International Journal of Pharmaceutics 422 (2012) 356–363

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Optimization of rapamycin-loaded acetalated dextran microparticles for immunosuppression

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ARTICLE INFO

Article history:
Received 6 August 2011
Received in revised form
28 September 2011
Accepted 17 October 2011
Available online 21 October 2011

Keywords:
Acid sensitive polymer
Rapamycin
Microparticle optimization
Macrophages
Immunosuppression

ABSTRACT

Immunosuppressive drugs can treat autoimmune disorders and limit rejection with organ transplants. However, delivering immunosuppressants like rapamycin systemically can have harmful side-effects. We aim to potentially reduce these toxic side-effects by encapsulating rapamycin in a polymeric microparticle to passively target phagocytes, the cells integral in immunosuppression. Acetalated dextran (Ac-DEX) is a recently described, biocompatible polymer which undergoes tunable burst degradation at the acidic conditions present in the phagosome (pH 5) but slower degradation at extracellular conditions (pH 7.4), thereby making it an ideal candidate for immune applications. Rapamycin-loaded microparticles were fabricated from Ac-DEX through a single emulsion (water/oil) technique. Optimized microparticles were determined by varying the chemical and physical parameters during particle synthesis. Microparticles synthesized from Ac-DEX with a molecular weight of 71k had higher encapsulation efficiency of rapamycin and slower overall degradation than microparticles synthesized from 10k Ac-DEX. To evaluate the ability of rapamycin-loaded Ac-DEX microparticles to reduce a pro-inflammatory response, they were incubated with lipopolysaccharide-stimulated RAW macrophages. RAW macrophages treated with rapamycin-loaded microparticles exhibited reduced nitric oxide production and favorable cell viability. Overall, we have shown optimization of immunosuppressive rapamycin-loaded microparticles using the novel polymer Ac-DEX. These particles will be advantageous for future applications in immune suppression therapies.

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1. Introduction

Immunosuppression can be useful in the treatment of autoimmune diseases and for patients undergoing organ transplants. Various immunosuppressive drugs have been developed to treat autoimmune diseases ranging from rheumatoid arthritis (Drosos, 2002) to multiple sclerosis (Hartung et al., 2002). Also, immunosuppressive drugs such as rapamycin or cyclosporine are administered to patients receiving organ transplants to discourage organ rejection, greatly increasing the survival rates of these patients (Khan, 2009). As a point of therapeutic intervention, phagocytes like dendritic cells (DCs) and macrophages can be targeted because they are considered as gateways to immune suppression responses. Several small molecules have been shown to skew phagocytes' immune responses *ex vivo*, such as rapamycin (Fischer et al., 2009; Turnquist et al., 2007), dexamethasone (Barrat et al., 2002; Bosma et al., 2008), Vitamin D3 (Griffin et al., 2000), dimethylfumaric acid ester (Litjens et al., 2004, 2006; Zhu and Mrowietz, 2001), retinoic acid (Manicassamy and Pulendran, 2009; Wada et al., 2009), and curcumin (Cong et al., 2009).

Rapamycin, also known as sirolimus, is an inexpensive immunosuppressive drug that has been well-studied in renal transplantation (Kahan, 2003) and in drug-eluting stents as an anti-inflammatory/anti-proliferation agent (Venkatraman and Boey, 2007). Rapamycin is typically administered as an oral solution under the commercial name Rapamune; however, regular administration of rapamycin even in low doses (1–2 mg/day) can produce side-effects in humans including diarrhea and headaches, but more seriously, myelosuppression, hyperlipidemia, and

Abbreviations: PLGA, poly(lactic-*co*-glycolic acid); Ac-DEX, acetalated dextran; MW, molecular weight; (71k), 71,400 Da; (10k), 10,500 Da; DCs, dendritic cells; DMSO, dimethyl sulfoxide; DCM, dichloromethane; PBS, phosphate buffered saline; PVA, poly(vinyl alcohol); BCA, bicinchoninic acid assay; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; EE, encapsulation efficiency; TLR, toll-like receptor.

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doi:10.1016/j.ijpharm.2011.10.034

over-immunosuppresion (Saunders et al., 2001). These side-effects could potentially be reduced by using a polymeric micro- or nano-particle system to deliver rapamycin by passively targeting phagocytes. The phagocytosis of rapamycin-loaded microparticles would allow for localized drug release to phagocytes, thereby reducing side-effects and perhaps requiring less drug (Bachelder et al., 2010). The polymer most commonly used to create microparticles for drug delivery applications is poly(lactic-*co*-glycolic acid) (PLGA). Previous research has shown that rapamycin-loaded PLGA microparticles significantly increased drug efficacy and reduced T-cell activation when the particles were incubated with phagocytic dendritic cells (DCs) for four days (Jhunjhunwala et al., 2009). Similarly, rapamycin-loaded PLGA nanoparticles have been shown to inhibit DC maturation far better than the free drug form (Haddadi et al., 2008). These results indicate that rapamycin has immunosuppressive effects on phagocytes when passively targeted to the cells.

Although PLGA and other polyesters (e.g., poly(lactic acid), polycaprolactone) are commonly used in drug delivery applications, they have several disadvantages. Most notably, they have fixed degradation rates on the order of months, degrade into acidic byproducts which can significantly lower the local pH (Lu et al., 2000), and release nearly half of their drug payloads in a burst fashion (Allison, 2008). Acetalated dextran (Ac-DEX) is a new biodegradable polymer that is formed by replacing the hydroxyl groups on dextran, a water-soluble homopolysaccharide of glucose, with cyclic and acyclic acetal groups to render the polymer water-insoluble (Bachelder et al., 2008). Ac-DEX is particularly promising because it can overcome many of the inherent disadvantages of polyesters for application in the body. In contrast to fixed degradation rates, Ac-DEX exhibits tunable degradation rates which can range from minutes to months, due to the differing rates of hydrolysis for cyclic and acyclic acetal groups. The relative ratio of these acetal groups can easily be controlled by the reaction time to form Ac-DEX, and the degradation rates can then be tailored to suit varying applications (Broaders et al., 2009). Specifically, Ac-DEX microparticles undergo tunable burst release in acidic phagosomal conditions (pH 5) but have a slower, tunable release profile in extracellular conditions (pH 7.4) (Bachelder et al., 2008). The pH-sensitivity of Ac-DEX microparticles allow them to release their drug payloads in the phagolysosome once they are passively targeted to the phagocyte. Importantly, this pH-sensitivity may minimize systemic toxic side-effects of the encapsulated drug by ensuring that the maximum amount of drug is released intracellularly in the phagolysosome instead of systemically, which could also lead to the administration of less drug for equivalent drug effect (i.e. dose sparing). Indeed, we have shown that with the delivery of the adjuvant imiquimod, significantly less drug is needed for equal macrophage and DC activation when delivered in an Ac-DEX microparticle (Bachelder et al., 2010). Ac-DEX also degrades into fully biocompatible and, more notably, pH-neutral byproducts including dextran and very low levels of an alcohol and acetone, a common metabolic byproduct (Bachelder et al., 2008). In addition, PLGA is not ideally suitable for immunotherapy applications since it has been shown to activate DCs *in vitro* and *in vivo* (Norton et al., 2010; Kou and Babensee, 2011). Furthermore, we have shown that materials sensitive to the acidic environment present in the phagolysosomal compartments of macrophages and DCs (pH 5) (i.e. Ac-DEX and polyacrylamide) have a significantly increased efficacy of the immunological synapse compared to degradable (PLGA) and non-degradable (iron oxide) materials (Broaders et al., 2009). These characteristics make Ac-DEX microparticles a more desirable vehicle to deliver rapamycin to phagocytes than microparticles made from other available biopolymers.

Previously, Ac-DEX had been synthesized from dextran with a MW of 10,500 (10k) (Bachelder et al., 2008). However, production of Ac-DEX with an increased MW of 71,400 (71k) was hypothesized to increase drug encapsulation efficiency, particle size, and degradation rates primarily due to its higher viscosity and longer chain lengths; it has been previously shown that increased molecular weight of polymer correlates to increased encapsulation efficiency with chitosan (Xu and Du, 2003) and lengthened degradation rates with PLGA and poly(lactic acid) (Alexis, 2005). Because 71k Ac-DEX has not yet been investigated in literature, optimized parameters were first established to create the best rapamycin-loaded Ac-DEX microparticles in terms of yield and encapsulation efficiency, similar to work completed by Mao et al. with PLGA (2007). Additionally, the release of rapamycin from Ac-DEX microparticles was characterized in acidic (pH 5) and neutral conditions (pH 7.4). Lastly, cell studies were performed with RAW macrophages stimulated with pro-inflammatory lipopolysaccharide (LPS) to determine the efficacy of the drug in its free and encapsulated form. In summary, the objective of this study was to synthesize 71k Ac-DEX rapamycin-loaded immunosuppressive microparticles, which could potentially reduce the toxic side-effects of the encapsulated immunosuppressant *in vivo* due to the controlled and localized release of drug.

2. Materials and methods

All materials were purchased from Sigma–Aldrich (St. Louis, MO) and used as received unless otherwise noted.

2.1. Ac-DEX synthesis

Ac-DEX was prepared with some modifications to the procedure described by Broaders et al. (2009). Lyophilized dextran (MW = 71,400) and pyridinium p-toluenesulfonate (0.0617 mmol) were dissolved in anhydrous DMSO and reacted with 2-methoxypropene (37 mmol) under nitrogen gas. After six hours, the reaction was quenched with triethylamine, precipitated in basic water (pH 9), vacuum filtered, and lyophilized for two days to yield a fluffy white solid. The product was then purified by dissolving it in ethanol (200 proof) and centrifuged (5 min, 10,000 × *g*, Beckman RA-21, Los Angeles, CA, USA). The supernatant was precipitated in basic water and lyophilized for two days to yield Ac-DEX (1.5 g).

2.2. Ac-DEX NMR analysis

NMR analysis provided the relative cyclic:acyclic ratio of acetal groups of the Ac-DEX polymer. Ac-DEX was suspended in deuterium oxide and hydrolyzed with deuterium chloride. After two hours, the samples were read with a 300 MHz ^{1}H-NMR (Bruker 300 Ultrashield). The hydrolysis of one cyclic acetal produces one acetone molecule and the hydrolysis of one acyclic acetal produces one acetone and one methanol molecule. Therefore, from the relative ratio of the peaks from acetone, methanol, and the anomeric carbon on dextan, the cyclic:acyclic ratio of acetal substitution and degrees of substitution per 100 glucose could be determined (Broaders et al., 2009). A further description of NMR analysis and examples of NMR spectra of degraded Ac-DEX may be found in the Supporting Information by Bachelder et al. (2008)

2.3. Particle formation

2.3.1. Ac-DEX particle formation via homogenization

Homogenized microparticles were prepared via a single-emulsion technique (water/oil) (Bachelder et al., 2010). To fabricate the homogenized microparticles, Ac-DEX (100 mg) and rapamycin (1 mg, LC Laboratories, Woburn, MA) were dissolved in dichloromethane (DCM, 1 mL) and added to 3% poly(vinyl alcohol) (PVA) in PBS (17 mL). The resultant mixture was homogenized for

30 s (Polytron PT 10-35 Homogenizer, 20,500 RPM) and the emulsion was immediately poured into a spinning solution of 0.3% PVA (40 mL). The reaction mixture was allowed to spin for two hours to evaporate the solvent and allow for particle hardening. To recover the microparticles, each formulation was subjected to centrifugation (5 min, 20,000 × *g*, 4 °C). The supernatant was discarded, and the resulting microparticle sediment was washed with basic water to remove excess drug and PVA (2 × 5 min, 20,000 × *g*, 4 °C). The microparticles were then suspended in basic water and lyophilized for two days to yield rapamycin-loaded Ac-DEX microparticles. To produce blank microparticles, the same procedure was followed, except no rapamycin was added.

2.3.2. Ac-DEX particle formation via sonication

To synthesize sonicated microparticles, Ac-DEX (100 mg) and rapamycin (1 mg) were dissolved in DCM (1 mL) and added to 3% PVA (2 mL). The mixture was sonicated for 30 seconds (Misonix Ultrasonic Liquid Processor, amplitude 2, 4, or 8%, duty cycle 50%) and the formed emulsion was immediately pipetted into a spinning solution of 0.3% PVA (40 mL). The same washing procedure was performed as described for the homogenized microparticles.

2.4. Particle analysis

2.4.1. Scanning electron microscopy

Microparticles were suspended in basic water at 20 mg/mL, and a small amount (20 μL) was added to a silicon wafer. The samples were allowed to air dry, and then sputter coated with a layer of palladium/gold alloy. The samples were imaged with a FEI NOVA NanoSEM 400.

2.4.2. Particle size analysis

The size and size distribution of the prepared microparticles were measured with a light scattering size analyzer by suspending the microparticles in basic water (Brookhaven, 25 °C, 90° angle).

2.4.3. Ac-DEX particle degradation analysis

Blank Ac-DEX microparticles were suspended in triplicate in sodium acetate buffer (pH 4.90) or in PBS (pH 7.4). Blank microparticles were used because our preliminary findings indicated that the addition of encapsulated drug has no effect on the degradation of the particle (data not shown). The samples were kept at 37 °C on a shaker plate at 150 RPM. At various timepoints (0 to 240 h), aliquots were withdrawn and centrifuged (15,000 × *g*, 4 °C, 5 min). Microparticles collect on the sides of the tubes, so when aliquots were withdrawn, the mass of microparticles in the medium was unchanged. The supernatants were then stored at −20 °C in a 96-well polystyrene plate. The supernatants were analyzed with a microplate reductometric bicinchoninic acid based assay (BCA) according to the manufacturer's protocol (Protein Assay Kit; Pierce, Rockford, IN). The resultant absorbances were measured at 562 nm with a platereader (FlexStation 3 Benchtop Multi-Mode Microplate Reader). The assay measured the amount of the degradation product dextran in the supernatant with time. A curve was fit to this data, and the polymer degradation $t_{1/2}$ was defined to be the time on the curve at which 50% of the Ac-DEX had degraded.

2.4.4. Determination of rapamycin loading

Rapamycin-loaded Ac-DEX microparticles were fully dissolved in acetonitrile (5 mg/mL). The solution was vortexed, bath sonicated for ten minutes, centrifuged (5 min, 15,000 × *g*, 4 °C) and the supernatant was withdrawn and analyzed with HPLC (Agilent 1100 series, column: C18 × 5 μm × 150 mm × 4.6 mm, 1 mL/min, 20 min, mobile phase: 65% acetonitrile/35% water) at 278 nm. The experimental rapamycin concentration in each particle was determined by comparison with a standard curve of rapamycin in acetonitrile. The encapsulation efficiency was determined by the equation: EE (%) = 100 × (experimental rapamycin concentration)/(theoretical rapamycin concentration). Also, the rapamycin percent weight loading (w/w) was determined by the equation: weight loading (%) = 100 × (loaded rapamycin in mg)/(amount of polymer in mg).

2.4.5. Drug release from microparticles

The release of rapamycin from Ac-DEX microparticles was performed by collecting aliquots in the same manner used for the degradation analysis. The aliquots were centrifuged (15,000 × *g*, 4 °C, 5 min) and the supernatants were analyzed via HPLC at 278 nm, using the same parameters as described in determining the encapsulation efficiency. After plotting the concentration of rapamycin versus time, a curve was fit to the data and the drug release $t_{1/2}$ was defined to be the time on the curve at which 50% of the encapsulated rapamycin was released.

2.5. Cell studies

2.5.1. Cell study preparation

Macrophages (RAW 264.7; ATCC, Manassas, VA) were grown and maintained as per the manufacturer's guidelines. To make the cell media, 50 mL of fetal bovine serum (Hyclone, Pittsburgh, PA) and 5 mL of penicillin-streptomycin (Fischer, Pittsburgh, PA) were added to 500 mL of Dulbecco's Modified Eagle's Medium (Fischer, Pittsburgh, PA). Cells were maintained at 100% relative humidity, 37 °C, and 5% CO_2 for the duration of the experiments.

Macrophages were plated at a concentration of 1×10^4 cells/mL and incubated for 24 h in two 96-well plates. After 24 h, the media in each well was replaced with media containing optimized rapamycin-loaded Ac-DEX microparticles ranging from 0.001 to 0.1 μg rapamycin/mL, blank Ac-DEX microparticles, or free rapamycin at the same concentrations, all in triplicate. The media used in one plate was spiked with lipopolysaccharide (LPS) (100 ng/mL) to promote nitric oxide (NO) production, whereas the media used in the other plate contained no LPS to act as a control. The cells were incubated for another 24 h at these conditions and nitric oxide production was assessed.

2.5.2. Nitrite analysis

A Griess assay was performed to determine the nitric oxide production by the macrophages. The supernatants from each well were removed and centrifuged (15,000 × *g*, 4 °C, 10 min), and 50 μL of the resulting supernatant was withdrawn. The Griess reagents (Promega, Fitchburg, WI) were added as per the manufacturer's instructions, and standard nitrite concentrations were prepared. The absorbance was measured at 540 nm and compared with the standard curve to determine nitrite concentration and subsequent nitric oxide production. The NO concentrations for the microparticles and free drug were standardized with respect to 0 μM.

2.5.3. Cell toxicity analysis

A MTT assay was performed to ensure variation in nitric oxide production was not the result of disparity in cell lethality. Fresh media (150 μL) and a solution of MTT in media (5 mg/mL, 20 μL) were added to each well after supernatants were withdrawn, and the plate was incubated for 3 hours until formazan crystals had formed. The supernatants were removed and isopropanol (200 μL) was added to each well to dissolve the purple formazan crystals. The plate was analyzed using a platereader (FlexStation 3 Benchtop Multi-Mode Microplate Reader) at an absorbance of 560 nm, and background absorbance at 670 nm was subtracted to yield the cell viability. Respective controls, such as wells with dead cells grown in absence of media and blank wells were also analyzed.

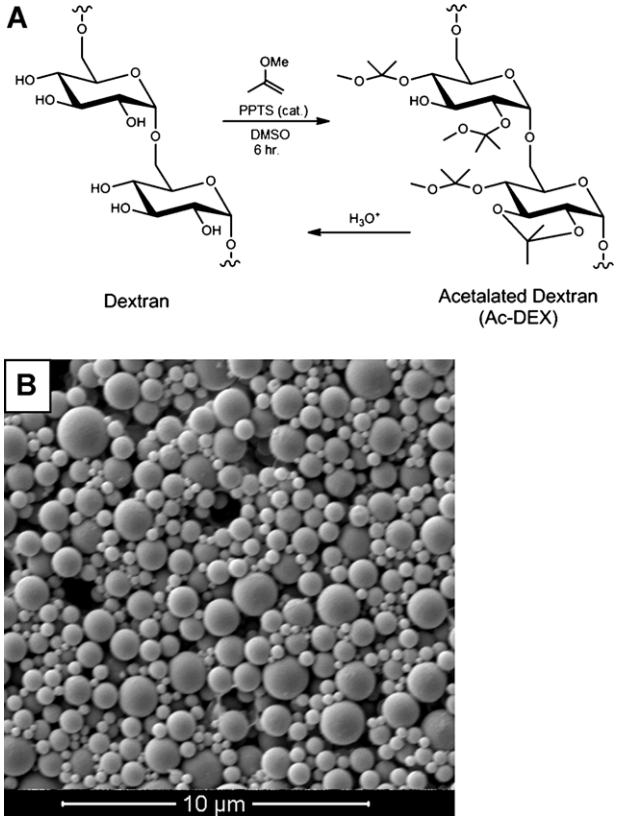

Fig. 1. (A) The reaction scheme to synthesize acetalated dextran (Ac-DEX) polymer. (B) A representative SEM micrograph of 71k Ac-DEX microparticles. The average diameter of the pictured particles was 829 nm, determined by Dynamic Light Scattering. The scale bar is 10 μm.

The absorbance measurements were standardized with respect to 0 μM to obtain percent viability.

3. Results and discussion

3.1. 71k versus 10k Ac-DEX particle degradation

Fig. 1 shows the reaction scheme to synthesize Ac-DEX and a representative SEM micrograph of 71k Ac-DEX microparticles. Ac-DEX polymer was synthesized according to Fig. 1A. The average diameter of the microparticles in Fig. 1B was 829 nm with a polydispersity of 0.17, indicating that the particles were fairly uniform, and the shape of the particles was spherical with no observable porosity at higher magnification.

In Fig. 2, the degradation of 71k and 10k Ac-DEX microparticles at two different pHs (4.9 – phagosomal, and 7.4 – physiological) is shown. Table 1 also quantifies various properties of the Ac-DEX polymer and microparticles. The 71k Ac-DEX microparticles require about three times longer to fully degrade at pH 5 than 10k Ac-DEX particles at the same pH. Furthermore, 71k Ac-DEX microparticles exhibit an initially quick degradation period that results in approximately 50% degradation in 40 h, followed by a slower degradation until about 210 h when the particles become fully degraded. At pH 7.4, the 71k Ac-DEX particles degrade much slower and in a more linear fashion, with only approximately 20% degradation in 240 h. The results from the 10k Ac-DEX particles mirror the degradation profiles of 10k Ac-DEX particles as reported by Broaders et al. (2009). Qualitatively, the particle degradation in both pH 5 and pH 7.4 looked to be in agreement with the time-lapse pictures showed by Bachelder et al. (2008); the suspension

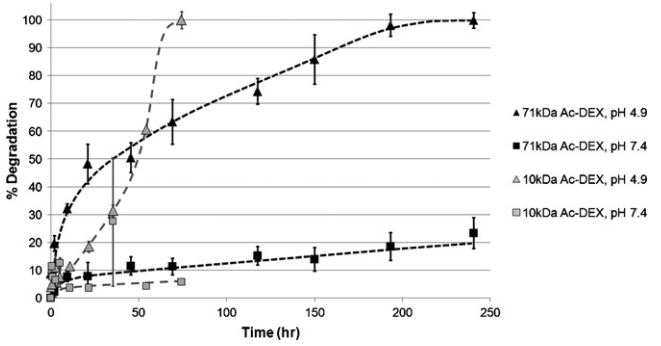

Fig. 2. Degradation rates of 10k and 71k Ac-DEX microparticles in acidic and pH-neutral buffers as determined by a reductometric bicinchoninic acid based assay, which measured the amount of degraded dextran in the sample. Each data point is presented as the mean ± standard error mean ($n = 3$).

of particles and buffer change from cloudy-white to a transparent solution as the particles degrade.

The initial quick degradation of Ac-DEX microparticles is due to hydrolysis of acyclic acetal groups on the polymer, followed by the steady and rate-limiting hydrolysis of the cyclic acetal groups (Broaders et al., 2009). In addition, Fig. 2 indicates that increasing the molecular weight of the polymer also varies the degradation rate, since both the 10k and 71k Ac-DEX were synthesized with the same reaction time of 6 h. The lengthened degradation of Ac-DEX with a higher molecular weight is consistent with other polymers such as PLGA and poly(lactic acid) (Alexis, 2005).

As shown in Table 1, NMR results indicated that the 71k Ac-DEX had a cyclic acetal coverage of 0.86 per glucose residue, with a relative cyclic acetal coverage of 61%, whereas the 10k Ac-DEX had a cyclic acetal coverage of 0.89 per glucose residue and a relative cyclic acetal coverage of 64%. Because the cyclic acetal coverage of the 10k and 71k Ac-DEX were approximately the same, the lengthened degradation rate is possibly due to the higher viscosity and longer chain length of the 71k Ac-DEX and not differing cyclic acetal coverage. Broaders et al. displayed the tunable degradation rates of 10k Ac-DEX polymer via simple variance of reaction time (2009); here we were able to expand the range of degradation rates by also altering the MW of the dextran.

3.2. Encapsulation of rapamycin in 71k and 10k Ac-DEX microparticles

The default 71k Ac-DEX microparticles were determined to have an encapsulation efficiency (EE) of rapamycin of 64 ± 2%, whereas the 10k Ac-DEX microparticles had an EE of 19 ± 9%. The positive correlation between EE and molecular weight is in contrast to PLGA, for which the molecular weight and encapsulation efficiency are generally inversely related (Hans and Lowman, 2002). One such example of this trend is the increasing EE of encapsulated albumin for decreasing PLGA molecular weight (Song et al., 1997). This is, however, not necessarily indicative of all polymers and all types of encapsulated drugs; lowering the molecular weight of PLGA increases the number of its carboxylic end groups, possibly resulting in more covalent bonding with the drug and thus a better EE (Fernandez-Carballido et al., 2004). The polymer chitosan exhibits a positive correlation between EE and molecular weight, perhaps because the longer chains of polymer make entrapment of drug easier (Xu and Du, 2003). In addition to longer chain lengths, the greater viscosity of 71k Ac-DEX may facilitate encapsulation of the drug by preventing the diffusion of the drug into the continuous phase after emulsification. 71k Ac-DEX is also less soluble in DCM solvent than 10k Ac-DEX, and lower solubility of polymer in organic solvent leads to faster solidification of microparticles

Table 1
Ac-DEX polymer and microparticle properties, including molecular weight (MW), reaction time, cyclic acetal coverage (per glucose residue and relative) as determined by NMR analysis, and polymer degradation $t_{1/2}$ (the time for 50% of the microparticle to degrade) and drug release $t_{1/2}$ (the time for 50% of encapsulated rapamycin to be released) for pH 5 and pH 7.4 buffers. For pH 7.4 buffers, 50% polymer degradation and drug release were not observed, so the percentage of polymer degraded or drug released is reported at its respective time. Drug release $t_{1/2}$ data for 71k refers to the optimized microparticle system described in Section 3.3.

MW (kDa)	Reaction Time (h)	Cyclic acetal coverage (per glucose residue)	Cyclic acetal coverage (relative)	Polymer degradation $t_{1/2}$ (h)		Optimized drug release $t_{1/2}$ (h)	
				pH 5	pH 7.4	pH 5	pH 7.4
10	6	0.89	64%	45	5% at 75	n/a	n/a
71	6	0.86	61%	40	20% at 240	65	5% at 240

resulting in a higher EE (Yeo and Park, 2004). Although it can be concluded that higher molecular weight Ac-DEX results in a higher EE of rapamycin, this trend may be different with other types of drugs due to different drug molecular weights, functional groups, and hydrophilicities.

3.3. Optimization of 71k Ac-DEX microparticles

Based on previous research by Mao et al. (2007), seven parameters in the single-emulsion particle formation procedure were chosen to be varied in order to optimize the rapamycin-loaded Ac-DEX microparticles in terms of size, yield, and encapsulation efficiency. The parameters included Ac-DEX molecular weight, Ac-DEX concentration, continuous phase PVA concentration, continuous phase PVA volume, spinning PVA concentration, spinning PVA volume, and either homogenization speed or sonication amplitude. Each particle was compared to the default particle, which was synthesized using the parameters given in the particle preparation section. Table 2 presents the yield, encapsulation efficiency, size, and polydispersity for each particle synthesized with the given parameters varied.

Of the seven parameters investigated, the most apparent trend was observed in varying the MWs of Ac-DEX, as seen in Fig. 3, and no clear correlations were generally observed between the other six parameters and EE, yield, and particle size. As discussed previously, 71k Ac-DEX microparticles had a superior EE to 10k Ac-DEX microparticles (64 ± 2% compared to 19 ± 9%). However, microparticles synthesized from even higher MWs of Ac-DEX of 150k and 2 million (m) did not increase either EE or yield. This could be attributed to fact that the 150k and especially the 2 m Ac-DEX

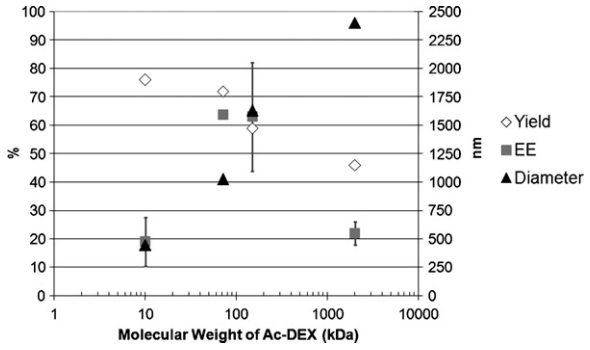

Fig. 3. Effect of molecular weight of Ac-DEX on yield, encapsulation efficiency (EE), and diameter of the resultant particles. Yields and diameters are presented as means, and EEs are presented as the mean ± standard error mean (n = 3). Increasing the molecular weight resulted in decreasing the yield and increasing the diameter of the particles; EE is maximum at 71k to 150k.

Table 2
Yield, encapsulation efficiency (EE), diameter, and polydispersity of all twenty batches of Ac-DEX particles. EE is presented as the mean ± standard error mean (n = 3). Each parameter changed is given with its respective default value in parenthesis.

	Yield (%)	EE (%)	Diameter (nm)	Polydispersity
Parameter changed (default value)				
Default unloaded (Blank)	61	0	1034	0.23
Default loaded Rapamycin	72	64 ± 2	1027	0.19
Ac-DEX concentration (100 mg/mL)				
50 mg/mL	78	31 ± 5	829	0.17
200 mg/mL	57	43 ± 5	1396	0.34
Continuous phase volume (17 mL)				
10 mL	49	79 ± 11	696	0.29
25 mL	57	67 ± 3	946	0.14
Spinning PVA volume (40 mL)				
100 mL	71	79 ± 6	1093	0.19
250 mL	60	67 ± 6	1200	0.29
RPM homogenization speed (20500 RPM)				
8850 RPM	66	71 ± 7	1123	0.24
23,750 RPM	64	44 ± 5	972	0.20
Continuous phase PVA concentration (3%)				
0.3% PVA	52	55 ± 8	338	0.24
1%PVA	54	81 ± 9	2187	0.28
Spinning solution PVA concentration (0.3%)				
1%PVA	60	72 ± 13	1039	0.16
3% PVA	102[a]	52 ± 4	1038	0.16
Molecular weight Ac-DEX (71k)				
10k Ac-DEX	76	19 ± 9	446	0.30
150k Ac-DEX	59	63 ± 19	1631	0.11
2 m Ac-DEX	46	22 ± 4	2405	0.52
Sonication amplitude (n/a)				
2%	21	15 ± 2	370	1.05
4%	50	48 ± 14	132	0.09
8% (optimized)	63	91 ± 1	473	0.56

[a] Elevated yield is likely due to contamination from PVA solution.

exhibited high enough viscosity that the homogenizer was unable to impart sufficient energy to form a perfect emulsion of Ac-DEX, drug, PVA, and solvent. Regardless, the 150k and 2 m Ac-DEX would likely have such lengthy degradation times that their microparticles would be impractical for the delivery of immunosuppressive agents as proposed.

Although the yields and encapsulation efficiencies were generally consistent, a few deviated significantly. For example, the particle system with 3% PVA spinning solution had a yield of over 100%, likely due to residual shards of PVA remaining in the microparticles since the PVA concentration was 10 times larger than the default value. Also, the particle system with 2% sonication amplitude exhibited a low yield of 21% and EE of 15 ± 2%, possibly because too little energy was imparted by the sonicator to effectively emulsify the solution.

The optimized particle was determined to be the particle system synthesized via sonication with 8% amplitude. This particle synthesis method had 63% yield, 91 ± 1% EE, 0.91% weight loading, and an average particle diameter of 473 nm. The weight loading of our particles were much higher than reported weight loadings of rapamycin-loaded PLGA nanoparticles and microparticles, while also having slightly higher EE. Haddadi et al. (2008) achieved 81 ± 8% EE for rapamycin-loaded PLGA nanoparticles 150–450 nm in diameter with a 0.05% weight loading. Jhunjhunwala et al. (2009) reported 74 ± 5% EE for rapamycin-loaded PLGA microparticles 3 to 4 μm in diameter with a 0.37% weight loading.

3.4. Characterization of drug release for optimized particle system

The drug release of the optimized rapamycin-loaded Ac-DEX particle system was characterized in acidic and pH-neutral conditions, and the $t_{1/2}$ values are shown in the last column of Table 1. Due to the acid sensitivity of Ac-DEX, rapamycin was released at a much faster rate in burst at pH 5 conditions (50% in 45 h), which simulate phagosomal conditions, compared to minimal release in the pH 7.4 conditions (5% in 240 h), which simulate extracellular conditions. Since rapamycin has no ionizable functional groups, pH has no effect on its aqueous solubility (Simamora et al., 2001). Therefore, it may be concluded that the pH-sensitivity of Ac-DEX microparticles is responsible for the differing drug release rates and not pH-dependent drug solubility.

Additionally, it should be noted that the rapamycin is very poorly soluble in the aqueous buffers (2.6 μg/mL) (Simamora et al., 2001). Haddadi et al. (2008) circumvented this problem for PLGA nanoparticles by using a 9:1, v/v buffer:ethanol mixture in which rapamycin is more soluble (2008); however, Ac-DEX is soluble in ethanol, so aqueous pH 5 and pH 7.4 buffers were used in our studies. Moreover, it was important to most accurately model *in vivo* conditions, so using ethanol or any other organic solvent would not be appropriate.

The pH-sensitivity of the drug release implies that rapamycin-loaded Ac-DEX microparticles that passively target phagocytes would release their drug payload upon phagocytosis, and drug would be released at a significantly reduced rate while residing in the extracellular environment. The pH-sensitive release of rapamycin from the Ac-DEX microparticles also suggests that the maximum amount of drug could be delivered intracellularly to the macrophage with a minimum amount of drug being released in other pH-neutral locations in the body, meaning drug-loaded Ac-DEX microparticles could both maximize the efficacy of the drug and minimize systemic and local toxicity. Therefore, a smaller amount of drug could be needed, which would reduce both harmful side-effects and cost. Bachelder et al. (2010) have demonstrated dose sparing by treating macrophages and DCs with imiquimod-encapsulated Ac-DEX microparticles, finding that less drug was required when encapsulated in Ac-DEX microparticles.

3.5. NO production in LPS-stimulated raw macrophages exposed to rapamycin-loaded Ac-DEX microparticles

Two commonly used methods to ensure phagocytosis of the microparticles are ligand-mediated targeting and passive targeting via particle sizing. Beaudette et al. (2009) have previously shown that it is possible to chemoselectively ligate alkoxyamine-bearing molecules such as cell-penetrating peptides to Ac-DEX particles (2009). Other researchers have proposed ligand-mediated targeting with polymeric particles to increase uptake in phagocytic cells. Hamdy et al. (2011) ligated mannan, a toll-like receptor 4 (TLR-4) agonist, to the surface of antigen-loaded PLGA nanoparticles and noted a non-statistically significant increase in particle uptake (<5%) in DCs. For tolerance applications, TLR activation would be counter-productive and exasperate the disease, but this study shows the impact of ligand-mediated particle uptake by phagocytic cells. Alternatively, the passive targeting of phagocytes has been well-established to be dependent on the size of the particle. Phagocytes like DCs and macrophages will phagocytose microparticles less than approximately 10 μm in diameter (Johansen et al., 2000). Unlike most cell types *in vivo*, macrophages have phagocytic properties (Foged et al., 2002) allowing them to internalize particles larger than 100 nm (Foged et al., 2005; Hirota et al., 2007). Studies have shown that particles in the range of 100–1000 nm are more likely to be phagocytosed by CD11c- cells (macrophages) than CD11c+ cells (DCs); results indicated that smaller particles are cleared by resident macrophages (100–1000 nm) and larger particles are predominately transported by DCs from the injection site to the lymph nodes (Manolova et al., 2008). Therefore, it was determined that the diameter of optimized rapamycin-loaded Ac-DEX microparticles (473 nm) was ideal for passive targeting of macrophages. Although ligation to the particles is possible, we determined that phagocytosis could be best optimized through passive targeting and particle sizing; the small increase (<5%) of particle uptake with ligation shown by Hamdy et al. (2011) is only a marginal improvement. Additionally, preliminary *in vivo* work has shown that we have downstream signaling such as production of antigen specific antibodies and T-cells without ligation (data not shown). For these experiments, microparticles were injected into the sub-cutaneous area of the flank or nap of neck. Ac-DEX microparticles can also be introduced through both needle and needle-free methods, including intraperitoneal, intravascular, intratracheal, or internasal introduction.

Free rapamycin and Ac-DEX microparticles with and without encapsulated rapamycin were incubated with RAW macrophages, and LPS was added or not added to evaluate the formulations' efficacy. Macrophages identify LPS through TLR-4 on their surface (Paul, 1994) and phagocytose the microparticles through non-opsonic receptors. The addition of LPS was used to stimulate an inflammatory response in the macrophages and thereby induce pro-inflammatory signaling such as nitric oxide production. The production of nitric oxide and related reactive oxygen species by macrophages has also been associated with diseases such as artherosclerosis and sepsis (Dröge, 2002). Rapamycin, an immunosuppressant, should reduce inflammatory responses, thereby decreasing nitric oxide levels. Thus, the efficacy of the drug is inversely related to the concentration of nitric oxide measured. A Griess assay was used to measure the nitric oxide concentrations for each formulation after 24 h of incubation with the rapamycin formulations.

Fig. 4A shows the nitric oxide production from RAW macrophages as determined with the Griess assay. For LPS-stimulated RAW macrophages, there was a dose-dependent response for both the rapamycin-loaded Ac-DEX microparticles and free rapamycin. Although dose sparing was not observed, there was no significant difference in nitric oxide production in LPS

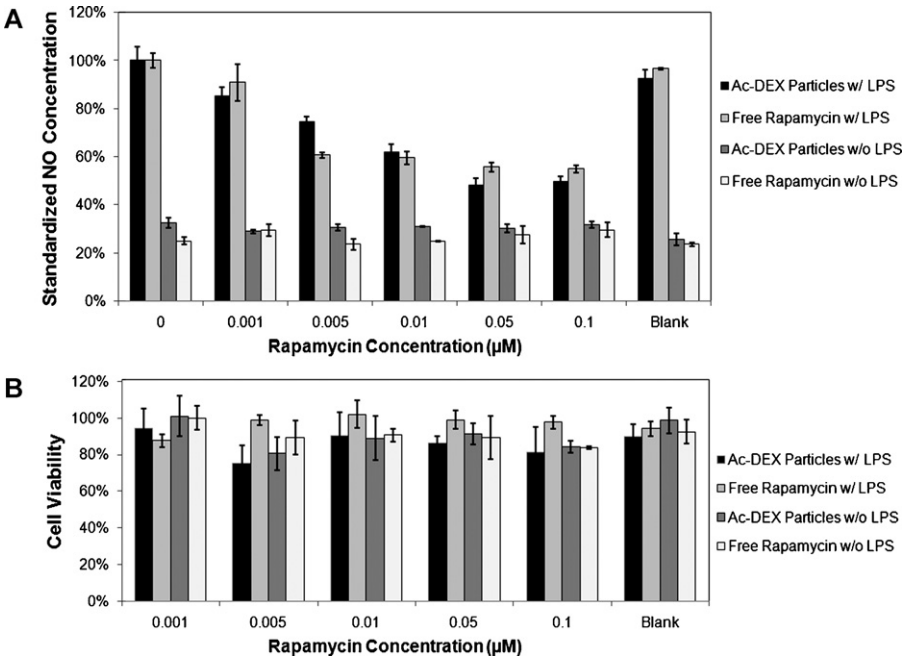

Fig. 4. (A) Nitric oxide production from RAW 264.7 macrophages incubated with optimized 71k Ac-DEX particles loaded with and without rapamycin, and free rapamycin. Each bar is presented as the mean ± standard error ($n = 3$). The figure shows a dose-dependent response for LPS-stimulated RAW macrophages (w/LPS), and also includes no LPS (w/o LPS) as a control. (B) Cell viability for Ac-DEX particles and free rapamycin with and without LPS stimulation. Each bar is presented as the mean ± standard error mean ($n = 3$).

treated macrophages between the Ac-DEX microparticles and free drug, with the sole exception at the rapamycin concentration of 0.005 μM. There was constant and low nitric oxide production from RAW macrophages which were not stimulated with LPS. Additionally, Fig. 4B shows that Ac-DEX microparticles and free rapamycin demonstrate favorable cell viability with and without LPS, indicating that the rapamycin and microparticles had no cytotoxic effects on the cells and variance in nitric oxide production was not the result of disparity in cell viability.

The Griess assay results indicate that immunosuppresion can be achieved with rapamycin-loaded Ac-DEX microparticles just as efficiently as with free rapamycin. The Ac-DEX microparticle advantage over free drug, however, is that the drug could be passively targeted to the macrophages *in vivo* rather than delivered systemically throughout the body. Furthermore, once the microparticles are phagocytosed, the drug payload would be released in a burst fashion intracellularly in the phagosomal acidic environment. Unlike PLGA microparticles, Ac-DEX microparticles have a relatively low release of rapamycin at pH 7.4, which could potentially reduce toxic systemic side-effects of the drug *in vivo*. The encapsulation of immunosuppressive drugs with harmful side-effects like rapamycin in microparticles made from acid-sensitive, biocompatible polymers like Ac-DEX could improve health in patients with autoimmune diseases or patients undergoing organ transplants.

4. Conclusions

Microparticles synthesized from 71k Ac-DEX exhibit better encapsulation efficiency of rapamycin than 10k Ac-DEX, possibly because of the polymer's higher viscosity and longer chain length. Also, 71k Ac-DEX microparticles require approximately three times longer to fully degrade in pH 5 conditions and have larger particle diameters than 10k Ac-DEX microparticles. These results add to the versatility of Ac-DEX microparticles as a drug-delivery vehicle. Degradation times can now be controlled through both reaction time and molecular weight of dextran used, allowing for greater flexibility, which may be tailored to specific applications. Furthermore, this study demonstrated that immunosuppresion can be achieved with rapamycin-loaded Ac-DEX microparticles at levels similar to free drug in culture. Rapamycin-loaded Ac-DEX microparticles could potentially be passively targeted to macrophages and subsequently phagocytosed in the acidic environment of the phagolysosome; this intracellular environment would trigger the intracellular burst release of the drug payload due to the acid-sensitivity of the Ac-DEX polymer with minimal drug released in extracellular pH-neutral environments. Because of their immunosuppressive capabilities, it is possible that immunosuppressant-loaded Ac-DEX microparticles could be applied *in vivo* to treat autoimmune diseases or prevent organ rejection while reducing the toxic side-effects of the drug. Future work includes investigating scalable particle production methods and evaluating particle trafficking *in vivo* in both immune cells and tissues.

Acknowledgements

This work was supported in part by The Ohio State University College of Pharmacy Summer Undergraduate Research Fellowship Program. The authors also thank Yu Jeong Kim, Claire Parker, and Dr. Sadhana Sharma for their collaboration.

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