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Research Paper

Protamine-based nanoparticles as new antigen delivery systems

José Vicente González-Aramundiz [a,c], Mercedes Peleteiro Olmedo [b], África González-Fernández [b], María José Alonso Fernández [a], Noemi Stefánia Csaba [a,*]

[a] Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), Health Research Institute of Santiago de Compostela (IDIS), Dept. of Pharmacy and Pharmaceutical Technology, School of Pharmacy, Univ. of Santiago de Compostela, Santiago de Compostela, Spain
[b] Immunology, Biomedical Research Center (CINBIO) and Institute of Biomedical Research of Vigo (IBIV), Universidad de Vigo, Campus Lagoas Marcosende, Vigo, Pontevedra, Spain
[c] Departamento de Farmacia, Facultad de Química, Pontificia Universidad Católica de Chile, Santiago, Chile

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ABSTRACT

The use of biodegradable nanoparticles as antigen delivery vehicles is an attractive approach to overcome the problems associated with the use of Alum-based classical adjuvants. Herein we report, the design and development of protamine-based nanoparticles as novel antigen delivery systems, using recombinant hepatitis B surface antigen as a model viral antigen.

The nanoparticles, composed of protamine and a polysaccharide (hyaluronic acid or alginate), were obtained using a mild ionic cross-linking technique. The size and surface charge of the nanoparticles could be modulated by adjusting the ratio of the components. Prototypes with optimal physicochemical characteristics and satisfactory colloidal stability were selected for the assessment of their antigen loading capacity, antigen stability during storage and *in vitro* and *in vivo* proof-of-concept studies. *In vitro* studies showed that antigen-loaded nanoparticles induced the secretion of cytokines by macrophages more efficiently than the antigen in solution, thus indicating a potential adjuvant effect of the nanoparticles. Finally, *in vivo* studies showed the capacity of these systems to trigger efficient immune responses against the hepatitis B antigen following intramuscular administration, suggesting the potential interest of protamine–polysaccharide nanoparticles as antigen delivery systems.

1. Introduction

Since their implementation in 1926, aluminum salts have had a crucial role in making vaccination the most important human intervention in the improvement of global health. But despite its great value, alum has important shortcomings, such as (i) insufficient adjuvant effect for subunit vaccines and peptides, (ii) lack of effect when administered via non-parenteral routes, (iii) unwanted side effects, i.e. local reactions or hypersensitization in allergic patients and (iv) limited thermostability [1,2]. Due to these limitations, significant efforts have been made to develop alternative adjuvants.

In a broad sense, an adjuvant could be defined as a molecule or structure that can increase and/or modulate the immunogenicity of an antigen, allowing it to induce a potent and persistent immune response at low doses [3]. Some authors divided the adjuvants into two groups: immunostimulants (adjuvants that interact with specific receptors of antigen presenting cells) and delivery systems [4]. Particulate delivery systems may act as adjuvants because they can modify the uptake, trafficking and processing of antigens, which results in better and more adequate immune responses [5,6].

In the design of novel "alum-free" vaccine particulate delivery systems, it is necessary to consider the nature of the antigen and its intrinsic immunogenicity, the administration route and the availability of biomaterials with an adequate safety profile. On the other hand, optimal particle size in antigen delivery still remains a controversial issue [4]. Nanometric systems have raised hope for better adjuvants because of their ability to control the release and increase the trans-epithelial transport of antigens, thus being considered as promising strategies for the development of single dose and needle-free vaccines [7,8].

Different materials have been studied in order to develop particulate antigen delivery systems, lipids, polymers and

* Corresponding author. Tel.: +34 881815450.
E-mail addresses: jvgonzal@uc.cl (J.V. González-Aramundiz), mercepool@gmail.com (M. Peleteiro Olmedo), africa@uvigo.es (Á. González-Fernández), mariaj.alonso@usc.es (M.J. Alonso Fernández), noemi.csaba@usc.es (N.S. Csaba).

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polysaccharides among them. In particular, chitosan, polylactic and polylactic-co-glycolic acid have been the polymers most widely applied in the development of vaccine nanocarrier [9,10]. More recently, polyaminoacids and polypeptides have been considered for their versatility and biocompatibility [11]. In particular, protamine, an arginine-rich peptide with cell-penetrating properties, has shown a synergistic adjuvant effect with other immunomodulatory molecules, i.e. CpG [12]. This effect was also observed when protamine was used in combination with PLGA microparticles. The resulting system was able to stimulate the proliferation of antigen-specific T cells and the secretion of IFN-γ [13]. So far, this polypeptide has been used as biomaterial for antigen delivery and immunomodulation in microparticles [14], DNA-loaded liposomes (LPD) [15] and complexes [16]. In addition to antigen delivery, protamine has also been combined with other biopolymers such as modified polyacrylic acids for mucosal delivery [17,18].

Other materials, such as hyaluronic acid (HA) and alginate (ALG) have also shown immunoadjuvant activity, as indicated by macrophage and dendritic cell recruitment plus activation and/or induction of cytokine production [19–22].

Based on this information, the aim of this work was to develop and characterize a new protamine based nanometric antigen delivery system to harness its adjuvant properties in association with a polysaccharide such as alginate or hyaluronic acid. The combination of these biomaterials, in a nanoparticulated form, generates new nanocarriers with safer materials and great adjuvant activity.

The ability of these systems to encapsulate and deliver antigens was assessed using recombinant hepatitis B surface antigen (rHBsAg) as a model antigen. Finally, the *in vitro* and *in vivo* performance of this novel delivery approach was assessed in macrophage cell cultures and upon administration to mice either by intramuscular or by nasal administration.

2. Materials and methods

2.1. Chemicals

Protamine sulfate was purchased from Yuki Gosei Kogyo, Ltd. (Japan) and Hyaluronic acid (HA) of 162 kDa and 29 kDa was provided by Bioiberica (Spain) and by Soliance (France), respectively. Sodium alginate (ALG) (PRONOVA UP VLVG) of <75 kDa was supplied by Novamatrix (Norway). Recombinant hepatitis B surface antigen (rHBsAg) was kindly donated by Shantha Biotechnics Limited (Hyderabad, India). Enzyme linked immunosorbent assay (ELISA) kit (Murex rHBsAg Version 3) was obtained from Diasorin (United Kingdom). Chicken polyclonal antibody to hepatitis B virus surface antigen and rabbit polyclonal antibody to chicken conjugated with horseradish peroxidase were purchased from Abcam pcl (United Kingdom). Rabbit IgG and mouse monoclonal IgG used as controls were purchased from Biokit (Spain) and Acris Antibodies GmbH (Germany), respectively. Secondary Abs conjugated to horseradish peroxidase were from Southern Biotech (USA). 5-TAMRA, 5-Carboxytetramethylrhodamine, Succinimidyl Ester (single isomer) and Alexa Fluor 488-phalloidin were obtained from Invitrogen (United Kingdom). Triton X-100, glucose, trehalose, PBS, hyaluronidase, heparin and aluminum hydroxide gel were obtained from Sigma–Aldrich (Spain). All other chemicals used were of reagent grade or higher purity.

2.2. Preparation of protamine:polysaccharide nanoparticles and loading of rHBsAg

Nanoparticles were prepared by an ionic cross-linking technique as described by Calvo et al. [23]. Briefly, 1 ml of protamine and either hyaluronic acid or alginate was kept under magnetic stirring at a concentration of 1 mg/ml. The counter ion (0.4 ml) was also dissolved in purified water at different concentrations (0.417; 0.5; 0.625; 0.833; 1.25 and 2.5 mg/ml) and particles with protamine:polysaccharide ratios ranging from 1:6 to 6:1 (w/w) were prepared upon mixing; corresponding to 0.83; 0.85; 0.89; 0.95; 1.07 and 1.43 mg/ml of final nanoparticle concentrations. Nanoparticles were instantaneously obtained upon interaction of protamine with the polysaccharide. For the encapsulation of rHBsAg, the antigen was mixed with the polysaccharide at a theoretical loading of 2.5 and 5% (with respect to the total amount of polymers), prior to the production of nanoparticles.

2.3. Characterization and stability of nanoparticles

2.3.1. Size and zeta potential

The hydrodynamic diameter and polydispersity index of the particles were determined by photon correlation spectroscopy (PCS) after dilution of the samples in ultrapure water. The zeta potential was measured by laser-Doppler anemometry diluting the samples with KCl 1 mM (Zetasizer®, NanoZS, Malvern Instruments, Malvern, UK).

2.3.2. Morphological analysis

The morphological examination of the nanoparticles was carried out by transmission electron microscopy (TEM, CM12 Philips, the Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid solution and dried on a copper grid.

2.3.3. Production yield and nanoparticle composition

Nanoparticles were isolated by centrifugation for 40 min, at 18000*g* and 15 °C (Universal 32R, Hettich Zentrifugen, Germany) and the supernatants were discarded. The nanoparticle pellets were freeze-dried (Genesis SQ freeze drier, Virtis, US) and weighed. The production yield was calculated as follows (Eq. (1)):

$$\text{Production Yield } (\%) = \frac{\text{Nanoparticle weight}}{\text{Theoretical weight (total solids)}} \times 100 \quad (1)$$

In order to determine the incorporation rate of the different components, nanoparticle samples (non-isolated and isolated nanoparticles) and the raw materials (hyaluronic acid and protamine) were freeze-dried and analyzed by elemental analysis (FISONS EA 1108, United Kingdom). Protamine and hyaluronic acid content in the nanoparticles samples was analyzed by a mathematical model, comparing the nitrogen and carbon content of the nanoparticles with that of protamine and hyaluronic acid as raw materials. As additional controls, nanoparticle supernatants (product of the isolation process) and physical mixtures of the components (dry mixing) were also analyzed by the same method.

2.3.4. Colloidal stability of the nanoparticles

The stability of non-isolated blank nanoparticles was evaluated in an aqueous suspension under storage conditions (4 °C) by measuring nanoparticle size and zeta potential at different time points (1, 7, 14, 21, 30, 60 and 90 days). Particle stability was also evaluated in PBS and cell culture medium (RPMI supplemented with 10% FBS) at 37 °C for 48 h by monitoring the evolution of particle size at different time points (0, 15, 30, 45 min and 1, 2, 6, 24 and 48 h).

2.4. Association efficiency and structural integrity of rHBsAg

Nanoparticles were loaded with the viral antigen rHBsAg as detailed in Section 2.2. The efficiency of the antigen association was determined indirectly through calculation of the difference between the total amount of antigen added to the nanoparticle