik Corporation, Wilmington, DE) at 10 mg g^{-1} alginate using aqueous carbodiimide chemistry [42,45]. Briefly, 1% (w/v) partially oxidized alginate was dissolved in 0.1 M MES buffer with 0.3 M NaCl (pH 6.5) for 4 h at room temperature. Subsequently, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (Thermo Fisher Scientific) and N-hydroxysulfosuccinimide (sulfo-NHS) (Thermo Fisher Scientific) were dissolved in MES buffer immediately prior to the reaction and added to the alginate to obtain a 2:1 sulfo-NHS:alginate molar ratio. The alginate solution was allowed to react for 5 min prior to addition of peptide [9]. The resulting solution was continuously mixed at room temperature for 20 h prior to being transferred to 3500 MWCO dialysis cassettes (Thermo Fisher Scientific) for 3 day dialysis against ultrapure water, followed by lyophilization.

2.2. Cell culture and encapsulation

Stable C2C12 cells were previously prepared by transfection with a furin-cleavable, B10-modified human insulin gene expressed downstream of a CMV promoter and by selection with puromycin [46]. Stable C2C12 cells were cultured in 25 mM glucose Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, West Sacramento, CA), 1% penicillin/streptomycin (Mediatech) and 1 μg ml^{-1} puromycin (Sigma) in order to maintain constant selective pressure on the cells. Cells were cultured under subconfluent conditions in order to prevent cell differentiation [42] in a 37 °C humidified incubator with 95% air/5% CO_2. All salt solutions used for encapsulation/coating were adjusted to 300 mOsm by changing the amount of the major salt component in the solution. For encapsulation, cells were detached from flasks using 0.25% trypsin (Mediatech) and cell number determined using trypan blue dye exclusion. The cell suspension was then centrifuged, and 3.5% oxidized RGD-modified LVM, reconstituted in 0.85% NaCl (w/v), was added to the cell pellet to obtain an encapsulation density of 3×10^6 cells ml alginate^{-1}. This low cell density was used to promote cell–matrix interactions over cell–cell interactions, thus allowing us to study the effect of the former and minimize the effect of the latter on the cryopreservation outcome. Beads of 300–600 μm diameter were formed using an electrostatic bead generator (Nisco Engineering AG, Zurich, Switzerland) and crosslinked in a 1.1% (w/v) $CaCl_2$ bath. Beads were immediately coated according to the procedure of Sun [47], with modifications. Beads were placed in 0.1% (w/v) CHES in 1.1% (w/v) $CaCl_2$ for 3 min, washed with 1.1% (w/v) $CaCl_2$, and then incubated in 0.1% poly-L-lysine (PLL) (molecular weight 15,000–30,000, Sigma) in 0.85% (w/v) NaCl, with mixing, for 5 min. Beads were then exposed to successive washes in 0.1% (w/v) CHES in 1.1% (w/v) $CaCl_2$, 1.1% (w/v) $CaCl_2$, and 0.85% (w/v) NaCl (two washes). Beads were then incubated in 0.2% (w/v) Pronova UP LVM (unmodified) alginate in 0.85% (w/v) NaCl for 4 min. After one additional wash in 0.85% (w/v) NaCl, beads were washed with culture medium and placed in T-25 flasks. Beads were not exposed to sodium citrate. Flasks were placed on a platform rocker (Stovall, Greensboro, NC) in an incubator at 37 °C. Encapsulated cells were cultured in the same medium as monolayers.

2.3. Cryopreservation

2.3.1. Vitrification

Vitrification was carried out with the cryoprotectant (CPA) cocktail solution DPS as in Ahmad et al. [48]. DPS consists of 3 M dimethylsulfoxide (DMSO), 3 M 1,2-propanediol, and 0.5 M sucrose in a modified version of the EuroCollins carrier solution, the latter consisting of 34.95 g l^{-1} glucose, 0.84 g l^{-1} $NaHCO_3$ (Fisher Chemical, Fisher Scientific, Pittsburgh, PA), 1.12 g l^{-1} KCl and 1.68 g l^{-1} NaCl [48]. For CPA addition approximately 0.7 ml of beads were placed in 40 μm cell strainers (BD Biosciences, Bedford, MA) and sequentially transferred through CPA solutions of increasing concentration at 4 °C in a 6-well plate (BD Biosciences) (Table 1). After incubation in the last CPA solution, the beads and solution were transferred to pre-siliconized 20 ml borosilicate glass vials (Fisherbrand/Fisher Scientific) and a layer of isopentane (EMD Chemicals, Gibbstown, NJ) was placed on top of the CPA and