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Location of molecules in layer-by-layer assembled microcapsules influences activity, cell delivery and susceptibility to enzyme degradation ☆

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ABSTRACT

Layer-by-layer assembled microcapsules have the potential to be versatile cell delivery systems incorporating multiple activities and functions. However, it is necessary to determine the influence that different capsule locations have on activity of bioactive molecules in order to optimise delivery and for generation of multifunctional capsules. In this study we examine the influence that locating the bioluminescent enzyme luciferase in different microcapsule locations has on activity in intact synthetic and biodegradable microcapsules before and after cell delivery as well as its susceptibility to protease degradation. We also examine the effect of microcapsule position on cell transfection with plasmid DNA. Based on the findings of experiments in this study we also demonstrate co-delivery of luciferase protein and plasmid DNA encoding a fluorescent protein from two different locations within the same microcapsule. Our studies confirm that, the core, subouter layer, and outer layer are optimal for cell delivery but these positions offer least protection from protease activity. By contrast middle layer molecules remain entangled with capsule layers preventing their release which is inefficient for cell delivery but this provides better protection from protease degradation. The findings of this study will enable more rationale layer-by-layer assembly of microcapsules containing biologically active molecules for cell delivery and aid in the generation of multifunctional microcapsules.

1. Introduction

Due to the native conditions utilised in layer-by layer (LbL) assembly [1,2] the process is well suited for the inclusion of biologically active molecules into both biologically functionalised films [3] and micro- and submicron sized capsules [4]. LbL assembled capsules also have the potential to enter cells where they can in their simplest form deliver a cargo with gradually controlled or stimuli-triggered release as well as act as cell residing biosensors to report on intracellular processes [5,6], for recent review on biological application of LbL see [7,8]. These capsules also have the potential to be engineered with multiple activities by incorporating molecules with distinct activities into different layers or within the capsule core. In order to achieve optimum cell delivery or for the successful generation of multi-active capsules it is important to determine the activity/availability of molecules when they are positioned at different sites within capsules so that construction can be optimised. Previous studies with LbL assembled films have shown that enzyme activity typically decreases as they are placed in deeper layers due to reduced access of substrate through the covering layers of polyelectrolytes [9,10]. When enzymes such as lipase B and glucose oxidase have been incorporated into synthetic microcapsules the same observation has been made, with lower activity of enzyme in the core attributed to reduced mass transfer of substrate [11,12]. We have recently utilised the bioluminescent enzyme firefly luciferase to examine the influence that different locations in biodegradable microcapsules has on activity. In intact microcapsules, luciferase activity was lowest in the core and highest as an outer layer [13], observations that are in agreement with other enzyme reports. When microcapsules are delivered to cells there are additional factors such as uptake efficiency, degradation processes, pH, and exocytosis that can influence a molecules activity. When luciferase containing microcapsules were engulfed by cells outer layer luciferase was available to react with the fastest kinetics and core luciferase was similarly active, but luciferase in a middle layer was much less active [13]. We now further extend our observations on how location affects activity of luciferase enzyme and assess

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protection from protease degradation, in addition we examine the effect of microcapsule location on cell transfection with plasmid DNA. These studies further inform us about the potential of using different capsule positions for delivering bioactive molecules to cells and will facilitate generation of multifunctional microcapsules.

2. Materials and methods

2.1. Reagents

Poly(allylamine hydrochloride) (PAH, molecular weight ~56 kDa), Poly-L-arginine Hydrochloride (PLA, molecular weight 15–70 kDa), Poly(sodium 4-styrenesulfonate) (PSS, molecular weight ~70 kDa) and Dextran Sulfate sodium salt (DS, molecular weight ~100 kDa), Polyethylenimine, branched (PEI, 25 kDa), Ethylenediaminetetraacetic Acid disodium salt (EDTA), Sodium Hydroxide and all salts were purchased from Sigma. Other reagents included recombinant American firefly luciferase (Roche Diagnostics GmbH, Mannheim, Germany), luciferase assay system, D-luciferin K^+ salt, passive lysis buffer and Fugene® 6 transfection reagent all from Promega Corp (Madison, WI, USA). DMEM, FBS, penicillin and trypsin were purchased from Lonza Biologics Inc (Newington, NH, United States).

2.2. Preparation of plasmid DNA

Plasmid $pcLuc^+$ encoding firefly luciferase from a CMV promoter [14] was propagated in DH5α E. coli and was purified using the Qiagen EndoFree mega kit (Qiagen Ltd., Crawley, West Sussex, UK) with plasmid DNA resuspended in sterile distilled water.

2.3. Microcapsules construction and characterization

Microcapsules for all experiments were prepared using the layer-by-layer assembly technique [15] by alternate deposition of oppositely charged polyelectrolytes on calcium carbonate particles. Plasmid DNA and luciferase were incorporated into the cores by co-precipitation or in the shells as layers of negative charge. Sacrificial $CaCO_3$ templates were synthesized immediately before microcapsule preparation by mixing of 0.33 M $CaCl_2$ and Na_2CO_3 solutions whilst vigorously stirring, according to a well established protocol [15]. When luciferase or plasmid DNA was incorporated into the microcapsules, an appropriate amount of these substances was mixed with $CaCl_2$ prior to Na_2CO_3 addition. Luciferase incorporation methods and calculations of incorporation efficiency were previously described [13]. Both molecules were used at a concentration of 1 mg/ml for co-precipitation and half of this concentration for inclusion in layers, with a total amount of 200 μg for each inclusion. PAH and PSS were used as synthetic polyelectrolytes, whilst PLA and DS were used as biodegradable ones. To buffer endosomal acidification and promote release of microcapsules from endosomes into the cytosol, PEI was utilised to create an outermost layer for some samples. Polyelectrolytes were adsorbed from 2 mg/ml solutions in 0.5 M NaCl for synthetic layers (PAH, PSS, PEI) and 0.15 M NaCl for biodegradable layers (PLA, DS). After the shells were fully constructed, $CaCO_3$ cores were dissolved in 0.2 M EDTA (pH 6.5) followed by triple washing and centrifugation steps first in EDTA and then in water.

To determine the amount of DNA incorporated into capsules the amount remaining in supernatants collected from the adsorption of the plasmid and also from subsequent layers, as well as from dissolution of calcium carbonate cores was determined. Total losses of DNA were then subtracted from the initially added amount to calculate the total amount incorporated in the capsules. Plasmid DNA concentration in solutions was measured using the high-sensitivity double stranded DNA concentration assay kit and Qubit fluorimeter (Invitrogen. Leek, The Netherlands) according to the manufacturer's instructions. The DNA incorporation ratio was found to be 96, 34% and 45% for core, middle (4th) and subouter (6th) layers, respectively.

During measurement of luciferase incorporation efficiency it was found that the synthetic polyelectrolyte PSS (but not PAH or PEI) had a drastic inhibitory effect on luciferase activity (Supplementary Fig. 1), and this was taken into account when calculating the amount of luciferase incorporated. The percentage incorporation of luciferase used both for co-precipitation and for shell layers, was measured to be close to 100%. If a mass-to-surface area factor would be calculated, it would likely seem to be a bit higher than normal, (25 mg/m^2 compared to usual 0.1–1.5 mg/m^2 [16,17] and up to 26 mg/m^2 in some cases [18,19]). Together with the fact that incorporation was measured based on activity of enzyme in solution, this can potentially indicate, that activity of enzyme is being inhibited by the encapsulation process.

Incorporation of layers including luciferase and plasmid DNA was also confirmed by zeta-potential measurements using Nano-ZS zetasizer (Malvern Instruments, UK) (Supplementary Fig. 2), that showed a good charge overcompensation in the course of layer adsorption.

Data on incorporation ratio for each prepared sample was then used to prepare comparable solutions of all microcapsules. It was assumed, that microcapsule dispersions with co-precipitated substances and substances in the layers had the same initial number of particles. Microcapsule concentration in suspension was determined by counting on a haemocytometer and volumes were adjusted so that the numbers were equal for different samples. Afterwards, similar volumes of dispersions were used for experiments. See Table 1 for the full list of the microcapsules assembled in this study.

2.4. Capsule degradation with trypsin

For experiments on the effect of trypsin on the activity of encapsulated enzyme, 10 μl of microcapsules suspension at a concentration used for all other experiments, were introduced to wells of a 96 well plate. Then 50 μl of trypsin at the desired concentration (range 2.5–250 ng/ml prepared in PBS) was introduced and the plate was incubated at 37 °C (5 min). Immediately followed by the addition of 70 μl of complete DMEM medium to the same wells to neutralise trypsin action then luciferase activity in microcapsules was measured using the standard luciferase assay. To study the effect of trypsin on microcapsule shell structure, microcapsules were visualized using an FEI Inspect F scanning electron microscope. After trypsin treatment (2.5 μg/ml) microcapsules were washed with distilled water and ~10 μl of dispersion was taken to a sample holder with attached glass slide. After drying at room temperature, samples were sputtered with gold and visualized.

2.5. Cells culture and transfection

The human embryonic kidney epithelial cell line 293T and human epithelial carcinoma cell line HeLa were routinely cultured in DMEM (BioWhittaker, Wokingham, UK) supplemented with penicillin (100 U/ml)(BioWhittaker), streptomycin (100 μg/ml) (BioWhittaker) glutamine (2 mM) (BioWhittaker) and 10% heat inactivated foetal bovine serum (GibcoBRL, Paisley, UK), in a humidified incubator containing 10% CO_2 at 37 °C. For experiments, cells were plated in 96 or 24 well plates prior to addition of microcapsules.

2.6. Luciferase activity assay

The kinetics of luciferase activity in intact microcapsules was monitored as described before [19]. Following addition of luciferase substrate to microcapsules light emission in 10 s was monitored (MLX Microtiter® Plate Luminometer, Dynex Technologies, USA) and was repeated at various time points over a period of 300 min. In experiments with transfected luciferase plasmid, cells were lysed in 100 μl of passive lysis buffer and activity in 10 μl of centrifuged lysate was measured.