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Cellular uptake of multilayered capsules produced with natural and genetically engineered biomimetic macromolecules

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ABSTRACT

Multilayered microcapsules of chitosan and biomimetic elastin-like recombinamers (ELRs) were prepared envisaging the intracellular delivery of active agents. Two ELRs containing either a bioactive RGD sequence or a scrambled non-functional RDG were used to construct two types of functionalized polymeric microcapsules, both of spherical shape ~4 μm in diameter. Cell viability studies with human mesenchymal stem cells (hMSCs) were performed using microcapsule/cell ratios between 5:1 and 100:1. After 3 and 72 h of co-incubation, no signs of cytotoxicity were found, but cells incubated with RGD-functionalized microcapsules exhibited higher viability values than RDG cells. The internalization efficacy and bioavailability of encapsulated DQ-ovalbumin were assessed by monitoring the fluorescence changes in the cargo. The data show that surface functionalization did not significantly influence internalization by hMSCs, but the bioavailability of DQ-ovalbumin degraded faster when encapsulated within RGD-functionalized microcapsules. The microcapsules developed show promise for intracellular drug delivery with increased drug efficacy.

1. Introduction

In the field of drug delivery, there has been much interest in developing active agent nano- and micro-carriers, such as micelles, liposomes, polymersomes and polymer capsules [1–4]. Such devices may be used in the release control of encapsulated biomolecules and to increase their efficacy by delivering them to the location where they are needed most. In particular, nanostructured nano- and micro-capsules made using layer-by-layer (LbL) strategies have been studied to encapsulate and deliver drugs in a controlled manner [5–9]. The principle behind LbL adsorption lies in the existence of intermolecular interactions that potentiate attraction among distinct polymer chains. Some examples include electrostatic contacts, hydrophobic interactions and hydrogen bonding, which drive the assembly of layers of a few nanometers onto a surface [10–12]. With this simple and versatile technique, robust coatings can be assembled on bidimensional templates and even complex three-dimensional substrates. For example, multilayer microcapsules have already been proposed as biosensors and as drug carriers—including stem cell differentiation promoters—with tunable release kinetics dependent on the number of layers or on external stimuli [13–17].

Multilayer capsules may encapsulate and deliver bioactive agents to cells or tissues by conventional mechanisms of release to the biological environment [18] or by digestion within the cells themselves [19]. The latter possibility is particularly interesting: delivering drugs to the intracellular environment, where the whole metabolic machinery is found, ensures that large amounts are delivered to the target tissue, ensuring their high efficacy. Moreover, it could be possible to circumvent undesirable side effects to the surrounding cells or tissues resulting from premature drug leakage. Dendrimers, micelles and biodegradable nanoparticles have been developed for the intracellular delivery of bioactive agents [20–23]. In comparison, multilayer-coated capsules present several advantages that are not often found in other conventional

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fabrication techniques: (i) they can be prepared in mild conditions, allowing large amounts of sensitive or easily denatured biological molecules [24] and even cells [25] to be encapsulated; (ii) it is possible to encapsulate drugs with low water solubility [6]; (iii) the release rate can be controlled better by varying the number of layers and their composition [26]; and (iv) the shell can provide a high degree of functionality by selecting biofunctional building blocks that may participate in biological mechanisms [27].

The primary objective of the present work was the design of multilayer microcapsules made of natural and nature-inspired biomaterials exhibiting bioactive sequences for intracellular drug delivery: namely, chitosan (CHI) and elastin-like recombinamers (ELRs) were used as building blocks for multilayer shells, assembled on spherical particles of calcium carbonate. CHI is a well-known polycationic polysaccharide of marine origin, exhibiting biocompatibility, adhesiveness and non-toxicity, as well as bacteriostatic, fungistatic, antimicrobial and hemostatic activities [28]. ELRs, a recombinant type of elastin-like polymer, are temperature-responsive and biomimetic polypeptides. They are soluble in aqueous media below a critical transition temperature (T_t), but undergo phase separation above it [29]. Their recombinant and modular nature is perhaps their most attractive property, because their physicochemical properties can be tuned, and introducing specific relevant amino acid domains can extend their physiological functionality [30,31]. LbL adsorption can be performed using ELRs alongside polysaccharides such as CHI and alginate by resorting to strong electrostatic interactions and the stabilizing effect of weaker hydrophobic interactions [32].

In the present study, human mesenchymal stem cells (hMSCs) were co-incubated with microcapsules made with an ELR exhibiting the cell adhesion motif arginine–glycine–aspartic acid (RGD). RGD is a motif of the human structural protein fibronectin, and is the minimal sequence required for recognition by cell adhesion receptor integrins [33]. Using RGD has previously enhanced the internalization of nano-/micro-particles [34–36]. However, such assessment has been often made with controls that do not exhibit any sort of functionalization. Therefore, in addition to RGD, the present authors also constructed microcapsules with an ELR exhibiting a non-functional scrambled RDG sequence (referred to as $RDG^{(-)}$, for the sake of distinction), instead of completely eliminating the functional layer. To the present authors' knowledge, this is the first time that the internalization efficacy of RGD-functionalized LbL microcapsules has been compared with a microcapsule type that substitutes the bioactive group with a non-functional analogue.

The synthesized microcapsules were characterized in terms of morphology and cytotoxicity toward hMSCs. The internalization efficacy of microcapsules was quantified by flow cytometry in order to identify the existence—or absence—of a receptor-mediated uptake. Capsules loaded with DQ-ovalbumin enabled an assessment of its degradation stage by monitoring fluorescence changes inside the cells.

2. Materials and methods

2.1. Materials

Medium molecular weight CHI (Ref. 448877), sodium carbonate anhydrous (Na_2CO_3, Ref. 222321) and ethylenediaminetetraacetic acid (EDTA) (Ref. E9884) were purchased from Sigma–Aldrich. CHI was purified in a series of filtration and precipitation in water and ethanol steps, adapted from the method described elsewhere [37], followed by freeze-drying. The degree of deacetylation was determined as 82% by ^{1}H-NMR. Calcium chloride ($CaCl_2$) was purchased from VWR (Ref. 1.02378.0500). Two custom-made ELRs containing either RGD or $RDG^{(-)}$ peptide sequences were purchased from Technical Protein Nanobiotechnology (Spain) (a schematic representation can be found in Supplementary Figure 1, in Appendix B). Molecular weight and amino acid composition of the acquired batches were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy and amino acid content analysis, respectively, and can be found elsewhere [32]. DQ-ovalbumin (Ref. D12053) was obtained from Invitrogen. Rhodamine B isothiocyanate (Ref. 283924) was purchased from Sigma–Aldrich.

Paraformaldehyde (Ref. P6148), Triton X-100 (Ref. T9284) and Accutase™ (Ref. A6964) were purchased from Sigma–Aldrich. The hMSCs from human adipose tissue (Ref. R7788-115), its basal medium, Dulbecco's modified Eagle's medium (DMEM, Ref. 31966-021), fetal bovine serum (FBS Ref. 16000-044), penicillin streptomycin solution (ref SV30010), trypsin-EDTA (Ref. SV30010), Dulbecco's phosphate buffered saline (DPBS) (Ref. 14190-136), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Ref. 15630049), Trypan Blue stain 0.4% (Ref. 15250061), Alexa Fluor 488 phalloidin (Ref. A12379), LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells (Ref. L3224), Alamar Blue® (Ref. DAL1025) and DAPI (Ref. D21490) were supplied by Invitrogen. All cell culture plastic ware and consumables were acquired from NUNC.

2.2. Measurement of the charges

The zeta (ζ)-potentials of the ELRs were determined using Nano-ZS equipment from Malvern (UK), at 25 °C. Aqueous solutions of each ingredient in phosphate buffered saline (PBS) (pH = 7.4) were used, with a concentration of 300 $\mu g \cdot ml^{-1}$.

2.3. Synthesis of calcium carbonate sacrificial templates

Aqueous solutions of Na_2CO_3 and $CaCl_2$ were prepared with a concentration of 1 M. Typically, the co-precipitation of both solutions was performed by adding 1 ml of Na_2CO_3 solution, followed by 1 ml of $CaCl_2$ to 4 ml of ultrapure water (Milli-Q) under heavy stirring for 30 s. The stirring was then stopped, and the newly synthesized calcium carbonate ($CaCO_3$) microparticle suspension was left to precipitate and react for 15 min. The supernatant was removed, and the particles were washed three times with 0.15 M sodium chloride (NaCl) adjusted to pH 5.5. To produce microcapsules preloaded with protein cargo, the medium where the co-precipitation occurs consisted of an aqueous solution of DQ-ovalbumin with a concentration of 160 $\mu g \cdot ml^{-1}$. Unless otherwise stated, all changes of solutions after the template synthesis reaction and during the construction of microcapsules were made by leaving the particle suspension precipitating for 10 min, followed by removal of the supernatant and replacement with the next solution—polymeric or washing.

2.4. Construction of the multilayer shells and microcapsule formation

The $CaCO_3$ sacrificial templates were immersed alternately in solutions of CHI and of ELR (300 $\mu g \cdot ml^{-1}$, pH 5.5, 0.15 M NaCl) for 10 min under mild stirring. Each adsorption step was followed by three steps of washing with 0.15 M NaCl, pH 5.5: to disaggregate the substrates, the first washing step was accompanied by four sequential ultrasound pulses of 3 s each, while the subsequent steps were only rinsing. This process was repeated until two CHI/ELR bilayers were assembled. After the construction of the multilayers, the $CaCO_3$ core was dissolved using EDTA at 0.2 M, pH 5.5. From this point on, the precipitation of the microcapsules was aided by centrifugation (1000 rpm, 25 min) and ultrasound pulses timed as described above. The newly formed hollow microcapsules