

Acetal-Derivatized Dextran: An Acid-Responsive Biodegradable Material for Therapeutic Applications

Eric M. Bachelder, Tristan T. Beaudette, Kyle E. Broaders, Jesse Dashe,
And Jean M. J. Fréchet*

Department of Chemistry, University of California, Berkeley, California 94720-1460

*Corresponding Author: (phone) 510.643.3077; (fax) 510.643.3079;
(email) frechet@berkeley.edu

Supporting Information

General Procedures and Materials	S1
Experimental Methods	S2

General Procedures and Materials

All reagents were purchased from commercial sources and used without further purification unless otherwise specified. Water (dd-H₂O) for buffers and particle washing steps was purified to a resistance of 18 MΩ using a NANOpure purification system (Barnstead, USA). When used in the presence of acetal containing materials, dd-H₂O was rendered basic (pH 8) by the addition of triethylamine (TEA) (approximately 0.01%). ¹H NMR spectra were recorded at 400 MHz and ¹³C spectra were recorded at 100 MHz. To prevent acid catalyzed hydrolysis of acetal containing compounds, CDCl₃ was passed through a plug of basic alumina prior to recording NMR spectra. Multiangle light scattering (MALS) experiments were performed with a Waters 510 pump, a 7125 Rheodyne injector, a Wyatt Optilab differential refractive index detector and a Wyatt DAWN-EOS MALS detector. Absolute molecular weights determined from light scattering data were calculated using Astra software from Wyatt assuming a quantitative mass recovery (online method). Columns were thermostatted at 35 °C. MALS experiments run with THF as a solvent were performed using two 7.5 x 300 mm PLgel

mixed-bed C columns with a 5 micron particle size. MALS experiments run in aqueous conditions were performed using dd-H₂O with 5% acetic acid as a solvent and Viscotek C-MBMMW-3078 and C-MBHMW-3078 cationic columns (7.8 mm x 300 mm) in series. Fluorescence measurements were obtained on a Fluorolog FL3-22 spectrofluorometer (Horiba Jobin Yvon) or a Spectra Max Gemini XS (Molecular Devices, USA) for microplate-based assays, usage courtesy of Prof. Jonathan Ellman. Fourier transform infrared spectroscopy (FT-IR) was carried out on a 3100 FT-IR spectrometer (Varian, USA). UV-Vis spectroscopic measurements were obtained from samples in quartz cuvettes using a Lambda 35 spectrophotometer (Perkin Elmer, USA) or using a Spectra Max 190 (Molecular Devices, USA) for microplate-based assays, usage courtesy of Prof. Carolyn Bertozzi. RAW 309 and HeLa cells were obtained from ATCC (Manassas, VA) and grown according to ATCC's directions.

Experimental Methods

Synthesis of Acetalated Dextran (Ac-DEX). A flame-dried flask was charged with dextran ($M_w = 10\ 500$ g/mol, 1.00 g, 0.095 mmol) and purged with dry N₂. Anhydrous DMSO (10 mL) was added and the resulting mixture was stirred until complete dissolution of the dextran was observed. Pyridinium *p*-toluenesulfonate (15.6 mg, 0.062 mmol) was added followed by 2-methoxypropene (3.4 mL, 37 mmol). The flask was placed under a positive pressure of N₂, then sealed to prevent evaporation of 2-methoxypropene. After 3 h, the reaction was quenched with TEA (1 mL, 7 mmol) and the modified dextran was precipitated in dd-H₂O (100 mL). The product was isolated by centrifugation at 4 600 x *g* for 10 min and the resulting pellet was washed thoroughly with dd-H₂O (2 x 50 mL, pH 8) by vortexing and sonication followed by centrifugation and removal of the supernatant. Residual water was removed by lyophilization, yielding “acetalated dextran” (Ac-DEX) (1.07 g) as a fine white powder. IR (KBr, cm⁻¹): 3444, 2989, 2938, 1381, 1231, 1176, 1053, 853. ¹H NMR (400 MHz, CDCl₃): δ 1.39 (s, br, 25H), 3.25 (br, 6H), 3.45 (br, 2H), 3.60-4.15 (br, 12H), 4.92 (br, 1H), 5.13 (br, 1H).

Preparation of Double Emulsion Particles Containing OVA. Microparticles containing ovalbumin (OVA) were made using a double emulsion water/oil/water (w/o/w) evaporation method similar to that described by Bilati et al.¹ Briefly, OVA (10

mg) was dissolved in phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4, 50 μ l). Ac-DEX (200 mg) was dissolved in CH₂Cl₂ (1 mL) and added to the OVA solution. This mixture was then emulsified by sonicating for 30 s on ice using a probe sonicator (Branson Sonifier 450) with an output setting of 3 and a duty cycle of 10%. This primary emulsion was added to an aqueous solution of poly(vinyl alcohol) (PVA, M_w = 13 000 – 23 000 g/mol, 87-89% hydrolyzed) (2 mL, 3% w/w in PBS) and sonicated for an additional 30 s on ice using the same settings. The resulting double emulsion was immediately poured into a second PVA solution (10 ml, 0.3% w/w in PBS) and stirred for 3 h allowing the organic solvent to evaporate. The particles were isolated by centrifugation (14 800 x g, 15 min) and washed with PBS (50 mL) and dd-H₂O (2 x 50 mL, pH 8) by vortexing and sonication followed by centrifugation and removal of the supernatant. The washed particles were resuspended in dd-H₂O (2 mL, pH 8) and lyophilized to yield a white fluffy solid (135 mg).

Preparation of Empty Double Emulsion Particles. Particles that did not contain protein were made in the same manner as above omitting OVA.

Preparation of Empty PLGA Particles. Particles prepared from poly(DL-lactide-co-glycolide) (PLGA, 85% lactide, 15% glycolide) were made in the same manner as above substituting PLGA for Ac-DEX.

Preparation of Double Emulsion Particles Containing FITC-Dextran. Particles containing fluorescein isothiocyanate (FITC) labeled dextran were made in the same manner as above substituting FITC-dextran (M_w = 66 100 g/mol, 10 mg) for OVA.

Quantification of Encapsulated OVA. Ac-DEX particles containing OVA were suspended at a concentration of 2 mg/mL in a 0.3 M acetate buffer (pH 5.0) and incubated at 37 °C under gentle agitation for 3 d using a Thermomixer R heating block (Eppendorf). After the particles had been fully degraded, aliquots were taken and analyzed for protein content using the fluorescamine reagent and a microplate assay as described by Lorenzen et al.² Empty Ac-DEX particles were degraded in a similar fashion and used to determine a background fluorescence level. The results were compared to a standard curve and the mass of OVA encapsulated was calculated. The protein loading was 3.7 ± 0.4 wt % and the loading efficiency was 74%.

Single Emulsion Particle Preparation. Single emulsion particles encapsulating pyrene were prepared according to a procedure adapted from Jung et al.³ Briefly, Ac-DEX (49.9 mg) and pyrene (5.5 mg) were dissolved in CH₂Cl₂ (1 mL). This solution was added to a PVA solution (3 mL, 1% w/w in PBS) and emulsified by sonicating for 30 s on ice using a probe sonicator (Branson Sonifier 450) with an output setting of 5 and a duty cycle of 70%. The resulting emulsion was poured into a second PVA solution (50 ml, 0.3% w/w in PBS) and stirred for 4 h allowing the organic solvent to evaporate. The single emulsion particles were isolated in the same manner as described for the double emulsion particles above. The washed particles were resuspended in dd-H₂O (2 mL, pH 8) and lyophilized to yield a white fluffy solid (38 mg).

Quantification of Encapsulated Pyrene. The amount of encapsulated pyrene in single emulsion microparticles was determined by measuring pyrene's absorbance at 335 nm. Ac-DEX particles were weighed out in triplicate and dissolved in THF by sonicating the solutions for 2 min. The resulting solutions were diluted and the absorbance at 335 nm was determined. The loading of pyrene in the particles was calculated using pyrene's molar absorptivity in THF as reported by Venkataramana et al.⁴ The pyrene loading was 3.6 ± 0.5 wt % and the loading efficiency was 36%.

Scanning Electron Microscopy. Microparticles were characterized by scanning electron microscopy using a S-5000 microscope (Hitachi, Japan). Particles were suspended in dd-H₂O (pH 8) at a concentration of 1 mg/mL and the resulting dispersions were dripped onto silicon wafers. After 15 min, the remaining water was wicked away using tissue paper and the samples were allowed to air dry. The particles were then sputter coated with a 2 nm layer of a palladium/gold alloy and imaged. An SEM image of single emulsion particles is presented in Figure S1.

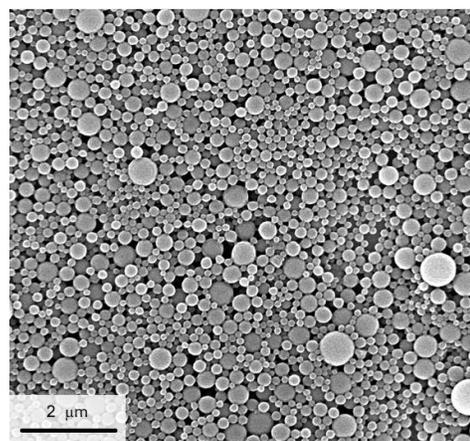


Figure S1. Representative SEM image of single emulsion Ac-DEX particles.

Particle Size Analysis by Dynamic Light Scattering. Particle size distributions and average particle diameters were determined by dynamic light scattering using a 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. Size distribution histograms are presented in Figure S2. The results in the text are presented as average particle diameters \pm half width of the distribution at half maximal height.

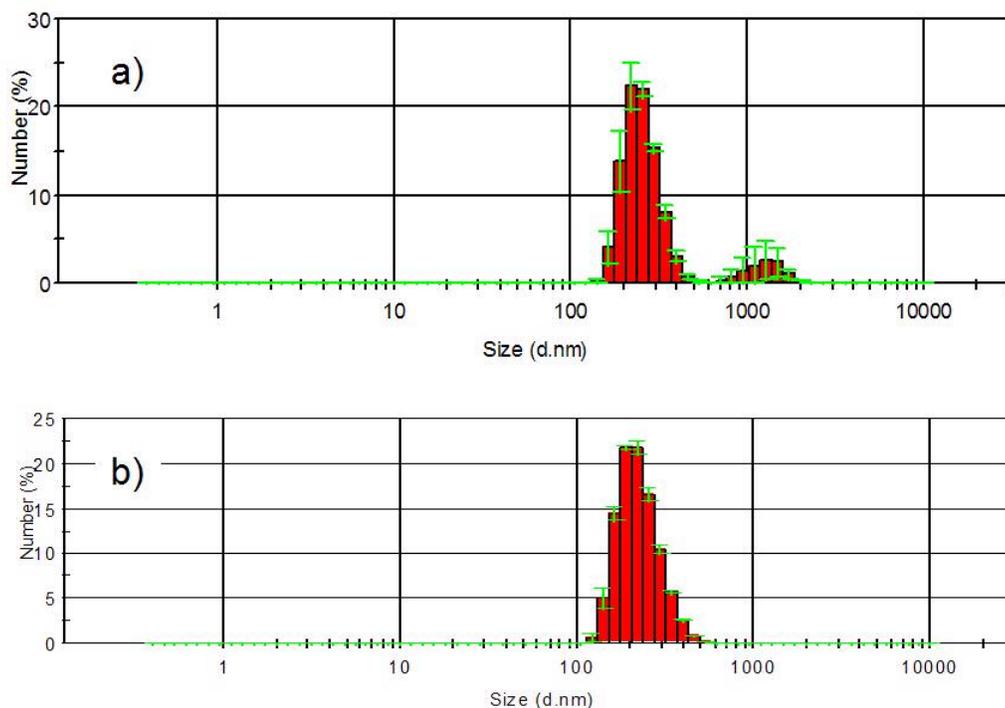


Figure S2. Size distribution histograms of (a) double emulsion particles encapsulating OVA or (b) single emulsion particles encapsulating pyrene.

Particle Degradation: Detection of Soluble Polysaccharides via BCA assay. Empty Ac-DEX particles were suspended in triplicate at a concentration of 2 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 using a Thermomixer R heating block (Eppendorf). At various time points, 120 μ l aliquots were removed, centrifuged at 14 000 \times g for 10 min to pellet out insoluble materials and the supernatant was stored at -20 °C. The collected supernatant samples were analyzed for the presence of reducing polysaccharides using a microplate reductometric bicinchoninic acid based assay according to the manufacturer's protocol (Micro BCA Protein Assay Kit, Pierce, USA; Figure 2a).⁵

pH-Dependant Release of FITC-Dextran from Ac-DEX Particles. This experiment was performed essentially in the same manner as above except FITC-dextran loaded particles were used instead of empty particles. The quantity of FITC-dextran in the supernatant samples was determined by measuring the emission at 515 nm with an excitation of 490 nm. The amount of FITC-dextran in each sample was calculated by fitting the emission to a calibration curve. The results of this experiment are presented in Figure S3.

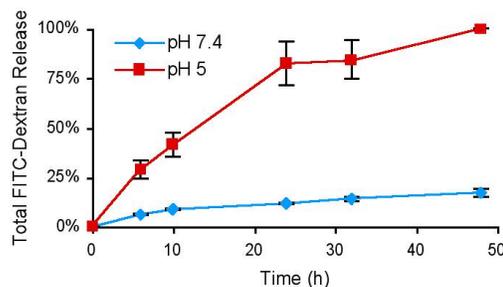


Figure S3. Release profile of FITC-dextran encapsulated in Ac-DEX particles at 37°C and in pH 5 or pH 7.4 buffer.

Particle Degradation: ^1H NMR Study. Empty Ac-DEX particles (9.5 mg) and deuterated PBS buffer (850 μL , pH 5.5) were added to an NMR tube, which was immediately flame sealed. This slightly higher pH value (compared to pH 5 used in the BCA experiment) was chosen to allow for the observation of degradation in better detail at earlier time points. An ^1H NMR spectrum was taken (initial time point) and the tube was placed in an oil bath heated to 37 °C. After various time points additional ^1H NMR

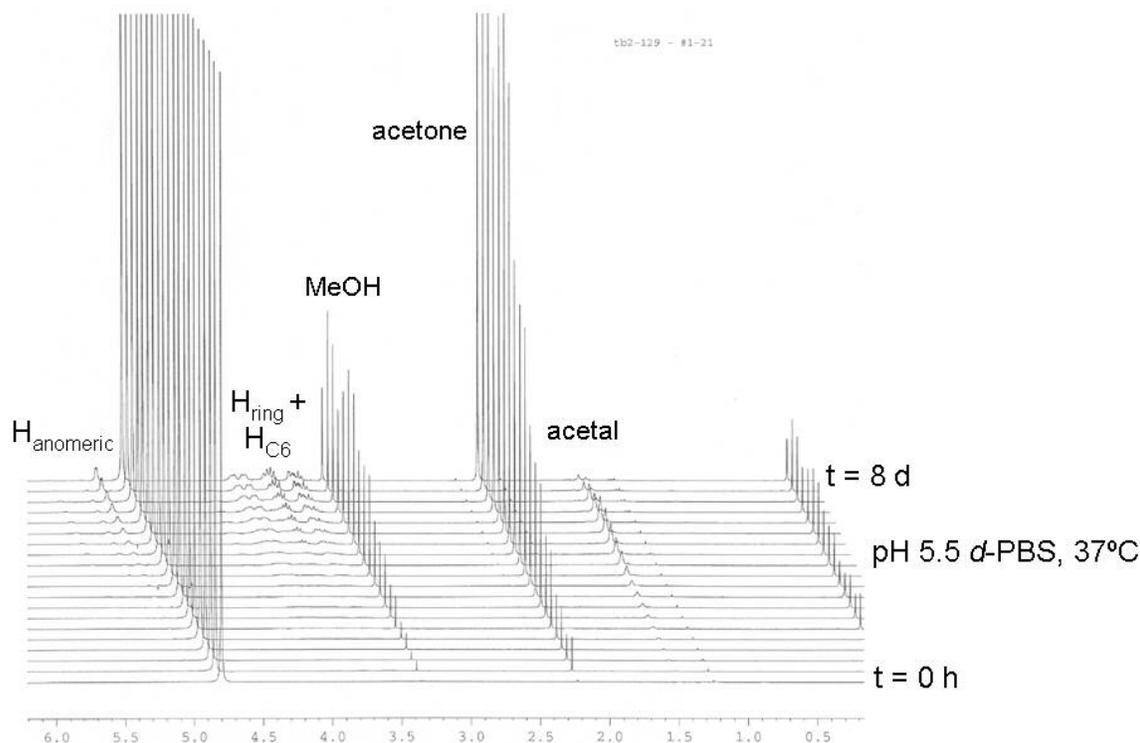


Figure S4. Stack plot of ^1H NMR spectra of empty Ac-DEX particles incubated in deuterated pH 5.5 buffer over time. Spectra are shown for the first eight days and are normalized with respect to the integration of the TMS peak.

spectra were taken and the appearance of acetone, methanol, and signals assigned to the methyl groups of cyclic isopropylidene acetals⁶ was measured as a ratio of these peaks' integral to the integral of the internal standard peak (3-(trimethylsilyl) propionic-2,2,3,3,d₄ acid, sodium salt). The data was normalized by dividing the values for acetone and the cyclic acetals by six and the values for methanol by three. A stack plot of the NMR spectra at various time points is presented in Figure S4 and spectrum of the final time point, which shows signals only from dextran, methanol and acetone is presented in Figure S5.

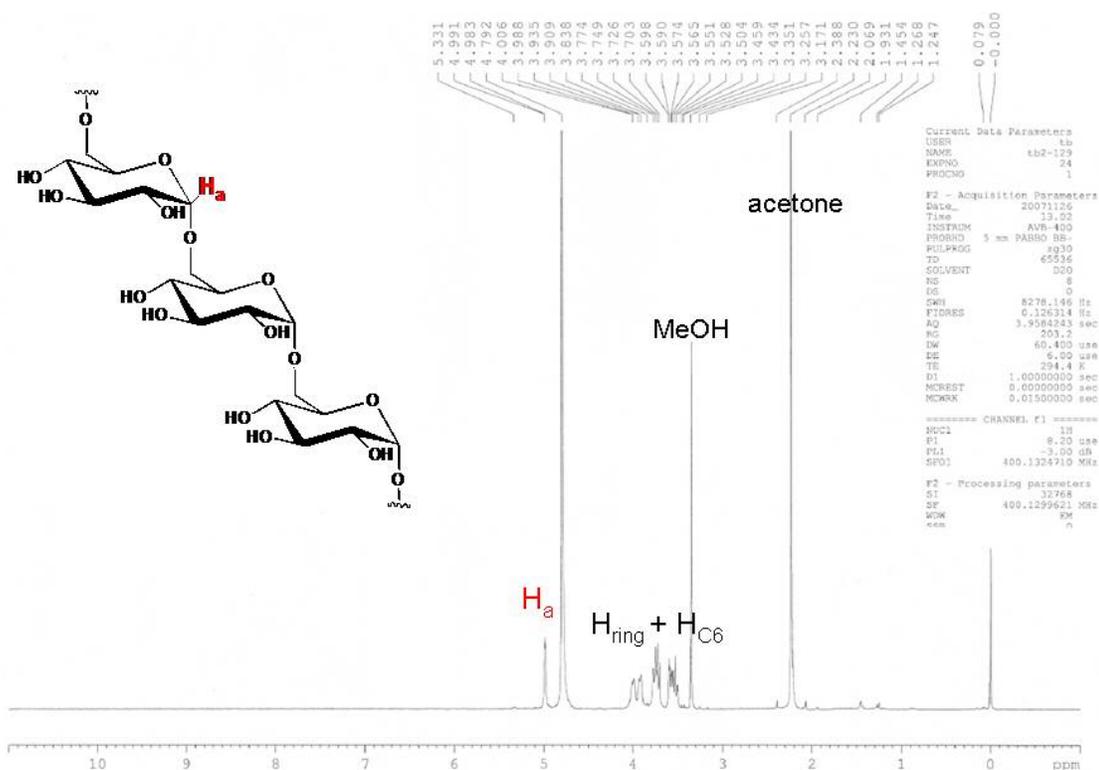


Figure S5. Final ¹H NMR spectrum of degraded Ac-DEX particles.

Particle Degradation: Digital Photography. Empty Ac-DEX particles were suspended at a concentration of 2 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 stirring. Digital photographs of the samples were obtained after various time points. The white object visible in some of the vials is a magnetic stir bar.

Cytotoxicity Studies. For cell viability experiments, degradation products of empty Ac-DEX particles were tested using RAW macrophages (Figure S6a). Additionally, empty Ac-DEX particles and empty PLGA particles were incubated with either HeLa cells (Figure S6b) or RAW macrophages (Figure S6c). The degradation products were obtained by incubating Ac-DEX particles in a 0.3 M acetate buffer (pH 5.0) at 37 °C under gentle agitation for 3 d. The resulting solution was desalted using a Microcon 3 centrifugation filter (Millipore, USA) and lyophilized. During the desalting and lyophilization steps the methanol and acetone released during degradation was removed. Before use in the viability experiment, the lyophilized degradation products were dissolved in medium, and methanol and acetone were added corresponding to the maximum amount of these byproducts released, as found in the ¹H-NMR degradation study described above.

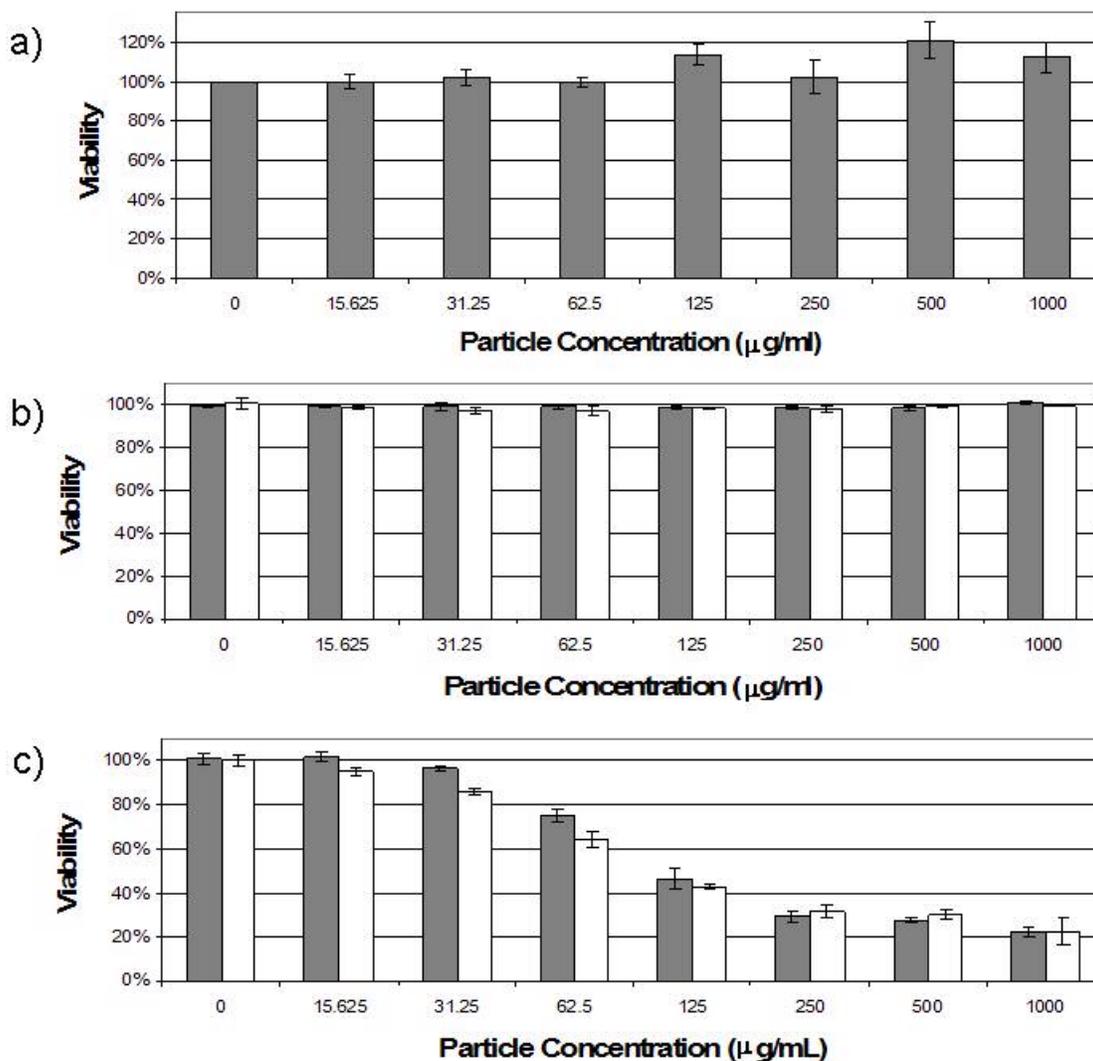


Figure S6. Cell viability as measured by LDH release after overnight culture with (a) Ac-DEX degradation products and RAW macrophages (b) Ac-DEX particles (shaded) or PLGA particles (white) with HeLa cells (c) Ac-DEX particles (shaded) or PLGA particles (white) with RAW macrophages. Correction for spontaneous LDH release was obtained from untreated cells. Maximum cell death was determined by freeze/thaw lysis.

For each viability experiment, 1×10^4 RAW macrophages or HeLa cells were seeded in a 96 well plate and allowed to grow overnight. Serial dilutions of the degradation products, empty Ac-DEX or PLGA particles were added to the cells, which were then incubated for 20 hours per the manufacturer's instructions. The next morning, the medium was removed and an LDH assay was performed to measure toxicity according to the manufacturer's protocol (Cytotox 96 NonRadioactive Cytotoxicity Assay, Promega, USA). Correction for spontaneous LDH release was obtained from untreated cells.

Maximum cell death was determined by freeze/thaw lysis. Results are presented as the mean of triplicate cultures \pm 95% confidence intervals.

MHC Class I Presentation (B3Z) Assay. B3Z cells, a CD8⁺ T-cell hybridoma engineered to secrete β -galactosidase when its T-cell receptor engages an OVA₂₅₇₋₂₆₄:K_b complex,⁷ generously donated by Prof. N. Shastri (University of California, Berkeley), were maintained in RPMI 1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum, 2 mM Glutamax, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin. 1×10^4 RAW macrophages were seeded overnight in a 96 well plate and subsequently incubated with OVA-containing Ac-DEX particles or free OVA. After 6 h, the cells were washed and 1×10^5 B3Z cells were added to the macrophages and cocultured for an additional 16 h. The medium was removed and 100 μ L 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 in 1 L of PBS) was added to each well. After 30 min, the absorbance at 595 nm was measured using a microplate reader. Results are presented as the mean of triplicate cultures \pm 95% confidence intervals (Figure 3).

References

1. Bilati, U.; Allemann, E.; Doelker, E., *Pharm. Dev. Technol.* **2003**, 8, 1-9.
2. Lorenzen, A.; Kennedy, S. W., *Anal. Biochem.* **1993**, 214, 346-348.
3. Jung, J.; Lee, I. H.; Lee, E.; Park, J.; Jon, S., *Biomacromolecules* **2007**, 8, 3401-3407.
4. Venkataramana, G.; Sankararaman, S., *Eur. J. Org. Chem.* **2005**, 2005, 4162-4166.
5. Doner, L. W.; Irwin, P. L., *Anal. Biochem.* **1992**, 202, 50-53.
6. (a) Cai, J. Q.; Davison, B. E.; Ganellin, C. R.; Thaisrivongs, S., *Tetrahedron Lett.* **1995**, 36, 6535-6536 (b) Debost, J. L.; Gelas, J.; Horton, D.; Mols, O., *Carbohydr. Res.* **1984**, 125, 329-335.
7. (a) Karttunen, J.; Sanderson, S.; Shastri, N., *Proc. Natl. Acad. Sci. U. S. A.* **1992**, 89, 6020-4 (b) Karttunen, J.; Shastri, N., *Proc. Natl. Acad. Sci. U. S. A.* **1991**, 88, 3972-6.