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Multilayered Microcapsules For the Sustained-Release of Angiogenic Proteins From Encapsulated Cells¹

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Abstract

Background—Multilayered alginate microcapsules with a permselective poly-L-ornithine membrane can be used for the dual purpose of encapsulating cells in the inner core and sustained release of angiogenic proteins from the outer layer. In this study, we examined the encapsulation and release of a novel chimeric form of FGF-1 (HB-GAM/FGF-1) from the outer layer of alginate microcapsules.

Methods—Radiolabeled HB-GAM/FGF-1 was encapsulated in the outer layer of multilayered alginate microbeads constructed using varying alginate conditions. The encapsulation and release of the chimera was quantified.

Results—The outer layer was able to encapsulate and release HB-GAM/FGF-1 for up to thirty days. The outer layer made with 1% alginate of high manuronic acid content provided the fastest release, while 1.25% high guluronic acid content alginate displayed the longest duration of release.

Conclusions—The outer layer of multilayered alginate microbeads can be used for the encapsulation and long-term release of HB-GAM/FGF-1.

Keywords

alginate; neovascularization; FGF-1; heparin-binding growth associated molecule

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Introduction

In recent years, islet transplantation has been used clinically as a treatment for type 1 diabetes. This course of treatment is limited, however, because large volumes of islets are required for clinical efficacy, and patients must be prescribed immunosuppressive drugs to prevent transplant rejection (1,2). Encapsulation of islets in a permselective biomaterial may reduce the need for immunosuppressive drugs. Moreover, stimulating increased neovascularization at the transplant site may improve islet viability, thereby reducing the number of islets required to achieve functional graft outcomes.

Alginate is a negatively-charged polysaccharide extrated from algae, consisting of (1–4)-linked β -D-mannuronic acid (M-unit) and α -L-guluronic acid (G-unit) monomers that is crosslinked into a solid gel when exposed to divalent cations, such as Ca^{2+} . Materials based on alginate have been used for a wide variety of biomedical applications due to its biocompatibility, hydrophilicity, non-immunogenicity and ability to gel at physiologic pH and temperature (3). Alginate microcapsules with a permselective poly-L-ornithine (PLO) membrane have been studied for cell encapsulation (4,5). The PLO coating formed around the microbead, influences solute transport, allowing the diffusion of vital solutes while preventing the entry of larger molecules and cells that may initiate immune rejection. The viability of transplanted islets is diminished, however, due to poor vascularization. The co-encapsulation of islets and a protein that stimulates neovascularization, such as fibroblast growth factor-1 (FGF-1), may increase islet functionality due to enhanced local microvascular network formation (6,7).

Our lab has developed a technique for the synthesis of multilayered alginate microcapsules that could serve the purpose of simultaneously encapsulating islets and delivering therapeutic molecules that stimulate local neovascularization (9) (Figure 1). The inner alginate core can be used to encapsulate islets, while the outer layer can be used as a region for protein encapsulation. This drug delivery method has been shown to release FGF-1 for up to thirty days (9). However, the released FGF-1 retained its biological activity only when co-encapsulated with heparin. Biologically enhanced chimeras of FGF-1 could allow for the release of FGF-1 that is active in the absence of exogenous heparin. A chimera of heparin-binding growth associated molecule to FGF-1 (HB-GAM/FGF-1) has been shown to display heparin-independent mitogenic activity and relative endothelial cell (EC) specificity (10). In the present study, our aim was to quantify the encapsulation and release of HB-GAM/FGF-1 from the outer layer of multilayered alginate microcapsules.

Materials and Methods

Materials

Low viscosity (20–200 mPa·s) ultra-pure sodium alginate with either high mannuronic acid (LVM) or high guluronic acid (LVG) content was purchased from Nova-Matrix (Oslo, Norway with molecular weights 75–200 kDa and G/M ratios of ≤ 1 and ≥ 1.5 , respectively. Iodo-beads used to radiolabel HB-GAM/FGF-1 were purchased from Pierce Chemical Co. (Rockford, IL) and a buffer solution containing 1mCi of ^{125}I was acquired from Perkin Elmer (Waltham, Massachusetts). PLO hydrochloride (molecular weight 15,000–30,000), was purchased from Sigma-Aldrich (St. Louis, Mo). Chemicals used to make solutions are listed as follows: HEPES, NaCl, and MgCl_2 (Fisher Scientific); CaCl_2 (Acros).

Preparation of HB-GAM/FGF-1

The HB-GAM/FGF-1 chimera was expressed and purified as described previously (11). Briefly, full-length human HBGAM was linked to the amino-terminus of human FGF-1. The protein was expressed using the BL21-pLysS strain of *E.coli* and purified by heparin-

sepharose affinity chromatography and reversed-phase high performance liquid chromatograph. The chimera was verified using gel electrophoresis and amino acid analysis.

Radiolabeling of HB-GAM/FGF-1

Iodo-beads were washed with reaction buffer, and then incubated in a buffer solution containing 69 ng/ μ L ^{125} I for five minutes. The solution was added to 30 μ g of HB-GAM/FGF-1 and allowed to react for fifteen minutes. The Iodo-beads were removed and the solution was diluted to a final volume of 3 mL. Unconjugated ^{125} I was removed by dialyzing the solution against de-ionized water for 48 hours.

Synthesis of multilayered alginate microbeads

Alginate microbeads were fabricated using conditions previously shown to support islet encapsulation (5). 1.5% (w/v) LVM alginate was injected into a two-channel air microencapsulator, at an air jacket pressure of 15 psi and an alginate jacket pressure of 12.5 psi, fitted with a 25-gauge needle. Droplets were expelled into a 100mM CaCl_2 solution for crosslinking, and the microbeads were incubated for 15 minutes, before being washed thrice, with a solution of 22mM CaCl_2 and 0.9% NaCl. The microbeads were then transferred to a 0.1% (w/v) solution of PLO in normal saline, and placed on a vortex mixer for thirty minutes. The supernatant PLO solution was removed, and the beads were washed thrice with 22mM CaCl_2 in normal saline.

Radiolabeled HB-GAM/FGF-1 was added to alginate solutions of varying concentrations (1% and 1.25% LVM and LVG). After addition of the PLO layer, excess PLO was removed, and the beads transferred to a flat surface. The outer alginate solution containing radiolabeled HB-GAM/FGF-1 was then added to the microbeads, and placed on a shaker for forty-five minutes. Excess alginate solution that did not adhere to the microbeads was removed. The microbeads were then carefully transferred into a solution of 22mM CaCl_2 in normal saline to crosslink the outer layer. Two more washes were performed using a solution of 2mM CaCl_2 in saline to remove any unbound alginate.

Microbeads were placed in a solution of 0.9% NaCl supplemented with 2mM of CaCl_2 , and incubated at 37°C. The entire supernatant incubation solution was removed at predetermined time points, and the HB-GAM/FGF-1 quantified using a gamma-counter (Perkin-Elmer Packard Cobra II Auto Gamma). Following each measurement, the incubation medium was replaced with fresh solution in order to mimic infinite sink conditions. Encapsulation efficiency was determined by quantifying the amount of radiolabeled HB-GAM/FGF-1 contained in the alginate microbeads versus the amount loaded in the alginate solution used to create the outer layer.

Statistical Analyses

The data are expressed as mean $\pm$ standard deviation. Significant differences were determined by performing an analysis of variance (ANOVA) with a Student-Newman-Keuls post-test. In all cases, $p < 0.05$ was considered to be statistically significant.

Results

Quantitative analysis

For all four conditions studied, a distinct outer layer of alginate was formed on the surface of the microbeads. As previously reported (9), the size of the outer layer was found to increase using alginate solutions in the following order: 1% LVM < 1% LVG < 1.25% LVM < 1.25% LVG. For both LVM and LVG alginate, the size of the outer layer increased with increasing concentration. Moreover, LVG alginate formed thicker outer layers than LVM

alginate at equal concentrations. The outer layer remained stable for all conditions over the course of thirty days.

The encapsulation efficiency was determined by measuring the amount of HB-GAM/FGF-1 in the microbeads relative to the total protein added to the alginate solution. The encapsulation efficiencies did not vary with the alginate composition or concentration: $64.3 \pm 3.1\%$ and $62.6 \pm 4.5\%$ for 1% LVM and 1% LVG, respectively; $65.3 \pm 2.6\%$ and $66.2 \pm 4.1\%$ for 1.25% LVM and 1.25% LVG, respectively.

HB-GAM/FGF-1 release

All four groups studied showed an initial burst release of protein followed by a sustained low release of protein over the course of thirty days (Figure 2). Microbeads with a 1% LVM outer layer provided the fastest rate of release: 95% released in 3 days, with an initial burst release of 94% in the first 2 hours. Microbeads with an outer layer constructed using 1.25% LVG alginate provided the slowest initial burst release after two hours, as well as the most sustained release thereafter. Under these conditions, seventy percent of the encapsulated HB-GAM/FGF-1 was released within 2 hours, 80% within 4 days, and 82% within 30 days.

Discussion

Previous researchers have investigated alginate/poly-L-ornithine/alginate (APA) microcapsules for the encapsulation of cells using a thin coat of alginate on the surface to mask the positive charge of PLO so as to reduce the inflammatory response following implantation. Our technique for synthesizing multilayered alginate microcapsules is novel because an outer layer is generated that can be used for encapsulation and release of proteins. Previous methods for vascularizing transplanted islets have added proteins as a single high dose bolus which has the potential to lead to hyperpermeable vessels, stimulate of tumor growth, or promote ectopic neovascularization (12). The encapsulation and long-term release of low levels of protein from the outer layer of multilayered alginate microcapsules may promote formation of stable microvascular networks and localize the effects of the protein, thus reducing potential ectopic side effects.

The rate of release of HB-GAM/FGF-1 varied with the alginate composition of in the outer layer. LVM alginate provided a faster rate of protein release than LVG alginate for both 1% and 1.25%. The porosity of alginate gels differ for LVM and LVG alginates, which could lead to different transport rates within the gels. The release rates of FGF-1 with heparin versus HB-GAM/FGF-1 differ when encapsulated in the outer layer composed of the same alginate composition (Figure 3). A greater burst release is exhibited for HB-GAM/FGF-1 than FGF-1 with heparin. Overall there is more FGF-1 retained in the beads at 30 days than HB-GAM/FGF-1. This may be due to opposite charges of the two molecules, as FGF-1 has a net negative charge at physiologic pH while HB-GAM/FGF-1 is positive. This may result in increased electrostatic interactions between FGF-1 and the PLO coat leading to greater retention in the outer layer.

Our lab has previously shown that a sustained delivery of FGF-1, in contrast to a high bolus delivery of the protein results in a persistent microvascular network response (8,13). The encapsulation of HB-GAM/FGF-1 in the outer alginate layer provides a long-term release that may lead to stable vessel formation. The release is likely to be longer *in vivo* than the *in vitro* findings. Previous studies using a similar alginate system found that release that occurred over a period of hours *in vitro* lasted for weeks *in vivo*, likely due to diffusive resistance of the surrounding tissue (13). We have previously determined that 0.5 ng/day of FGF-1 promotes persistent neovascularization compared to higher doses of 5 ng/day and 50 ng/day (8). A high initial burst release may be required to instigate the initial formation of

new vessels (14), while continued low-levels help stabilize the microvascular network and form proper vessel morphology.

Clinical trials using islets encapsulated in alginate microcapsules have shown some promise as a treatment for type 1 diabetes. However, large numbers of islets are required for efficacy due to poor survival following transplantation. The ability to encapsulate islets in multilayer microcapsules in which an angiogenic protein is released from the outer layer may enhance the viability of transplanted islets. This improved viability could reduce the number of islets required for treatment, increasing the potential clinical impact of these therapies.

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Figure 1.

A visual schematic of the concept behind multilayered alginate microbeads. Islets are encapsulated in the inner core, and an angiogenic protein (HB-GAM/FGF-1) in the outer alginate layer. The permeable PLO membrane prevents the diffusion of large molecules such as antibodies, but allows for the exchange of solutes such as glucose, oxygen and insulin. When implanted the protein is released from the outer layer and stimulates neovascularization towards the bead.

Figure 2.

The release of HB-GAM/FGF-1 from the outer layer of multilayered alginate microbeads varied based on the composition and concentration of alginate used. An initial burst release of HB-GAM/FGF-1 is exhibited for all four groups studied (A), and release at low-levels is continued for up to thirty days (B).

Figure 3.

A comparison of the release profiles of FGF-1 with heparin and HB-GAM/FGF-1 encapsulated in alginate microbeads with a 1.25% LVG outer layer. HB-GAM/FGF-1 has a much higher initial burst release than FGF-1, but its continued long-term release that would serve to achieve persistent neovascularization.