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Biodistribution and lymph node retention of polysaccharide-based immunostimulating nanocapsules

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ABSTRACT

The adjuvant properties of polyglucosamine/squalene-based nanocapsules (PG-nanocapsules) associated with different subunit antigens has been previously reported. Thus, the aim of the present study was to monitor the biodistribution of PG-nanocapsules and their affinity for the draining lymph nodes after subcutaneous (s.c.) injection. The nanocapsules were efficiently radiolabeled with indium-111 (^{111}In) (labeling efficiency of 98%). The diameter and zeta potential values of the unlabeled nanocapsules was preserved after the radiolabeling process and only 20% of the ^{111}In dissociated from the nanocapsules after 48 h of incubation in serum. The radiolabeled nanocapsules and the control $^{111}InCl_3$ in saline solution (18.5 MBq (500 μCi) in 100 μL) were injected s.c. in New Zealand White rabbits. The γ-scintigraphy imaging analysis revealed a slow clearance of the nanocapsules from the injection site and their progressive accumulation in the popliteal lymph node over time (3.8% ± 1.2 of the injected dose at 48 h). Indeed, the clearance rate of the nanocapsules from the injection site was significantly slower than that of the control (free $^{111}InCl_3$), which rapidly drained into systemic circulation and accumulated mainly in excretion organs (*i.e.* kidneys and liver). In contrast, the biodistribution of nanocapsules was preferably limited to the lymphatic circulation. These results suggest that the immune potentiating effect previously observed for PG-nanocapsules is mainly due to the formation of a depot at the injection site, which was followed by a slow drainage into the lymphatic system and a prolonged retention in the lymph nodes.
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1. Introduction

The development of polymeric nanocarriers has helped to overcome biopharmaceutical hurdles associated with novel therapeutic macromolecules, such as inadequate biodistribution, short half-life and limited bioavailability upon administration through non-invasive routes [1]. For vaccine development, nanotechnology has emerged as a new approach for the rational design of vaccines [2,3]. Overall, specific advantages of nanovaccines include (i) facilitated interaction with immune cells, thus improving and modulating antigen-specific immune responses (adjuvant effect); (ii) ability to overcome mucosal barriers in order to generate both, systemic and mucosal immune responses (needle-free vaccination) [4–6].

Previously, we described an adjuvant system based on polysaccharide/lipid (shell/core) nanocapsules [7,8]. The purpose of designing these core–shell nanocapsules for vaccine delivery has been to rationally modify the squalene-based emulsions in order to incorporate the different components (oil, surfactants and antigen) within the same nanocarrier, thanks to the polymeric coating of polyglucosamine (PG). This multifunctional structure enabled the association of antigens onto their cationic surface and their improved presentation to the immune system, whereas the squalene core may confer the immunostimulant properties to the system. Thus, it could be possible for a concomitant delivery of both

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the antigens and immunoacting molecules to antigen presenting cells (APCs), with the final goal of promoting and modulating the immune response.

Recently, these nanocapsules composed of PG and squalene (shell–core, respectively) were developed and their efficacy for the delivery of the recombinant hepatitis B surface antigen (rHBsAg) and the hemagglutinin of the influenza virus (HA) was shown [9]. Moreover, in the case of rHBsAg, PG-nanocapsules were capable of eliciting a protective and sustained specific immune response after a single immunization [9].

In general it is accepted that vaccine delivery systems (liposomes, microspheres, nanoparticles, emulsions) are recognized by antigen presenting cells (APCs) and promote the uptake of the associated antigen [10]. However, their migration from the injection site toward the lymph nodes has been much less explored, with the drainage of the injected vaccine into downstream lymph nodes potentially being a crucial step in the initiation of the adaptive immune response.

Therefore, the overall goal of this study was to provide insights on the adjuvant mechanisms of PG-nanocapsules. Taking this goal into account, we studied the biodistribution of PG-nanocapsules after s.c. injection and their lymphatic migration and retention in draining lymph nodes. For this purpose, we have used sensitive scintigraphic imaging techniques, which can provide additional quantitative information about *in vivo* fate of PG-nanocapsules in real time, permitting correlation with the immune responses previously observed.

2. Materials and methods

2.1. Materials

2.1.1. Polymers and chemicals

Ultrapure highly deacetylated chitosan in base form (Ultrasan, Mw 276 kDa and deacetylation degree 95.5%) was acquired from Bio Syntech Canada Inc. (Quebec, Canada) and further named as polyglucosamine (PG). The emulsifier soybean L-α-lecithin Epikuron 145 V was a gift from Cargill (Barcelona, Spain). Squalene oil (density 0.855 g/mL) was obtained from Merck (Darmstadt, Germany). 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-diethylenetriaminepentaacetic acid (14:0 DTPA-PE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Indium-111 chloride ($^{111}InCl_3$) was obtained from Nordion (Ottawa, ON, Canada) and Technetium-99m (^{99m}Tc or Tc-99m) as sodium pertechnetate ($^{99m}TcO_4Na$) was acquired at the local radiopharmacy (GE Healthcare San Antonio, TX). The lipophilic chelator hexamethylpropyleneamine oxime (HMPAO) was provided as the commercial kit Ceretec™ (GE Healthcare; Little Chalfont, UK). HPLC grade solvents (2-propanol, chloroform and acetone) were purchased from Fisher Scientific (Pittsburg, PA).

2.2. Preparation and radiolabeling of polyglucosamine nanocapsules (PG-nanocapsules)

PG-nanocapsules were prepared by the solvent displacement technique, as previously described [9] with slight modifications. Briefly, lecithin (10 mg) and squalene (25 μL) were co-dissolved in 0.5 mL of 2-propanol. This organic phase was poured under constant magnetic stirring into an aqueous solution of PG (0.25 mg/mL in acetic acid solution 0.1%), resulting in the spontaneous formation of PG-nanocapsules. The organic solvents were removed under reduced pressure using a rotary evaporator (Büchi; Switzerland) and the final suspension volume was corrected to 5 mL using water (pH 5.5).

PG-nanocapsules were radiolabeled following two different protocols:

2.2.1. Labeling with Tc-99m

Ceretec™ vial was reconstituted following manufacturer's instructions. Briefly, 4 mL of sodium pertechnetate ($^{99m}TcO^-_4$) (592 MBq/16 mCi) were injected into the vial and incubated for 5 min in order to form the chelation complex ^{99m}Tc–HMPAO. Aliquots (50, 100 or 200 μL) of ^{99m}Tc–HMPAO were immediately added to the acetone phase during the preparation of PG-nanocapsules in order to incorporate it within the oily core. Then, the procedure was followed as described above. The radionuclide was thus incorporated within the oily core (Fig. 1A).

Quality control procedures were performed by paper chromatography as described in the Ceretec™ package insert to monitor formation of the ^{99m}Tc–HMPAO complex immediately after preparation and once mixed with the organic phase.

2.2.2. Labeling with In-111

DTPA-conjugated phosphoethanolamine (DTPA–PE) was dissolved in a mixture of 2-propanol:chloroform (60/30 v/v) at 10 mg/mL. This solution was incubated with 1 or 2 μL of $^{111}InCl_3$ containing 7.77 or 189 MBq (0.21 or 5.11 mCi for *in vitro* or animal experiments, respectively) for 30 min. Then, 100 μL of ^{111}In–DTPA–PE was incorporated in the organic phase with lecithin and squalene, and the preparation procedure for PG-nanocapsules was followed as described above. The radionuclide was thus incorporated in the phospholipid phase (Fig. 1B).

2.3. Physicochemical characterization

Diameter and polydispersity index were measured by photon correlation spectroscopy (PCS) using a ZetaPlus/90Plus (Brookhaven Instruments Corp.; Holtsville, NY). Nanocapsules were diluted in filtered water and analyzed at 25 °C with a detection angle of 90°. ζ potential measurements were performed by laser doppler anemometry (LDA) using the same equipment. Samples were diluted in an aqueous solution of KCl 10^{-3} M.

2.4. Radiolabeling efficiency

PG-nanocapsules radiolabeled with either ^{99m}Tc or ^{111}In were centrifuged at 21,460 × *g* for 90 min at 4 °C (Allegra 21R, Beckman-Coulter; Brea, CA). Supernatant was collected and filtered using Vivaspin 500 MWCO 1,000,000 (Sartorious; Goettingen, Germany) in order to remove any remaining nanocapsules. The activity of each filtered fraction, filter and isolated radiolabeled PG-nanocapsules, was measured using a gamma-counter (Packard). The labeling efficiency was calculated as follows: ([activity of isolated PG-nanocapsules] + [activity of filters])/total activity.

2.5. Assessment of the radiolabeling stability

PG-nanocapsules radiolabeled with either ^{99m}Tc or ^{111}In were incubated with fetal bovine serum (FBS) in a proportion 1/1 (v/v) at 37 °C. At indicated time points (baseline, 0.5, 1, 2, 4, 24 and 48 h) samples were withdrawn and centrifuged in order to separate the free radionuclide. Radioactivity of both separated fractions was counted using a gamma-counter.

In a different experiment one sample of ^{99m}Tc– or ^{111}In–PG-nanocapsules was incubated with FBS (1/1) at 37 °C for 30 min. The sample was centrifuged and the supernatant collected in order to quantify the free radionuclide. Isolated nanocapsules were then resuspended with the same volume of FBS and incubated for