

Acetalated dextran is a chemically and biologically tunable material for particulate immunotherapy

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Materials that combine facile synthesis, simple tuning of degradation rate, processability, and biocompatibility are in high demand for use in biomedical applications. We report on acetalated dextran, a biocompatible material that can be formed into microparticles with degradation rates that are tunable over 2 orders of magnitude depending on the degree and type of acetal modification. Varying the degradation rate produces particles that perform better than poly(lactic-co-glycolic acid) and iron oxide, two commonly studied materials used for particulate immunotherapy, in major histocompatibility complex class I (MHC I) and MHC II presentation assays. Modulating the material properties leads to antigen presentation on MHC I via pathways that are dependent or independent of the transporter associated with antigen processing. To the best of our knowledge, this is the only example of a material that can be tuned to operate on different immunological pathways while maximizing immunological presentation.

acid-sensitive | biocompatible | encapsulation | polymer | vaccine

Vaccines are among the most specific, effective, and efficient tools developed to prevent a multitude of illnesses. Despite their many successes, vaccines often suffer from either inadequate immunogenicity or safety. An ideal vaccine would be highly stimulatory, yet completely safe, and would allow for the prevention or treatment of diseases that are otherwise difficult to treat. Particle-based immunotherapy holds great promise for the development of such a system because particles can mimic active pathogens but are not infectious (1). The safe and efficient activation of cytotoxic T lymphocytes (CTLs) for CTL-mediated cell killing remains a significant challenge for particulate vaccines (2). CTL activation is difficult because antigens must be internalized by antigen-presenting cells (APCs), the most effective of which are dendritic cells (DCs) (3). Furthermore, once internalized, antigens must be properly processed and presented via MHC I in a process that may require lysosomal escape (4) or rapid carrier degradation (5) to be effective. This process of presentation of extracellular antigens via MHC I is referred to as cross-presentation, and although it is an accepted phenomenon, the mechanisms of presentation are not completely understood (6). Being able to control the pathway through which cross-presentation occurs may have important biological or therapeutic ramifications.

Encapsulation of protein antigens in particulate delivery vehicles may help to realize an optimal CTL response by sequestering an antigen until it reaches the target cell, delivering it to a particular class of cells, and/or aiding in its presentation via the desired pathways at appropriate rates. Iron oxide microparticles have been successfully used for the prevention of model tumors (7), but iron oxide is completely nondegradable, which could potentially lead to toxic bioaccumulation. Another material used in particulate vaccines is poly(lactic-co-glycolic acid) (PLGA), which is biocompatible and easily processed into microparticles (8, 9). However, one possible drawback to PLGA in immunotherapy is that its degradation rate is slow and not tunable on timescales relevant to antigen presentation, potentially limiting optimal behavior. Previous work from our group has focused on making fast-degrading pH-responsive particles

based on cross-linked polyacrylamide (PA), which increase CTL activation relative to nondegradable materials (10–12). Although this system showed promise in tumor prevention studies, it is not likely to be viable therapeutically because of the nonbiodegradable linear polyacrylamide by-products that remain after particle degradation.

Acetalated dextran (Ac-DEX) is a new biocompatible polymer that can be prepared in a single step by reversibly modifying dextran (a biocompatible, biodegradable, Food and Drug Administration-approved, homopolysaccharide of glucose) with acetal-protecting groups (13). Ac-DEX has solubility properties orthogonal to those of the parent dextran, such that it is only soluble in organic solvents and completely insoluble in water. This, in turn, allows for the processing of Ac-DEX into microparticles by using standard emulsion techniques. Acid-catalyzed hydrolysis of the pendant acetals regenerates native dextran, and innocuous amounts of acetone and methanol as small molecule by-products. We sought to determine the potential of Ac-DEX for use as an immunotherapeutic agent by studying protein loading, chemical modification, degradation rate, and its ability to elicit both MHC I and MHC II presentation.

Results and Discussion

Synthesis of Ac-DEX Particles. The reaction of dextran with 2-methoxypropene in the presence of an acid catalyst leads to the formation of both cyclic and acyclic acetals (14, 15), causing a switch in solubility and providing a trigger for acid-mediated degradation (Fig. 1A) (13). Particles prepared by using a standard double-emulsion technique were isolated by centrifugation then dried by lyophilization without the need for cryoprotectant. Scanning electron microscopy (SEM) revealed spherical particles that ranged primarily between 100 and 400 nm in diameter in the dry state (Fig. 1B). Dynamic light scattering (DLS) was used to confirm the particle dimensions in an aqueous environment, and, in agreement with the SEM data, showed that the particles exist as a homogeneous population having a z-average diameter of 270 ± 115 nm (Fig. 1C). These physical properties were found to be consistent for all double-emulsion particles used in this work.

Loading of Ac-DEX Particles with Ovalbumin (OVA). Achieving an efficient and reproducible loading of proteins into Ac-DEX particles is important because immunotherapeutic efficacy may be antigen-limited (12). To study protein loading, Ac-DEX particles were generated in triplicate encapsulating OVA at feed values ranging from 5 to 100 μg of protein per mg of polymer. Following encapsulation, particles were degraded at pH 5 and the protein content was quantified by using fluorescamine (Fig. 2A) (16). Loadings of up to 74 μg of protein per mg of particle were achieved reproducibly. Loading efficiency was found to be

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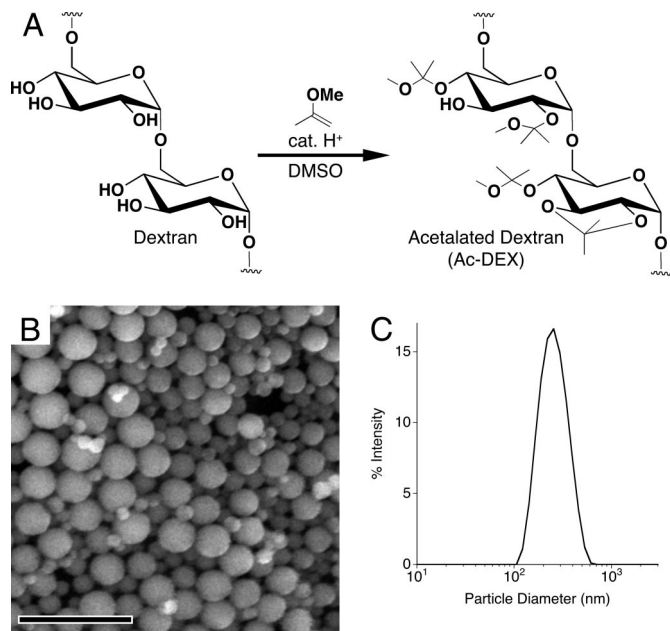


Fig. 1. Synthesis and characterization of Ac-DEX. (A) Single-step procedure for modification of water-soluble dextran to organic-soluble Ac-DEX. The resulting polymer contains acyclic acetals, cyclic acetals, and some remaining hydroxyls. (B) Typical SEM micrograph of particles made from Ac-DEX by using a double-emulsion procedure. (Scale bar, 1 μm .) (C) Typical DLS data for Ac-DEX particles suspended in water.

nearly quantitative at feed values up to 40 μg of protein per mg of polymer, which may be particularly beneficial for the encapsulation of proteins that are expensive or difficult to obtain in large quantities. At higher attempted protein loadings, the efficiencies decreased somewhat, but remained above 50%.

Because there may be a threshold amount of protein that must be delivered before efficient presentation can be observed, we determined the effect of particle loading on presentation efficiency. MHC I presentation of antigen-derived epitopes from bone-marrow-derived dendritic cells (BMDCs) was determined by using the B3Z CD8⁺ T cell hybridoma assay (17, 18). B3Z cells express a T cell receptor specific for the complex of MHC I and SIINFEKL (OVA_{258–265}), the immunodominant class-I restricted peptide derived from OVA, and express β -galactosidase on complexation, allowing for T cell activation to be quantified colorimetrically. Increasing the total amount of protein led to an

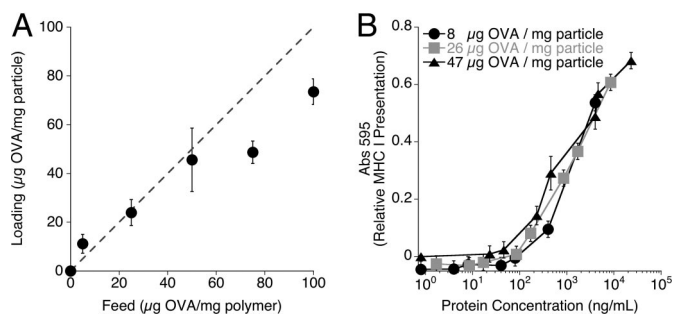


Fig. 2. Optimized protein loading in particles and its effect on MHC I presentation. (A) Protein encapsulation in particles compared with the feed amount of protein used in particle synthesis. Dashed diagonal line represents 100% loading efficiency. ($n = 3$, mean \pm SD). (B) Relative MHC I presentation from BMDCs versus protein concentration for particles with varying protein loadings. MHC I presentation correlates with protein concentration, not particle loading ($n = 3$, mean \pm SD).

increase in MHC I presentation regardless of the amount of protein encapsulated in the particles (Fig. 2B). No minimum threshold for protein loading was observed and all protein loadings demonstrated effective presentation. The lack of dependence of MHC I presentation on particle loading is indicative that Ac-DEX itself does not significantly affect presentation.

Acetal Content and Type Dictate Degradation Rate. Because acyclic and cyclic acetals have significantly different rates of hydrolysis (19), tuning the extent of modification with each kind of acetal might have important implications for the rate of particle degradation, which, in turn, could modulate biological activity. Acyclic acetals are formed quickly, whereas more stable cyclic acetals become prevalent after some equilibration period (19). To study the effect of the nature and proportion of each type of acetal on the properties of Ac-DEX we prepared a small library of Ac-DEX by using reaction times ranging from 0.5 to 1,500 min for high acyclic or high cyclic acetal content, respectively. Samples subjected to a reaction time of 2 min or longer precipitated on addition to water, indicating that acetalization had caused sufficient hydrogen bond disruption to abrogate water solubility. After isolation and purification of the various library compounds, the polymers were suspended in D₂O and the acetals were fully hydrolyzed by addition of DCl to determine both the extent of coverage of hydroxyls as well as the type of acetals that had been formed by using ¹H-NMR. As expected, initial acetal formation was found to be very rapid, reaching a maximum coverage of 89% of hydroxyl groups at 2.5 min and settling to a final coverage of 83% of hydroxyls over a longer period (Fig. 3A). The small decrease in coverage over the course of the reaction may be due to partial hydrolysis by trace water in the reaction. The relative proportion of cyclic and acyclic acetals was determined by comparing the relative concentrations of acetone and methanol since hydrolysis of an acyclic acetal yields one molecule each of acetone and methanol, whereas the hydrolysis of a cyclic acetal yields only a single acetone molecule (Fig. 3B). At reaction times of up to 5 min, acyclic acetals dominate the acetal population. However, as the reaction progresses, cyclic acetals replace a large portion of the acyclic acetals attaining a final ratio of two cyclic acetals for each acyclic acetal. After 1,500 min, 66% of all hydroxyls were found to be protected as cyclic acetals, which is the maximum theoretically possible given that only two of the three hydroxyls on each glucose repeat unit can be used to form a single cyclic acetal. To better understand the exact composition of the polymer over time, the degree of hydroxyl substitution (DS, number of hydroxyl modifications per 100 glucose units) of each type of acetal was determined (Fig. 3C). The initial burst of coverage was caused by the rapid formation of acyclic acetals, but with additional reaction time, the acyclic acetal content declined rapidly and the proportion of cyclic acetals increased. Given that cyclic acetals are significantly more stable than their acyclic counterparts, it is expected that the half-life of hydrolysis of any Ac-DEX can be regulated by controlling the amount of each type of acetal present, hence the synthetic protocol.

Based on this, particles were prepared from Ac-DEX samples that had been reacted for 2, 5, 10, 60, 360, and 1,500 min (designated as Ac-DEX2, Ac-DEX5, etc.) and were then suspended in either pH 5 or pH 7.4 buffered water at 37 °C. At given times, aliquots were removed, centrifuged, and the supernatants were assayed for the release of soluble dextran by using a bicinchoninic acid-based assay (Fig. 4A) (13, 20). Qualitatively, all particles were seen to degrade almost completely within the first 48 h at pH 5, but materials that had been acetalated over a longer period were significantly slower to degrade in solution. Half-lives of degradation were determined by empirical curve fitting for all samples except Ac-DEX2, which degraded too quickly to enable an accurate measurement. The calculated

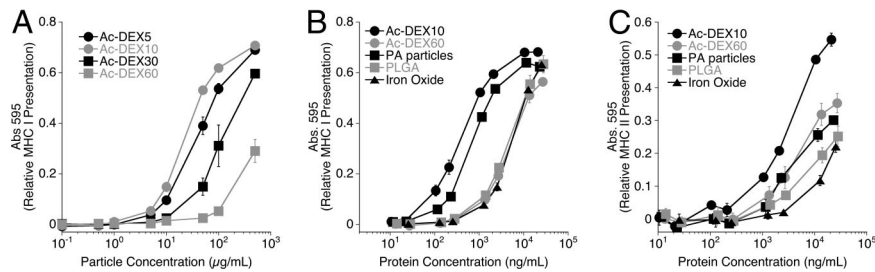


Fig. 5. Degradation rate is important for antigen presentation. (A) Relative MHC I presentation from BMDCs for OVA-containing particles made from Ac-DEX5, Ac-DEX10, Ac-DEX30, and Ac-DEX60 (corresponding to degradation half-lives of 0.27, 1.7, 11, and 16 h) ($n = 3$, mean \pm SD). (B) Relative MHC I presentation from BMDCs for OVA-containing particles made from Ac-DEX10, Ac-DEX60, PA particles, PLGA, and iron oxide. Quickly degrading materials (Ac-DEX10 and PA particles) show presentation at significantly lower protein concentrations ($n = 3$, mean \pm SD). (C) Relative MHC II presentation from BMDCs for OVA-containing particles used in B ($n = 3$, mean \pm SD).

from the materials that degrade more slowly. Despite this, MHC II presentation was higher with Ac-DEX10 particles compared with all other particle types tested. Because PLGA has been successfully used as a delivery vehicle for generating humoral responses to diseases such as tetanus and tuberculosis, it appears that Ac-DEX might be a more effective alternative for such therapeutic applications (23, 24).

Ac-DEX Induces TAP-Dependent or Independent Antigen Presentation.

The most accepted mechanism of MHC I cross-presentation is said to involve transport of antigen from the cytosol to the endoplasmic reticulum via the transporter associated with antigen processing (TAP) (4). However, another pathway of possible importance for antigen presentation from particulate vehicles has been shown to be TAP independent (4, 25, 26). We therefore sought to gain some insight into the pathway through which antigens encapsulated in Ac-DEX particles are presented on MHC I. It has been hypothesized that quickly degrading materials may cause enough change in osmotic pressure on degradation to disrupt lysosomal compartments and release protein into the cytosol (12). Presentation of peptides derived from proteins processed in the cytosol requires TAP; therefore, a study of TAP dependence may help to partially support or refute this osmotic disruption theory. B3Z cells were used to quantify MHC I presentation from BMDCs from either TAP^{-/-} mice or

WT mice that had been incubated with the particle sets described above (Fig. 6). In agreement with literature precedent, we found that presentation of SIINFEKL from PLGA particles was TAP independent, whereas iron oxide particles require TAP (26). Interestingly, TAP dependence does not correlate with the degradation rate of the material: acetal-cross-linked PA particles require TAP for presentation, but Ac-DEX10 does not, even though both materials degrade quickly and lead to similar levels of MHC I presentation in WT mice. These findings suggest that osmotic disruption of the lysosome cannot fully account for the observed behavior; otherwise, both materials would depend on TAP for presentation. We hypothesize that it is the chemical nature of a material, not necessarily its degradation properties, that may determine the processing and presentation pathways, that may determine the processing and presentation pathways used. This is supported by the observation that particles made from PLGA can present in a TAP-independent fashion and materials made from it are known to cause DC maturation (27, 28). Thus, it seems likely that not all materials used for protein delivery are inert to cellular recognition. It may be one such recognition event that determines which materials are able to access TAP-independent MHC I presentation pathways.

Seemingly contrary to this hypothesis, Ac-DEX10 does not require TAP for presentation, but Ac-DEX60 does. This difference might be rationalized as follows: even though Ac-DEX10 and Ac-DEX60 are both made from acetalated dextran, the two

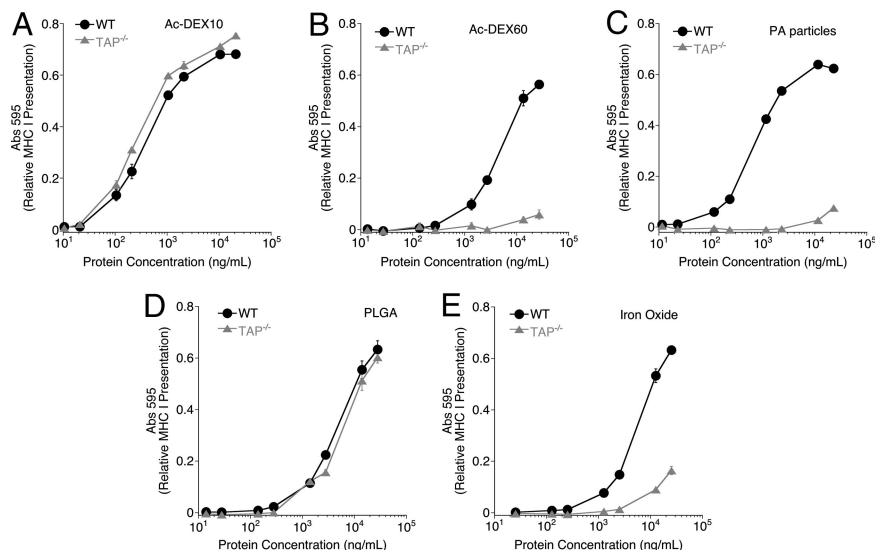


Fig. 6. TAP dependence of MHC I presentation for Ac-DEX particles compared with other known particulate formulations. Relative MHC I presentation from WT or TAP^{-/-} BMDCs incubated with OVA-containing particles made from Ac-DEX10 (A), Ac-DEX60 (B), PA particles (C), PLGA (D), or iron oxide (E) ($n = 3$, mean \pm SD).

materials may have surfaces with vastly different chemical characteristics. Because Ac-DEX10 particles degrade relatively rapidly, there may be significant acetal hydrolysis on the particle surface at pH 7.4 leading to the formation of glucose moieties in an otherwise hydrophobic context which may bind to cell surface receptors such as Toll-like receptors or lectin receptors and initiate endocytosis and presentation through a TAP-independent pathway. In contrast, Ac-DEX60 particles with their high proportion of cyclic acetals may not degrade quickly enough at pH 7.4 to present a significant concentration of unmodified glucose residues on their surface to bind to the same receptors. This unique ability to chemically determine the processing pathways available to Ac-DEX is extremely appealing for the prospect of achieving fine control over immune responses as well as gaining insight into the pathways involved in MHC I presentation.

Conclusion

Ac-DEX is a unique addition to the class of materials that can be used to fashion protein-encapsulating particulate delivery vehicles for immunotherapy. Because it is synthesized by a reversible modification of dextran, its biological fate is expected to be the same as that of unmodified dextran, which has been thoroughly vetted as a plasma expander (29). It can be processed into particles in the same ways as PLGA and other hydrophobic polymers and shares their high efficiencies of protein loading, but it possesses considerably increased flexibility in terms of degradation properties and biological processing. Tuning of the degradation rate of Ac-DEX results in greater than an order of magnitude improvement in MHC I presentation efficiency over PLGA or Ac-DEX with suboptimal degradation properties. Additionally, proteins encapsulated in quickly degrading Ac-DEX particles were presented via a TAP-independent pathway, whereas a slowly degrading Ac-DEX was not. To our knowledge, Ac-DEX is the only example of a material that can be tailored to use or avoid a particular pathway for processing, and presentation.

Methods

Animals and Cell Lines. Female C57BL/6, B6CBAF1, and TAP^{-/-} (B6.129S2-*Tap1*^{tm1Atp}) mice 6–8 weeks of age were purchased from the Jackson Laboratory. Mice were housed in accordance with National Institutes of Health guidelines. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, Berkeley. B3Z (CD8⁺) and KZO (CD4⁺) cells were generously donated by Nilabh Shastri (University of California, Berkeley). Cells were cultured in RPMI medium 1640 supplemented with 10% FBS, 2 mM GlutaMAX, 100 units/mL penicillin, 100 μ g/mL streptomycin, 1 mM sodium pyruvate, and 0.055 mM 2-mercaptoethanol (all from Invitrogen with the exception of the serum, which was from HyClone).

Preparation of BMDCs. Bone marrow was flushed from the tibia and femurs of euthanized mice by using a needle, passed through a 40- μ m nylon cell strainer, and cultured in 1 mL of Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 10% FBS, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 6 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF, Peprotech) at 1×10^6 cells/mL in 24-well tissue culture plates. On day 2, 1 mL of medium was added to each well. On days 4, 6, and 7, 1 mL of medium in each well was replaced with 1 mL of fresh medium. On day 6 or 7, BMDCs were isolated by using magnetic separation beads specific for CD11c⁺ cells (Miltenyi Biotec) according to the manufacturer's instructions. Isolated cells were used immediately in subsequent assays.

Synthesis of Acetalated Dextran (Ac-DEX) (13). In flame-dried flask under N₂, dextran ($M_w = 10,500$ g/mol, 1.00 g, 0.095 mmol) was dissolved in anhydrous DMSO (10 mL). 2-methoxypropene (3.4 mL, 37 mmol) was added followed by pyridinium *p*-toluenesulfonate (15.6 mg, 0.062 mmol). The reaction was quenched with triethylamine (1 mL, 7 mmol) after 4 h and the modified dextran was precipitated in dd-H₂O (100 mL). The product was isolated and washed by repeated centrifugation (14,800 $\times g$, 15 min) and thorough washing with dd-H₂O (2 \times 50 mL, pH 8). Residual water was removed by lyophilization,

yielding "acetalated dextran" (Ac-DEX) (1.07 g) as a fine white powder. Ac-DEX was further purified by dissolving in acetone (5 mL) and precipitating into water (500 mL, pH 8) then filtering and lyophilizing the resulting white powder.

Synthesis of Acetalated Dextran with Varying Acetal Coverage. A flame-dried flask under N₂ was charged with dextran ($M_w = 10,500$ g/mol, 1.30 g, 0.124 mmol) and dissolved in anhydrous DMSO (13 mL). 2-methoxypropene (4.6 mL, 48 mmol) was added and an aliquot (≈ 1.4 mL) was removed as a zero time point and added to H₂O (10 mL) containing triethylamine (200 μ L). Pyridinium *p*-toluenesulfonate (20.2 mg, 0.0803 mmol) was added and time points were taken as above for 10 s, 30 s, and 1, 2.5, 5, 10, 30, 60, 120, 180, 360, and 1500 min. Samples were placed in a -20 $^{\circ}$ C freezer immediately after quenching until all time points were collected. Each sample was isolated and washed by repeated centrifugation (4,600 $\times g$, 30 min) and thorough washing with dd-H₂O (2 \times 50 mL, pH 8). Residual water was removed by lyophilization.

Preparation of Double-Emulsion Particles Containing OVA. Microparticles containing OVA were made by using a double-emulsion water/oil/water (w/o/w) evaporation method similar to that described by Bilati et al. (30). In brief, a stock solution of OVA in PBS (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4, 40 μ L) was prepared. Ac-DEX (160 mg) was dissolved in CH₂Cl₂ (0.8 mL) and added to the OVA solution. This mixture was then emulsified by sonicating for 30 s on ice by using a probe sonicator (Branson Sonifier 450, 1/2" flat tip) with an output setting of 5 and a duty cycle of 80%. To this primary emulsion was added an aqueous solution of vinyl alcohol (PVA, $M_w = 13,000$ – $23,000$ g/mol, 87–89% hydrolyzed) (1.6 mL, 3% wt/wt in PBS) and sonicated for an additional 30 s on ice by using the same settings. The resulting double emulsion was immediately added to a second PVA solution (8 mL, 0.3% wt/wt in PBS) and stirred for 3 h, allowing the organic solvent to evaporate. The particles were isolated by centrifugation (14,800 $\times g$, 15 min) and washed with PBS (50 mL) and dd-H₂O (2 \times 50 mL, pH 8) by vortexing and bath sonicating (VWR Ultrasonic Cleaner 750) followed by centrifugation and removal of the supernatant. The washed particles were lyophilized to yield a white powdery solid (yields 85 \pm 4%, $n = 15$).

Preparation of Empty Particles. Particles that did not contain protein were made in the same manner as above omitting the first emulsion with the OVA solution.

Preparation of PLGA Particles. Polylactic-co-glycolic acid (lactide:glycolide 50:50, $M_w = 5,000$ g/mol, Sigma) particles were formed by using the same double-emulsion procedure as above, but by using PLGA in place of Ac-DEX.

Preparation of Iron Oxide Particles. Iron oxide particles (BioMag Amine, Polysciences) were purchased and conjugated to OVA according to the manufacturer's protocol.

Preparation of Polyacrylamide Particles. PA particles were synthesized and quantified as previously reported (31).

Quantification of Encapsulated OVA. Ac-DEX particles containing OVA were suspended at 2 mg/mL in 0.3 M acetate buffer (pH 5.0) and incubated at 37 $^{\circ}$ C under gentle agitation for 2 days by using a Thermomixer R heating block (Eppendorf). After complete degradation, aliquots were taken and analyzed for protein content using fluorescamine (0.3 mg/mL in acetone) and a microplate assay as described by Lorenzen et al. (32). Empty Ac-DEX particles were degraded in a similar fashion and used to determine background fluorescence.

Scanning Electron Microscopy. Microparticles were characterized by scanning electron microscopy with a S-5000 microscope (Hitachi) after sputter coating with 2 nm of a palladium/gold alloy.

Dynamic Light-Scattering Measurements. Particle size distributions and average particle diameters were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, United Kingdom). Particles were suspended in dd-H₂O (pH 8) at a concentration of 1 mg/mL and three measurements were taken of the resulting dispersions.

NMR Determination of Acetal Content. Ten milligrams of Ac-DEX was placed in an NMR tube and D₂O (0.7 mL) was added. Tubes were tilted to roughly 20 $^{\circ}$ from horizontal and a single drop of DCI was placed inside the rim of the tube. After capping and sealing the tube with parafilm, the tube was righted to vertical and vortexed for 1 min. Acyclic acetals were determined by comparing

the integration of the methanol peak (3.34 ppm, 3H) to the average of the dextran peaks (3.4–4.0 ppm, 6H). Cyclic acetals were determined by comparing the difference in the integration of the acetone (2.08 ppm, 6H) and methanol peaks to the average of the dextran peaks. All integrations were normalized to the number of protons on each molecule.

Particle Degradation and Detection of Soluble Polysaccharides. Empty Ac-DEX particles were suspended in triplicate at a concentration of 5 mg/mL in either a 0.3 M acetate buffer (pH 5.0) or PBS (pH 7.4) and incubated at 37 °C under gentle agitation by using a Thermomixer R heating block (Eppendorf). At desired time points, 100- μ L aliquots were removed, centrifuged at $14\,000 \times g$ for 10 min, and the supernatant was stored at -20 °C. The collected supernatant samples were analyzed for the presence of reducing polysaccharides by using a microplate redoxometric bicinchoninic acid based assay according to the manufacturer's protocol (Micro BCA Protein Assay Kit, Pierce).

MHC Class I and II Presentation Assays. For MHC class I presentation assays, BMDCs from C57BL/6 or TAP^{-/-} mice were plated at 5×10^4 cells per well (100 μ L per well) in 96-well tissue culture plates (Falcon, BD Biosciences), and cultured overnight. Particle samples were prepared by suspending particles at

5 mg/mL in BMDC medium (without GM-CSF). The samples were then diluted in medium, and in each well of the 96-well plate, medium was replaced with 100 μ L of diluted sample. After 6 h of incubation, the cells were washed with 150 μ L of B3Z/KZO medium, and freshly cultured B3Z cells were added to the wells at 1×10^5 cells per well (200 μ L per well in B3Z/KZO medium). After overnight cocubation, the medium in each well was replaced with 100 μ L of a solution containing 0.155 mM chlorophenol red β -D-galactopyranoside (Roche), 0.125% Nonidet P-40 Alternative (EMD-Calbiochem), and 9 mM MgCl₂ (Sigma) in PBS (pH 7.4, Invitrogen). The plates were developed at room temperature in the dark and the absorbance of each well was read at 595 nm by using a SpectraMax 190 microplate reader (Molecular Devices). Samples were measured in triplicate. MHC II assays were performed as above, with the exception that B6CBAF1 mice were used as the source of BMDCs, and KZO cells were used to detect antigen presentation.

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