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Cell-compatible covalently reinforced beads obtained from a chemoenzymatically engineered alginate

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Abstract

A chemoenzymatic strategy has been exploited to make covalently linked alginate beads with high stability. This was achieved by grafting mannuronan (alginate with 100% mannuronic acid (M)) with methacrylate moieties and then performing two enzymatic steps converting M to guluronic acid (G) in alternating sequences (MG-blocks) and in G-blocks. In this way a methacrylate grafted alginate with better gel-forming ability was achieved. Covalent bindings were introduced into the beads by using a photoinitiating system that initiated polymerization of the methacrylate moieties. The covalent links were demonstrated by beads remaining intact after treatment with EDTA. The new chemoenzymatic photocrosslinked (CEPC) beads were compatible with cells with low post-encapsulation ability like C2C12 myoblasts and human pancreatic islets. The islets continued secreting insulin after encapsulation. On contrary, cells with a high post-encapsulation proliferative ability like 293-endo cells died within 2-week post-encapsulation. The exceptional stability and the cell compatibility of the new CEPC beads make them interesting as bioreactors for delivering therapeutic proteins in future applications.

Keywords: Alginate beads; Chemoenzymatic engineering; Photocross-linking; Stability; Cell viability; Islets

1. Introduction

Encapsulation of cells producing therapeutic proteins into semi-permeable alginate capsules may be one potential way of protecting the transplanted cells from the host immune system. However, several issues need to be addressed and stringent demands as long-term stability and biocompatibility (to host as well as to the encapsulated cells) need to be met for the successful design of a microcapsule for cell therapy.

Although synthetic-polymer-based microcapsules have been proposed for cell encapsulation [1], alginate, a collective term for a family of polysaccharides produced by brown algae [2] and bacteria [3,4], is, to date, the most extensively used material for capsule formation due to its excellent ionotropic properties [5]. However, alginate-based hydrogel beads meet the requirements of biocompatibility, but lack of long-term stability, since the hydrogel rests upon the presence of the cross-linking ion. This is dramatically evident, for instance, in presence of biological fluids, where competing ions (sodium) and divalent cations chelators (citrate) are present.

Improvements in increasing alginate bead stability have been achieved by coating the gel with a poly L-lysine (PLL) layer. Successful long-term implantations of microencapsulated allografts in alginate-polylysine microcapsules have been reported for spontaneous diabetic dogs [5] and man [6]. However, the main drawback of this strategy is represented by the presence of the polycation itself, since PLL has been reported to be toxic towards different cell lines, to activate complement and macrophages towards the production of interleukin-1 (IL-1) and to induce

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fibrosis [7–9]. Alternatively, a strategy based on a chemical introduction of photopolymerizable groups [10–12] on alginate backbone has already been proposed. The latter seems very appealing in view of the increase in stability of the hydrogel determined by the introduction of additional (covalent) cross-links. However, the lack of selectivity characterizing chemical modifications [13], that hence generally occur both on guluronic acid (G) and mannuronic acid (M) residues, seriously hampers the ability of the modified alginate to form instantaneous calcium gels and thus its applicability for cell encapsulation.

A new chemoenzymatic approach has recently shown to be able to overcome this limit [13]. It is based on coupling a chemical step for the modification of mannuronan with two epimerization reactions which introduce G residues both in alternating and block sequences. The final result is an alginate sample with side-chain moieties exclusively on M residues. Following these results, we succeeded in producing an alginate sample bearing methacrylate groups exclusively on M residues and containing enough G groups, both in alternating and block sequences, to allow for gel formation. The selectively modified alginate obtained was used to prepare calcium beads and cylinders that showed a higher-dimensional stability and an increased mechanical strength with respect to a randomly modified alginate.

Beads obtained from the selectively modified alginate were additionally crosslinked by using a photoactivated reaction to obtain stable chemoenzymatic photocrosslinked (CEPC) beads under conditions that allowed for cell survival. Two cell lines differing in post-encapsulation growth abilities, the murine C2C12 myoblast and the human 293 endo cells, were encapsulated within the CEPC beads, demonstrating different growth-pattern within the beads. Moreover, human pancreatic islets of Langerhans were entrapped within CEPC beads showing both survival and secretion of insulin after immobilization.

2. Materials and methods

2.1. Materials and reagents

High molecular weight mannuronan (fraction of guluronic residues, F_G, <0.001) was isolated from an epimerase-negative mutant (AlgG-) of *Pseudomonas fluorescens* [14]. The recombinant mannuronan C-5 epimerases were produced by fermentation of these recombinant *Escherichia coli* strains: AlgE4 in JM 105 and AlgE6 in SURE, respectively, as previously reported [15]. *Laminaria hyperborea* stipe (UP-LVG) alginate was from Pronova Biopolymers (Oslo, Norway) with $F_G = 0.65$ and M_w of 200 kDa ($[\eta] = 8.90$ dL/g). Methacrylic anhydride was from Fluka. Calcium carbonate (average particular size 4 μm), triethanol amine (TEA) and dimethyl sulfoxide (DMSO) for spectroscopy were purchased from Merck (Darmstadt, Germany). D-glucono-δ-lactone (GDL), ethylenediaminetetraacetyc acid (EDTA), eosin Y 5% (EY), MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] and dithizone (DTZ) (1.5 diphenylthiocarbazone) were purchased from Sigma-Aldrich Chemical Co, Nordic (sigma-aldrich.com). 1-vinyl-pyrrilidinone (VP) was purchased from Acros Organics (Geel, Belgium). Live/Dead Viability/Cytotoxicity kit for animal cells was purchased from Molecular Probes, InVitrogen detection technologies, Europe (www.invitrogen.com).

2.2. Methacrylate-grafted mannuronan (6PAn)

Methacrylic anhydride (7eq. per polymer repeating unit) was added dropwise to a 1% (W/V) solution of mannuronan in deionized water at 0 °C adjusting the pH to ~9 by means of a concentrated (5 M) NaOH solution. The mixture was stirred at 8 °C for 24 h and a 5 M NaCl solution was added (final concentration 1%). The solution was treated with ethanol and the precipitate was centrifuged and washed three times with ethanol. The precipitate was dissolved in deionized water, dialyzed (cut-off molecular weight of the membrane approximately 12,000) against deionized water until the conductivity was below 2 μS at 4 °C and the pH adjusted to 7. The polymer solution was filtered through 0.45 μm Millipore filters and freeze dried. A ^{1}H-NMR on the final polymer revealed a degree of substitution of 6%, calculated as reported in the caption to Fig. 1 (per polymer repeating unit).

2.3. Epimerization with AlgE4 (6PAnE4) and with AlgE6 (6PAnE4E6)

The methacrylate-modified mannuronan, 6PAn, was epimerized with AlgE4, yielding 6PAnE4, and successively with AlgE6 obtaining, as the final product, the sample 6PAnE4E6 (Table 1 and Scheme 1). In both cases, already reported procedures were followed [13].

2.4. Methacrylate-modified alginate from L. hyperborea (6LhypAn)

The modification of alginate from *L. hyperborea* was performed under the same reaction conditions reported for mannuronan. The degree of substitution, evaluated from ^{1}H NMR, was found to be approximately 6–7%.

2.5. NMR spectroscopy

Samples were prepared as described by Grasdalen et al. [16]. The ^{1}H and ^{13}C NMR spectra were recorded in D_2O at 90 °C with Bruker WM 300. The chemical shifts are expressed in parts per million (ppm) downfield from the signal for 3-(trimethylsilyl)propane-sulfonate.

2.6. Bead formation

Calcium alginate gel beads from *L. hyperborea*, 6LhypAn and 6PAnE4E6 were prepared by allowing a 1.8% alginate solution to drip into a gelling bath containing 50 mM of $CaCl_2$ and 0.1% of Tween 20. The size of the droplets was controlled by means of an electrostatic bead generator [17] (7 kV, polymer solution flow 10 mL/h, steel needle with 0.4 mm outer diameter, 1.7-cm distance from the needle to the gelling solution). The beads were stirred for 30 min in the gelling and rinsed to remove the excess of calcium.

2.7. Preparation of gel cylinders

Homogeneous calcium gels from *L. hyperborea*, 6LhypAn and 6PAnE4E6 were prepared in 24-well tissue culture plates ($\varnothing = 16$ mm; $h = 18$ mm; Costar, Cambridge, MA) by blending a 1% (w/v) polymer solution with $CaCO_3$ (15 mM) followed by the addition of the slowly hydrolyzing GDL (a molar ratio GDL/$Ca^{2+} = 2$ was maintained). The gels were taken out from the wells after 24 h and their Young's modulus (E) was calculated from the initial slope of the force/deformation curve [18], as measured with a Stable Micro Systems TA-XT2 texture analyzer at 20 °C. For all gels exhibiting syneresis, the final polymer concentration was determined and E was corrected by adaptation of $E \propto c^2$ [19].