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Synthesis, Optimization, and Characterization of Camptothecin-Loaded Acetalated Dextran Porous Microparticles for Pulmonary Delivery

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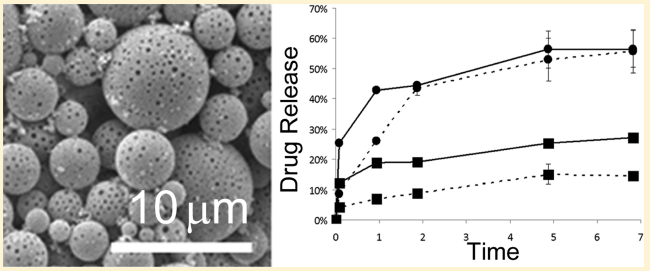
Supporting Information

ABSTRACT: We propose the use of a new biopolymer, acetalated dextran (Ac-DEX), to synthesize porous microparticles for pulmonary drug delivery. Ac-DEX is derived from the polysaccharide dextran and, unlike polyesters, has tunable degradation from days to months and pH neutral degradation products. Ac-DEX microparticles fabricated through emulsion techniques were optimized using a variety of postprocessing techniques to enhance the respirable fraction for pulmonary delivery. Tangential flow filtration resulted in a maximum 37% respirable fraction for Ac-DEX porous microparticles, compared to a 10% respirable fraction for poly(lactic-co-glycolic acid) (PLGA) porous microparticles. Ac-DEX microparticles were of an optimum diameter to minimize macrophage clearance but had a low enough theoretical density for deep lung penetration. Transepithelial electrical resistance (TEER) measurements showed that the particles did not impinge on a monolayer of lung epithelial cells in either air or liquid conditions. Also, the release of the chemotherapeutic camptothecin was shown to be tunable depending on Ac-DEX degradation time and molecular weight, and drug release was shown to be bioactive over a range of concentrations. Our results indicate that both release kinetics and fraction of burst release of drug from Ac-DEX porous microparticles can be tuned by simply changing the Ac-DEX polymer properties, affording a large range of formulation options for drug delivery to the pulmonary cavity. Overall, Ac-DEX porous microparticles show promise as an emerging carrier for pulmonary delivery of drugs to the alveolar region of the lung, particularly for the treatment of lung diseases.



KEYWORDS: *local drug delivery, inhalable dry particles, pulmonary microparticles, camptothecin, optimization of emulsion chemistry*

INTRODUCTION

Local pulmonary drug delivery of inhalable dry-powder (e.g., microparticles) systems is highly favorable because it can concentrate the drug at the site of disease, which reduces systemic toxicity, and can also result in smaller doses and less dosing frequency.[1] Also, pulmonary delivery is a preferred administration route over intravenous delivery, because it can be self-administered, which can lead to increased patient compliance and reduced health care costs. Currently, nebulizers, metered-dose inhalers (MDIs), and dry powder inhalers (DPIs) are used to deliver drugs to the pulmonary cavity; however, the absence of controlled release can result in local toxicity in the lungs.[2,3] Liposomal carriers and polymeric nanoparticles are also used to deliver drugs to the lungs, but instability problems of liposomal dispersions can result in drug leakage, and polymeric nanoparticles can lead to toxicity via phenomena like phagocytosis by alveolar macrophages.[1,4−14]

To achieve efficient drug delivery to the lung, the particle size and controlled drug release profile plays a crucial factor. For ideal pulmonary drug delivery, the physical characteristics of particles (carriers) are crucial.[7,8] There are two particle size considerations: geometric and aerodynamic diameters. Geometric diameter is the actual physical size of the particle, whereas aerodynamic diameter defines how a particle acts once it enters the air stream into the lungs and also determines the place in the lung where it will be deposited. Particle size should be in a very specific size range because if particles are too small, they will get exhaled; alternatively, if they are too large, they do not leave the inhaler. Furthermore, alveolar macrophages can phagocytose small particles for clearance through the mucociliary elevator; thus, particles should be large enough to inhibit phagocytosis by the macrophages. It is crucial to consider particle aerodynamic diameter and density which can indicate the activity of particles in the lung as well as the location in the lung where the particles deposit.[1,7] An optimal pulmonary particle would have an ideal geometric and

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aerodynamic diameter, while still offering controlled release of the drug payload.

We have developed a porous microparticle made of the new biodegradable polymer acetalated dextran (Ac-DEX) that can release the drug in a controlled fashion. Ac-DEX was chosen in contrast to commonly used materials like polyesters (e.g., poly(lactic-co-glycolic acid (PLGA)) because these polyesters produce acidic byproducts that may alter the local pH.[15] Conversely, Ac-DEX has a pH-neutral byproduct of dextran, an alcohol and acetone, a metabolic byproduct.[4] Also, Ac-DEX has a broader range of tunable degradation rates ranging from hours to months, so it can be formulated to release the drug over an extended amount of time and reduce polymeric buildup and potential toxicity in the lungs.[4,6] Moreover, Ac-DEX is acid-sensitive: at lower pH Ac-DEX degradation occurs more quickly, whereas at higher pH, the polymer degrades more slowly, resulting in a more controlled release of drug.[4,16] Here we show Ac-DEX microparticles can be made with geometric and aerodynamic properties ideal for alveoli lung deposition. To optimize the pulmonary delivery of these microparticles, we evaluated several particle processing conditions and measured the respirable fraction of the particles in an Andersen Cascade Impactor (ACI). The degradation and model chemotherapeutic (camptothecin) release rates of these microparticles have been characterized to display the flexibility of Ac-DEX as a polymer for this application. To evaluate toxicity, we examined whether our microporous particulate system results in any damage to the lung epithelial cells by measuring the transepithelial electrical resistance (TEER) of a lung epithelial cell monolayer. Finally, we evaluated the bioactivity of the chemotherapeutic camptothecin released from Ac-DEX microparticles by monitoring the viability of lung epithelial cancer cells cultured with drug loaded microparticles. This work aims to optimize Ac-DEX pulmonary carriers to deliver drugs to the lungs.

EXPERIMENTAL SECTION

Materials. The following materials were obtained from Sigma Aldrich (St. Louis, MO, USA): dextran from *Leuconostocmesenteroides* (71 400 and 9000−11 000 MW), pyridinium *p*-toluenesulfonate (PPTS, 98%), 2-methoxypropene (2-MOP, 97%, stabilized), tetraethylamine (TEA, ≥99%), poly(vinyl alcohol) (PVA, 87−89% hydrolyzed, 13 000−23 000 MW), poly(DL-lactide-*co*-glycolide) (PLGA, 85:15 lactide/glycolide, 50 000−75 000 MW), sodium acetate (SA), potassium phosphate (KP), dimethyl sulfoxide (DMSO; anhydrous, 99.9%), deuterium oxide (D_2O, 99.9 atom % D, 0.75 wt % TSP), deuterium chloride (DCl, 35 wt % solution in D_2O, 99 atom % D), and thiazolyl blue tetrazolium bromide (MTT). Glacial acetic acid (ACS grade), anhydrous ethanol, and phosphate buffer (PBS, pH 7.4) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Dichloromethane (DCM) and (*S*)-(+)-camptothecin (384.3 MW) were purchased from Honeywell Burdick & Jackson (Muskegon, MI) and Alexis Biochemicals (Enzo Life Sciences, Plymouth Meeting, PA), respectively. All reagents were used as received.

Synthesis and Characterization of Acetalated Dextran. *Synthesis of Acetalated Dextran with Varying Acetal Coverage and Molecular Weight.* The synthesis of acetalated dextran was completed as previously described.[4] Briefly, dextran with varying molecular weights (71 400 MW, denoted as 71 000, or 9 000−11 000 MW, denoted as 10 000) was used for the reaction. An oven-dried glass vial was charged with dextran and pyridinium *p*-toluenesulfunate (0.12 mmol). Under positive pressure of dry N_2, anhydrous DMSO was added to the glass vial, and the resulting mixture was stirred until complete dissolution of the dextran was observed. The reaction was initiated by adding 2-methoxypropene (74 mmol). Each MW dextran was reacted for either 5 min (fast) or 6 h (slow), resulting in four acetalated dextran (Ac-DEX) polymers thereby denoted as 10k-fast, 10k-slow, 71k-fast, and 71k-slow based on the molecular weight and reaction time. After the reaction was complete, the solution was quenched with triethylamine in excess. The product was then precipitated in nanopure water with approximately 0.02% triethylamine to render the water at a pH ∼9, isolated via vacuum filtration, and lyophilized to remove residual water. To further purify the polymer, it was then dissolved in anhydrous ethanol, reprecipitated, filtered, and lyophilized, resulting in a white powder.

NMR Analysis of Ac-DEX Acetal Content. The relative cyclic to acyclic ratio of acetal substitution of the Ac-DEX systems was determined via ^{1}H NMR using a 300 MHz Bruker 300 Ultrashield NMR, as described previously.[6] Briefly, Ac-DEX was weighed into an NMR tube, and D_2O and DCl were then added. Upon complete degradation of Ac-DEX, the solution was analyzed via NMR. Acyclic acetals hydrolyze to produce methanol and acetone whereas cyclic acetals produce only acetone. The cyclic acetal coverage of Ac-DEX was then determined via the relative ratio of acetone to methanol by comparing the integration of the acetone peak (2.08 ppm) to the methanol peak (3.34 ppm). All integrations were normalized to the number of protons on each molecule.

Preparation of Porous Pulmonary Microparticles. *Preparation of Camptothecin-Loaded Ac-DEX Microparticles.* Ac-DEX porous microparticles containing camptothecin were fabricated via a double emulsion water/oil/water (w/o/w) evaporation method. A stock solution of camptothecin was made by dissolving the drug in DMSO (7 mg/mL). This solution was then added to DCM to produce a final concentration of 0.7 mg/mL of camptothecin. Ac-DEX was dissolved in the DCM solution. PBS was added to this mixture, which was then emulsified by sonication for 10 s using a probe sonicator (1/2" flat tip, Misonix Ultrasonic Liquid Processor) at a 5% amplitude and power output of 30 W. This primary emulsion was added to an aqueous solution of PVA (3% w/w in PBS) and emulsified by homogenization for 30 s using Polytron PT 10-35 Homogenizer (Westbury, NY). 10k-5 min and 10k-6 h Ac-DEX particles were homogenized at 5200 rpm, whereas 71k-5 min and 71k-6 h systems were homogenized at 12 500 rpm. The mean particle size of microparticles was inversely proportional to homogenization speed; as a result, elevation of the stirring speed decreased the size of microparticles to make them more uniform between the different molecular weight and polymers (e.g., PLGA, Ac-DEX). On the basis of the study done by Yang et al.,[1] the level of breakage of the second stage emulsion depends on the amount of input power. Therefore, because 71k Ac-DEX is prone to irregular particle agglomeration because of its viscosity and longer chain length, it has to be stirred at the higher speed to get the desired particle size range. The resulting double emulsion was then added to another aqueous solution of PVA (0.3% w/w in PBS) and stirred for 2 h to allow for organic solvent evaporation and particle hardening. The microparticles were isolated by centrifugation (500*g*, 10 min) and washed once with PBS and twice with nanopure water (pH ∼9). The particles were then lyophilized (−70 °C and 25 mTorr) overnight to yield a white powdery solid.

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Preparation of PLGA Microparticles. Porous poly(DL-lactide-*co*-glycolide) microparticles were fabricated for comparison to the Ac-DEX microparticles. They were formed using the double emulsion method described above, but by using PLGA instead of Ac-DEX and homogenized at 19 400 rpm to ensure a particle size near that of the Ac-DEX particles.

Preparation of Blank Microparticles. Particles that did not contain camptothecin were made in the same methods as above by omitting camptothecin in the organic phase.

Post-Processing of Microparticles for Aerosolization Studies. After the porous microparticles had undergone the stirring/evaporation step, particles were postprocessed using various methods to determine whether or not this would have an effect on the aerosolization properties of the particles. Briefly, "lyophilize only" refers to the standard method as described previously; "sonicated, lyophilized" refers to particles that were transferred to a microcentrifuge tube after centrifugation and sonicated in a water bath for one hour prior to lyophilization; "resonicated" refers to particles that were initially lyophilized, then resuspended in 1 mL of basic water, sonicated in a water bath for one hour, and lyophilized again; "tangential flow filtration" refers to microparticles that were washed via tangential flow filtration after the spinning/evaporation step (no centrifugation) using a MiniKros Sampler Follow Fiber Filter Module (PES, 0.2 μm, 1050 cm^2, Spectrum Laboratories, Rancho Dominguez, CA), followed by lyophilization.

Characterization of Microparticles. *Determination of Microparticle Morphology via SEM.* The morphology of the microparticles was characterized by scanning electron microscopy (SEM) with a FEI NOVA NanoSEM 400. Microparticle samples were prepared by suspension in nanopure basic water, dropping on SEM preparation mount (Ted Pella; Redding, CA) and drying overnight at ambient conditions. The SEM preparation mount was then sputter coated with a palladium/gold alloy prior to imaging.

Size Analysis of Microparticles via Dynamic Light Scattering. The geometric diameter of the porous microparticles was determined via dynamic light scattering using a Nicomp 370 submicrometer particle sizer (Port Richey, FL). Particles were suspended at 0.5 mg/mL in basic water, vortexed for one minute, and sonicated in a water bath prior to analysis. The volume weight diameter and standard deviation values were recorded.

Analysis of Microparticle Density. The apparent porous microparticle density was determined via tap density.[8,14] A potion of 25−35 mg of particles was weighed into a glass tube, capped, and inverted. The tube was tapped on a hard surface 200 times to ensure efficient packing of the particles. The height of the particles of the tube was then measured using a caliper and the resulting volume was calculated. The density was then calculated as:

$$\rho = \frac{m}{V} \quad (1)$$

where ρ is the tap density, m is the recorded mass of the particles, and V is the volume determined by measuring the height of the particles in a tube with a known diameter.

Degradation Analysis of Ac-DEX Microparticles. Ac-DEX microparticles were analyzed for the presence of dextran using a bicinchoninc acid (BCA) assay which quantifies the amount of Ac-DEX degradation.[4,6] Blank particles were suspended in triplicate in either 0.3 M sodium acetate buffer (pH 5) or modified potassium phosphate buffer (pH 6.9). The samples were incubated at 37 °C on a shaker plate at 150 rpm. At desired time points, aliquots were removed and centrifuged at 20 000*g* for 10 min, and supernatant from each sample was stored in a 96 well plate at −20 °C until all time points were collected. After the completion of degradation, the supernatant samples were analyzed using a BCA assay according to the manufacturer's instructions (Micro BCA Protein Assay Kit: Pierce, Rockford, IL).

Quantification of Encapsulated Camptothecin. Ac-DEX microparticles containing camptothecin were suspended in triplicate in 0.3 M sodium acetate buffer (pH 5). Aliquots were taken and analyzed for camptothecin content by measuring the fluorescence of camptothecin at 360 nm/430 nm (excitation/emission) via a plate reader (FlexStation 3 benchtop multimode microplate reader). Blank Ac-DEX particles were analyzed in a similar fashion and used to determine background fluorescence. A standard curve of camptothecin in sodium acetate buffer (pH 5) was prepared to relate fluorescence to drug concentration. The encapsulation efficiency of camptothecin was determined using the following equation:

$$\text{encapsulation efficiency} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \quad (2)$$

In Vitro Release Studies of Camptothecin from Ac-DEX Microparticles. The release of camptothecin from microparticles was studied in a modified potassium phosphate buffer (pH 6.9) to represent the pH of the alveoli. Particles with and without camptothecin were suspended in triplicate in potassium phosphate buffer and were incubated at 37 °C on a shaker plate at 150 rpm. At desired time points, aliquots were removed, centrifuged at 20 000*g* for 10 min, and supernatant from each sample was stored in a 96 well plate at −20 °C until all time points were collected. The amount of camptothecin in the sample at a given time was analyzed by measuring the fluorescence of camptothecin at 360 nm/430 nm (excitation/emission) via a plate reader, subtracting the fluorescence background due to degraded particles without camptothecin and comparing to respective standard curve prepared in potassium phosphate buffer.

Microparticle Aerosolization Analysis Using an Andersen Cascade Impactor. The aerodynamic particle size and distribution was determined using an eight-stage Andersen Cascade Impactor (ACI, Courtesy of Battelle Memorial Institute, Columbus, OH). Hard gelatin capsules (size 3; Capsule Connection, Prescott, AZ) were loaded with 10−11 mg of microparticles, placed into a low-resistance dry powder inhaler (Rotahaler), and split open via the inhaler mechanism to release the particles after onset of flow. Glass filters (81 mm; Pall Life Sciences, Exton, PA) were placed on the ACI stages to minimize particle bounce or re-entrapment.[7] The microparticles were drawn through the ACI at a flow rate of 28.3 L/min for 30 s. The amount of particles deposited onto each stage was then determined by measuring the difference in mass of the glass filters before and after particle deposition. For the ACI, the effective cutoff diameters for each impaction stage were: Stage 0, 9.0 μm; Stage 1, 5.7 μm; Stage 2, 4.7 μm; Stage 3, 3.3 μm; Stage 4, 2.1 μm; Stage 5, 1.1 μm; Stage 6, 0.7 μm; Stage 7, 0.4 μm. The respirable fraction (RF) and total amount of particles collected were determined as:

$$\text{respirable fraction} = \frac{\text{mass of particles} < 4.7\ \mu\text{m(stages 2 through 7)}}{\text{total particle mass on all stages}} \quad (3)$$

$$\text{total collected} = \frac{\text{total particle mass on all stages}}{\text{initial particle mass loaded into capsule}} \quad (4)$$

The experimental mass mean aerodynamic diameter ($MMAD_T$) and geometric standard deviation (GSD) were determined using a Mathematica program written by Warren Finlay.

Cell Culture. A549 human lung adenocarcinoma cells were purchased from American Type Culture Collection (Manassas, VA). A549 cells were grown and maintained as per the manufacturer's guidelines. A complete cell medium was comprised of 450 mL of Ham's F-12 medium (Hyclone, Pittsburgh, PA), 50 mL of fetal bovine serum (Hyclone, Pittsburgh, PA), and 5 mL of penicillin-streptomycin (Fischer, Pittsburgh, PA). Cells were maintained at 100% relative humidity, 37 °C, and 5% CO_2 for the duration of the experiments.

TEER Analysis of Lung Adenocarcinoma Epithelial Cells. Transepithelial electrical resistance (TEER) analysis was performed on A549 human alveolar adenocarcinoma cells to determine the integrity of the cell monolayer upon exposure to porous pulmonary microparticles. The cells were seeded onto Transwell clear permeable filter inserts (0.4 μm polyester membrane, 12 mm, Corning, NY) at a density of 1×10^4 cells/cm^2. A portion of 500 μL of media was added to the apical side of the membrane with the cells, whereas 1500 μL was added to the basolateral side. TEER across the cell monolayers was measured via an epithelial volt ohm meter (World Precision Instruments; Sarasota, FL) using an EVOM2 chopstick electrode. TEER was measured from days 2 through 5 to allow for a baseline reading to be reached, ensuring a confluent cell monolayer. The presence of the cell monolayer for all samples was confirmed via light microscopy. The baseline TEER from a Transwell insert containing no cells was subtracted from all samples. On day 5, two sample conditions were induced including air-interfaced culture (AIC) where the supernatant on the apical side of the cells was removed to expose the layer to air and the basolateral side was reduced to 700 μL of medium. For the liquid-covered culture (LCC), 500 μL of media was maintained on the apical and 1500 μL on the basolateral sides of the culture. On day 6 prior to microparticle impingement, TEER was measured for both AIC and LCC conditions. For the appropriate measurement, the volume of AIC condition was adjusted with prewarmed medium so that both AIC and LCC were at the same conditions when measuring the resistance. Then, AIC was returned to its original condition, but LCC was maintained the same. Microparticle impingement consisted of spraying 0.7−1 mg of particles onto the cells via a syringe (1 mL syringe, 21 G needle). Light microscopy was used to verify dispersion of the particles across the cell monolayer. TEER was measured immediately following microparticle impingement and again at 4 and 24 h. After this time, cells grew beyond a monolayer.

***In Vitro* Cytotoxicity Analysis of Camptothecin-Loaded Microparticles.** A549 cells were plated at 1×10^4 cells/mL and incubated for 24 h in a 96-well plate. After 24 h, the media in each well were replaced with media containing camptothecin-loaded Ac-DEX microparticles ranging from 0.001 to 1 μM camptothecin, blank Ac-DEX microparticles, or free camptothecin at the same concentrations, all in triplicate. Because of the solubility considerations of camptothecin, a stock solution of 1 mM of camptothecin in DMSO was prepared. Because of the known cytotoxicity of DMSO and to maintain consistency, appropriate volumes of DMSO were added to all wells (including microparticles and 0 μM) to obtain a final concentration of 0.1% v/v DMSO in cell medium. The cells were incubated for another 48 h at these conditions.

After 48 h, an MTT assay was performed to determine cell viability. All supernatants were removed, and fresh media (150 μL) and a solution of MTT in media (5 mg/mL, 20 μL) were added to each well. The plate was incubated for 5 h to allow for the formation of purple formazan crystals. The supernatants were then removed, and isopropanol (200 μL) was added to each well to dissolve the crystals. The plate was analyzed using a plate reader (FlexStation 3 Benchtop Multi-Mode Microplate Reader) at an absorbance of 560 nm, and a background absorbance at 670 nm was subtracted to yield the cell viability. All viabilities were normalized with respect to 0 μM. Respective controls, such as wells with dead cells grown in the absence of media and blank wells, were also analyzed.

RESULTS

Characterization of Acetalated Dextran Polymer and Porous Microparticles. The synthesis of Ac-DEX from dextran and 2-methoxypropene resulted in final products consisting of both differing cyclic acetal coverage and molecular weight. Table 1 shows the Ac-DEX systems, their molecular weight, reaction time, the corresponding cyclic acetal coverage, and degradation at two different pH values. The cyclic acetal coverage for both 10k-fast and 71k-fast Ac-DEX was approximately 59%, whereas for 10k-slow and 71k-slow, the cyclic coverage was approximately 92%.

Table 1. Ac-DEX Polymer and Porous Particle Properties Including Cyclic Acetal Coverage for Ac-DEX Polymer via NMR and Degradation Analysis for Ac-DEX Porous Pulmonary Microparticles at pH 5 and pH 6.9

system	dextran MW	Ac-DEX reaction time	cyclic acetal coverage (%)	50% degradation at pH 5.0 (hrs)	degradation at pH 6.9 (hrs)
10k-fast	10 000	5 min	58.9	0.77	50% at 126
10k-slow	10 000	6 h	92.3	24.0	42% at 648
71k-fast	71 000	5 min	58.5	1.8	50% at 126
71k-slow	71 000	6 h	92.0	74.8	21% at 648

Ac-DEX porous pulmonary particles were degraded at both pH 5 and pH 6.9 to demonstrate their acid-labile nature. As shown in Table 2, at pH 5.0, 10k-fast and 71k-fast microparticles degraded 50% in 0.77 and 1.8 h, respectively, whereas 10k-slow and 71k-slow particles degraded at 1 day and 3.1 days. At pH 6.9, 10k-fast and 71k-fast microparticles degraded 50% in 126 h, whereas 10k-slow degraded 42% and 71k-slow degraded 21% in 27 days.

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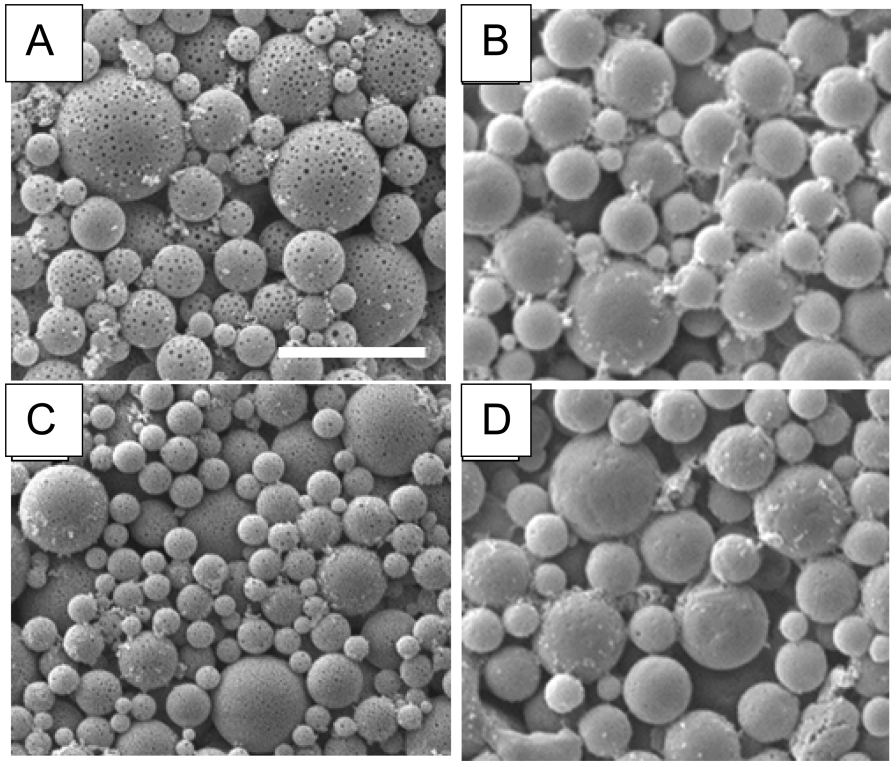

Figure 1. Representative SEM micrographs of Ac-DEX porous pulmonary microparticles fabricated from (A) 10k-fast, (B) 10k-slow, (C) 71k-fast, and (D) 71k-slow Ac-DEX. The scale bar for SEM images represents 10 μm.

Table 2. Physical Parameters of Ac-DEX Porous Microparticles Including: Camptothecin Encapsulation Efficiency (EE), Geometric Diameter Determined via Dynamic Light Scattering, Particle Density Determined via Tap Density Measurement, Theoretical Mass Mean Aerodynamic Diameter ($MMAD_T$) Determined from Geometric Diameter and Particle Density Calculations, Experimental Mass Mean Aerodynamic Diameter ($MMAD_E$), and Geometric Standard Deviation (GSD) Determined via ACI Studies

system	camptothecin EE (%)	diameter (μm)	density (mg/cm^3)	$MMAD_T$ (μm)	$MMAD_E$ (μm)	GSD (μm)
10k-fast	10.7 ± 0.019	5.0 ± 1.8	0.134	1.8	5.3	1.6
10k-slow	7.9 ± 0.019	5.3 ± 1.6	0.089	1.6	8.7	2.0
71k-fast	11.8 ± 0.020	4.6 ± 1.5	0.111	1.5	6.0	1.4
71k-slow	37.1 ± 0.019	6.7 ± 2.0	0.089	2.0	8.7	1.8
PLGA		7.2 ± 2.8	0.152	2.8	11.9	2.1

Ac-DEX porous microparticle morphology was confirmed via SEM imaging as demonstrated in Figure 1A–D. 10k-fast microparticles exhibited a porous external structure. 71k-fast microparticles also exhibited external porosity, although it was not as prevalent as with 10k-fast. Neither 10k-slow nor 71k-slow demonstrated external porosity. Select SEM micrographs displayed particles which had broken open to show their internal external structure. Examples of this for 10k-fast and 71k-fast can be seen in the Supporting Information (Figure S1), and similar internal morphology was seen for 10k-slow and 71k-slow (data not shown).

Camptothecin Encapsulation and Release Analysis. Microparticles were loaded with camptothecin, which is a model drug and chemotherapeutic. 71k-fast and 10k-fast exhibited a similar encapsulation efficiency (EE) for camptothecin (11.8 and 10.7%, respectively). However, the EE for 71k-slow was greater than that of 10k-slow (37.1 and 7.9%, respectively).

Figure 2A displays the release profiles of camptothecin from Ac-DEX porous microparticles. 10k-fast Ac-DEX microparticles released camptothecin faster than 71k-fast (up to 1 day), whereas after this time their release profiles were statistically the same. Both samples had a release of ~55% of the camptothecin after 5 days. Similarly, 10k-slow microparticles exhibited faster camptothecin release up to two days in comparison with 71k-slow samples. Approximately 40% of camptothecin was released for these samples after one week. All Ac-DEX microparticles demonstrated a burst release of camptothecin within the first day or two, followed by a more controlled release profile. Both 71k polymers exhibited a smaller burst release compared to their 10k counterparts.

As seen in Table 2, the geometric diameter of all microparticles was above the 1–4 μm size range, indicating that the particles will resist phagocytosis by alveolar macrophages.[17] Microparticles made of 10k-fast and 71k-fast were smaller than 10k-slow and 71k-slow (5.0, 4.6, 5.3, and 6.7 μm in diameter, respectively) whereas the PLGA porous microparticles were 7.2 μm in diameter. The density of the particles was determined via tap density measurements. Microparticles fabricated from 10k-fast and 71k-fast exhibited similar density values of 0.134 and 0.111 mg/cm^3; 10k-slow and 71k-slow both were 0.089 mg/cm^3, and PLGA was largest at 0.152 mg/cm^3.

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The theoretical MMAD ($MMAD_T$) was calculated from the geometric diameter and density of the microparticles. This relationship can be described through the following equation:

$$MMAD_T = \sqrt{\rho}\, d \quad (5)$$

where d is the geometric diameter and ρ is the density of the particle.[7] As seen in Table 2, the theoretical aerodynamic diameters for all porous microparticle systems was within the size necessary for effective pulmonary delivery deep within the lung, which is 1−5 μm. The calculated particle sizes for both Ac-DEX and PLGA porous microparticles ranged from 1.5 to 2.8 μm. The experimental MMAD and geometric standard deviation was determined via analysis of the ACI data. These values all ranged between 5.3 and 11.9 μm.

Aerosol Analysis of Microparticles. The aerosol properties of the microparticles were analyzed in regards to pulmonary delivery using an ACI and tap density measurements. The respirable fraction of particles collected in an ACI corresponds to those with a diameter less than 4.7 μm, which can be inhaled deep within the lung for drug delivery. Particles were postprocessed following their fabrication to determine this effect on their aerosol properties. From Table 3, it can be seen that 10k-fast and 71k-fast particles processed via tangential flow filtration (TFF) exhibited the highest respirable fraction values (37% and 27%, respectively), whereas all other systems (including those treated at different conditions and the PLGA samples) were much lower.

Table 3. Particle Processing Optimization of Ac-DEX Porous Microparticles for Andersen Cascade Impactor (ACI) Studies Where Particle Systems Were Exposed to Various Final Processing Conditions[a]

system	particle processing condition	respirable fraction (%)	total collected (%)
10k-fast	lyophilized only	13	44
10k-fast	sonicated, lyophilized	14	60
10k-fast	resonicated	18	51
10k-fast	tangential flow filtration	37	65
71k-fast	tangential flow filtration	27	58
PLGA	tangential flow filtration	10	70

[a]The respirable fraction includes particles which impacted on ACI stages corresponding to sizes below 4.7 μm, and the total collected includes all particles collected on the ACI stages.

TEER Analysis of A549 Cells. TEER measurements were performed on A549 lung adenocarcinoma cells to determine the effect of Ac-DEX and PLGA porous microparticles on cells exposed to either air or liquid culture conditions. The presence of an effective cell monolayer was confirmed by steady TEER values (20.5−28.0 Ω cm^2) after 5 days of culturing and the visible presence of cells seen via light microscopy (data not reported). Immediately after exposure to the microparticles, cells at AIC and LCC were measured for TEER. They were then measured again at 4 and 24 h after particle exposure. As seen in Figure 2C,D, for both AIC and LCC conditions, the TEER values remained the same or increased, indicating that the cell monolayer stayed intact and was not affected by the presence of either Ac-DEX or PLGA porous microparticles in comparison to the control of no microparticles.

Cytotoxicity Analysis of A549 Cells Exposed to Camptothecin-Loaded Ac-DEX Microparticles. A549 lung adenocarcinoma cells were exposed to camptothecin in its free form and encapsulated into Ac-DEX microparticles. After microparticle exposure, cell viability was analyzed via an MTT assay, and the results are shown in Figure 1B. For free camptothecin, a dose response curve was seen from 0.01 to 1 μM. Similarly, both 10k-fast and 10k-slow exhibited dose response curves in the same concentration range. Corresponding blank particles had no effect on the cells, showing the same viability as cells not exposed to particles.

DISCUSSION

Degradation Study of Ac-DEX Microparticles. To achieve enhanced pulmonary delivery, Ac-DEX is particularly promising as a new biodegradable polymer. Many experimental studies apply PLGA or other polyesters (e.g., poly(lactic acid), polycaprolactone) for pulmonary drug delivery applications;[7,8] however, several downsides still exist with their use. One concern is the fact that they have narrow range of degradation rates on the order of months resulting in a "slow release" of drug payload and potential buildup of polymer.[15] Ac-DEX overcomes this problem with tunable degradation rates which can range from hours to months, due to the differing rates of hydrolysis for cyclic and acyclic acetal groups.[6] Previously we have shown that the cyclic acetal coverage can be controlled by reaction time resulting in a broad range of degradation rates of polymeric nanoparticles at 10k MW.[18] Table 1 further supports this data where porous microparticles comprised of Ac-DEX polymer reacted for 5 min (fast) are compared to polymers reacted for 6 h (slow). At lung pH (~6.9), the microparticles degrade after hours or months, allowing for a broad range of degradation rates to accommodate the controlled release of the drug. Here we also display the ability to expand the range of microparticle degradation rates by also altering the MW of the dextran and therefore the MW of Ac-DEX. For the fast formulations of Ac-DEX, MW does not appear to have a significant influence on the degradation rates of the porous microparticles; however slow Ac-DEX seems to have significantly different rates of degradation at both pH 5 and 6.9. Perhaps this is due to the porosity of the particles, since degradation rates vary with MW for less porous nanoparticles (data not shown). The degradation rate for the fast polymers at both MWs is on the same order of magnitude at pH 5.0, further supporting this conclusion. In the comparison of porous microparticles formulated from 10k and 71k MW Ac-DEX, microparticles comprised of 71k Ac-DEX degraded over a longer length of time compared to 10k Ac-DEX. This difference could be accounted for in the increased porosity of the 10k microparticles. The discrepancy for surface porosity observed among the particles is most likely due to partial hydrolysis of fast-Ac-DEX during the particle synthesis step. After homogenization the particles are stirred for two hours in a pH 7.4 buffer. Since Ac-DEX can start degrading at this pH, the surface erosion of these particles would reveal the porosity inherent in these particles, and fast-Ac-DEX would degrade more quickly than slow-Ac-DEX. The tunability of microparticles fabricated with Ac-DEX illustrates the flexibility of this new material as well as its superiority as a polymeric material for porous microparticles for pulmonary delivery.

Camptothecin Encapsulation Efficiency and Release Profile. Drug encapsulation efficiency and *in vitro* drug release profiles studies were performed using camptothecin as a model chemotherapeutic agent (Table 2 and Figure 2A). The 71k-slow profile had a much higher EE compared to 10k-slow. We have shown that the 71k polymer can encapsulate the model

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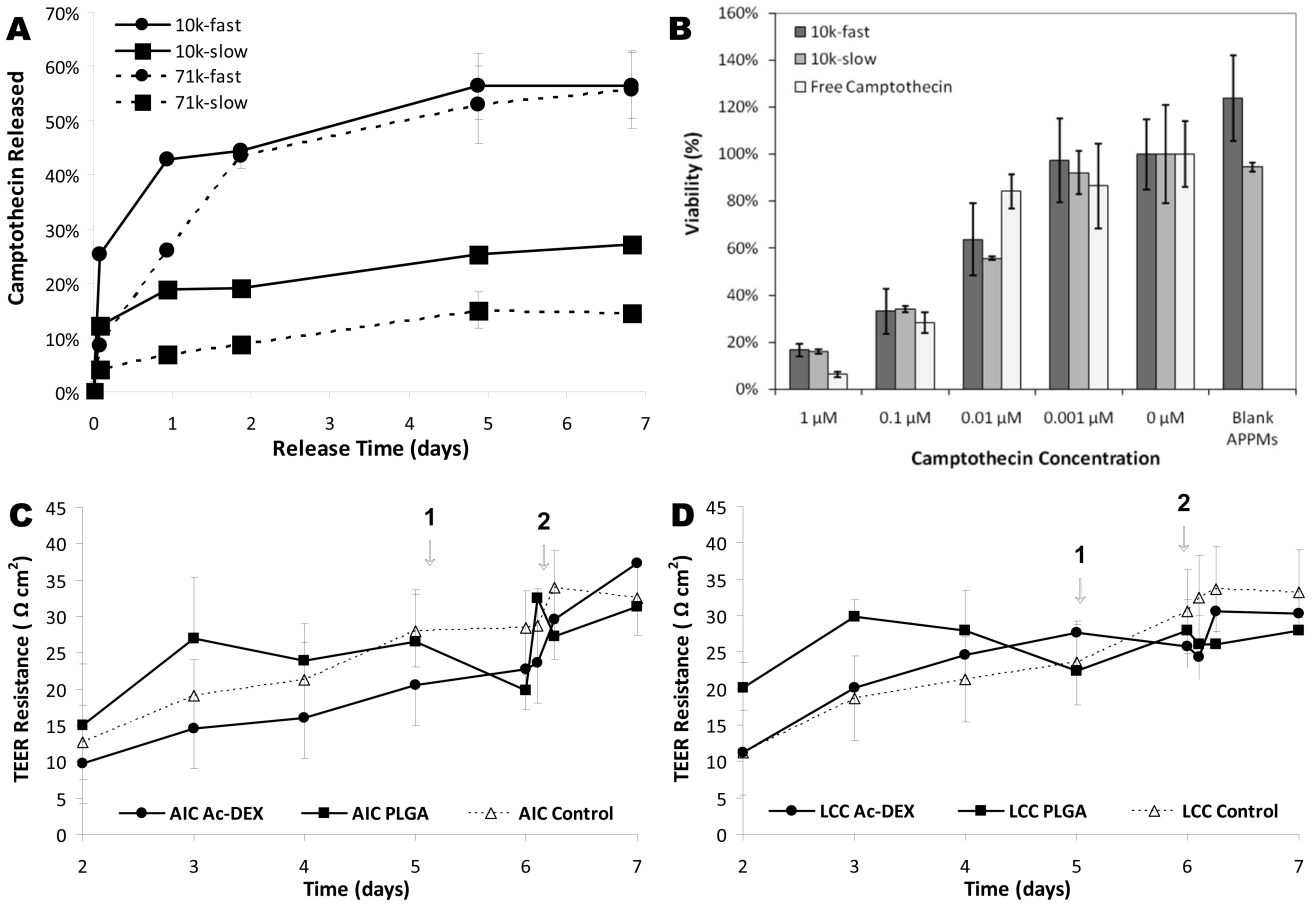

Figure 2. (A) Camptothecin drug release profiles for Ac-DEX porous pulmonary microparticles at lung pH 6.9 for one week. Data are presented as the average ± standard deviation. (B) Cytotoxicity analysis of A549 lung adenocarcinoma cells exposed to camptothecin in its free form and encapsulated in 10k Ac-DEX porous pulmonary microparticles at two different degradation rates. The cell viability is normalized with respect to 0 μM camptothecin concentration. (C–D) TEER analysis of A549 lung adenocarcinoma epithelial layers exposed to microparticles over time where C demonstrates air-interfaced culture (AIC) conditions and D is liquid-covered culture (LCC) conditions. TEER resistance was measured at 2, 3, 4, and 5 days to ensure a tight monolayer had been achieved. At day 5 (arrow labeled 1), AIC samples were exposed to air on the apical side of the cells (LCC samples remained the same), and all cells were exposed to microparticle samples on day 6 (arrow labeled 2). TEER measurements were then made immediately after microparticle exposure and again at 4 and 24 h. Ac-DEX samples were 10k-fast microparticles, whereas the control samples were cells not exposed to microparticles but exhibited to the same conditions otherwise. All data are presented as the average ± standard deviation. n = 3 for all analysis.

drug rapamycin at a higher efficiency compared to the 10k polymer.[16] Due to the relatively higher MW, 71k-slow has a higher encapsulation efficiency. However, for 71k-fast and 10k-fast there was no major difference in the encapsulation efficiency. We hypothesize that, during the formation of particles, there is partial degradation that would negate any increase in encapsulation efficiency due to MW.

In addition, by varying encapsulation efficiencies and thus loadings with Ac-DEX porous microparticles, camptothecin loaded Ac-DEX microparticles have variable release kinetics at pH 6.9 that depend on the polymer's molecular weight as well as the degradation rate. The slowest degrading microparticle (71k-slow) had the smallest amount of burst release and then maintained a linear release rate, achieving 10% total release after 7 days. In contrast, 71k-fast released roughly 50% of its drug payload in the same amount of time, comparable to the release kinetics of 10k-fast. The 10k-slow displayed a similar steady state linear trend as 71k-slow but had a burst release almost four times greater than the higher molecular weight particles. The variation in burst release is most likely correlated to the surface porosity of the particles. This sort of dosage flexibility that burst release offers would not be available with other polymers like PLGA. A comparable small molecule budesonide had a burst release rate from porous PLGA microparticles of less than 5% of drug at 2 h (compared to 4.1–25.4% with Ac-DEX at 2 h) for microparticles laden with variable amounts of porogens.[19] We have illustrated here that through simple changing of the molecular weight or reaction time of the Ac-DEX polymer, unique release kinetics result, which are not afforded with PLGA porous microparticles.

Two Particle Size Considerations: Geometric and Aerodynamic Diameters. Previous studies have shown that ideal microparticles for pulmonary drug delivery should be highly porous with a density less than ~0.4 mg/cm^3.[7,8] Additionally, the geometric diameter of the microparticles should be greater than 4 μm to minimize phagocyte clearance but have a small aerodynamic diameter around 1–3 μm.[7,8] Our results (Table 2) indicate that microparticles fabricated from Ac-DEX have theoretical aerodynamic diameters that fall within the ideal range, whereas the experimental aerodynamic diameters were all above 5 μm. It is expected that experimental diameters are greater than theoretical because it has been

shown that with the ACI there exists an indirect relationship between particle aerodynamic diameter and the stage of the impactor that the particle deposits.[7,8] The discrepancy in particle diameter is accounted for by the fact that porous particles are lighter in mass and correspondingly larger than those used for impactor calibration.[8] Furthermore, porous particles exhibit a reduced lubrication layer repulsion than small nonporous particles of similar aerodynamic size so that deposition by inertial impaction may increase.[7] Overall, this indicates that the experimental Andersen Impactor diameter ($MMAD_E$) results may not be true for porous particles since the plate to size ratio is not based solely on aerodynamic size but on a combination of aerodynamic and geometric sizes. Thus, others have concluded that particle mass density can be the main determinant of aerodynamic diameter of microparticles.[7] The reported Ac-DEX microparticle densities were all less than 0.25 mg/cm^3, and the geometric diameters were large enough to avoid phagocytic uptake, illustrating that these particles could be ideal for deep lung depositions of drug.

For most polymers, large porous microparticles of the type presented here are fabricated through a double emulsion procedure. With this fabrication method, several factors influence the size of particles such as polyvinyl alcohol (PVA) concentration in the external water phase, homogenization speed, and polymer concentration.[1,13] In addition to particle diameter, which can be reasonably tuned by changing process parameters, the aerosolization efficiency is also a key consideration since microparticles are prone to agglomerate when formed through emulsion processes (Table 3). To this end, all Ac-DEX microparticles were synthesized through a standard double emulsion method and various post processing techniques were performed to improve the respirable fraction. The respirable fraction is an indicator of the aerosolization efficiency of the particle. In an ACI, the respirable fraction represents the total weight on all the plates corresponding to sizes below 4.7 μm. Although ACI studies are not ideal for porous particle deposition as previously discussed, it can be used for valid, albeit conservative, comparison between similarly porous microparticles, as presented here. Based on our ACI study, among the post modifications, tangential flow filtration was the optimal condition, resulting in the highest respirable fraction (37%) with the dry powder formulation being released from a Rotahaler into a modified mouthpiece and then to the ACI. This is significantly greater than a roughly 10% respirable fraction observed with the commercially prevalent metered dose inhaler.[20] Also the respirable fraction of Ac-DEX 10k fast particles is almost 4-times greater than observed with PLGA particles. The highest respirable fraction of PLGA microparticles was observed when the particles were washed through centrifugation and lyophilized (11%) (Table S.1 of the Supporting Information). Most notably, the more visually porous fast degrading Ac-DEX microparticles had elevated respirable fractions compared to the slower degrading microparticles (Table S.1 of the Supporting Information), although even these values were higher than with PLGA.

Particle Biocompatibility and Drug Bioactivity. As a part of the respiratory tissue barrier, lung epithelial cells play a crucial role when characterizing microparticles for pulmonary delivery.[21] When microparticles fabricated from polyesters degrade, they release acidic byproduct which can shift the local pH of the pulmonary mucosa that will cause abnormal increases in the osmolarity.[15] If this happens, the lung can become vulnerable to bacterial invasion through the pulmonary epithelium, which can be deadly.[22] Furthermore, Fiegal et al. have shown that porous PLGA microparticles resulted in changes in cell barrier properties and tight junctions *in vitro* in cell cultures that were immersed in liquid covered culture media.[23] To evaluate the toxicity of Ac-DEX, we examined whether our microporous particulate system causes any damage to the lung epithelial cells by measuring the TEER of a lung epithelial cell monolayer that successfully mimics the physiological lung condition by expressing proper protein patterns for the formation of tight junctions.[21,23] Our study has shown that Ac-DEX particles do not disrupt the electrical resistance of an epithelial cell layer whether in liquid or air exposed conditions. Although Fiegal et al. reports property changes of LCC conditioned lung epithelial cells by impingement of PLGA particles,[23] Ac-DEX porous microparticles have no significant change in the integrity of lung epithelial cells *in vitro* as a result of the introduction of the microparticles to the lung cell culture.

As shown in the MTT assay (Figure 1B), at almost all concentrations, there was no statistical difference between the cytotoxicity of the Ac-DEX microparticles and free camptothecin, meaning that the microparticles were bioactive and just as effective as free drug at killing lung cancer cells *in vitro*. However, we hypothesize that once nebulized Ac-DEX microparticles and free camptothecin are compared *in vivo*, the microparticles will exhibit greater cytotoxicity to lung cancer cells than free drug since drug is released all at once with the free formulation, compared to the encapsulated formulation. Furthermore, since the microparticles are ideally sized for deep lung deposition (shown in Table 2), the drug released from the microparticles would be localized to the lungs, whereas if the drug were delivered intravenously it would be distributed systemically throughout the body.

■ CONCLUSION

Here we report formulations of porous Ac-DEX microparticles that have been processed for efficient deposition in the lungs. In contrast to polyester porous microparticle formulations, the degradation rate of Ac-DEX can be varied from hours to months, which could reduce polymeric build-up and side effects caused by the polymer as well as facilitate the tuning of drug release to optimize drug action. With these formulations, we have achieved acceptable drug-loading and sustained drug-release *in vitro* of camptothecin. The theoretical aerodynamic and actual diameters of the particles are ideal for deep lung delivery and avoidance of phagocytosis. Furthermore, after the post processing of these microparticles, the respirable fraction increased which indicates the improvement of aerosolization. Finally, no significant damage was caused by the Ac-DEX porous microparticles to lung epithelial cells either in liquid or air exposed conditions. Overall, we have created a novel porous microparticle system with promising qualities for deep lung deposition of drugs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Comparison of respirable fraction and total collected amounts for porous pulmonary microparticles (Table S.1); degradation profiles (Figure S.1); and representative SEM micrographs (Figures S.2 and S.3). This material is available free of charge via the Internet at http://pubs.acs.org.

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