International Journal of Pharmaceutics 527 (2017) 103–114

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Association of chitosan and aluminium as a new adjuvant strategy for improved vaccination

F. Lebre[a,b], D. Bento[a,b], J. Ribeiro[d], M. Colaço[a,b], G. Borchard[c], M.C. Pedroso de Lima[a], O. Borges[a,b,*]

[a] CNC–Center for Neuroscience and Cell Biology, University of Coimbra, 3004-0504, Coimbra, Portugal
[b] University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548, Coimbra, Portugal
[c] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Quai Ernest Ansermet 30, 1211, Geneva, Switzerland
[d] ADAI-LAETA, Department of Mechanical Engineering, University of Coimbra, Portugal

ARTICLE INFO

*Article history:*
Received 25 March 2017
Received in revised form 11 May 2017
Accepted 12 May 2017
Available online 15 May 2017

*Keywords:*
Chitosan nanoparticles
Aluminium
Delivery system
Hepatitis B antigen

ABSTRACT

The use of particulate adjuvants offers an interesting possibility to enhance and modulate the immune responses elicited by vaccines. Aluminium salts have been extensively used as vaccine adjuvants, but they lack the capacity to induce a strong cellular and mucosal immune response. Taking this into consideration, in this study we designed a new antigen delivery system combining aluminium salts with chitosan. Chitosan-aluminium nanoparticles (CH-Al NPs) exhibited a mean diameter of 280 nm and a positive surface charge. The newly developed CH-Al NPs are more stable at physiological environment than classical CH NPs, showing no cytotoxic effects and revealing potential as a delivery system for a wide range of model antigens. *In vivo* studies showed that mice immunized with hepatitis B surface antigen (HBsAg)-containing CH NPs display high anti-HBsAg IgG titers in the serum, as well as the highest antigen-specific IgG on vaginal washes. Furthermore, in contrast to mice receiving antigen alone, mice immunized with the particulate adjuvant were able to elicit IgG2c antibody titers and exhibited higher antigen-specific IFN-γ levels in splenocytes. In conclusion, we established that CH-Al NPs, combining two immunostimulants to enhance both humoral and cellular immune responses, are a safe and promising system for antigen delivery. Our findings point towards their potential in future vaccination approaches.
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## 1. Introduction

The use of vaccine adjuvants provides an interesting approach to modulate the type and magnitude of immune responses. Aluminium salts, often designated as alum, have been widely used as adjuvants in licensed vaccine due to their immunomodulatory properties (Kool et al., 2012; Marrack et al., 2009; Oleszycka and Lavelle, 2014). Even though they were first introduced to the market in the 1930s, the mechanism of adjuvanticity of aluminium salts is still unclear. Originally it was suggested that alum would allow a sustained released of the antigen, resulting in a prolonged and effective stimulation of the immune system. However, this so-called "depot-effect" (Glenny et al., 1926) was later challenged (Holt, 1950; Hutchison et al., 2012). Indeed, Hutchison et al. reported that, 2 h after immunization, excision of the depot site did not alter the humoral response elicited by alum, thus suggesting that this was dispensable for adjuvanticity. In addition to the depot theory, several other mechanisms of alum action have been proposed, namely the controversial requirement of the NLRP3 inflammasome activation and the downstream effects elicited on the adjuvant effect of alum *in vitro* and *in vivo* (Eisenbarth et al., 2008; Franchi and Nunez, 2008; Li et al., 2008; Oleszycka et al., 2016). More recently, aluminium salts have been shown to induce cell death at the injection site leading to the release of host DNA, which would act as a danger associated molecular pattern (DAMP), boosting the immune response (Marichal et al., 2011). Aluminium-containing adjuvants are known to induce a strong humoral response, mainly characterized by the secretion of antigen-specific antibodies; however they lack the capability to induce a Th1-type response, which is important for protection against diseases that require strong cell-mediated immune response (e.g. HIV, tuberculosis, malaria) (Marrack et al., 2009; Oleszycka and Lavelle, 2014). Therefore, an attractive approach to solve this issue would be to combine aluminium salts with a Th1-biased immunostimulant. Previous studies have demonstrated that chitosan has the ability to

* Corresponding author at: Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548, Coimbra, Portugal.
*E-mail address:* olga@ci.uc.pt (O. Borges).

http://dx.doi.org/10.1016/j.ijpharm.2017.05.028
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induce dendritic cell (DC) maturation, antigen-specific Th1 responses and, in contrast to other adjuvants, it does not promote inhibition of the Th1 cell-polarizing cytokine IL-12 (Carroll et al., 2016; Mori et al., 2012). This makes chitosan an ideal candidate to combine with aluminium salts towards the generation of effective parenteral as well as mucosal vaccines.

Chitosan is a natural cationic polymer of β-(1–4)-linked glucosamine and N-acetyl-glucosamine obtained after the alkaline deacetylation of chitin, which is naturally present in the exoskeletons of crustacean and fungal cell walls. Chitosan is a very versatile molecule and, depending on the deacetylation process, the resulting polymer varies in molecular weight and degree of deacetylation, which has a strong impact on its physicochemical and biological properties (Huang et al., 2005; Kiang et al., 2004; Zhou et al., 2008). In addition to its common use as a drug and gene delivery system, chitosan has a number of other promising applications in the biomedical field, in particular, as a vaccine adjuvant (Arca et al., 2009; Hu et al., 2013; Lebre et al., 2016a), due to its safety profile, biodegradability, mucoadhesive properties and immunostimulatory potential (Baldrick, 2010; Borges et al., 2007; Carroll et al., 2016; Illum, 1998). Moreover, by making use of simple and mild processes (e.g. ionotropic gelation, precipitation/coacervation), it is possible to produce particles with controlled physicochemical characteristics.

The use of particles as vaccine adjuvants offers several advantages, including enhanced uptake by antigen presenting cells (APCs), protection of antigen from enzymatic degradation, promotion of a depot effect with gradual release of the antigen and the ability to facilitate antigen cross-presentation and co-delivery of antigens and immunomodulators to the same cell population (Schijns and Lavelle, 2011; Smith et al., 2013). When developing a new particulate adjuvant, physicochemical characteristics such as size, surface charge/chemistry and shape are known to play an important role in the way the body interacts and responds to it (Hotaling et al., 2015; Lebre et al., 2016b; Oyewumi et al., 2010). For example, Lunov et al. showed that amino-functionalized, but not carboxy or non-functionalized polystyrene particles with a comparable mean diameter of 110 nm, activate the NLRP3 inflammasome in macrophages through the lysosomal rupture model (Lunov et al., 2011). Moreover, while there is still a great deal of debate on whether small or large particles are more effective in inducing specific immune response, particles with a size within the viral range (<500 nm) are known to be more extensively internalized by APCs, drain better through the lymphatics and activate the inflammasome, which can be beneficial for the induction of cell mediated immunity (Hotaling et al., 2015). Kobiasi et al. addressed the relationship between chitosan particle size and dispersity in *in vivo* cell trafficking and uptake and found that 150 nm chitosan nanoparticles drained approximately 50 times faster to the lymph nodes after 24 h than their 1.3 μm microparticle counterparts (Al Kobiasi et al., 2012).

The main aim of this study was first to develop a particulate adjuvant that combines chitosan and aluminium in the same particle and second to investigate whether the combination of the recognized immunostimulatory properties of aluminium salts and chitosan would result in enhanced adjuvant effects, enabling a mixed Th1/Th2-type of immune response, while additionally facilitating the antigen draining to local lymph nodes. For this purpose, we optimized the production of chitosan nanoparticles using aluminium sulfate in place of the conventionally used sodium sulfate as a cross-linking agent for chitosan. Remarkably, chitosan particles prepared with aluminium salts demonstrated to be much more stable than traditional particles, critical for formulation development. We then evaluated the cytotoxicity of chitosan-aluminium nanoparticles in 3 distinct cell cultures and then investigated their capacity to be internalized and to act as a delivery system for 6 different model antigens. Finally, we tested the ability of CH-Al NPs to modulate the immune response following subcutaneous immunization using the recombinant hepatitis B surface antigen (HBsAg). Collectively, our data show that CH-Al NPs can be used as adjuvant towards effective vaccination, combining their stability and ability to mediate delivery of various model antigens with the immunomodulatory properties of aluminium salts and chitosan.

## 2. Experimental section

### *2.1. Chitosan nanoparticle optimization and characterization*

Chitosan (CH) (ChitoClear, 95% degree of deacetylation; Primex Bio-Chemicals AS) was purified using a previously described method, with some modifications (Gan and Wang, 2007; Lebre et al., 2016a). To optimize the preparation method of the CH-Al NPs, nine CH/Al ratios were tested. Therefore, chitosan-aluminium nanoparticles (CH-Al NPs) were prepared by adding equal volumes of 0.05–0.15% CH (in 25 mM sodium acetate buffer (AcB)) solution and 0.25–1.50% aluminium sulfate (Al) aqueous solution, under high-speed vortexing for 20 s, followed by incubation at room temperature for 1 h. For the preparation of chitosan nanoparticles (CH-Na NPs), equal volumes of a chitosan solution (0.1% in 25 mM AcB, pH 5.0) and a sodium sulfate solution (0.625%) were mixed under high-speed vortexing for 20 s. The resulting nanoparticle suspensions were centrifuged for 30 min at 4500*g*, the supernatants were discarded and the pellet resuspended in 25 mM AcB, pH 5.5.

Size and zeta potential of CH NPs were measured by dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively, in a Delsa Nano C (Beckman Coulter, USA). The analysis was performed at 25 °C, in 25 mM AcB, pH 5.5.

The presence of aluminium in the particles was evaluated by energy-dispersive X-ray spectroscopy (EDS) analysis using a high resolution FEI Quanta 400 FEG E Scanning Electron Microscope coupled to an EDAX Genesis X4M X-Ray Energy Dispersive Electron Spectrometer (EDAX Inc., USA). Aluminium quantification was performed indirectly, by determining the amount that remained free in the particle supernatants, using the eriochrome cyanine R method, after minor modifications (Clesceri et al., 1998). Briefly, the volumes described on protocol were reduced allowing the reaction to be performed with 90% less sample.

Particle morphology was evaluated by scanning electron microscopy (SEM) (JSM-700 1FA, JEOL, Japan). Prior to image acquisition, one drop of nanoparticle suspension was placed over a copper surface and let to dry overnight. Afterward, samples were mounted on microscope stub, coated with gold, and then observed under the microscope.

### *2.2. Stability studies*

To assess the physical integrity of the NPs, 2 mL of nanoparticle suspensions were centrifuged for 30 min at 4500*g*, the supernatants were discarded and the pellet resuspended in 1 mL of either PBS, pH 7.4; 200 mM phosphate buffer (PB), pH 7.4; 10 mM HEPES, pH 7.4 or 25 mM AcB, pH 5.5, at 20 °C or 37 °C. Transmittance at 500 nm of the resulting suspension was measured straight after, in a spectrophotometer (UV-1601, Shimadzu Corporation, Japan).

The thermal degradation process and the stability of the nanoparticles were investigated. The CH-Al NPs and CH-Na NPs curves were obtained followed by thermogravimetric analysis (TGA) and differential thermal analysis (DTA) (DTA/TGA – Scientific Rheometrics 1500, UK). Approximately 3 mg to 6 mg of the freeze-dried samples were weighed into ceramic pans, which were heated

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