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Research paper

Chitosan:β-glucan particles as a new adjuvant for the hepatitis B antigen

Edna Soares, Sandra Jesus, Olga Borges*

Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal
Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

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ABSTRACT

The development of new vaccine adjuvants is urgently needed not only to enable new routes of vaccine administration but mostly to go beyond protective humoral immunity, often insufficient to fight infectious diseases. The association of two or more immunopotentiators or mimicking pathogen physicochemical properties are strategies that can favor powerful and more balanced Th1/Th2 immune responses. Therefore, the present work aimed to combine both chitosan and β-glucan biopolymers in the same particle, preferably with surface β-glucan localization to simulate the cell wall of some pathogens and to stimulate the immune cells expressing the Dectin-1 receptor. Chitosan:β-glucan particles (ChiGluPs) were developed through a chitosan precipitation method. The chitosan was precipitated into a β-glucan alkaline solution followed by genipin crosslink. The optimized method produced particles with a mean diameter of 837 nm for ChiPs and 1274 nm for ChiGluPs. β-glucan surface location was confirmed by zeta potential measurements (+24 mV for ChiGluPs and +36 mV for ChiPs) and zeta potential titration. These new particles showed high antigen loading efficacy and low cytotoxicity. Mice vaccination studies revealed that both ChiPs and ChiGluPs had an adjuvant effect for the hepatitis B surface antigen (HBsAg), with ChiGluPs resulting in serum anti-HBsAg total IgG 16-fold higher than ChiPs, when administered with 1.5 μg HBsAg per dose. Specifically, IgG1 subclass was 5-fold higher and IgG3 subclass was 4-fold higher for ChiGluPs comparing to ChiPs. Overall, the preparation method developed allowed the advantageous combination of β-glucan with chitosan, without chemical functionalization, which represents an additional step toward tailor-made adjuvants production using simple precipitation techniques.

1. Introduction

Vaccination is one of the great successes of public health, still intangible for many infectious diseases [1]. Advancing technology allowed the development of subunit vaccines as a safer alternative to the traditional inactivated or live-attenuated [2]. However, subunit antigens (e.g. proteins, peptides or nucleic acids) are generally poorly immunogenic and require the addition of immunostimulatory agents (adjuvants) to obtain long-lasting protective immunity [2–4]. Vaccine adjuvants have multiple mechanisms of action that often include depot effect, facilitated antigen presentation, increased secretion of immunomodulatory cytokines to control T and B cells response and the ability to stimulate innate immunity and indirectly modulate adaptive immune responses [5]. Few adjuvants are currently licensed for human use, being aluminum salts (alum) the oldest and most used. The challenge is to develop new improved and safe adjuvants that will confer immunomodulatory properties, allowing vaccines to evoke other than antigen-specific antibodies, often insufficient for some infectious diseases [6]. The antigen-specific cellular immune response is rarely induced by recombinant antigen-based prophylactic vaccines and is a key-feature for therapeutic vaccines not only for cancer but also for chronic infections diseases like the hepatitis B.

One interesting strategy is vaccine tailoring to look like pathogens, mimicking their properties responsible for immunity often including size, shape and surface molecule organization [2]. Both nano- and microparticles have been used with this aim and natural polysaccharides are potential candidates to be their main constituents due to intrinsic immunomodulatory, biocompatibility and biodegradability properties [3]. In some cases, they can act as pathogen-associated molecular patterns (PAMPs) that recognize innate immune system pattern recognition receptors (PRRs), triggering and regulating adaptive immunity [7]. Indeed, a particulate nature is a consistent principle for adjuvant design, often originating a pro-inflammatory environment to recruit and prolong the interaction with immune cells [8]. Moreover, the combination of PAMPs with a particulate form will definitely contribute to the potency of the vaccine adjuvant resulting in proper vaccine targeting and improved immune responses [2–5,7].

Most innate immune responses are directed against three classes of

* 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).

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fungal cell wall glycans, recognized by dendritic cells (DCs) receptors: β-glucans, mannans, and chitin/chitosan [9]. Therefore, the aim of the present work consisted in the development of a "pathogen-like" adjuvant comprising both chitosan and β-glucan in a particulate form. Both biopolymers are well tolerated by the human body representing a safe approach with reduced side effects [4,7]. First, chitosan is a product of chitin deacetylation widely recognized as an excellent choice for the preparation of drug delivery systems. The presence of protonated primary amine groups, especially in acidic conditions, enable the interaction with negatively charged biomolecules (e.g. protein antigens) [10]. Additionally, chitosan intrinsic immunomodulatory properties have been associated with NRLP-3 inflammasome activation [9,11]. Second, β-glucan surface localization was a desired feature for the new developed adjuvant. β-glucans are specifically recognized by the Dectin-1 receptor, a PRR widely expressed in macrophages and DCs, whose activation leads to the modulation of both innate and adaptive immunity, usually promoting Th17 cell differentiation (Kagimura, da Cunha et al. 2015)[12,13]. Although both soluble and particulate β-glucans bind to this receptor, the dowstream signaling is only activated by the latter one [14], resulting in tumor necrosis factor (TNF)-α release, the hallmark of Dectin-1 activation [15]. Consistently, curdlan was our choice for the β-glucan source as it can be solubilized in NaOH and the particulate form is a more robust activator of inflammatory response than the yeast derived β-glucan zymosan [16]. Previous results from our group showed that chitosan-based particles resulted in a predominantly HBsAg-specific Th2-biased immune response through the subcutaneous route [17,18]. Therefore, the main intention of the present study was to understand if the addition of β-glucan would benefit the strength and the modulation of the elicited immune response towards a more balanced Th1/Th2 immune response, comparing simple chitosan particles (ChiPs) to blend chitosan:β-glucan particles (ChiGluPs) as adjuvants for the hepatitis B surface antigen (HBsAg). Apart from particle development, some *in vitro* assays were done to understand particle cytotoxicity to target cells as well as their ability to load and deliver model antigens.

2. Materials and methods

2.1. Materials

Chitosan (ChitoClear™ – 95% DD and 8 cP viscosity measured in 1% solution in 1% acetic acid) was purchased from Primex BioChemicals AS (Avaldsnes, Norway) and purified as previously described [19]. Curdlan (Lot 60201) produced by *Alcaligenes faecalis*, the β-1,3-glucan (β-glucan) source used, was acquired from Megazyme (Bray, Ireland) and CBC Genipin from Challenge Bioproducts Co., Ltd. (Taiwan, China). The hepatitis B surface antigen (HBsAg) (purity > 98% by SDS-Page tested in ELISA with anti-HBsAg antibodies), subtype adw, a virus-like-particle, with an approximate size of 25 nm, was purchased from Aldevron (Fargo, ND, USA). Bovine serum albumin (BSA, 96% fraction V), ovalbumin (OVA, 98%), myoglobin from equine skeletal muscle (95–100%), α-casein (> 70%), lysozyme (≥ 80%), thiazolyl blue tetrazolium bromide (MTT) reagent, Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI 1640), heat-inactivated fetal bovine serum (FBS), HEPES, sodium pyruvate, 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF), Lipopolysaccharide (LPS), Concavalin A, MEM amino acids, MEM non-essential amino acids and FITC-BSA (A9771) were purchased from Sigma Aldrich Corp. (MO, USA). Image-iT™ LIVE Plasma Membrane and Nuclear Labeling Kit was acquired from Life Technologies Corporation (Paisley, UK). IgG1, IgG2c, IgG3 and IgE horseradish peroxidases (HRP) were purchased to Rockland Immunochemicals Inc. (Limerick, PA, USA.). IgG HRP was obtained from Bethyl Laboratories (Montgomery, TX, USA). Goat anti-mouse HBsAg (ad/ay) HRP was acquired from Meridian, Life Science, ®Inc. (Memphis, TN, USA). Murine interferon (IFN)-γ, IL-4 and IL-17 standard ABTS ELISA development kits were acquired from PeproTech (Rocky Hill, NJ, USA). Fluorescein isothiocyanate (FITC) was purchase to Santa Cruz Biotechnology (Santa Cruz, CA, USA). DC™ Protein Assay from Bio-Rad (Hercules, CA, USA). All other chemicals and reagents used were described in methods and are of analytical grade.

2.2. Particle experimental design

Chitosan (ChiPs) and chitosan:β-glucan (ChiGluPs) particle production was based on a precipitation method followed by genipin crosslink. For particle optimization, the effect of chitosan concentration and genipin maturation conditions were the size influencing factors evaluated. Briefly, a volume of 18 mL of several chitosan solutions (0.05, 0.025 or 0.01% (w/v) in acetic acid 0.1% (v/v)) were added dropwise to 18 mL of a 0.025 M NaOH solution containing or not 0.5% (w/v) Tween™ 80 (ChiPs), under a high-speed homogenizer (homogenizer Ystral X120, Ballrechten-Dottingen, Germany). Additionally, some of the conditions with Tween™ 80 also included 0.025% (w/v) β-glucan in the NaOH solution (ChiGluPs). After 1 h of maturation, the resultant particles were washed twice in 10 mL of phosphate buffered saline pH 7.4 (PBS) (centrifuging 10 min at 2000*g*) to remove soluble exceeding compounds. The particles were suspended again in a volume of 10 mL of 0.1% (w/v) genipin. The optimization of the crosslink conditions was performed for three different size influencing factors: maturation time (3 h, 6 h or overnight (ON)), buffer (PBS or Tris Buffer pH 9 (TRIS)) and temperature (4 °C or room temperature (RT)). Size was measured after washing the particles twice with milliQ water (10 min at 720*g*). For studies which required particles stained with fluorophores, part (25%) of the chitosan was labeled with a fluorescein (FITC), following a method previously described by our group [19] and used to particle production.

2.3. Particle characterization

2.3.1. Size, zeta potential and morphological analysis

Delsa™ Nano C particle analyzer (Beckman Coulter, CA, USA) was used to measure HBsAg-loaded and unloaded particles mean hydrodynamic diameter by dynamic light scattering (DLS) and zeta potential by electrophoretic light scattering. Size analyses were performed at 25 °C and scattered light collected at a 165° angle. Unloaded particles were submitted to a size and zeta potential titration measured through a wide range of pH values in water, adding NaOH or HCl for pH changes. Transmission electron microscopy (TEM) of unloaded particles suspended in water was performed using a JEOL JEM 1400, 120 kV (JEOL, Peabody, MA, USA), placing a drop of the sample in a mesh grid which was dried out before visualization.

2.3.2. Quantification of β-glucan incorporation

To assess β-glucan incorporation into ChiGluPs, curdlan was fluorescently labeled with DTAF as previously described [20] with minor modifications. Briefly, β-glucan was solubilized at 0.025% (w/v) in 0.1 M NaOH, vacuum filtered (0.45 μm) and added to 0.05 M sodium tetraborate in a proportion to originate the borate buffer pH 10.8. DTAF solubilized in dimethyl sulfoxide (DMSO) was added to the previous mixture in a proportion of 1:1 (w/w) DTAF:Curdlan. After an overnight incubation in the dark, 96% ethanol was added for curdlan precipitation in a proportion of 2:1. The precipitate was centrifuged 25 min at 2000*g*, the resultant pellet was solubilized in NaOH 0.1 M, and this process repeated until the supernatant was free of unbound DTAF. DTAF-labeled β-glucan was solubilized in NaOH 0.025 M in a final concentration of 0.025% (w/v). ChiGluPs were produced as described above, centrifuged and DTAF-β-glucan was measured either in the pellet or in the supernatant using a fluorescence microplate reader (Synergy HT, Biotek®) (absorption/emission 492/512 nm). The standard curve represented a linear relationship between the fluorescence output and the DTAF-labeled β-glucan concentration.