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Functionalization of polyanhydride microparticles with di-mannose influences uptake by and intracellular fate within dendritic cells

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ABSTRACT

Innovative vaccine delivery platforms can facilitate the development of effective single-dose treatment regimens to control emerging and re-emerging infectious diseases. Polyanhydride microparticles are promising vaccine delivery vehicles due to their ability to stably maintain antigens, provide tailored release kinetics and function as adjuvants. A major obstacle for the use of microparticle-based vaccines, however, is their limited uptake by dendritic cells (DCs). In this study, we functionalized the microparticle surface with di-mannose in order to target C-type lectin receptors (CLRs) on DCs. Polyanhydride particles based on sebacic acid (SA), 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) were evaluated. Co-incubation of di-mannose-functionalized microparticles up-regulated the expression of CLRs on DCs. More importantly, di-mannose functionalization increased the uptake, as measured by the percentage of cells internalizing particles. The uptake of CPH:SA microparticles increased ~20-fold, from 0.82% (non-functionalized) to 20.2%, and internalization of CPTEG:CPH microparticles increased ~7-fold from 1.35% (non-functionalized) to 9.3% upon di-mannose functionalization. Both di-mannose-functionalized and non-functionalized particles trafficked to lysosomes. Together, these studies demonstrate that employing rational vaccine design principles, such as the targeting of CLRs on antigen-presenting cells, can enhance delivery of encapsulated antigens and potentially induce a more robust adaptive immune response.

1. Introduction

Vaccines have played a critical role in improving public health over the last century. Traditional vaccination strategies, including the use of killed or live attenuated organisms, subunit fragments and recombinant proteins, have been successful in preventing and even eradicating multiple diseases [1]. However, the high reactogenicity of these vaccines, the need for multiple immunizations and/or the requirement for parenteral administration have led researchers to seek alternative strategies. In this regard, the use of novel biomaterials as vaccine delivery platforms has emerged as a rational alternative [2,3]. In particular, particle-based vaccines offer several advantages over traditional vaccines. They can be formulated to tailor the release of encapsulated immunogens over time to provide antigen persistence, thus eliminating the need for booster immunizations. The encapsulated protein may also be protected from degradation, which would allow for lower immunogenic doses to be administered. For example, particles composed of polyanhydrides have been shown to protect fragile immunogens, possess immunomodulatory properties and confer protective immunity in a single dose [4–9].

A key challenge in particle-based vaccine development is the efficient targeting of antigen-presenting cells (APCs) such as dendritic cells (DCs), into which the encapsulated antigen can be released, processed and presented via major histocompatibility complex (MHC) molecules. Increasing the number of APCs that internalize antigen-loaded microparticles would increase the presentation of antigen via MHC II to $CD4^+$ T cells. C-type lectin receptors (CLRs), a type of pattern recognition receptor that recognizes pathogen-associated molecular patterns, are an attractive target to facilitate this strategy. Pathogens such as *Yersinia pestis* and *Mycobacterium tuberculosis* display conserved carbohydrate structures on their cell surface and are detected by CLRs [10,11]. Previous studies have shown successful targeting of CLRs on APCs

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by functionalizing particles or antigen with mannoproteins [12], antibodies [13,14] or glycans [15]. For example, carbohydrate modification of a model antigen, ovalbumin, resulted in a 50-fold increase in antigen presentation to $CD4^+$ T cells [14]. However, detailed studies on the effect of functionalization on uptake, intracellular trafficking, and persistence of particles have not been reported.

The rationale for the work described herein comes from two lines of reasoning. First, the surface functionalization of microparticles with chemical moieties such as carbohydrates or linker molecules offers the opportunity to confer new physical (charge) and chemical (hydrophilicity) properties to the particles that enhance cellular uptake. Second, the knowledge that carbohydrates are designed to interact with specific cellular receptors may be exploited to facilitate microparticle internalization. The functionalization strategies described in this work allow both non-specific and specific (receptor-mediated) interactions of microparticles with immune cells. Polyanhydrides based on sebacic acid (SA), 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) were evaluated in this present study. Functionalization of microparticles with 2-[α-D-mannopyranosyl-(1 → 2)-α-D-mannopyranosyloxy]-acetic acid (di-mannose) markedly increased the number of DCs that internalized microparticles. Morphometric analysis of intracellular fate revealed that the linker-functionalized microparticles eroded faster than the non-functionalized or di-mannose-functionalized particles. Lastly, it was demonstrated that the uptake of di-mannose-functionalized microparticles was not dependent on the mannose receptor-mediated pathway.

2. Materials and methods

2.1. Materials

Sebacic acid (99 %), *p*-carboxybenzoic acid (99+ %) anhydrous, 1-methyl-2-pyrrolidinone (99+ %), and anhydrous dimethylacetamide were purchased from Aldrich (Milwaukee, WI); 4-*p*-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2 pyrrolidinone, triethylene glycol, and FITC (fluorescein isothiocyanate)–dextran were purchased from Sigma–Aldrich (St. Louis, MO); 4-*p*-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethylformamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, pentane and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ).

2.2. Polymer synthesis and microparticle fabrication

CPH and CPTEG monomers were synthesized as previously described [16,17]. Copolymers containing a 50:50 molar ratio of CPH:SA or CPTEG:CPH were synthesized by melt polycondensation, as previously described [5]. Their chemical structure was characterized with ^{1}H nuclear magnetic resonance (NMR) and the molecular mass was determined using gel permeation chromatography (GPC).

Microparticles of 50:50 CPH:SA and 50:50 CPTEG:CPH copolymers were fabricated by cryogenic atomization, as described elsewhere [17,18]. Briefly, 100 mg of copolymer was dissolved in methylene chloride. For FITC-loaded microparticles, 1 mg of the fluorescent payload was added to the dissolved polymer and dispersed by sonication at 40 Hz for 30 s. The polymer solution was pumped through an 8700–1200 MS ultrasonic atomizing nozzle (SonoTek Corporation, Milton, NY) into 200 ml of frozen ethanol (with an excess of liquid nitrogen). This procedure was performed at 4 °C. After storage at −80 °C for 24 h, 200 ml of cold ethanol was added to reduce particle aggregation; the solutions were then stirred at 300 rpm for 15 min and placed back in the freezer at −80 °C for an additional 48 h. Next, vacuum filtration was used to collect the microparticles and they were dried overnight under vacuum. Scanning electron microscopy (SEM; JEOL 840 A, JEOL Peabody, MA) was used to observe the morphology of the microparticles. The particle size distribution was obtained from SEM images using ImageJ v1.36b image analysis software (National Institutes of Health, Bethesda, MD).

2.3. Microparticle functionalization and characterization

Synthesis of carboxy-linked di-mannose was performed as previously described [19,20]. Glycolic acid linker and di-mannose were conjugated on the surface of polyanhydride microparticles by a two-step amine-carboxylic acid coupling reaction [19,20]. In the first step, microparticle suspensions (100 mg ml^{-1}) were incubated with 10 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 12 equivalents of *N*-hydroxysuccinimide (NHS) and 10 equivalents of ethylenediamine in distilled water. Reagent equivalents were related to the average concentration of carboxylic acid groups on the surface of the non-functionalized microparticles. For example, the –COOH surface concentration was determined to be 6.7×10^{-4} mmol mg^{-1} of non-functionalized microparticles and was calculated theoretically using end-group quantification from ^{1}H NMR spectra. The first reaction was carried out at 4 °C for 9 h, with end-over-end rotation. After completion of the first attachment reaction, particles were centrifuged at 12,000*g* for 10 min and the supernatant was discarded. A wash step with cold distilled water was then performed in order to remove unreacted reagents. The second attachment reaction involved the incubation of microparticles with 12 equivalents of EDC, 12 equivalents of NHS and 10 equivalents of di-mannose or glycolic acid in distilled water using constant end-over-end rotation for 9 h at 4 °C. After completion of the reaction, microparticles were collected by centrifugation (12,000*g* for 10 min), washed twice with cold water and dried under a vacuum for at least 2 h.

SEM and quasi-elastic light scattering (QELS; Zetasizer Nano, Malvern Instruments Ltd., Worchester, UK) were utilized to analyze the morphology and size, and the ζ-potential of non-functionalized and functionalized microparticles, respectively. To determine the surface composition of the functionalized and non-functionalized particles, X-ray photoelectron spectroscopy (XPS; PHI 5500 Multi-technique System, Physical Electronics, Inc., Chanhassen, MN) was used. Deconvolution of high-resolution carbon peaks (C1*s*) using CasaXPS software (RBS Instruments, Bend, OR) was performed to identify the main carbon-bound components on the microparticle surface. Finally, an optimized high throughput version of a phenol–sulfuric acid assay [19] was used to estimate the amount of sugar attached to the microparticles.

2.4. Mice

C57BL/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and mannose receptor-deficient ($MR^{-/-}$) C57BL/6 mice were a generous gift from Dr. Mary Ann McDowell of the University of Notre Dame. All animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee. Mice were housed in pathogen-free conditions, with the bedding, feed and caging sterilized prior to use.

2.5. DC culture

Bone-marrow DCs were derived from tibias and femurs of C57BL/6 mice and cultured in vitro in the presence of murine