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Statistical approach in alginate membrane formulation for cell encapsulation in a GMP-based cell factory

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Abstract

A cell encapsulation technology in alginate has been developed with the aim of obtaining cell controlled release or three-dimensional cultures. The aim of this work is to verify the predictability of alginate capsules for large-scale production by Good Manufacturing Practice (GMP) standardized procedures in a cell factory. A cell-free capsule model was performed following the GMP guidelines: an opaque agent suspension in a bivalent cation solution (Ca^{2+}, Ba^{2+}, Sr^{2+}) was dropped in a sodium alginate solution, obtaining capsules presenting a liquid core surrounded by a gel alginate membrane. The concentration of the ion, and the treatment with protamine, can considerably vary the characteristics of the capsules (weight, whole diameter, core diameter, gel capsule thickness, capsule strength). It is therefore possible to optimize the performance of the capsules, relating the molecular structure and size of the polymeric membrane to the desired functional properties. Technological resources are available for large-scale cell encapsulation intended for advanced therapies (gene therapy, somatic cell therapy and tissue engineering) in a cell factory, following GMP guidelines.
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1. Introduction

Since the pioneering study by Chang [1], the aim of encapsulating living cells has been to entrap them within semi-permeable membranes. These membranes must be permeable to molecules such as oxygen, nutrients and growth factors, which are essential for the cell's survival; furthermore, the elimination of cell secretions and catabolic products must be possible and, indeed, for some applications, the cell itself must be able to exit from the capsule. According to the technique described by Lim and Sun [2], cells are suspended in alginate solution: droplets are then converted into a rigid bead through the gelation of the polymer upon contact with bivalent ions, such as Ca^{2+}. In a second phase, a permanent semi-permeable membrane is created on the bead surface by treatment with a polycation. These microcapsules have been the most widely employed systems for a variety of applications (*in vivo* and *in vitro* three-dimensional (3D) cell cultures, clonal selection of desired cell phenotypes, bioengineering, large-scale production of cell-derived molecules in the biotechnology industry, reproductive biotechnology, gene or cell therapy, etc.) [3–5].

Many attempts have been made to optimize the performance of the capsules, and numerous encapsulation

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techniques have been developed over the years: however, there are two main kinds of capsules: (a) homogeneous beads, manufactured by gelling alginate droplets in a solution containing a bivalent ion followed by a surface treatment with a polycation (multi-step technique) [1,2,6]; (b) inhomogeneous, liquid-core capsules, produced by dropping a cell suspension containing bivalent ions into an alginate solution (one-step technique). This was described by Klein et al. [7], and it was developed for controlled release of semen for artificial insemination in swine [8–10] and for 3D culture of ovarian follicular cells [11–13]. Recently, this technique has also been employed in 3D culture of other cell types: nucleus pulposus for regenerative therapy of intervertebral disc and adipose derived mesenchymal stem cells for in vitro expansion [14]. The barium alginate capsules are formed from a core containing the live cells surrounded by a gel membrane which may, if desired, be coated by an external layer of protamine-reacted alginate. Depending on cell type, the core properties can be modified, and different suitable polymers can be employed to mimic an artificial extracellular matrix. Alternatively, cells in the core can be maintained into their physiological matrix.

The first aim of this study was to obtain a reliable statistical model in order to select the technological conditions to obtain capsules with the desired properties, depending on the cell type and capsule use. The use of a proper statistical design in pharmaceutical formulation development is well reported in the literature: for example, tablet formulations [14–16], micro- or nanocapsules [17–20], fluid bed spray coating [21], hydrocolloid dressing [22] and suspension [23].

The same approach could be followed also for cell encapsulation: in fact, a number of formulation parameters must be defined: these parameters can be simultaneously considered independent variables in the statistical model. In a previous note, a single process variable (e.g., barium concentration) was considered: a linear correlation was found between ion concentration and capsule properties (weight and morphology); in this work, three different cations (calcium, barium and strontium) at different concentrations were employed, and the capsule surface was treated with protamine or not treated: the capsules were characterized in terms of weight, morphology and mechanical properties.

The second goal of this study is the validation of the encapsulation process by adjusting to the current European guidelines [24] on advanced therapy medicinal products (gene therapy, somatic cell therapy and tissue engineering). The development of safe, efficient, reproducible and traceable method for 3D cell culture production based on Good Manufacturing Practice (GMP) is mandatory in the context of translational clinical research. In order to guarantee quality, efficiency and safety, several specific procedures were set up to ensure the absence of environmental and product microbial contamination according to European guidelines.

2. Methods

2.1. Capsule production in a GMP-based cell factory

Capsules were produced in a 'cleaning room' (class B, GMP guidelines). Each suite is supported by single-pass, positive pressure (60 Pa) HEPA filtered air, at a temperature of 18 °C and relative humidity 50%. The flow suite is unidirectional, with entry and exit air locks. The cleaning room environment was monitored by wireless probes directly interfaced with an external computer in order to assure the same operating conditions during each process step, as required by the European Community Guidelines that rule for the product in advanced therapies. Low contamination levels were guaranteed by differential pressures, absolute filtration systems and unidirectional laminar air flow. Personnel were trained in respect of the GMP guidelines. All the encapsulation steps were conducted in certified ISO Class 5 (Class 100) laminar flow biosafety cabinets. Environmental cleanliness controls were performed before operation in the unmanned state (t_0), and in the manned state during normal use (t_1). Particulate contamination control tests were conducted on the air sample (determination of CFU m^{-3} air) using sedimentation settle plates (CFU $(4\ h)^{-1}$), agar contact plates (UFC $plate^{-1}$) and the operator's gloved hand test.

For the production of the capsules, a mix of 20 mL xanthan gum 1%, w/v, aqueous solution (Satiaxane CX 2, SKW Biosystems, I) and titanium dioxide 0.5%, w/v, as an opaque agent was prepared. A saturated solution of chloride of a bivalent cation was then added to this suspension to obtain different Ba^{2+}, Ca^{2+} or Sr^{2+} ions concentrations ranging from 5 to 150 mM; all formulations were produced in replicate.

The resulting suspension was added dropwise into 100 mL of a 0.5%, w/v, sodium alginate aqueous solution (sodium alginate medium viscosity from *Macrocystis pyrifera*, mannuronic/guluronic ratio = 1.50, Sigma–Aldrich, D). An extrusion technique with a peristaltic pump was employed, whereby the suspension was forced through a needle (25GX5/8′) at a flow rate of 60 droplets min^{-1}. The height of the needle above the solution was 120 mm. Ions diffused out of the droplets and, when they reached the interface, reacted with the alginate by ionic interaction, leading to the formation of an alginate gel around the droplet [25]. Gel capsules (Gel) were obtained, collected by filtration, rinsed twice and suspended in water.

A second kind of capsule was obtained as follows: gel capsules at a concentration of 20%, v/v, were suspended in a 1%, w/v, aqueous solution of protamine sulphate (Sigma–Aldrich, D) and stirred for 3 min at room temperature using a magnetic stirrer. The operating conditions lead to a semipermeable membrane that allows – in barium alginate gel – the diffusion of model molecules as glucose and haemoglobin, according to previously reported results [12]. This treatment produces cross-linked capsules (protamine-reacted) presenting a rough external semi-opaque

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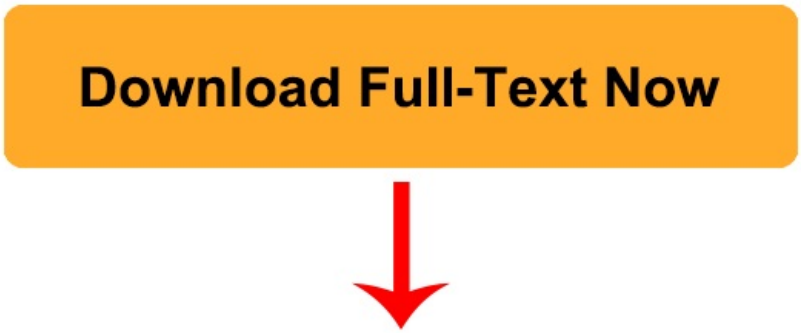

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