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Solid sorbitan esters nanoparticles are efficient and low-cost vehicles for subunit vaccines: Proof of concept with *Neisseria meningitidis* protein Mip

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ABSTRACT

In this study we present evidence of the vaccine potential of nanoparticles based on sorbitan esters (sorbitan monooleate, Span® 80), solid sorbitan esters nanoparticles (SSN) which are the lowest cost antigen nanovehicles proposed to date. We choose the *Neisseria meningitidis* macrophage infectivity potentiator (Mip) protein as a proof of concept target. The rMip-SSN nanosystems were evaluated for their physico-chemical properties, antigen loading and integrity as well as physical stability in various storage conditions. The immune response elicited by rMip-SSN was compared to that elicited by rMip-SSN with the conventional adjuvant $Al(OH)_3$ or with rMip in saline. SSN were able to associate, at a wide range of antigen concentrations with 100% efficiency, preserving the association and integrity of the antigen after long-term storage. Antibodies elicited by rMip-SSN mediated deposition of complement factors on the surface of meningococcal isolates and protective bactericidal titres even after rMip-SSN had been stored for nine months lyophilized at 4 °C. The association of different antigens to SSN can be easily achieved by electrostatic interactions by mixing the preformed nanosystems with the antigens of choice. Thus we can envisage the use of these nanoplatforms for the development of low-cost immunization kits ready for use for flexible vaccination strategies.

1. Introduction

Vaccination is one of the most successful tools for protecting the public's health from infectious diseases. Despite the success of vaccination, new safe strategies are required to improve the immunogenicity of vaccine antigens. In addition, greater stability without the need for cold chain delivery would be a great advantage. Current vaccine developments are mainly focused on isolated components, but these tend to be poorly immunogenic and thus need an appropriate delivery vehicle and/or adjuvant. Nanoparticles, in addition to improving antigen stability, have been proposed to act as either a delivery system increasing the efficiency of uptake by antigen presenting cells (APCs), or as immunostimulators [1] enhancing the immune response and promoting cell-mediated immunity [2]. The great challenge in nanovaccine development is the development of novel vaccines with reduced production cost and greater stability, even outside of the cold chain. Although many different nanosystems have been tested as vaccine vehicles, safety concerns, sophisticated production requirements at industrial scale and stability limitations of the final formulations have hampered the regulatory approval of nanovaccines [3]. In

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addition, most nanosystems proposed to date as antigen vehicles will dramatically increase vaccine cost, such that manufacturers cannot offer the vaccines at affordable prices. Thus to produce low-cost vaccines offering a great potential for high volume production we have recently described nanoparticles based on the cheapest available technology and components currently used in nanotechnology for vaccine development [4]. These nanoparticles can be tailored specifically in order to modulate relevant parameters affecting the association of different compounds, antigens and bioactive molecules, as well as *in vivo* performance, such as surface charge and composition. The production method of these nanoparticles is simple, takes place in one step and is easily scalable. We refer to these nanosystems as solid sorbitan esters nanoparticles (SSN) because they are based on sorbitan monooleate (Span® 80) and are characterized by a solid structure [5,6]. To evaluate the potential of these nanoplatforms we have selected *Neisseria meningitidis* protein macrophage infectivity potentiator (Mip) as a first proof of concept antigen for our nanovaccine design.

Vaccines containing either outer membrane vesicles (OMVs) in combination with recombinant antigens [7–9] or recombinant factor H alone [10,11] are the leading strategies to prevent serogroup B *N. meningitidis* disease. However, the recombinant antigens are poorly immunogenic requiring very large doses (~50μg) which are 10-fold greater than doses of native vaccine antigens such as those used in acellular pertussis vaccines [12]. These vaccines have further important limitations, such as their variable composition, low stability [13] and strain-specific protection [14]. The development of novel meningococcal vaccines has led to the search for new minor antigens to produce subunit vaccines. Recently, the meningococcal Mip, has been reported as a promising vaccine candidate [15,16]. The Mip family includes proteins with peptidyl-prolyl cis/trans isomerase (PPIase) enzymatic activity. These proteins seem to be involved in the initiation of the infection of macrophages and they have been described as virulence factors in different pathogens [17]. The *N. meningitidis* Mip is highly conserved with only three significantly different alleles and induces a cross-bactericidal activity against all of them [15,16]. Also, previous studies showed cross-reactive opsonophagocytosis and complement deposition activity using this antigen [18]. In addition, studies in our laboratory have shown that when the recombinant Mip was incorporated into liposomes this decreased the opsonophagocytosis and complement deposition antibody activity that was observed (unpublished data). Liposomes are a versatile vehicle especially useful for proteins which need to be presented in their native conformation, since these proteins are refolded into the lipid bilayer [19]. However, the immune properties of Mip appear to be independent of native conformation because bactericidal activity is induced when it is denatured in saline solution. Also, to preserve their stability, liposomes incorporate a low concentration of antigen so a larger volume is needed for immunization.

Thus we have developed new immunogenic compositions based on SSN bound to recombinant Mip (rMip) as an immunogenic, stable, scalable, with well-defined composition and inexpensive vaccine with appropriate antigen presentation to the immune system which can be stored as a lyophilized product.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The Mip protein from *N. meningitidis* H44/76 was cloned using pTrcHis2 TOPO expression vector (Invitrogen, USA). OneShot® BL21 (DE3) *E. coli* was used for the recombinant Mip expression. The plasmid also contains a gene for resistance to ampicillin and a His-tag. This *E. coli* clone was grown in YTx2 broth containing 50 μg/ml ampicillin.

Characteristics of the *N. meningitidis* strains used for the immunological assays were previously reported [20]. Strains H44/76, 3061 and 3063 were kindly provided by Dr. Ian Feavers (National Institute for Biological Standards and Control, UK). Strain NZ98/254 was kindly donated by Dr. D. Martin (Institute of Environmental Science and Research, New Zealand). Strains Nm26 and NmP27 were isolated from a patient and a carrier, respectively, in Galicia, Spain.

For production of OMVs *N. meningitidis* strains were grown at 37 °C and 180 rpm under iron restriction in Mueller–Hinton broth with 100 μM Desferal added (MH-Desferal) and were recovered during the late exponential growth phase (12 h). Meningococcal OMVs were obtained by French Press using a modification of a previously described protocol [21]. For bactericidal assays meningococcal strains were grown in Mueller Hinton broth with 50 μM Desferal.

Bacteria used for flow cytometry assays were killed using a protocol designed to minimize chemical alteration of surface epitopes. Briefly, live meningococci were incubated with 0.2% (w/v) sodium azide and 17 μg/mL of phenylmethylsulphonyl fluoride for 48 h at 37 °C.

2.2. Expression and purification of recombinant Mip

Expression and purification of rMip was performed using a using a modification of the method described by Sambrook *et al* [22]. Briefly, the *E. coli* clone containing the expression vector was grown for 24 h at 37 °C on 2xYT agar plates containing 50 μg/mL ampicillin. Colonies were then inoculated into tubes containing 10 mL of 2xYT broth with ampicillin, grown for 18 h at 37 °C with 180 rpm constant shaking, transferred to flasks with 500 mL of the same medium, and incubated for 1 h at 37 °C. Expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, USA) to a final concentration of 0.5 mM. Cells were recovered by centrifugation, resuspended in 8 M urea, 0.1 M NaH2PO4, 10 mM Tris-HCL, and 20 mM imidazol, pH 8, and sonicated. Insoluble material was removed by centrifugation at 20,000 × g for 1 h at 4 °C and the protein was purified using a 5 ml nickelnitrilotriacetic acid column (Ni-NTA; HisTrapTM FF, GE Healthcare). Eluted protein was washed and concentrated in ultrapure water using Amicon Ultra-15 centrifugal Filters (Merck Millipore Ltd., IRL). Protein identity was confirmed by mass spectrometry and protein concentration was quantified using the bicinchoninic acid (BCA) assay (Pierce, UK) and the protein was characterized by SDS-PAGE.

2.3. Preparation of rMip-SSN system

Sorbitan monooleate nanoparticles were produced as previously reported [5,6]. Briefly, a solution of 6.6 mg/ml of sorbitan monooleate (Span® 80, Sigma, Spain), in 30 ml of ethanol (organic phase) was prepared, and it was subsequently added under magnetic stirring to a 60 ml aqueous phase, thereby leading to spontaneous nanoparticle formation. A positive surface charge, necessary for the association of the rMip to the nanoparticles, was provided by dissolving oleylamine (OA) (Sigma, Spain) in the organic phase at a concentration of 0.33 mg/ml. The ethanol was subsequently removed under reduced pressure on a rotary evaporator, and the SSN were concentrated to a final volume of 10 ml.

The negatively charged rMip was associated to the SSN surface at different concentrations (from 0.1 to 3 mg/ml) by incubation with the nanoparticles at a 1/1 (v/v) ratio for 1 h at room temperature under magnetic stirring.

2.4. Characterization of rMip-SSN system

The association of the rMip to the nanoparticles was measured

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