NOTE

Novel Layer-by-Layer Deposition Technique for the Preparation of Double-Chambered Nanoparticle Formulations

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ABSTRACT: In this work, we report a novel method of layer-by-layer (LbL) deposition using concentration tubes that enables faster process and less damage to fragile nanocores than previously described methods. Such methods are generally based on continuous cycles of centrifugation/resuspension for long times and at high speeds, which may eventually lead to the aggregation of the deflocculated suspension of nanoparticles into a compact, non-resuspendable cake. The new method was applied to the preparation of a double-chambered nanocarrier system, which was successfully loaded with a fluorescently labeled model protein (lysozyme) and a model small molecule (fluorescein) in two defined and separate compartments, namely the poly lactide-co-glycolide (PLGA) core (~110 nm) and an outer shell obtained by LbL surface coating. The new method yielded stable suspensions of drug-loaded, LbL-coated PLGA nanoparticles, while centrifugation at high speeds and long time intervals leads to a compact cake of non-resuspendable aggregates. These nanocarriers were taken up by MDCK cells *in vitro*, where a colocalization of both model compounds was shown by confocal imaging. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2637–2640, 2015

Keywords: layer-by-layer; nanoparticles; codelivery; protein delivery; biomaterials; alginate; PLGA

INTRODUCTION

With the development of active biological therapeutics, ranging from small peptides to monoclonal antibodies, many combination therapies suggest the use of a protein and a small molecule drug together, as exemplified by the use of small interfering RNA molecules in combination with anticancer drugs to treat different resistant tumors.[1–3] The inclusion of protein therapeutics adds more challenges to the delivery vehicle as protein stability and activity become of high concern.[4] Therefore, one strategy toward a more effective codelivery of protein/small molecule drug combinations may be the careful design of a nanodelivery system that is able to efficiently encapsulate and colocalize both cargo species in the target cells.

Layer-by-Layer (LbL) technology, a technique based on the layer wise deposition of oppositely charged polyelectrolytes on the surface of interest, has gained increasing interest in the field of drug delivery. Not only because of its ability to build a layered system of tunable characteristics in terms of composition, nanometer-range thickness, surface charge, permeability, and elasticity, but more particularly relevant to protein formulation, LbL deposition has the advantage of utilizing mild conditions (i.e., aqueous solutions), which are more favorable to preserve correct protein folding and activity in contrast to organic solvents typically employed in the fabrication of many other protein formulations.[5]

Current published techniques aiming to deposit polyelectrolyte layers on nanoparticulate cores employ a fabrication protocol that is based on continuous cycles of centrifugation/resuspension for long times and at high speeds,[6] which may eventually lead to the aggregation of at least part of the deflocculated suspension of nanoparticles into a compact, non-resuspendable cake.

Hereby, we describe the preparation of a novel double-chambered, nanoparticulate system applying a simple and less time-consuming method using concentration tubes. Small poly lactide-co-glycolide (PLGA) nanoparticles loaded with fluorescein (Flu) base as a model small hydrophobic molecule represent the "internal chamber," whereas the "external chamber" is formed of alternating polyelectrolyte layers containing lysozyme (Lys) as a model protein (Fig. 1).

MATERIALS AND METHODS

Materials

Lysozyme from chicken egg white (14,600 Da) coupled to rhodamine was a kind gift from Capsulution Pharma (Berlin, Germany). Alginic acid sodium salt from brown algae (Alg), Flu base and poly (DL- PLGA) RG502H (molecular weight 7000–17,000 Da), were purchased from Sigma–Aldrich (Buchs, Switzerland). 4′,6-diamidino-2-phenylindole (DAPI) staining solution was purchased from Vector Laboratories Inc. (Burlingame, California). Vivaspin concentration tubes fitted with polyethersulfone (PES) 300 kDa molecular weight cutoff membranes were purchased from Sartorius Stedim (Goettingen, Germany). Other chemicals were of analytical grade.

Methods

Fabrication of Flu-Loaded PLGA Nanoparticles by Nanoprecipitation

Fluorescein-loaded PLGA nanoparticles were prepared using a slightly modified nanoprecipitation procedure described by Govender et al.[7] PLGA (100 mg) was dissolved in 9 mL of acetone, and 1 mL acetone containing 0.5 mg/mL Flu was added,

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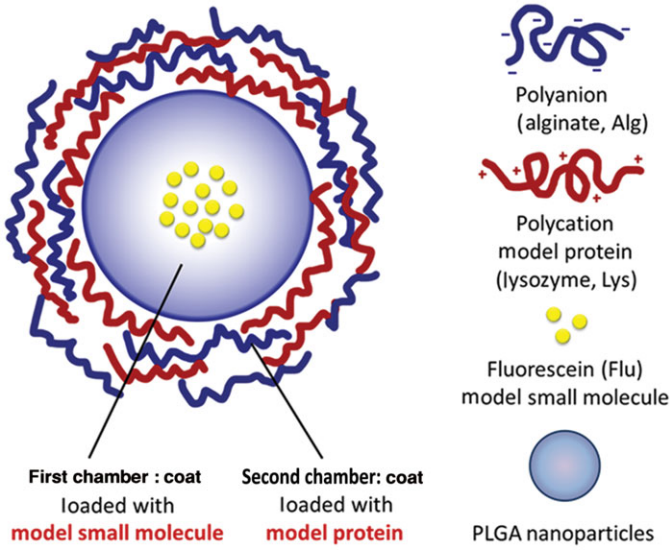

Figure 1. Schematic diagram of the double-chambered nanoparticles.

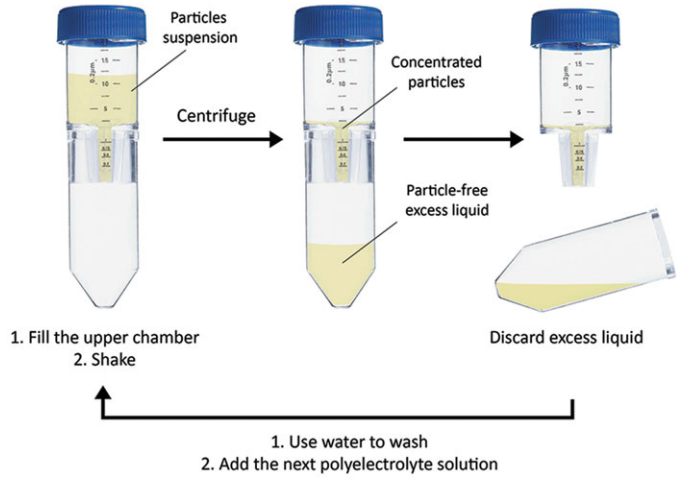

Figure 2. Schematic diagram showing the use of concentration tubes in deposition of polyelectrolyte layers.

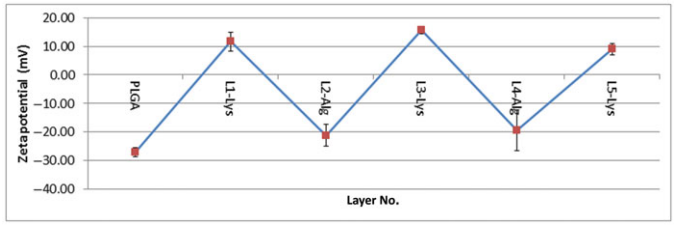

Figure 3. Reversal of zeta potential with addition of successive polyelectrolyte layers ($n = 6$, error bars represent SDs).

giving a 1% (w/v) final concentration of PLGA in acetone. The acetone solution was injected slowly via a syringe needle into 40 mL of water under high stirring in a wide-mouth beaker, and left under stirring overnight to remove acetone.

LbL Deposition over PLGA Nanoparticles: New Versus Conventional Protocols

The new and conventional LbL deposition methods were investigated and compared. To test the new method, the core suspension of formed PLGA nanoparticles was added to the upper compartment of a suitably sized Vivaspin concentration tube. Core suspensions were concentrated to a few milliliters by centrifugation (1000 *g* for 5 min) and washed thrice with milliQ (Merck Millipore, Darmstadt, Germany) water. The excess fluid was collected in the bottom chamber and discarded. To the negatively charged PLGA core, a first layer of Lys was deposited by adding 2 mL of 1 mg/mL Lys, followed by 10 min gentle shaking on a rotatory shaker. Again, core suspensions were concentrated and washed thrice with milliQ water to remove excess Lys before the addition of the next layer. The coming layer was composed of the biocompatible, negatively charged polyelectrolyte Alg. Two milliliters of 1 mg/mL Alg solution in milliQ water was added, followed by 10 min gentle shaking before the cores were washed. The same process (illustrated in Fig. 2) was repeated until five layers were deposited giving the following structure: [PLGA], $(Lys, Alg)_2$, Lys. To minimize particles sticking to the side filter walls, the suspension in the upper chamber was mixed with a micropipette several times in each step. Finally, coated particles were aspirated from the upper chamber using a micropipette. The pH of all polyelectrolyte solutions used and washing water was adjusted to 5.5, to maintain high charge density on both polyelectrolytes (PI of Lys and Alg was 11 and 3.6, respectively).

On the contrary, to test the conventional LbL method, the concentrated cores suspension was added to 2 mL Eppendorf (Schönenbuch, Switzerland) tubes. Similar steps of layering as described above were followed with one exception; separation of coated cores after each polyelectrolyte addition/washing was performed by centrifugation at 30,000*g* for 30 min as described before.[6]

Monitoring of Layer Deposition via Zeta Potential Measurements

A sample of particles ($n = 6$) was used to measure particle size and zeta potential using a Malvern Zetasizer NanoZS (Malvern, Lausanne, Switzerland), before coating and after the addition of each layer to prove successful layer deposition. All measurements were performed in milliQ water (pH 5.5) without any added electrolyte.

Qualitative Cellular Uptake Study Using Confocal Fluorescence Imaging

MDCK cells were seeded on glass cover slips in a six-well plate at an initial seeding density of 3×10^5 cells/well and allowed to attach for 48 h. After 2 days, the medium was aspirated from three wells, and 3 mL of 1 mg/mL particle suspension in culture medium was added. From the other three wells acting as controls, the medium was aspirated and 3 mL of fresh medium was added. After 4 h incubation, the cellular uptake of the particles was stopped by aspirating all supernatants. Cells were washed with ice-cold phosphate buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 15 min. Cells were then washed again with PBS, and cover slips were inverted and fixed on glass slides after addition of one drop of DAPI solution. Confocal laser scanning fluorescence imaging was performed with a Zeiss LSM700 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). A series of optical sections was recorded by moving the focal plane of the instrument (1 μm steps) through the