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The effect of antigen encapsulation in chitosan particles on uptake, activation and presentation by antigen presenting cells

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ABSTRACT

Particle-based vaccine delivery systems are under exploration to enhance antigen-specific immunity against safe but poorly immunogenic polypeptide antigens. Chitosan is a promising biomaterial for antigen encapsulation and delivery due to its ability to form nano- and microparticles in mild aqueous conditions thus preserving the antigenicity of loaded polypeptides. In this study, the influence of chitosan encapsulation on antigen uptake, activation and presentation by antigen presenting cells (APCs) is explored. Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) and ovalbumin (OVA) were used as model protein antigens and encapsulated in chitosan particles via precipitation–coacervation at loading efficiencies >89%. Formulation conditions were manipulated to create antigen-encapsulated chitosan particles (AgCPs) with discrete nominal sizes (300 nm, 1 μm, and 3 μm). Uptake of AgCPs by dendritic cells and macrophages was found to be dependent on particle size, antigen concentration and exposure time. Flow cytometry analysis revealed that uptake of AgCPs enhanced upregulation of surface activation markers on APCs and increased the release of pro-inflammatory cytokines. Lastly, antigen-specific T cells exhibited higher proliferative responses when stimulated with APCs activated with AgCPs versus soluble antigen. These data suggest that encapsulation of antigens in chitosan particles enhances uptake, activation and presentation by APCs.

1. Introduction

Over the last several decades, vaccine development has shifted away from using attenuated or inactivated whole pathogens in favor of recombinant subunit antigens [1–3]. This shift is partly due to safety concerns over potentially harmful pathogens and partly due to an increasing interest in inducing immunity towards non-pathogenic self or self-like antigens such as tumor-associated antigens or overexpressed proteins implicated in disease (e.g. amyloid beta) [4–6]. While subunit antigens are much safer, they are also much less immunogenic than whole pathogens. Subunit antigens are rapidly degraded by proteases and lack the requisite secondary immune stimulus, i.e. co-stimulation and/or danger signals, required for the generation of antigen-specific immunity [7]. As a result, a great deal of effort has been spent developing delivery systems and/or adjuvants capable of enhancing vaccine responses to subunit and polypeptide antigens [7–9].

The encapsulation of polypeptide antigens in nano- and/or microparticles has been explored extensively as a strategy to enhance immunogenicity. The advantage of this strategy is three-fold: First, encapsulation of antigens in particles can prevent antigen degradation and enhance antigen persistence. Second, antigen presenting cells (APCs), such as macrophages and dendritic cells have been shown to readily phagocytose and process particles ranging in size from 150 nm to 4.5 μm [10,11]. Third, most particle-based platforms can be engineered to contain additional adjuvants and/or targeting moieties to further influence immunogenicity [1, 2]. In general, antigens in particulate form have been shown to be more immunogenic than their soluble counterparts [12,13]. A myriad of particle-based antigen delivery approaches including liposomes, immune stimulating complexes (ISCOMs), and polymeric particles are under development and have been reviewed elsewhere [1,14,15].

Chitosan-based vaccine delivery systems have received increasing attention due to chitosan's remarkable versatility and unique characteristics [16–23]. Chitosan is a natural polysaccharide derived primarily from the exoskeletons of crustaceans. Chitosan nano- and microparticles can be manufactured via either precipitation–coacervation [24] or ionotropic gelation [25]. Polypeptides can be encapsulated either during particle formation [26] or adsorbed to particle surfaces after formation [27]. Chitosan's mucoadhesiveness and ability to loosen epithelial gap junctions justifies its use in mucosal vaccines. Several studies have shown

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that chitosan nano- and microparticles loaded with antigens can generate mucosal immunity following intranasal vaccination [28,29]. However, chitosan particles are also expected to elicit robust immune responses via non-mucosal routes. Yet, in the only s.c. vaccination study to date, no significant immunity was generated with a vaccine comprised of ovalbumin (OVA) adsorbed to chitosan nanoparticles [27].

While the above study may have failed to induce immune activation due to a documented change in antigen conformation, it is important to note that vaccine responses are highly complex, involve multiple cell types and require successful completion of many interdependent processes including antigen uptake, cytokine release, immune cell trafficking, antigen presentation, co-stimulation, etc. To simplify the contributions of chitosan-based particles, in this study, we focused solely on APC function. In particular, we evaluated the ability of antigen-encapsulated chitosan particles (AgCPs) to enhance antigen uptake, APC activation and antigen presentation. The effect of particle size on antigen uptake by both bone marrow-derived dendritic cells and RAW 264.7 macrophages was quantified via spectrophotometry and flow cytometry. The ability of AgCPs to induce APC activation was determined by measuring upregulation of surface activation markers as well as cytokine release. Finally, APCs exposed to AgCPs or soluble antigens were compared for their ability to present antigen and induce proliferation of antigen-specific T cells.

2. Materials and methods

2.1. Reagents and antibodies

Chitosan (molecular weight: 95 ± 20 kDa), sodium sulfate, polysorbate 80 (Tween 80), acetic acid, fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA), and OVA were purchased from Sigma (St. Louis, MO). Cell culture media components, including L-glutamine, HEPES buffer, trypsin-EDTA, FBS, antibiotics, DMEM, and RPMI-1640, were purchased from Thermo Scientific (Rockford, IL). Recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was purchased from Peprotech (Rocky Hill, NJ). $OVA_{257-264}$ peptide was purchased from AnaSpec, Inc., Fremont, CA. All antibodies used for flow cytometry along with cytometric bead array kits were purchased from BD Biosciences (San Jose, CA).

2.2. Laboratory animals

Female C57BL/6 J, OT-1 and OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolator cages and used at 8–12 weeks of age. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Arkansas. Animal care was in compliance with The Guide for Care and Use of Laboratory Animals (National Research Council).

2.3. Cell culture

RAW 264.7 mouse macrophage cells obtained from American Type Culture Collection (Manassas, VA) were cultured in complete media consisting of DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. Bone marrow-derived dendritic cells (BMDCs) were cultured from bone marrow cells using an established protocol [30].

2.4. Preparation of AgCPs

AgCPs were prepared via precipitation–coacervation as described previously with slight modifications [24,26,27]. Briefly, chitosan was dissolved in 2% acetic acid and passed through a 0.2 μm filter. AgCPs were formed by adding a 10% w/v sodium sulfate solution containing either FITC-BSA or OVA as model protein antigens, henceforth referred to as BsaCPs or OvaCPs, respectively. Chitosan particles containing no antigen (CPs) were formed in the same manner but without BSA or OVA. Tween 80 was added as a nonionic stabilizer and particles were stirred for 2 h with intermittent sonication (S4000, Misonix, Farmingdale, NY). Chitosan particles were separated through centrifugation at 25,000*g* for 10 min and freeze dried before further use. Particles of various sizes were obtained by varying chitosan concentration, the rate of sodium sulfate addition, sonication power, and chitosan:antigen ratio as seen in Table 1. These parameters and the levels of these parameters were selected based on results from ongoing AgCP optimization studies (data not shown).

Table 1
Formulation parameters and measurements for AgCPs and CPs.

	"300 nm" AgCPs	"1 μm" AgCPs	"3 μm" AgCPs	"300 nm" CPs
Chitosan concentration (mg/ml)	1	5	8	1
Sodium sulfate addition rate (ml/min)	8	5	0.2	8
Sonication power (Watts)	40	10	0	40
Chitosan: FITC-BSA ratio	10:1	10:1	10:1	–
Mean diameter[a] (nm)	332 ± 19	1034 ± 74	2918 ± 333	289 ± 9
Polydispersity index[a]	0.09 ± 0.03	0.16 ± 0.09	0.33 ± 0.05	0.10 ± 0.06
Surface charge[a] (mV)	16.8 ± 0.8	17.8 ± 0.5	18.0 ± 0.7	36.8 ± 1.4
Protein loading efficiency[a] (%)	89.2 ± 0.1	91.0 ± 0.1	96.2 ± 0.2	–

[a] Measured data are presented as mean ± standard deviation of three independent experiments.

2.5. Characterization of AgCPs

Particle size and surface charge were measured via dynamic light scattering (DLS) (Nano ZS90, Malvern Instruments, Malvern, UK). Morphological characteristics were documented using scanning electron microscopy (SEM) (Nanolab 200, FEI, Hillsboro, OR). Briefly, AgCPs were dispersed in DI water and vacuum dried onto a glass slide. The slides were sputter coated with gold using an Emitech SC7620 (Quorum Technologies, Ashford, Kent, UK) prior to imaging. SEM images were acquired at a beam voltage of 10–15 kV. The encapsulation efficiencies of FITC-BSA in AgCPs were quantified spectrophotometrically (Synergy2, Biotek, Winooski. VT) by measuring the supernatant after centrifugation. Antigen encapsulation efficiency (EE) was calculated as:

$$\mathrm{EE} = \frac{(\text{Initial antigen conc.} - \text{Unencapsulated antigen conc.})}{\text{Initial antigen conc.}} \times 100$$

2.6. Uptake of AgCPs by APCs

RAW 264.7 macrophages or BMDCs were collected and seeded at a density of 50,000 cells/well in 24 well plates. To determine the effect of particle size on uptake, cells were co-incubated with of 300 nm, 1 μm, or 3 μm BsaCPs. To determine the effect of antigen concentration on uptake, cells were co-incubated with BsaCPs at an effective antigen concentration of 1, 5, 10, 20, or 30 μg/ml. To determine the effect of incubation time on uptake, cells were co-incubated with 1 μm BsaCPs at an effective antigen concentration of 30 μg/ml for 12, 24, or 48 h. After each co-incubation, cells were rinsed three times with PBS and lysed with 1% triton solution. The amount of FITC-BSA released was quantified via fluorescence spectroscopy. To assess the percentage of cells taking up BsaCPs, cells were rinsed three times with PBS and briefly trypsinized to form a single cell suspension prior to analyzing on a FACS-CantoII (BD biosciences, San Jose, CA).

2.7. APC activation

Activation markers on macrophages and BMDCs co-cultured with AgCPs were analyzed via flow cytometry. Briefly, RAW 264.7 macrophages and BMDCs were seeded onto 6 well plates at a density of 1×10^6 cells/well and cultured in their respective growth media for 2 h. Media containing BsaCPs was then added at a final antigen concentration of 30 μg/ml. Unloaded 300 nm CPs that contained no antigen were used at the same dry weight as 300 nm AgCPs. Media alone was used as a negative control. After 24 h, cells were rinsed three times with PBS and briefly trypsinized to form a single cell suspension. FcγII and FcγIII receptors were blocked via incubation with 1 μg purified anti-mouse CD16/CD32 (clone: 2.4G2) per 1×10^6 cells for 15 min on ice. Cells were stained for 30 min on ice with fluorescence-labeled antibodies (1 μg/1×10^6 cells) to the following markers: MHC I (clone: AF6-88.5), MHC II (clone: 2G9), CD11b (clone: M1/70), CD11c (clone: HL3), CD80 (clone: 16-10A1), CD86 (clone: GL1), CD40 (clone: HM40-3) and CD54 (clone: 3E2). Cells were then washed twice with cold PBS and analyzed on a six-color FACSCantoII. Data analysis was performed using BD FACSDiva software (BD biosciences, San Jose, CA).

Cytokines released from macrophages and BMDCs were quantified via cytometric bead array (CBA) analysis. In brief, RAW 264.7 macrophages and BMDCs were