

Mannosylated Dextran Nanoparticles: A pH-Sensitive System Engineered for Immunomodulation through Mannose Targeting

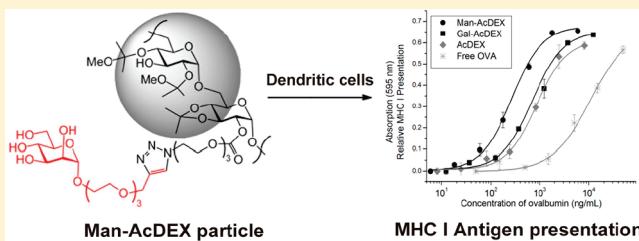
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 Supporting Information

ABSTRACT: Biotherapeutic delivery is a rapidly growing field in need of new materials that are easy to modify, are biocompatible, and provide for triggered release of their encapsulated cargo. Herein, we report on a particulate system made of a polysaccharide-based pH-sensitive material that can be efficiently modified to display mannose-based ligands of cell-surface receptors. These ligands are beneficial for antigen delivery, as they enhance internalization and activation of APCs, and are thus capable of modulating immune responses. When compared to unmodified particles or particles modified with a nonspecific sugar residue used in the delivery of antigens to dendritic cells (DCs), the mannosylated particles exhibited enhanced antigen presentation in the context of major histocompatibility complex (MHC) class I molecules. This represents the first demonstration of a mannosylated particulate system that enables enhanced MHC I antigen presentation by DCs *in vitro*. Our readily functionalized pH-sensitive material may also open new avenues in the development of optimally modulated vaccine delivery systems.



INTRODUCTION

Vaccination has been successful in preventing various diseases; however, the development of vaccines for illnesses such as cancer, AIDS, and malaria still suffers from ineffective or insufficient generation of protective immunity. One of the great challenges in the development of improved vaccines involves tailoring the type of immune response that a vaccine elicits to most effectively treat specific pathogens or cancers.¹ For example, cytotoxic T lymphocyte (CTL)-mediated immunity is believed to be critical for full protection from illnesses, such as HIV, malaria, and cancer.^{2,3} Therefore, much effort has been put into guiding antigen into major histocompatibility complex (MHC) class I processing pathways of antigen presenting cells (APCs), which is critical for the generation of CTL responses.⁴

Dendritic cells (DCs)—the most potent APCs—endocytose, process, and load antigens onto MHC class I or II molecules for presentation to naïve CD8⁺ or naïve CD4⁺ T cells, respectively.⁵ This MHC presentation, along with costimulatory signals, results in the activation of T cells, which then become effector T cells. DCs also express pathogen recognition receptors, such as Toll-like receptors (TLRs) and C-type lectins, with which DCs sense and/or capture microbes.^{6,7} Targeting DCs with ligands specific for particular TLRs or C-type lectins can provide opportunities to alter the signaling pathways of DCs during activation, thus modulating the ultimate form of the cellular and/or humoral immune response that is generated.^{8–10} Therefore, it is of great importance to develop a modular vaccine delivery system that can be used to address different activation mechanisms of the immune system.

Nanoparticles have received some attention for application as vaccine carriers because their size is in the range that can be taken up by phagocytic cells for the passive targeting of APCs. They can absorb, encapsulate, or be conjugated to antigens, and it is possible to incorporate ligands for the recognition and activation of APCs.^{11–13} Besides targeting antigens to APCs, it is equally important to deliver those antigens into desired intracellular compartments.¹⁴ Our group and others have reported acid-sensitive antigen delivery systems that allow antigens to be released under acidic conditions upon endocytosis by APCs and enter the MHC I presentation pathway.^{15–21} Recently, we have developed a simple approach to modify dextran, a clinically approved biopolymer, into an acid-sensitive micro- or nanoparticulate delivery system, where dextran is rendered water-insoluble by modifying its hydroxyl groups with acid-labile acetal groups, forming acetalated dextran (AcDEX).²² When tested *in vitro*, AcDEX particles encapsulating a model protein antigen led to significant enhancement of MHC class I presentation compared to the free protein or antigens delivered by commonly used materials such as poly(lactic-co-glycolic acid) (PLGA) or iron oxide particles, thus demonstrating the potential of AcDEX in vaccine development.¹⁶

We have now engineered the AcDEX system to display reactive groups on the particle surface, which can be readily functionalized with ligands capable of modulating immune

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responses. In this report, we demonstrate that introduction of mannose residues onto the surface of particles can enhance MHC I antigen presentation by DCs *in vitro*.

■ EXPERIMENTAL PROCEDURES

General Materials and Methods. All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as supplied, unless otherwise noted. Phosphate buffered saline (pH 7.4, PBS) was purchased from Invitrogen-Gibco (Carlsbad, CA). Water (dd-H₂O) was purified to a resistance of 18 MΩ using a NANOpure purification system (Barnstead, USA). All reactions were conducted under nitrogen. Organic solvents in reactions were purified by passing through two columns of neutral alumina on a commercial push still apparatus (Glass Contour, SG Water, NH). Analytical thin layer chromatography (TLC) was performed on silica gel 60-F₂₅₄ (Merck). Plates were visualized by ultraviolet light or charring with basic potassium permanganate solution, triphenylphosphine/ninhydrin solution or ethanolic sulfuric acid-anisaldehyde solution. Column chromatography used silica gel (SiliCycle, F60, 40–63 μm, 60 Å) and solvents were of reagent grade, and used as supplied. ¹H NMR spectra were recorded at 400 or 500 MHz, and ¹³C NMR spectra were recorded at 100 MHz. First-order chemical shifts are reported in δ (ppm) using residual solvent signals from deuterated solvents as references. For samples in D₂O, the chemical shifts are referenced to acetone (0.1% v/v) at δ 2.225. Signals in ¹H spectra were assigned with corresponding coupling constants or COSY spectra. NMR data reported are labeled according to IUPAC nomenclature for carbohydrate molecules. Mass spectrometry was performed under positive/negative-mode high-resolution electrospray ionization (ESI) on an Orbitrap instrument by the Chemistry Mass Spectrometry Facility of the University of California, Berkeley. Fourier transform infrared spectroscopy (FT-IR) was carried out on a Varian 3100 FT-IR spectrometer (Varian, USA). Particles used in the studies were suspended by alternately vortexing and sonicating in a VWR model 75 water bath. Fluorescence measurements for microplate-based assays were obtained on a Spectra Max Gemini XS (Molecular Devices, USA), usage courtesy of Professor Jonathan Ellman. UV-vis absorbances for microplate-based assays were measured using Spectra Max 190 (Molecular Devices, USA), and flow cytometry was performed using Becton-Dickinson Biosciences FACSCalibur flow cytometer, usage courtesy of Professor Carolyn Bertozzi.

Cell Lines and Culture. DC 2.4 cells were generously donated by Professor Kenneth Rock (University of Massachusetts Medical Center) and were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, from Hyclone), 2 mM GlutaMAX (Invitrogen), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 10 mM MEM Non-Essential Amino Acids (NEAA, from Invitrogen), 10 mM HEPES buffer (Invitrogen), and 0.055 mM 2-mercaptoethanol (Invitrogen). RAW 309 and RAW 264.7 macrophages were purchased from ATCC (Manassas, VA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen-Gibco 11960) supplemented with 10% FBS, 100 U/mL of penicillin, 100 μg/mL streptomycin, and 2 mM GlutaMAX. B3Z (CD8⁺) cells were kindly provided by Professor Nilabh Shastri (University of California, Berkeley) and were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM GlutaMAX, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate

(Invitrogen), and 0.055 mM 2-mercaptoethanol. Cells were incubated in a water-jacketed incubator at 37 °C with 5% CO₂.

Synthesis of Azido-Dextran. To a solution of dextran (MW = 10 500 g/mol, 1.57 g, 0.15 mmol) in anhydrous DMSO (30 mL) was added imidazole-1-carboxylic acid 2-[2-(2-azido-ethoxy)-ethoxy]-ethyl ester (2.61 g, 9.705 mmol) and 4-dimethylaminopyridine (DMAP, 1.30 g, 10.676 mmol). The reaction mixture was stirred for 2 days under argon and dialyzed using regenerated cellulose-based dialysis bag (MWCO = 3 kDa) against 8 × 4 L distilled water over 3 days. Lyophilization gave the product as a white fluffy powder (1.88 g, 85.3%). IR (KBr, cm⁻¹): 3409, 2929, 2362, 2340, 2115, 1751, 1268, 1150, 1104, 1017. ¹H NMR (400 MHz, D₂O): δ 5.01–4.88 (m, 1.4H, H-1), 4.40–4.26 (br, 1H, CH₂OC=O), 4.02–3.42 (m, 12.9H, H-2, H-3, H-4, H-5, H-6, CH₂O, CH₂N₃). ¹³C NMR (125 MHz, D₂O): δ 155.7, 155.0, 97.8, 80.3, 73.4, 71.4, 70.9, 70.2, 69.8, 69.7, 69.6, 69.4, 69.3, 68.4, 67.9, 67.5, 65.6, 50.2, 38.8.

Synthesis of Acetalated Azido-Dextran (Azido-AcDEX). Azido-dextran (MW = 14 700 g/mol, 1.47 g, 0.100 mmol) was dissolved in anhydrous DMSO (10 mL) in a 20 mL scintillation vial, followed by addition of 2-methoxypropene (3.4 mL, 37 mmol) and pyridinium *p*-toluenesulfonate (15.6 mg, 0.062 mmol). The reaction flask was sealed with parafilm, and the reaction was allowed to proceed for 2 h (fast-degrading azido-AcDEX) or 20 h (slow-degrading azido-AcDEX) at r.t.¹⁶ Triethylamine (0.1 mL, 0.7 mmol) was added to quench the reaction, and the dextran derivative was precipitated in dd-H₂O (pH 8, 100 mL). The product was collected by centrifugation at 14 000 × *g* for 10 min, and washed with dd-H₂O (pH 8, 2 × 50 mL). The resulting pellet was then lyophilized to yield a white power (1.07 g). ¹H NMR (500 MHz, CD₂Cl₂): δ 5.18 (1H), 4.95 (2H), 4.28 (2H), 3.97–3.65 (19H), 3.49 (2H), 3.38 (2H), 3.24–3.21 (4H), 2.50 (1H), 1.42, 1.38–1.34 (20H) (all reported as broad peaks).

Preparation of Nanoparticles Encapsulating Proteins (Azido-AcDEX). Ovalbumin (OVA) or bovine serum albumin (BSA) labeled by fluorescein isothiocyanate (FITC) was encapsulated into nanoparticles using a double emulsion water/oil/water (w/o/w) evaporation method similar to that described previously.¹⁶ Briefly, OVA or BSA-FITC (8 mg) was dissolved in phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4, 40 μL), and added to an azido-AcDEX (160 mg) solution in CH₂Cl₂ (1.6 mL). This mixture was then vortexed immediately and emulsified for 30 s on ice using a probe sonicator (Branson Sonifier 450, output setting at 5.5, duty cycle at 70%). An aqueous solution of poly(vinyl alcohol) (PVA, *M*_w = 13 000–23 000 g/mol, 87–89% hydrolyzed, 3.2 mL, 3% w/w in PBS) was added to the first emulsion, and sonicated for 30 s on ice using the same settings. The resulting double emulsion was immediately transferred into a second PVA solution (16 mL, 0.3% w/w in PBS) and stirred for 2 h to evaporate the CH₂Cl₂. The particles were isolated by centrifugation (14 000 × *g*, 15 min) and washed with dd-H₂O (pH 8, 3 × 15 mL). The particle pellets were sonicated to a nice suspension between each wash. The washed particles were suspended in dd-H₂O (pH 8, 2 mL) and lyophilized to give a white fluffy solid (105 mg).

Preparation of Empty Nanoparticles (Azido-AcDEX). Particles containing no protein were made in the same manner as above without the first emulsion with the OVA solution.

Preparation of Microparticles Using Emulsion Method. Micrometer-sized particles were prepared in a similar manner as that previously described.²³ Azido-AcDEX (50 mg) was dissolved

in ice-cold CH_2Cl_2 (1 mL) in a 15 mL round-bottom polypropylene tube. PBS buffer (50 μL) was added, and the first solution and the mixture were sonicated for 5–10 s to a milky emulsion using a Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT) with a 1/8 in microtip, an output setting of 1, and a continuous duty cycle. This primary emulsion was transferred dropwise to an ice-cold solution of PVA (12.5 mL, 3% w/w in PBS) that was being homogenized at 9600 rpm using an IKA T-25 Ultra-Turrax digital homogenizer with an S25N-10G generator. The homogenization was allowed to proceed for 30 s, and the resulting double emulsion was poured into a second PVA solution (25 mL, 0.3% w/w in PBS) and stirred for 2 h to evaporate CH_2Cl_2 . Particles were centrifuged ($4000 \times g$, 4 min), washed with dd-H₂O (3 \times 20 mL, pH 8), resuspended in dd-H₂O (1 mL, pH 8) and lyophilized to yield a white fluffy solid (46 mg, 92%).

Quantification of Encapsulated OVA. Particles containing OVA were suspended at 5 mg/mL in 30 mM acetate buffer (pH 5.0) and incubated at 37 °C under gentle agitation for 2 days using a Thermomixer R heating block (Eppendorf). After complete degradation, protein content was analyzed by a microplate assay using fluorescamine (1 mM in acetone) as described in the literature.²⁴ Empty Ac-DEX particles were degraded in a similar fashion and used to determine background fluorescence.

Quantification of Encapsulated BSA-FITC. Particles containing BSA-FITC were suspended at 5 mg/mL in dd-H₂O (pH 8) in a microplate, and fluorescence of FITC was measured in triplicate and analyzed against a standard fluorescence curve of BSA-FITC.

Size Measurements of Nanoparticles by Dynamic Light Scattering. Particles were suspended in dd-H₂O (pH 8) in a disposable sizing cuvette at a concentration of 1 mg/mL. Particle size distributions and average particle diameters were determined in three measurements by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, United Kingdom). Size distributions in the text are described as average particle diameters \pm half width of the distribution at half maximal height.

Size Measurements of Microparticles by Light Scattering. The size distribution of the microparticles was determined using a Horiba Partica LA-950 laser scattering particle size distribution analyzer after suspending the particles at 2 mg/mL in PBS. The reported results represent the geometric mean \pm standard deviation of the size distribution.

Scanning Electron Microscopy. Suspensions of nanoparticles or microparticles (1 mg/mL) in dd-H₂O (pH 8) were dripped onto silicon wafers and allowed to dry in the air. The particles were then sputtercoated with a 2 nm layer of a palladium/gold alloy and imaged using an S-5000 microscope (Hitachi, Japan) or TM-1000 microscope (Hitachi, Japan).

Preparation of Mannosylated or Galactosylated Particles (Man-AcDEX or Gal-AcDEX). Azido-AcDEX nanoparticles or microparticles (20 mg) were suspended in a 2 mL N₂ protected aqueous solution of copper sulfate (1 mM), potassium 5,5',5''-(2,2',2''-nitrilotris(methylene))tris(1*H*-benzimidazole-2,1-diyl)tripentanoate (BimC₄A)₃ (1.2 mM), ascorbic acid (10 mM), and HEPES buffer (0.1 mM, pH 8.5), and the reaction mixture was stirred for 1 h at r.t. The particles were then isolated by centrifuge (14 000 $\times g$, 10 min), washed with EDTA solution (10 mM in dd-H₂O, 3 \times 2 mL, pH 8) and dd-H₂O (3 \times 2 mL, pH 8), and resuspended in dd-H₂O (pH 8, 1 mL). The suspension was lyophilized to give white fluffy powder (18 mg, 90%).

Quantification of Mannose or Galactose Incorporation. Man-AcDEX or Gal-AcDEX particles (2 mg) were suspended in D₂O (0.7 mL) in an NMR tube, and DCl (5 μL) was added to the inside rim of the tube. The NMR tube was then capped and sealed immediately, and the suspension inside was mixed well until clear. After 10 min, NaOD (6 μL , 40 wt % in D₂O) was added to adjust the pH to 12. The solution was kept at r.t for 10 min, and ¹H NMR spectrum was acquired to observe the disappearance of the peak corresponding to ethylene protons adjacent to carbonate group (4.2–4.3 ppm), indicating the cleavage of the side chains from the dextran backbone. Spin–lattice relaxation times (T1) of the triazole proton of the mannoside and H-1 of glucose residue of dextran were measured (2.29 and 1.53 s, respectively), and the values were used in the acquisition of ¹H NMR spectrum of the mixture of dextran, mannoside, and azido-TEG. The ratio of the integration of triazole and glucose H-1 gave the ratio of mannoside residue and glucose residue of dextran.

Release of OVA from Particles. Particles containing OVA were suspended at 5 mg/mL in either a sodium acetate buffer (30 mM, pH 5.0) or PBS buffer (pH 7.4, GIBCO, 1×), and gently agitated at 37 °C using a Thermomixer R heating block (Eppendorf). At desired time points, aliquots (100 μL) were removed and centrifuged at 14 000 $\times g$ for 10 min. The supernatant was collected, stored at –20 °C, and analyzed for the presence of protein using a microplate fluorescamine assay as described before.

Confocal Microscopy. Azido-AcDEX, Man-AcDEX, Gal-AcDEX, and HO-AcDEX microparticles (100 μg) were incubated with ConA-FITC (1 mg/mL) in PBS buffer (pH 7.4, 100 μL) with BSA (3 wt %) in the dark for 10 min at r.t. The particle suspension was then centrifuged (10 000 $\times g$, 3 min), washed with PBS buffer (1 mL \times 3), resuspended in PBS buffer (2 mL), and loaded in a 35 mm #1.5 glass-bottomed tissue culture dish (MatTek, MA). Imaging was performed using a Zeiss LSM 510 Meta confocal microscope with an Apo 63× water dipping objective (Carl Zeiss, Germany) using an excitation wavelength of 488 nm for FITC.

Particle Uptake. DC 2.4 cells were seed to a 24-well tissue culture plate (Corning-Costar, NY) at 3.0×10^5 cells/well (1 mL/well) in growth medium. After overnight incubation at 37 °C, the medium was aspirated, and suspensions of particles encapsulating BSA-FITC at 2 mg/mL (250 μL /well) were added. At the indicated time points, medium was removed, and cells were washed with PBS buffer (Ca²⁺-, Mg²⁺-, 3 \times 300 μL), trypsinized (50 μL trypsin/well), collected in PBS (300 μL), centrifuged (3000 $\times g$, 5 min), and fixed in PBS buffer (300 μL) containing 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Cells were analyzed for particle uptake by a Becton-Dickinson Biosciences FACSCalibur flow cytometer (San Jose, CA) with 488 nm excitation. The fluorescence of cells incubated with different particles was normalized by the amount of BSA-FITC encapsulated in each type of particles.

MHC Class I Presentation (B3Z) Assay. B3Z cells²¹ were freshly thawed and cultured in growth medium over 5 days. Dendritic cells (DC2.4, 5 $\times 10^4$ /well) were cultured overnight in a 96-well plate and subsequently incubated with particles encapsulating OVA or free OVA. After 6 h, the cells were washed carefully with the medium for B3Z cells (3 \times 100 μL /well), and B3Z cells (1 \times 10⁵/well) were added to the dendritic cells and co-cultured for 16 h. The medium was replaced with 100 μL of CPRG buffer (91 mg of chlorophenol red β-D-galactopyranoside (CPRG, Roche, USA), 1.25 mg of NP40 (EMD Sciences, USA),

and 900 mg MgCl₂ per 1 L of PBS). The plates were kept in the dark at r.t. for 72 h, and the absorbance at 595 nm was measured (microplate reader, SpectraMax 190, Molecular Devices).

Mannose Inhibition MHC Class I Presentation (B3Z) Assay. Dendritic cells DC 2.4 (5×10^4 /well) were cultured overnight in a 96-well plate. The medium was then aspirated and OVA-containing particles suspended in medium containing various concentrations of free mannose were added to the cells. After 6 h, the cells were washed carefully and B3Z cells (1×10^5 /well) were added to the dendritic cells/macrophages and co-cultured for 16 h. The medium was replaced with 100 μ L of CPRG buffer, the plates were kept in the dark at r.t. for 72 h, and the absorbance at 595 nm was measured.

Cell Viability Assay. Viability of cells was assessed using a standard MTT assay. Briefly, dendritic cells were seeded onto 96-well microtiter plates (Corning-Costar, NY) at 5×10^4 cells/well, respectively, for 24 h. The growth medium was replaced with 100 μ L suspensions of particles or solutions of their degraded compounds at various concentrations. After incubation for 24 h, a 3 mg/mL solution of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) in growth medium (40 μ L) was added directly to each well and the plates were returned to incubator. After 30 min (for DC 2.4 cells), purple crystals were observed at the bottom of each well. The plates were centrifuged (500 $\times g$, 5 min), and the medium was replaced with DMSO (200 μ L/well) to dissolve the crystals of formazan. Glycine buffer (25 μ L/well, 0.1 M glycine, 0.1 M NaCl, pH 10.5) was added, and absorbances at 570 nm were measured (Spectra Max 190 plate reader, Molecular Devices). Cell viability was normalized in percentage as the ratio of the absorbances measured from treated and untreated cells.

■ RESULTS AND DISCUSSION

Preparation and Modification of “Clickable” pH-Sensitive Dextran Particles. We developed a general protocol to prepare pH-sensitive AcDEX particles that can be modified to introduce multiple copies of a ligand on their surface. In a first step, a fraction of the hydroxyl groups of dextran were modified with an amphiphilic linker bearing a reactive functional group. This was followed by a second step in which the majority of the remaining hydroxyl groups were protected by acetal moieties to render the modified dextran organic-soluble in order to enable particle fabrication via a double emulsion process. We hypothesized that these amphiphilic linkers would be oriented to the interface between water and organic solvent phase during particle formation, so that the reactive functional groups would largely be located on the surface of the particles, thus accessible for future modification (Figure 1).

We used azido groups as the reactive functionalities at the termini of the linkers due to their ease of conjugation in aqueous phase via a variety of bio-orthogonal approaches such as azide–alkyne cycloaddition, Staudinger ligation, and others.^{25,26} In this instance, dextran was modified using carbonyldiimidazole-activated azido-triethylene glycol (azido-TEG) to afford azido-dextrans with various degrees of modification (Figure 1 and Supporting Information Figure S1).²⁷ These azido-dextran materials were then acetalated by reaction with 2-methoxypropene to obtain the acid-sensitive azido-AcDEX polymer. The resulting water-insoluble polymer containing 0.4 azido-TEG/glucose residue was then used to prepare particles using standard emulsion-based techniques,²² to afford nanoparticles with low polydispersity

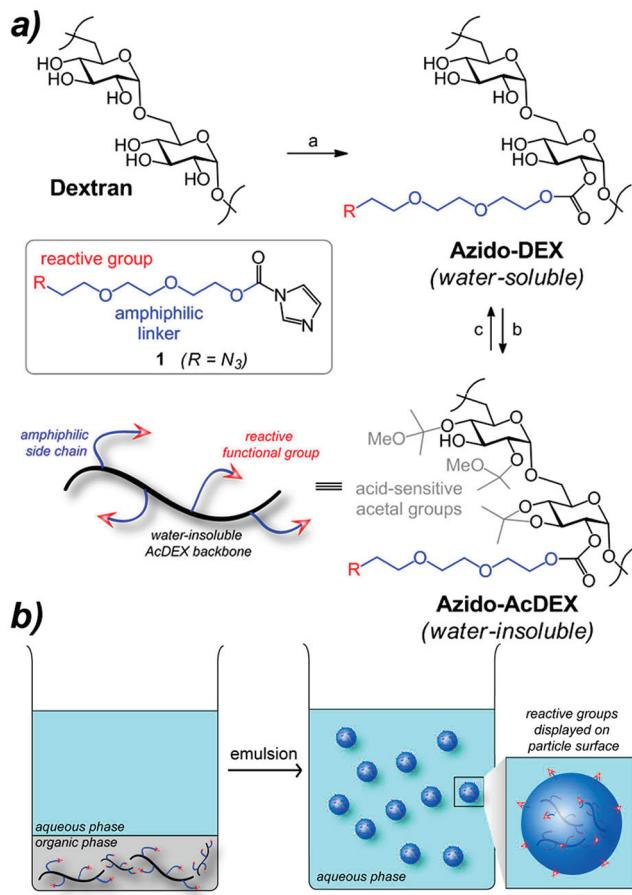


Figure 1. Preparation of “clickable” acid-sensitive dextran nanoparticles. (a) Synthesis of azido-AcDEX material. Reagents and conditions: a. 1, DMSO, DMAP; b. DMSO, 2-methoxypropene, pyridinium *p*-toluenesulfonate; c. acidic conditions (e.g., in endosomes). (b) Display of reactive functional groups on particle surface using emulsion.

(0.070) as measured by dynamic light scattering (DLS) (Figure 2). It should be mentioned that aggregation of particles was observed when using azido-AcDEX materials with degree of modification higher than 0.4 azido-TEG per glucose residue.

We then explored the surface modification of the azido-AcDEX nanoparticles with an appropriately functionalized mannose derivative. As a proof of concept, the ligand-assisted copper-catalyzed azide–alkyne cycloaddition (CuAAC) was used because it is a very efficient reaction for which Cu-ligands capable of significantly increasing the coupling rate are available.²⁸ Briefly, azido-AcDEX particles were suspended in HEPES buffer (0.1 M, pH 8.0) and stirred with an alkynylated mannose in the presence of Cu(I) and its ligand (BimC₄A)₃ (Figure 2).²⁹ To monitor the heterogeneous CuAAC reaction, aliquots of the particle suspension were removed at desired time points, washed to remove unbound molecules and salts, and lyophilized. The dry particles were then degraded in D₂O by addition of DCl to hydrolyze the acetal groups, followed by addition of NaOD solution to cleave the carbonate bonds linked to dextran (Supporting Information Figure S3). The solutions of degraded particles were then examined by ¹H NMR, and the signal corresponding to the triazole proton (the product of azide alkyne cycloaddition at 8.0 ppm was used to monitor the progress of the reaction (Supporting Information Figure S3). Using the integration of the triazole proton relative

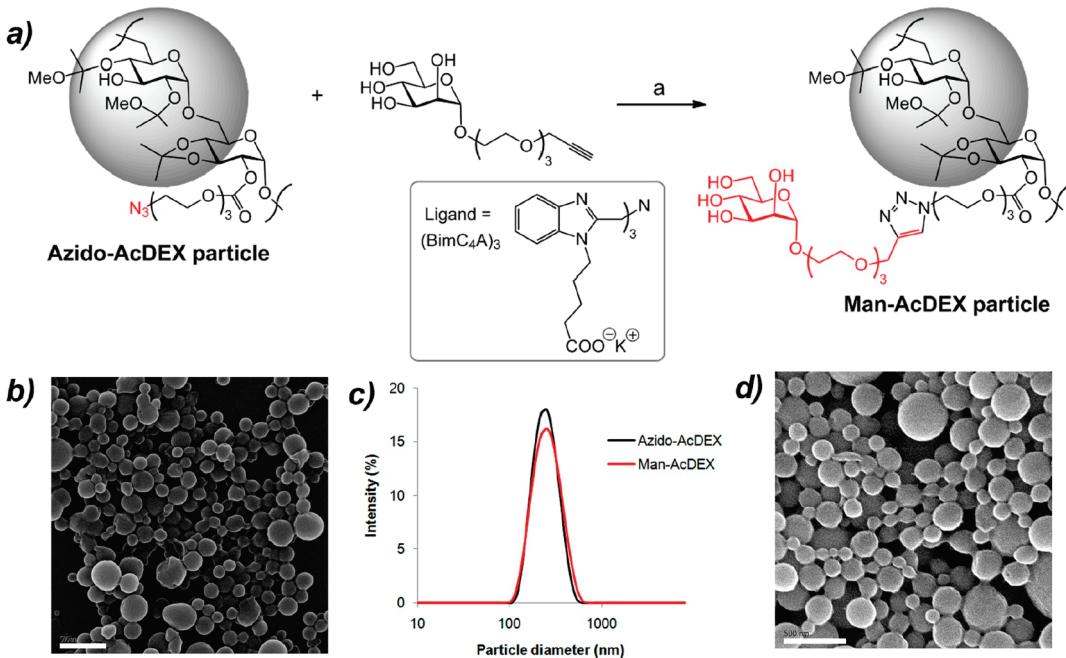


Figure 2. Synthesis of mannosylated AcDEX particles by CuAAC. (a) Synthetic scheme. Reagents and conditions: a. CuSO₄, ligand, ascorbic acid, HEPES buffer. (b) SEM image of Azido-AcDEX particles. (c) Size distribution of particles measured using DLS. (d) SEM image of Man-AcDEX particles (Scale bar = 500 nm).

to the anomeric proton, the number of mannose residues conjugated to particles were quantified by NMR and found to reach 2 mannose/100 glucose residues on dextran, leading to the estimate that a particle 200 nm in diameter would contain a few million mannose residues.

To verify that the reaction occurs at the surface of the particles, we prepared micrometer-sized particles large enough to visualize using confocal microscopy. These microparticles were subjected to the same reaction conditions as used for the nanoparticles and were surface-modified with mannoside, galactoside, or hydroxylated moieties, the latter two samples serving as negative controls. This was followed by incubation of the three types of modified microparticles with fluorescein isothiocyanate (FITC)-labeled concanavalin A (ConA), a reagent that specifically binds to α -mannose residues.³⁰ The particles were then washed to remove unbound reagents, and visualized using confocal microscopy (Figure 3). Only the mannosylated particles (Man-AcDEX) showed surface-associated fluorescence. In sharp contrast, galactosylated (Gal-AcDEX), hydroxylated, and unmodified azido-AcDEX particles showed no visible fluorescence, suggesting that the fluorescence halo around Man-AcDEX particles was due to specific binding between ConA and mannose residues on the particle surface.

Since all the above-mentioned particles could be used in the delivery of therapeutics, the cytotoxicity of the particles and that of the soluble materials obtained after their degradation were evaluated. All particles had a cytotoxicity comparable to that of PLGA and were fully degradable affording free dextran and nontoxic small molecules (Supporting Information Figure S4), supporting their potential use as biocompatible delivery vehicles.

Mannosylation of Particles Enhances MHC I Antigen Presentation by DCs. Recent reports have shown that soluble protein antigens taken up by DCs via mannose receptor³¹-mediated endocytosis bypass intracellular diversion and enter

the MHC I antigen presentation pathway directly.³² Therefore, targeting MRs of DC could constitute a useful route to enhance MHC class I antigen presentation. It is known that MRs contains 8 carbohydrate recognition domains (CRDs), 5 of which (CRDs 4–8) are involved in binding and endocytosis of mannose and related structures.^{33,34} Therefore, using multivalently displayed ligands to target the CRDs of MR or multiple MRs can be a reasonable strategy to deliver antigens into MR-mediated endocytosis pathway. A terminal mannose monosaccharide is known to be a favorable ligand for MRs, but other C-type lectins, including DEC-205, Endo180, dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN), Dectin-2 and langerin, can also interact with mannose-containing structures.³⁵ Hence, in practice, it is difficult to direct mannose-conjugates exclusively to MRs. However, differences in the spatial display of these mannose structures can produce a distinct set of carbohydrate recognition profile for each receptor. For example, DC-SIGN prefers to bind internal structures of high mannose oligosaccharides, therefore providing a certain degree of selectivity.^{36–40}

Particulate systems provide a scaffold to incorporate multiple copies of targeting groups, which can help to increase the overall binding avidity to the cell surface receptors.⁴¹ For example, mannose residues can be directed to the surface by self-assembly as seen in the preparation of liposomal or micellar carriers.^{42–45} However, few reports exist on the development of mannosylated polymer-based particles.^{46–51} Since it is not known if mannosylated particulate systems can improve the level of antigen presentation via the MHC class I pathway, we used our newly devised “clickable” particle system to study this effect. Mannosylation of our particles afforded a dense surface layer of mannose monosaccharide (up to 10⁶/particle) while also preserving the integrity of the particles and their acid-degradability, thus supporting their use as a carrier for the delivery of protein antigen to DCs.

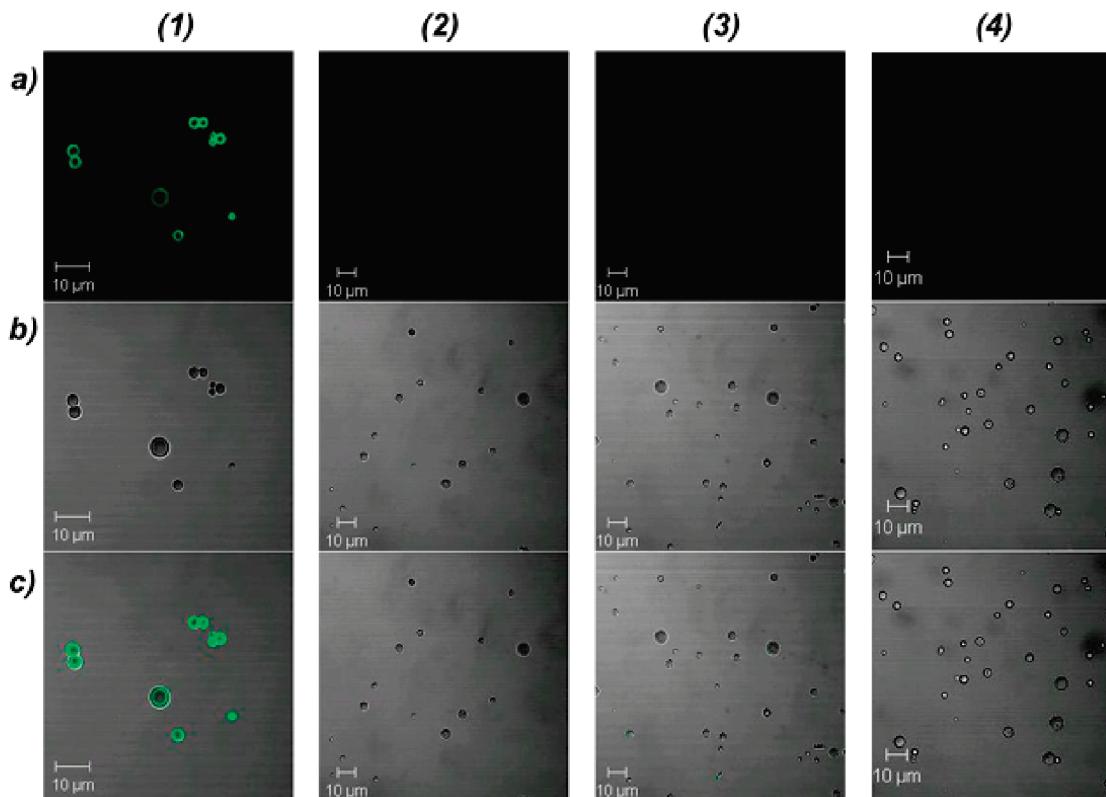


Figure 3. Confocal microscope images of microparticles ((1) Man-AcDEX particles, (2) azido-AcDEX particles, (3) Gal-AcDEX particles, (4) HO-AcDEX particles) after incubation with ConA-FITC. (a) Fluorescence image obtained using excitation wavelength of 488 nm; (b) differential interference contrast image; (c) merge of panels (a) and (b).

To test this hypothesis *in vitro*, we used a DC 2.4 cell line derived from murine bone marrow DCs⁵² that is known to express MRs on its cell surface.⁴³ We first performed a cellular uptake experiment to see whether the mannose residues on the particle surface were available to MRs, and how that might contribute to particle internalization. FITC-labeled bovine serum albumin (FITC-BSA) was encapsulated into AcDEX or azido-AcDEX particles, and the latter were modified by the protocol described above to yield dye-containing Man-AcDEX and, as a negative control, Gal-AcDEX particles (Figure 2). These particles were then incubated with DC 2.4 cells for fixed lengths of time before they were washed and collected. Flow cytometry was then used to analyze the cellular uptake of particles (Figure 4). At all time points, Man-AcDEX particles showed a 1.5- to 2-fold increased uptake when compared to Gal-AcDEX particles and unmodified azido-AcDEX or AcDEX particles, respectively. Given that DCs also perform phagocytosis, macropinocytosis, and other receptor-mediated uptake, mannosylation was not expected to cause a drastic increase in cellular internalization. However, this finding suggests that mannose residues were accessible to MRs on DCs and that mannose targeting groups may indeed help enhance cellular uptake to an appreciable extent.

MHC I presentation of antigen-derived peptides from DC 2.4 was examined using the B3Z cell assay.^{16,17,21,53,54} B3Z cells are CD8⁺ T cell hybridomas engineered to express β -galactosidase when their T cell receptor recognizes the OVA-derived SIINFEKL epitope (OVA_{257–264}) in the context of MHC I molecules. We first prepared AcDEX or azido-AcDEX particles encapsulating

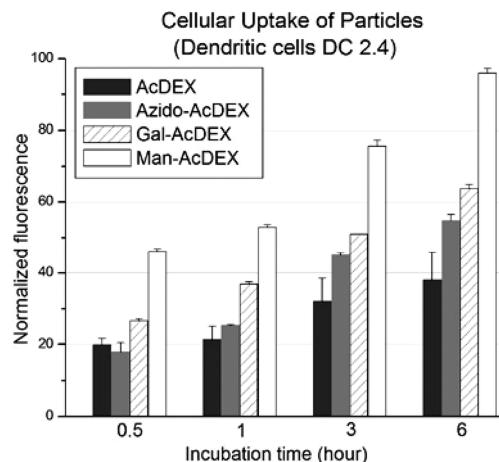


Figure 4. Cellular uptake of particles by DC 2.4 cells. Particles (2 mg/mL) were incubated with DC 2.4 at 37 °C. The fluorescence of cells was analyzed by a Becton-Dickinson Biosciences FACSCalibur flow cytometer with 488 nm excitation, and was normalized by the amount of BSA-FITC encapsulated in each type of particles.

OVA using a traditional double emulsion technique,⁵⁵ then surface-modified the latter with mannose or galactose, and incubated these particles with DC 2.4 cells (Figure 5). Antigen presentation was then determined using the B3Z assay. While all particulate formulations of OVA enhanced MHC I antigen presentation compared to free OVA, the performance of Man-AcDEX was about 5-fold better than that of Gal-AcDEX. This

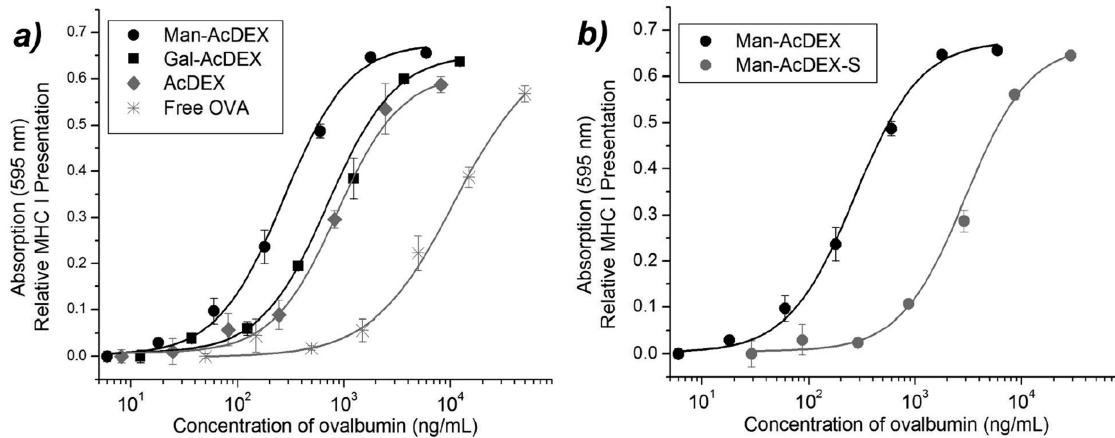


Figure 5. MHC I antigen presentation (B3Z assay) by DC 2.4. (a) Comparison of MHC I antigen presentation of OVA encapsulated in Man-AcDEX, Gal-AcDEX, and AcDEX particles, and free OVA. (b) Comparison of MHC I antigen presentation of OVA encapsulated in Man-AcDEX (fast-degrading) and Man-AcDEX-S (slow-degrading).

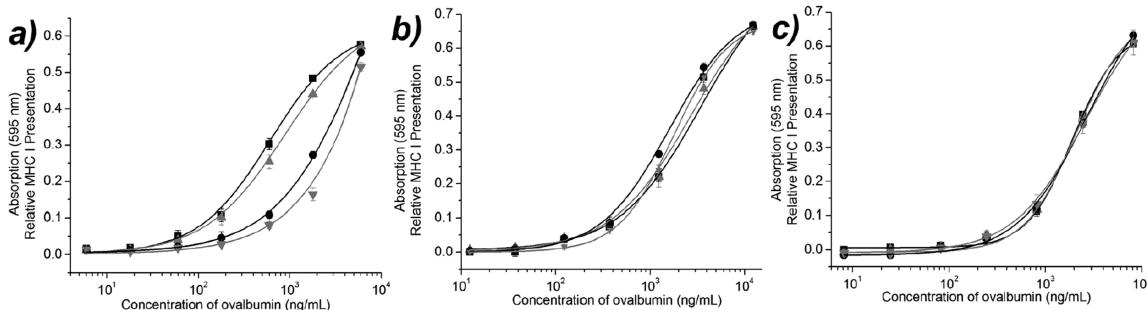


Figure 6. Mannose inhibition B3Z assay of DC 2.4. (a), (b), and (c) are Man-AcDEX, Gal-AcDEX, and AcDEX particles, respectively. Square, no addition of free mannose; triangle, with 10 mM free mannose; circle, with 50 mM free mannose; inverse triangle, with 100 mM free mannose.

finding could not be attributed to differences in antigen-release profiles, as both particles released antigen at similar rates at endosomal pH (Figure 5 and Supporting Information Figure S5). In addition, this may not be explained solely by the 1.5-fold difference in uptake of Man-AcDEX particles relative to Gal-AcDEX particles. One possible reason for this can be that these two types of particles go through different cellular uptake pathways, which causes differences in antigen presentation levels.

To verify that the enhanced MHC I antigen presentation from Man-AcDEX particles was indeed related to the surface-bound mannose groups, we incubated particles with DCs in the presence of free mannose, thus serving as a competitive ligand for the MRs (Figure 6). For Man-AcDEX particles, antigen presentation was suppressed upon addition of free mannose, and this inhibition was proportional to the concentration of free mannose. However, addition of free mannose did not inhibit antigen presentation from Gal-AcDEX or AcDEX particles. Collectively, these observations suggest that MRs and/or related C-type lectins were involved in the delivery and presentation of antigens in the case of Man-AcDEX particles, but not Gal-AcDEX or AcDEX particles.

In a previous report, we demonstrated that particle degradation and antigen release rates can influence the levels of antigen presentation observed—fast-degrading particles led to more efficient and enhanced class I presentation than slow-degrading particles.¹⁶ However, besides the differences in particle degradation

kinetics, particles made from the two types of materials may go through different internalization pathways due to their different surface chemistry. Hence, we prepared particles using a slower-degrading azido-AcDEX material (azido-AcDEX-S) and modified the particles with mannose to obtain slow-degrading Man-AcDEX particles (Man-AcDEX-S), to compare their antigen presentation efficiency to that of Man-AcDEX particles. Because both particles had the same surface modification, they should go through the same cellular uptake mechanism, so that the only difference would be their degradation rate. The use of the faster-degrading Man-AcDEX polymer led to a 10-fold increase in the levels of MHC I presentation compared to Man-AcDEX-S (Figure 5). AcDEX particles used in this study degraded faster than Man-AcDEX or Gal-AcDEX particles (Supporting Information Figure S5), and they showed similar antigen presentation abilities as Gal-AcDEX particles. Since receptors on DCs may also bind galactose, the difference in degrading speed between AcDEX particles and Gal-AcDEX particles might be partially offset.⁵⁶ On the other hand, possible uptake of Gal-AcDEX via receptors that recognize galactose may also compensate for the difference in antigen presentation compared to Man-AcDEX.⁵⁷

CONCLUSION

We have reported a particulate system made of a dextran-based pH-sensitive material that can be engineered to display

immunomodulatory properties. The feasibility of the novel system was evaluated by conjugating mannose to the particles using a simple procedure that complements the chemoselective ligation method we introduced earlier for dextran particles.⁵⁸ We have found that mannose monosaccharide-targeted cellular uptake by DCs led to enhanced MHC I antigen presentation compared to nontargeted particles. To our knowledge, this is the only example of a mannosylated particulate system leading to enhanced MHC I antigen presentation by DCs.

Delivery systems that can be engineered for immunomodulation are beneficial in a multitude of areas.⁵⁹ For vaccine development, conjugation of cell-surface receptor ligands can deliver antigens to specific intracellular pathways, providing opportunities to better control the antigen presentation to T cells. The materials used for these systems can also be modified by ligands to produce specific “danger” signals to manipulate the cytokine production and activation of APCs. Besides offering benefits for medical advances, such materials can also perform as model systems, providing help to better understand basic immunobiology.

■ ASSOCIATED CONTENT

S Supporting Information. Synthesis and characterization of small molecules. Quantification of mannose conjugated to particles. Cytotoxicity data of particles and their degraded products. Protein release curve from particles. Size of particles. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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