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Development of alginate–agarose subsieve-size capsules for subsequent modification with a polyelectrolyte complex membrane

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Abstract

Immunoisolatability, mechanical stability and biocompatibility of cell-enclosing microcapsules are controlled by the formation of external polyelectrolyte complex membranes. The present study aimed to develop cell-enclosing subsieve-size capsules of less than 100 μm in diameter for controlling these properties. We investigated the use of anionic polysaccharide alginate combined with thermosensitive agarose, and prepared particles composed of the two components. For this purpose, agarose in droplets suspended in liquid paraffin was first gelated by cooling, and followed by gelation of alginate in $CaCl_2$ solution. Treatment with a solution containing cationic polysaccharide chitosan inhibited the diffusion of bovine serum albumin into particles compared with non-treated particles. This observation showed that alginate–agarose particles were successfully coated with the polyelectrolyte complex membrane. In addition, cells enclosed in subsieve-size alginate–agarose capsules prepared via the gelation process adapted to the microenvironment and showed mitochondrial activity during 27 days of study. These results showed that alginate–agarose capsules prepared via the droplet breakup method in a water-immiscible co-flowing fluid and the subsequent ordered gelation process were effective vehicles for cell-encapsulation devices for cell therapy.

Keywords: Biomedical; Animal cell culture; Immobilisation; Immobilised cells; Subsieve-size capsule; Cell therapy

1. Introduction

The idea of encapsulating cells and tissues in spherical vehicles for protection from the host immune system, with retention of viability and metabolic functionality for in vivo implantation was first mentioned 40 years ago [1]. The approach is viewed as an alternative to viral or physical gene delivery [2]. Recent advances in genetic engineering have increased potential numbers of diseases for which this process can be used for clinical treatment [2,3]. To protect enclosed cells from the host immune system, the vehicle has to exclude immune molecules and immune cells. It must permit entry of small molecules such as oxygen, nutrients and electrolytes, and also allow exit of toxic metabolites, hormones and other small bioactive compounds. In addition, vehicles must have sufficient mechanical stability to prevent exposure of the enclosed cells to the host immune system, which would result in their destruction [4].

We recently developed a method for preparing cell-enclosing subsieve-size capsules of less than 100 μm in diameter with a narrow size distribution [5], and showed their effectiveness for tumor therapy [6]. The size of the subsieve-size capsules was about the tenth of the size of conventional microcapsules (500–1000 μm). Such a reduced size resulted in enhancements of molecular exchangeability, mechanical stability [7] and even biocompatibility [8]. We prepared subsieve-size capsules via emulsion of narrow size-dispersed polymeric aqueous droplets in liquid paraffin. We obtained the emulsion by injecting the polymeric aqueous solution into a co-flowing laminar stream of liquid paraffin. To date, we have successfully prepared subsieve-size capsules from temperature-sensitive marine uncharged polysaccharide agarose. However, it was impossible to make capsules from sodium–alginate aqueous solution because of the insolubility of multivalent cations for the formation of a three-dimensional hydrogel. Alginate has been one of the more successful components for bioencapsulation involving cell-encapsulation. An attractive advantage of alginate compared with agarose is that it has anionic charged carboxyl groups in its structure. Thus, it is easy to modify the resultant gel by cationic polymers such as poly-L-lysine [9] and chitosan

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[10], via electrostatic bindings for controlling biocompatibility and their molecular exclusion property. This study aimed to develop mammalian cell-enclosing subsieve-size capsules containing alginate for subsequent modification with cationic polymers.

Alginate and agarose differ from each other in their gelation mechanism, chemical stability and porosity. Gelation of alginate relies on the presence of multivalent cations such as calcium to crosslink the alginate molecules. In contrast, temperature change is the routine procedure to both dissolve and gel agarose, i.e., temperature is increased for dissolution or temperature is decreased for gelation. Gelation kinetics of alginate is much more rapid than that of agarose. Thus, two possible processes can be used to shape the spherical configuration of an aqueous solution containing agarose and alginate, i.e., the alginate gel can be made prior to gelation of agarose or agarose gel can be made prior to alginate. Orive et al. [11] prepared cell-enclosing alginate–agarose capsules on the basis of the latter approach. They dropped agarose into pre-cooled aqueous calcium chloride solution, and then prepared the polyelectrolyte complex of alginate and poly-L-lysine. In the present study, we prepared alginate–agarose capsules via the former approach due to insolubility of multivalent cations in a water-immiscible liquid; i.e., at first, the mixture was gelated in liquid paraffin by decreasing the temperature, and then the resultant capsules were placed into the aqueous solution containing calcium ions. To the best of our knowledge, there are no reports on cell-enclosing capsules prepared by this approach. Analyses of molecular permeation and mechanical properties were performed to evaluate the microscopic structure of the resultant composite gel and the possibility of modification by cationic polymers, respectively. In this study, we used chitosan as a model for the cationic polymers. Viability of the enclosed mammalian cells was determined to study the applicability of the particles for cell-encapsulation.

2. Materials and methods

2.1. Materials

Agarose with a low gelling temperature (26–30 °C, 1.5%) was purchased from Cambrex Bio Science Rockland (ME, USA). Sodium–alginate with a high content of guluronic acid and a molecular weight of 7.0×10^4 (Kimica I-1G), and chitosan with a molecular weight of 6.5×10^5 were kindly donated by Kimica (Tokyo, Japan) and Yaizu Suisankagaku Industry (Shizuoka, Japan), respectively. Liquid paraffin was obtained from Kanto Kagaku (Tokyo, Japan). The lecithin from soybean was purchased from Wako (Osaka, Japan). Feline kidney cells (CRFK cell line) were grown in DMEM (MO, USA) containing 10% fetal bovine serum, 400 mg/dl glucose, 75 mg/l penicillin and 50 mg/l streptomycin, and cultured in a humidified atmosphere at 37 °C with 5% CO_2.

2.2. Preparation of particles

Alginate–agarose particles were prepared from a mixture of 1 wt% sodium–alginate and 2 wt% agarose in calcium-free Krebs Ringer Hepes buffer solution (CF-KRH, pH 7.4). Two types of alginate–agarose particles of ca. 4 mm in diameter were prepared via two different gelation processes to study microscopic structure of the composite gel and mechanical properties of the gel coated with polyelectrolyte complex membrane: (1) the mixture solution was dropped from a 22-gauge needle into liquid paraffin pre-cooled at 4 °C. After 30 min of cooling at 4 °C, 100 mM $CaCl_2$ aqueous solution was added to the suspension for gelation of sodium–alginate and (2) the mixture solution was dropped from a 22-gauge needle into 100 mM $CaCl_2$ pre-cooled at 4 °C. After 3 h of soaking in 100 mM $CaCl_2$ at 4 °C, both particles were rinsed and soaked in KRH at 37 °C for 1 day. Non-coated alginate–agarose particles prepared via the former and latter processes were denominated as N-LIQ and N-CA, respectively. Alginate–agarose particles treated with a chitosan solution for the preparation of polyelectrolyte complexes of alginate/chitosan/alginate were prepared as follows: N-LIQ and N-CA were stirred in 0.05 wt% chitosan–acetic acid aqueous solution (pH 6.3) for 10 min, then the resultant particles were stirred in 0.05 wt% sodium–alginate in CF-KRH for 12 min. The N-LIQ and N-CA soaked in the aqueous chitosan solution were denominated as C-LIQ and C-CA, respectively. Different particle preparation processes and treatments with chitosan solution produced no significantly different particles ($p > 0.62$, similar diameters of particles).

Particles less than 200 μm in diameter were prepared using the droplet generator designed in our laboratory. Briefly, an aqueous alginate–agarose mixture solution (712 mPa s at 37 °C) in CF-KRH kept at 37 °C was extruded at a velocity of 2.9 cm/s from a stainless needle (inner diameter, 300 μm; outer diameter, 480 μm), into a co-flowing immiscible stream of liquid paraffin containing 3 wt% lecithin (68 mPa s at 37 °C). The liquid paraffin kept at 37 °C was allowed to flow in the same direction as the agarose solution. The resultant suspension was cooled at 4 °C for gelation of agarose, and then mixed with 100 mM $CaCl_2$. Subsieve-size particles enclosing CRFK cells were prepared from a mixture solution of 1.5 wt% alginate and 2.5 wt% agarose containing cells at 1.5×10^7 cells/ml.

2.3. Determination of transport characteristics

The four types of alginate–agarose particles, i.e., N-LIQ, N-CA, C-LIQ and C-CA, were stored for 12 h in KRH containing 0.1 wt% Na-azide at 37 °C. Na-azide was used as a bacteriostatic agent. Time-courses of diffusion of bovine serum albumin (BSA, M_W 67,000) into the particles were measured as follows. Particles (5 ml) were placed into a gently stirred KRH (10 ml) solution containing 0.1 wt% Na-azide and 0.4 mg/ml BSA (C_{L0}). Twenty-microlitre aliquots were withdrawn from the aqueous phase at various times, and concentrations of the substance at any time (C_L) were determined using a Bio-Rad Protein Assay kit (Bio-Rad, CA, USA). Results were expressed as means ± S.D. for three replicates.

2.4. Determination of mechanical properties

Mechanical properties of C-LIQ and C-CA were determined by compressing the particles using a Table-Top Material Tester