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Rational design of protamine nanocapsules as antigen delivery carriers

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ABSTRACT

Current challenges in global immunization indicate the demand for new delivery strategies, which could be applied to the development of new vaccines against emerging diseases, as well as to improve safety and efficacy of currently existing vaccine formulations. Here, we report a novel antigen nanocarrier consisting of an oily core and a protamine shell, further stabilized with pegylated surfactants. These nanocarriers, named protamine nanocapsules, were rationally designed to promote the intracellular delivery of antigens to immunocompetent cells and to trigger an efficient and long-lasting immune response. Protamine nanocapsules have nanometric size, positive zeta potential and high association capacity for H1N1 influenza hemagglutinin, a protein that was used here as a model antigen. The new formulation shows an attractive stability profile both, as an aqueous suspension or a freeze-dried powder formulation. In vitro studies showed that protamine nanocapsules were efficiently internalized by macrophages without eliciting significant toxicity. In vivo studies indicate that antigen-loaded nanocapsules trigger immune responses comparable to those achieved with alum, even when using significantly lower antigen doses, thus indicating their adjuvant properties. These promising in vivo data, alongside with their versatility for the loading of different antigens and oily immunomodulators and their excellent stability profile, make these nanocapsules a promising platform for the delivery of antigens.
Chemical compounds: Protamine sulphate (PubChem SID: 7849283), Sodium Cholate (PubChem CID: 23668194), Miglyol (PubChem CID: 53471835), α tocopherol (PubChem CID: 14985), Tween® 20(PubChem CID: 443314), Tween® 80(PubChem CID: 5281955), TPGS (PubChem CID: 71406).

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1. Introduction

Nanotechnology-based approaches are becoming increasingly important in the formulation and delivery of complex active molecules, such as protein and peptide antigens. In fact, the term nanovaccine has been recently coined to describe nano-scale antigen delivery systems [1]. The use of a nanocarrier may simultaneously address several of the critical challenges in vaccine development. Their particulate nature, together with their nanometric size can i) confer adjuvant properties to the associated antigen and ii) facilitate its transport across mucosal surfaces, thus enabling needle-free vaccination. In addition, the appropriate selection of biomaterials may iii) provide controlled release properties and iv) improved thermostability, allowing for the elimination of the currently required cold-chain for vaccine transport and storage [2].

Our group has previously reported the usefulness of chitosan nanocapsules, consisting of an oily core and a chitosan coating for the co-delivery of antigens in combination with immunostimulants. These chitosan nanocapsules were shown to be an adequate strategy for single-dose immunization against a hepatitis B surface antigen, while offering the possibility to be stored as a freeze-dried powder [3]. Considering these promising results, we decided to explore the feasibility of a new nanocapsule-type antigen delivery platform based on the natural cationic polypeptide, protamine.

The name protamine refers to a diverse family of aliphatic and strongly basic arginine-rich proteins (Fig. S1), with an average molecular mass of 4.5–5.5 kDa [4]. These proteins are synthesized during the late stage of spermatid development in different animals and plants, and their physiological function is the condensation and stabilization of the genetic material [5]. Interestingly, they also exhibit a number of properties that make them a very promising biomaterial for drug/antigen delivery [6]. First, protamine is an FDA approved drug indicated to

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revert the anticoagulant effects of heparin and is also used as an excipient in the formulation of NPH (Neutral Protamine Hagedorn) insulin to prolong its action. In addition, because of its high arginine content (about 70%), protamine can easily cross biological membranes [7] and promote the internalization of associated molecules [8]. Finally, protamine has also been found to help stabilizing and solubilizing proteins by electrostatic repulsion [9].

In addition to the products already in the market, protamine has attracted attention as a biomaterial for the design of drug delivery nanocarriers, including liposomes [10], nanoparticles [11], nanotubes [12] or layer-by-layer nanoshells, with a particular emphasis on its use as a gene delivery carrier [13]. In the immunization field, there is preliminary data that supports the idea that nanoparticles or microparticles containing protamine may enhance the immune response of an anthrax protective antigen or purified phospholipase A_2. Likewise, it has also been shown that protamine complexes including mRNA are able to stimulate TNF-α and INF-α secretion in human blood cells, thereby activating B cells [14], which suggests that Toll-like receptors (TLR) may recognize this complex (specifically TLR 7), acting, therefore, as a potential adjuvant [15].

With all this information in mind, our main goal was to design and develop an original antigen nanocarrier consisting of an oily core stabilized with one or more surfactants and surrounded by a protamine shell. For this, we first investigated the influence of critical formulation parameters on the physicochemical properties of the nanocapsules and also on the association efficiency of the influenza H1 antigen. The most promising prototype was then selected for studying its cytotoxicity and internalization in macrophages. Finally, we determined the in vivo response elicited by the selected prototype and the feasibility of its presentation as a freeze-dried powder.

2. Materials and methods

2.1. Chemicals

Protamine sulphate was kindly donated by LEO Pharma (Denmark). PEG-Stearate (Simulsol® M52) and Macrogol 15 hydroxystearate (Solutol® HS 15) were obtained from Seppic (France) and BASF (Germany) respectively. Miglyol® 812 was a kind gift from Sasol Germany GmbH (Germany), α-tocopherol (TCPH) was supplied by Merck (Germany). 5-Carboxytetramethylrhodamine succinimidylester single isomer (TAMRA) and Alamar blue were obtained from Invitrogen (United Kingdom). Antibodies for Western blot, mouse monoclonal antibodies against the 6xHis tag fused to the HI and goat anti-mouse IgG-HRP were supplied by Abcam (United Kingdom). Dulbecco's Modified Eagle's Medium (DMEM), glutamine, penicillin and streptomycin were supplied by Gibco (USA). Tween® 20, Tween® 80, D-α tocopherol polyethylene glycol 1000 succinate (TPGS), glucose, trehalose, Triton-X100, WGA (wheat germ agglutinin), PBS and sodium cholate were obtained from Sigma-Aldrich (Spain). All other products used were of reagent grade purity or higher.

2.2. Preparation of protamine nanocapsules

Blank protamine nanocapsules were prepared by the solvent displacement technique, following the procedure previously described by our group [16]. Different amounts of PEG-stearate (Simulsol® M 52) (0, 12, 24 and 48 mg) and sodium cholate (0, 5, 10 and 20 mg) were dissolved in 750 μl of ethanol, followed by the addition of Miglyol® (31.25, 62.5 or 125 μl) and 4.25 ml of acetone. This organic phase was immediately poured over 10 ml of an aqueous phase with 0.05% w/v protamine (one step procedure). The organic solvents were eliminated by evaporation under vacuum (Rotavapor Heidolph, Germany), to a constant volume of 5 ml.

For the nanocapsules preparation method in two steps, we used the same materials and amounts indicated above but omitted the protamine in the external aqueous phase. The resulting nanoemulsion (NE) was incubated in a subsequent step with a protamine solution (0.5% w/v). The volume ratio used was 4:1 (NE:protamine solution).

The same solvent displacement technique was used to prepare nanocapsules using different oils (Miglyol vs tocopherol) in combination with others surfactants, such as Solutol® HS 15, Tween® 20, Tween® 80 or TPGS with sodium cholate. The ratios between TCPH and the main surfactant used were 12:12, 60:12 and 60:60 (mass ratio).

Protamine nanocapsules were isolated by ultracentrifugation (Optima™ L-90 K, Ultracentrifuge, Beckman Coulter, USA) at 30,000 RPM for 1 h (at 15 °C) and then, resuspended in ultrapure water to a final theoretical protamine concentration of 1 mg/ml.

2.3. Physicochemical characterization of the protamine nanocapsules

The hydrodynamic diameter and polydispersity index of the systems were determined by photon correlation spectroscopy (PCS) (Zetasizer®, NanoZS, Malvern Instruments, Malvern, UK), after sample dilution with ultrapure water. The zeta potential was measured by laser-Doppler anemometry after diluting the samples with 1 mM KCl. Morphological examination of nanocapsules was carried out by transmission electron microscopy (TEM, CM12 Philips, The Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid solution.

2.4. Quantification of protamine association to the nanocapsules

The amount of protamine associated to the nanocapsules was indirectly measured through the quantification of the polypeptide remaining in the aqueous medium after the isolation of the blank nanocapsules by ultracentrifugation (described in Section 2.2). Protamine was quantified by Ultra Performance Liquid Chromatography (Acquity UPLC, Waters, Spain). The column Acquity UPLC® BEH C18 1.4 μm 2.1 × 50 mm was used with a flow rate of 0.3 ml/min and a gradient as follows: 0–2 min, 100% A; 2–2.1 min 70% A; 2.1–6 min 100% A. The column was maintained at 35 °C. Solvent A was water with 0.1% trifluoroacetic acid (v/v) and solvent B was acetonitrile with 0.1%TFA (v/v). The detector wavelength was set at 214 nm.

2.5. Stability of protamine nanocapsules upon storage

The stability of the protamine nanocapsules in suspension was evaluated by monitoring the evolution of particle size during storage at 4 °C as an aqueous suspension for up to 3 months and also upon incubation in PBS (37 °C, pH = 7.4) for up to 5 days. Particle size analysis was carried out as indicated in Section 2.3.

2.6. Freeze-drying of protamine nanocapsules

Different concentrations of protamine nanocapsules (1, 0.75, 0.5, 0.25 and 0.1% w/v) were lyophilized (Labconco Corp, USA) in presence/absence of trehalose or glucose at 5 or 10% (w/v) as cryoprotectors. Samples were frozen at −20 °C and then subjected to an initial drying step at −35 °C followed by a secondary drying at 0 °C, in a high vacuum atmosphere. The total time for both steps was 24 h. To end this process, the temperature was increased slowly to room temperature.

The freeze-dried formulations were resuspended with ultrapure water by manual resuspension and their physicochemical characteristics were evaluated as mentioned in Section 2.3.

2.7. Association of influenza hemagglutinin to the protamine nanocapsules and determination of its structural integrity

The 6xHis tagged globular domain of the hemagglutinin from the A/PR/8/34 influenza virus (aa 18–529) fused to the endoplasmic reticulum retention amino-acid sequence KDEL was expressed by a baculovirus

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