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Optimization of alginate microcapsules containing cells overexpressing α-L-iduronidase using Box-Behnken design

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ABSTRACT

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease caused by deficiency of α-L-iduronidase (IDUA), which results in the lysosomal accumulation of glycosaminoglycans (GAG) leading to widespread clinical manifestations. The microencapsulation of IDUA overexpressing recombinant cells has been considered as a promising strategy for the treatment of MPS I. This study aimed at the optimization of alginate microcapsules containing recombinant BHK (Baby Hamster Kidney) cells (rBHK) overexpressing IDUA produced by electrostatic extrusion technique. The alginate microcapsule (MC-A) optimization study was carried out by means of an experimental Box-Behnken Design that allowed the simultaneous evaluation of the influence of voltage (kV), alginate/cell suspension flow (mL/h), and alginate concentration (%) on size and IDUA activity. The optimal conditions of voltage (10 kV), flow (25 mL/h), and alginate concentration (1.3%) made possible to obtain the smallest microcapsules showing the highest IDUA activity. After optimization, the microcapsules were sequentially coated with PLL and alginate (MC-APA) to increase their stability. MC-A and MC-APA presented monodisperse populations (span < 1.22) with an average diameter of less than 350 μm. The coating increased the mechanical stability of MC-APA by about 6-fold and modulated the permeability to the enzyme. Surface analyzes of MC-APA showed the presence of PLL bands, suggesting that the last alginate layer appears to have only partially coated the PLL. After 30 days of subcutaneous implantation of the MC-APA microcapsules containing rBHK cells in a MPS I murine model, a significant increase in IDUA activity was observed in the skin near the implant. Histological analysis revealed an inflammatory infiltrate at the application site, which did not prevent the release of the enzyme under the conditions evaluated. Taken together, the overall results demonstrate the feasibility of MC-APA as a potential alternative for local treatment of MPS I.

1. Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease characterized by alpha-L-iduronidase (IDUA) deficiency, leading to lysosomal accumulation of the GAG dermatan and heparan sulfate (Beck et al., 2014). Several biochemical and physiological processes can be affected, leading to a chronic, multisystemic pathological condition with various clinical manifestations such as cardiorespiratory, musculoskeletal and central nervous system impairment (Cimaz and Torre, 2014; Martins et al., 2009; Munoz-rojas et al., 2011). The treatments available for MPS I are based on hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). However, HSCT is only effective if it is carried out early in life and is associated with high morbidity and mortality (D'Aco et al., 2012; Valayannopoulos and Wijburg, 2011). Despite the wide application of ERT, its effects are still limited to peripheral manifestations lacking neurocognitive benefits, since the enzyme is not able to overcome the blood-brain barrier. In addition, adherence to treatment is impaired by the need for intravenous administration and the high cost of the enzyme (Valayannopoulos and Wijburg, 2011; Wolf et al., 2015).

To date, there is no effective and curative treatment for all clinical manifestations of the disease, motivating the search for new treatment alternatives (Schuh et al., 2016). Cell microencapsulation, which consists on the immobilization of cells inside a semipermeable membrane

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allowing the release of biologically active products (Murua et al., 2008; Santos et al., 2013), has been investigated as a potential therapeutic strategy for MPS I since microencapsulated recombinant cells could release IDUA, which may be uptaken by the neighboring deficient cells (Matte et al., 2011). Several studies investigated the use of cell microencapsulation for diferent applications, as well as factors that may influence the characteristics of microcapsules and consequently the success of this treatment approach. (Gattás-Asfura et al., 2015; Mohanty et al., 2016; Nabavimanesh et al., 2015; Paredes-juarez et al., 2013; Tam et al., 2011; Veiseh et al., 2015).

In general, the preparation method and the qualitative and quantitative composition of the microcapsules may directly influence their physicochemical properties. These parameters may play a role on stability, ease of implantation, as well as inflammatory reactions that could occur near the implant. The main cell encapsulation technologies are based on extrusion and divided into the following techniques: air flow, vibration, mechanical dropping and electrostatic potential. In the specific case of MPS I, some cell types have already been encapsulated in alginate microcapsules, whether or not they were coated with poly-L-lysine (PLL). However, to date, these microcapsules were obtained exclusively by the air flow extrusion technique (Barsoum et al., 2003; Mayer et al., 2010; Piller Puicher et al., 2012). The air flow extrusion can lead to microcapsules with greater size dispersion, with more deformations and also with micro-air bubbles influencing cell viability and biocompatibility *in vivo*.

Electrostatic extrusion is based on the use of electrostatic forces to disrupt a liquid filament at the needle tip and form a charged stream of small droplets, which fall into a crosslinking solution (Manojlovic et al., 2006). This technique is more controllable, reproducible, easy to use under sterile conditions, and allows to obtain smaller and uniform microcapsules in large scale in comparison to the other means of production (Manojlovic et al., 2006). In this sense, the main objective of this study was to optimize the microencapsulation of rBHK cells overexpressing IDUA in alginate microcapsules through a Box-Behnken Design (BBD) to determine the ideal conditions and the relevant parameters of the electrostatic extrusion equipment to obtain the smallest and more permeable microcapsules. In a second phase, the study aimed to evaluate the effect of PLL coating on the properties of alginate microcapsules, as well as to evaluate IDUA activity after subcutaneous implantation in a MPS I murine model.

2. Materials and methods

2.1. Cell culture

BHK cells, transfected with the pR-IDUA plasmid (constructed on the commercial replicative plasmid pREP9) to generate rBHK cells overexpressing IDUA (Mayer et al., 2010), were cultured in Dulbecco's Modified Eagle Medium (DMEM) (LGC Biotechnology, São Paulo, BRZ) supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, USA) and 1% antibiotic ampicillin/streptomycin (Gibco, New York, USA). Cells were incubated in a humidified CO_2 incubator at 37 °C to about 90–95% confluence. For the encapsulation, cells were trypsinized for about 3 min in 0.25% trypsin-EDTA (Gibco, New York, USA) and counted in Neubauer's chamber.

2.2. Experimental design

2.2.1. Optimization of microcapsules using response surface methodology (RSM)

The response surface methodology was used to optimize alginate microcapsules and to investigate the correlation between responses and factors. This study aimed to minimize the size of microcapsules and maximize the permeability to the enzyme, analyzed through IDUA activity in the culture medium after 24 h of microencapsulation. The three-factor, three-level BBD, which consists of a set of points located at the midpoint of each end and the replicated central point of the multidimensional cube (Box and Behnken, 1960), was used to obtain the polynomial models. The independent variables used were alginate concentration (%), alginate/cell suspension flow (mL/h), and voltage (kV) (Table 1). A design with 15 runs (3 central points), for which the polynomial equation (Eq. 1) was generated by the statistical software Minitab v.17 (State College, PA, USA) can be expressed as:

Table 1
Variables in Box-Behnken 3^3 design.

	Levels		
Independent variables	Low (−1)	Medium (0)	High (1)
X_1 alginate concentration (%)	1.3	1.5	1.7
X_2 alginate/cell suspension flow (mL/h)	15	20	25
X_3 voltage (kV)	6	8	10
Dependent variables	Constraints		
Y_1 Size (μm)	Minimum		
Y_2 IDUA activity (%)	Maximum		

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_1X_3 + A_6X_2X_3 + A_7X_1^2 + A_8X_2^2 + A_9X_3^2 \quad (1)$$

where Y represents the dependent variables; $\times_1$, $\times_2$, and $\times_3$ are the independent variables; A_0 the regression coefficient; A_1–A_3 are the linear coefficients; A_4–A_6 the cross product coefficients; and A_7–A_9 are the quadratic coefficients.

2.2.2. Data analyzes and model validation

The results obtained from the proposed matrix were evaluated and used for the regression analysis and to generate the polynomial equations that describe the relationship between the responses and the considered factors. The results were statistically tested and the *p* value was used as a tool to verify the significance of each independent variable in the responses. The adequacy of the model was evaluated by the coefficient of determination (R^2). The contour plots were drawn as a function of two factors, keeping the other constant. In order to validate the model, five (5) formulations were produced under the optimized conditions and the results of the responses obtained experimentally were compared with the values of the responses predicted by the software.

2.3. Production of microcapsules

The rBHK cells were immobilized in microcapsules using an electrostatic encapsulation unit type V1 (Nisco, Zurich, SWZ) attached to a syringe infusion pump. Cells were suspended in an Ultra-Pure Low Viscosity Guluronic sodium alginate solution (UP LVG) (NovaMatrix FMC, Sandvika, NOR) containing 8.3×10^6 cells/mL. The suspension was extruded (0.35 mm needle) in a constant infusion rate. The droplets fell on a crosslinking solution of 80 mM $CaCl_2$ (Sigma-Aldrich, St. Louis, USA) and formed the alginate hydrogel matrix containing the incorporated cells. The alginate microcapsules (MC-A) were kept under stirring to complete the ionic gelation. After optimizing the preparation of the alginate microcapsules, they were sequentially coated with 0.05% poly-L-lysine hydrobromide (MC-APA) (PLL; Sigma-Aldrich, St. Louis, USA) for 10 min, followed by a final coating with 0.1% sodium alginate solution for 5 min. The voltage used on the electrostatic generator, the infusion flow conditions of the alginate/cell suspension, as well as the alginate concentration were determined by the experimental design. The cell encapsulation process was performed under sterile conditions and all solutions were sterilized by filtration through a 0.22 μm membrane.