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Mannitol-Guided Delivery of Ciprofloxacin in Artificial Cystic Fibrosis Mucus Model

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ABSTRACT: Abnormal airway mucus presents a significant challenge for inhalational drug delivery. Recognizing the thick and tenacious airway mucus seen in the cystic fibrosis (CF) patients as a critical barrier to effective drug delivery, both into the mucus layer itself as well as across that layer to the underlying airway epithelium, we hypothesize that mannitol or NaCl can form inhalable drug carriers, improve drug penetration into the mucus, and ultimately enhance the drug's therapeutic effect. The objective of this study is to test whether mannitol and NaCl particles, as inhalable drug carriers, improve drug delivery into and enhance a drug's activity within a mucus-like material. Microparticles containing Ciprofloxacin (Cipro), an active ingredient, and either mannitol or NaCl were produced by spray-drying. Cipro encapsulated in mannitol particles (Cipro–mannitol) was significantly more effective at killing *Pseudomonas aeruginosa* (*P. aeruginosa*) grown in artificial mucus (AM) than Cipro encapsulated in either NaCl particles (Cipro–NaCl) or in hydrophobic particles consisting of dipalmitoylphosphatidylcholine (DPPC), albumin, and lactose (Cipro–DAL). The relatively high antibacterial effectiveness of Cipro–mannitol was not due to the effect of mannitol on bacteria or on Cipro. Rather, the unique performance of the mannitol-based particles in AM is attributable to its ability to increase local water content in the AM and enhance drug penetration into it. Mannitol is a promising excipient for inhalable microparticles that facilitate the drug delivery into the CF mucus. Biotechnol. Bioeng.

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KEYWORDS: cystic fibrosis; mannitol; inhalable dry particles; mucus; drug delivery

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Additional Supporting Information may be found in the online version of this article.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene (Ramsey, 1996). This gene encodes a chloride channel in the apical membrane of epithelial cells, and mutations in it lead to disturbances in ion and water movement across the epithelial layers in many organ systems (Griesenbach and Alton, 2009; Ramsey, 1996). In the respiratory system, most CFTR mutations result in accumulation of dehydrated and tenacious mucus within the airways, poor airway clearance, recurrent or chronic bacterial infections, and persistent inflammation. This leads to progressive lung destruction and respiratory failure (Murphy and Rosenstein, 1998; Ramsey, 1996; Sanders et al., 2000).

The current CF therapies focus on attenuating disease progression and delaying the onset of irreversible damage in the respiratory system and other organs. In brief, antibiotics (antipseudomonals, antistaphylococcals, and others) are used to control the airway infection; steroids and nonsteroidal anti-inflammatories to control the inflammatory response (Marchall et al., 2000; Murphy and Rosenstein, 1998; Stern and Geddes, 2000); inhaled β-adrenergic agonists to optimize bronchodilation; and recombinant human deoxyribonuclease (rhDNase, Dornase alfa), a mucolytic, to promote mucus clearance (Goa and Lamb, 1997; Hodson et al., 2003; Murphy and Rosenstein, 1998).

The lungs are a major therapeutic target in CF, so inhalational drug delivery is at the center of the CF armamentarium, since it ensures deposition of medications at the site of action, which increases their local availability and decreases systemic side effects. However, CF mucus is thicker, more tenacious, and much less penetrable than

normal. This poses a significant challenge for effective inhalational delivery of many therapeutic agents (Hanes and Demeester, 2009). Negotiating this barrier is important in two important paradigms. In the case of inhalational delivery of antibiotics, it is crucial for the drug to penetrate into, but not necessarily across, the mucus and ensure an even distribution of the drug within the mucus to maximize its antibacterial effect. On the other hand, for the delivery of genetic therapeutics, theoretically the most ideal treatment of CF (Griesenbach and Alton, 2009), it is important to penetrate across the mucus layer and deliver the drug payload to the underlying epithelium.

Recognizing this, we previously hypothesized that co-delivery of the antibiotic ciprofloxacin (Cipro) with a mucolytic agent (rhDNase) would enhance Cipro's penetration into the mucus and showed, using an artificial mucus (AM) model, that co-delivery of Cipro with rhDNase significantly enhanced Cipro's diffusion and antipseudomonal activity in the *Pseudomonas*-laden AM (Yang et al., 2010). However, co-delivery with rhDNase is not a viable option for genetic therapeutics. Moreover, the substantial cost of rhDNase presents an additional challenge to its routine use. Therefore, in this study we explored alternative agents that would influence mucus rheology and enhance drug penetration. We chose mannitol and NaCl, both used clinically by inhalation due to their ability to increase water flux into the bronchial lumen and improve mucus clearance (Daviskas et al., 2008; Donaldson et al., 2006; Elkins and Bye, 2006; Elkins et al., 2006; Eng et al., 1996; Hirsh, 2002; Jaques et al., 2008; Nilsson et al., 2007; Wills, 2007).

We hypothesized that mannitol and/or NaCl particles can form an inhalable drug carrier and function as an osmotic agent, inducing influx of water into the mucus and enhancing the transmucus diffusion of the drug. To test this, microparticles containing Cipro and either mannitol or NaCl were produced by spray-drying, and the ability of the osmotic agents to enhance penetration of Cipro into the mucus-like material and its antibacterial activity were assessed. A previously described AM model (Yang et al., 2010) that resembles the CF mucus in chemical composition and rheological properties was used for the evaluation of drug diffusion.

Materials and Methods

Materials

See the Supporting information.

Particle Preparation

Microparticles were prepared with LabPlant SD-05 Spray Dryer (LabPlant Ltd, North Yorkshire, UK). The feed solution was prepared as 2 mg/mL solutions of excipient(s) in 70% ethanol. The inactive excipients were (i) mannitol, (ii) NaCl, (iii) a mixture of mannitol and leucine (4:1), or (iv) a mixture of dipalmitoylphosphatidylcholine (DPPC), albumin, and lactose (DAL) in the ratio of 3:1:1 by weight. For visualization of particle penetration through AM, Coomassie blue (CB) was added as 1.3% of total particle weight. For antibacterial activity testing, Cipro was included as 5% total particle weight. Cipro was first added to a small amount of water and homogenized with a Sonics Vibra-Cell Ultrasonic Processor (Sonics & Materials, Inc., Newtown, CT) for 8 min alternating on and off (4 min total sonication time) at 50% amplitude and then combined with the excipient solution prior to spray drying. The spray dryer was operated at the feed rate of 15 mL/min, inlet temperature of 150°C, and nozzle size of 1 mm. The outlet temperature was 85–95°C. The particles were named according to their active ingredient and the excipient. For example, CB-mannitol was mannitol particles containing 1.3 wt% CB, and Cipro–NaCl was NaCl particles containing 5 wt% Cipro.

Scanning Electron Microscopy (SEM)

The morphology of the prepared particles was examined with a JEOL JSM-840 scanning electron microscope (JEOL Ltd, Tokyo, Japan), using a 5 kV accelerating voltage, a 28 mm working distance, a 70 μm objective aperture, and a probe current of 6×10^{-11} A.

Aerodynamic Characteristics of Spray-Dried Particles

Aerodynamic particle size distribution was determined using an eight-stage Mark II Anderson Cascade Impactor (ACI) previously described (Yang et al., 2010). Detailed procedure is described in the Supporting information. The fine particle fraction (FPF) was defined as the amount of particles with an aerodynamic size <4.7 μm (particles deposited at stage 3 and lower) divided by the initial total particles loaded in the inhaler (10 mg; nominal dose). The cumulative mass of particles less than effective cutoff diameter as percent of total mass recovered in the ACI was plotted against the effective cutoff diameter. The mass median aerodynamic diameter (MMAD) was defined on this graph as the particle size at which the line crossed the 50th percentile.

Determination of the Drug Content in Spray-Dried Particles

1.5 mg of Cipro–mannitol, Cipro–NaCl, Cipro–mannitol/leucine, or Cipro–DAL particles was dissolved in 1 mL of HCl solution (pH 2) containing 0.5% SDS. This mixture was agitated using a vortex mixer and centrifuged at 8,000 rpm for 5 min. The supernatant was then diluted five times with water and analyzed using High Pressure Liquid Chromatography (HPLC 1100 series, Agilent Technologies, Palo Alto, CA) and an Atlantis analytical column (dC18; 4.6×250 mm; particle size 5 μm). See supporting information for the detailed HPLC condition.

Release Kinetics

One milliliter of phosphate buffered saline (PBS, pH 7.4) was used to disperse 1.5 mg dry particles (equivalent to 75 μg of Cipro) and incubated in 37°C with constant agitation. After 0.5 h of incubation, the suspension was centrifuged at 8,000 rpm for 5 min to separate 0.8 mL of supernatant, and an equal volume of fresh PBS was replaced. This sampling procedure was repeated after 1, 2, 3, and 4 h of incubation. The sampled supernatants were stored at −20°C and analyzed with HPLC.

Determination of Minimal Inhibitory Concentration of Cipro and Mixtures of Cipro and Excipients

The minimal inhibitory concentration (MIC) of Cipro and a mixture of Cipro and mannitol (or NaCl) was determined in accordance with the Clinical and Laboratory Standards Institute guideline for broth microdilution procedures (CLSI, 2009). See supporting information for the detailed inoculum preparation procedure. Ten microliters of the inoculum was added to wells of a 96-well plate, which contained 100 μL of serially diluted solutions of standard Cipro or a mixture of Cipro with either mannitol or NaCl in the ratio of 5:95 by weight to make the final Cipro concentrations ranging from 0.008 to 8 μg/mL. Bacterial growth was determined after overnight incubation at 37°C. The MIC was defined as the lowest drug concentration with no visible growth, as indicated by the lack of absorbance at 650 nm. The MIC measurement was performed in triplicate.

Preparation of Artificial Mucus

Sterile AM was prepared as previously described (Yang et al., 2010). Detailed procedure is described in the Supporting information.

Visual Inspection of Active Ingredient Penetration Through Artificial Mucus

First, a 10% gelatin solution was prepared in hot water. One milliliter of the gelatin solution was placed in each well of a 24-well plate, hardened at room temperature, and stored at 4°C until use. One milliliter of AM was placed atop the hardened gelatin gel. To compare the penetration of the particles through the AM, 10, 20, or 30 mg of CB-mannitol particles (equivalent to 0.13, 0.26, 0.39 mg CB, respectively), 30 mg of CB-DAL particles (equivalent to 0.39 mg CB), 30 mg of CB-NaCl particle (equivalent to 0.39 mg CB), and 0.13, 0.26, 0.39 mg of CB were placed on 1 mL of AM and incubated in a closed chamber maintaining 100% relative humidity, emulating the humid environment of the airways (Marini, 1998). After 1, 3, or 7 h, the AM and the particles were removed from the gelatin plates, which were then rinsed with water six times. The gelatin gel was melted at 100°C for homogenous mixing of gelatin and CB. The absorbance of CB in gelatin was measured at 590 nm.

Preparation of Artificial Mucus Containing *P. aeruginosa*

To mimic pathogen-laden CF mucus, AM containing *P. aeruginosa* was prepared. A single colony of *P. aeruginosa* grown on a tryptic soy agar plate was isolated, seeded in 25 mL of tryptic soy broth, and incubated at 37°C with gentle shaking. When the optical density of the broth at 420 nm reached 0.15, 10 μL of the broth was added to 1 mL of sterile AM and incubated at 37°C for 2 h prior to the treatment with particles.

Antibacterial Effectiveness of Dry Particles in *Pseudomonas*-Laden Artificial Mucus

To compare the antipseudomonal effectiveness of the particles in AM, 1.6 mg of each particle type (mannitol, NaCl, DAL, Cipro–mannitol, Cipro–NaCl, Cipro–DAL, or Cipro–mannitol/leucine) was placed on 1 mL of the *Pseudomonas*-laden AM and incubated at 37°C for 1 or 2 h. All the Cipro particles contained 80 μg of Cipro and were added as dry powder to *Pseudomonas*-laden AM (without an external water source). After the incubation, 1 mL of dilution medium (2 mg/mL cellulase, 400 μg/mL chloramphenicol in 0.05 mol/L citrate buffer, pH 4.6) was added to the particle-treated mucus, which was then incubated for 30 min at 37°C. Ten microliters of the diluted AM was further diluted with tryptic soy broth as needed, plated on a tryptic soy agar plate, and incubated at 37°C overnight, after which the bacterial colonies were counted.

Rheological Analysis of Artificial Mucus

1.6 mg of mannitol, NaCl, DAL, Cipro–mannitol, Cipro–NaCl, or Cipro–DAL particles were placed atop 1 mL of AM, and incubated at 37°C for 1 h and stored at −80°C until analysis. Rheological properties (storage modulus (G'), loss modulus (G''), and phase angle (δ)) of the AMs were determined as previously described (Yang et al., 2010) using the AR 2000 rheometer (TA Instruments, Leatherhead, Surrey, UK). Detailed procedure is described in the Supporting information.

Results

Particle Characterization

As shown in Figure 1, spray-dried NaCl particles were cubic crystals with sides 1–5 μm long. Mannitol formed spherical particles, comparable in size to the NaCl particles. The MMADs were 6.5 μm for the NaCl and 5.1 μm for the mannitol particles (Table I), slightly bigger than the apparent sizes estimated by SEM. The FPFs of the nominal dose of NaCl and mannitol particles were 17.6% and 22.4%, respectively.

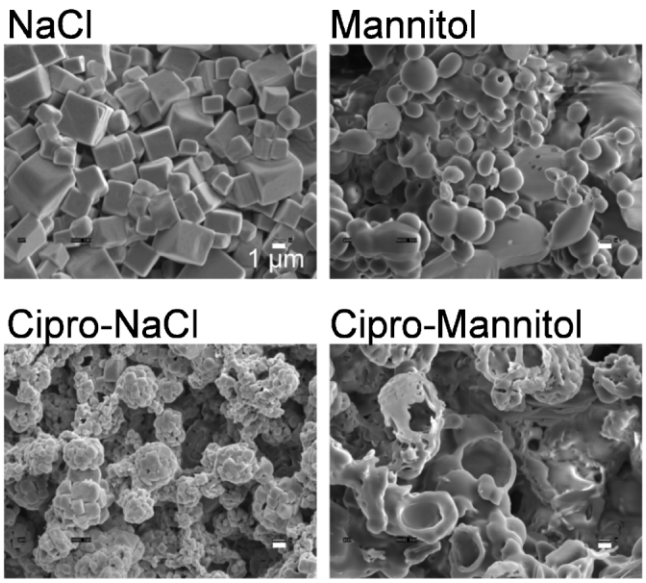

Figure 1. Scanning electron micrographs of spray-dried particles. Scale bar = 1 μm. Magnification: 5,000×.

Mannitol particles including 20 wt% leucine had MMAD and FPF of 4.5 μm and 27.3%, respectively. Leucine is frequently used to enhance the dispersion of spray-dried particles (Li et al., 2003, 2005; Seville et al., 2007; Ibrahim et al., 2010), but we did not observe statistical difference between mannitol and mannitol-leucine particles ($P > 0.05$, *t*-test).

The particle morphology changed significantly with the inclusion of Cipro. The Cipro–NaCl particles still looked crystalline but were aggregates of much smaller cubes (each side around 1 μm). This morphological change was reflected in the MMAD and FPF of the Cipro–NaCl particles, which were 4.0 μm and 27.3%, respectively, closer to values appropriate for inhalation than NaCl particles, although statistical difference was not seen (both $P > 0.05$ vs. NaCl particles by t-test). The Cipro–mannitol particles were bigger and hollow, which may be attributable to the hydrophobicity of Cipro. Hydrophobic ingredients tend to form hollow particles, because they get saturated easily during the drying process and are unable to move along with the receding droplet surface (Vehring, 2008). The presence of Cipro in mannitol particles did not significantly influence their aerodynamics (both FPF and MMAD $P > 0.05$ vs. mannitol particles by *t*-test). However, the FPF of Cipro–mannitol-leucine particles was improved to 54.3%, significantly better than mannitol-leucine particles ($P < 0.05$, *t*-test).

HPLC analysis shows that Cipro was loaded in spray-dried particles without loss (Table I). The Cipro content in Cipro–NaCl and Cipro–mannitol particles prepared with the target drug content of 5 wt% were 4.8 ± 0.2% and 5.2 ± 0.4%, respectively. Both Cipro–NaCl and Cipro–mannitol particles released the drug as soon as they were resuspended in PBS, similar to Cipro–DAL, a control particle system made of hydrophobic excipients (Supplementary Fig. 1).

Effect of Excipients on Active Ingredient Penetration Through Artificial Mucus

To compare the ability of excipients to enhance the diffusion of an active ingredient through the AM, NaCl, mannitol, and DAL particles containing 1.3 wt% CB were prepared. The CB-laden particles were placed atop a layer of AM. Underneath the AM, 10% gelatin gel was present to capture the CB that penetrated the AM layer (Fig. 2A). Therefore, the amount of CB in the gelatin gel was proportional to the extent of particle penetration. No volume change of gelatin gel was observed during the incubation with AM. Figure 2B and C shows that the CB penetration was dependent on the excipients. CB in CB-mannitol particles penetrated the AM faster than the dye alone, or that in CB-NaCl or CB-DAL particles. Over 3–7 h, CB-mannitol with 0.13 mg CB resulted in more CB penetration through AM and staining of the underlying gelatin than CB alone, CB-NaCl, or CB-DAL carrying three times more CB (0.39 mg).

Effect of Excipients on the Antibacterial Effectiveness of Cipro the *Pseudomonas*-Laden AM

To test whether the observed ability of mannitol to enhance the transmucus dye penetration translates to enhanced drug delivery into the mucus, we compared the antibacterial effectiveness of Cipro-particles in the *Pseudomonas*-laden AM model as described in our previous study (Yang et al., 2010). The bacterial counts in the AM after a 1 h incubation with Cipro-particles (all containing 80 μg Cipro) were the least for the Cipro–mannitol particles, intermediate for the Cipro–NaCl, and the greatest for Cipro–DAL (Fig. 3A).

Table I. FPF and MMAD of spray-dried particles.

Particles	Cipro content (wt%)	Encapsulation efficiency (%)	FPF (%)	MMAD (μm)
NaCl	—	—	17.6 ± 10.9	6.5 ± 2.0
Mannitol	—	—	22.4 ± 1.6	5.1 ± 0.2
Mannitol–leucine	—	—	27.3 ± 7.1	4.5 ± 0.7
Cipro–NaCl	4.8 ± 0.2	96.2 ± 3.6	27.3 ± 5.3	4.0 ± 0.8
Cipro–mannitol	5.2 ± 0.4	103.6 ± 8.3	24.4 ± 10.4	4.8 ± 0.5
Cipro–mannitol–leucine	5.0 ± 0.2	100.6 ± 4.8	54.3 ± 3.3	3.6 ± 0.4

[a]All data are expressed as averages ± standard deviations of three independent batches.

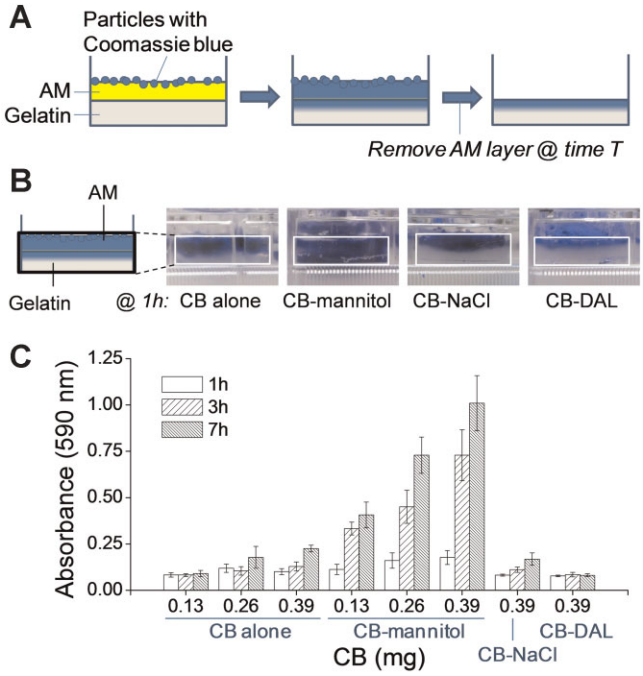

Figure 2. Ability of Coomassie blue (either as free dye or contained in particles) to penetrate AM. **A**: Schematic of visual inspection of CB penetration through artificial mucus. **B**: CB penetration into AM. The boxed area shows AM layer on top of gelatin gel. **C**: CB reaching the gelatin gel across the AM layer, represented by the absorbance of CB in gelatin. Data are expressed as averages and standard deviations of three independently and identically produced samples.

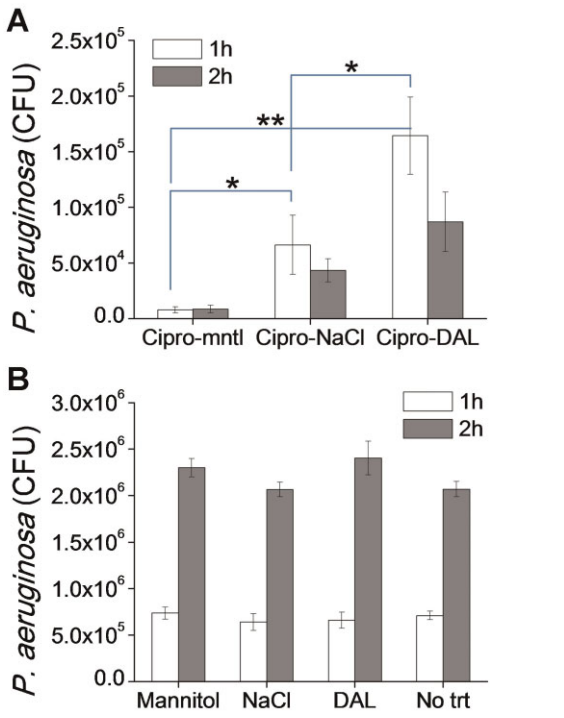

Figure 3. **A**: Antibacterial effectiveness of Cipro particles in the *Pseudomonas*-laden AM. $^{*}P<0.05$; $^{**}P<0.01$ by *t*-test. In 2 h, all pairs of data were significantly different ($P<0.01$). **B**: Effect of excipients on the bacterial growth. No trt, No treatment. Data are expressed as averages and standard deviations of three independently and identically produced samples.

Although the overall bacterial counts decreased after incubation for another hour, the trend remained the same. Blank mannitol, NaCl, and DAL particles did not demonstrate any intrinsic antibacterial activity at the same quantity (1.6 mg) as the Cipro-particles (Fig. 3B).

To examine whether the greater antibacterial effectiveness of the Cipro–mannitol and to a lesser degree the Cipro–NaCl particles was due to a pharmacologic interaction between Cipro and the excipients, MIC of Cipro mixed with each excipient in the ratio used in the particle was compared to that of Cipro alone. Both Cipro/NaCl and Cipro/mannitol mixtures showed the MIC of 0.25 μg/mL (Supplementary Fig. 2), comparable to standard Cipro (Madaras-Kelly et al., 1996), confirming that neither excipient potentiated the antibacterial effectiveness of Cipro. The result of the Cipro/mannitol mixture was also consistent with a recent study (Adi et al., 2010), which demonstrated that the presence of mannitol did not influence Cipro activity.

Effect of Excipients on Rheological Properties of Artificial Mucus

To evaluate each particle's influence on AM's rheology, the AM was incubated with DAL, Cipro–DAL, NaCl, Cipro–NaCl, mannitol, or Cipro–mannitol for 1 h at 37°C and analyzed with a rheometer. We previously confirmed that our AM model had similar G' to that of clinical CF mucus and maintained consistent G' at 37°C up to 7 h (Yang et al., 2010). DAL and Cipro–DAL particles had no significant effect on the G' of the AM, consistent with our previous report (Yang et al., 2010). NaCl and Mannitol particles decreased the G' of AM by 23% and 31%, respectively, compared to the non-treated AM, and Cipro–NaCl and Cipro–mannitol by 17% and 19%, respectively, at 1.995 Hz (Fig. 4A). The effects of Cipro–NaCl and Cipro–mannitol were not significantly different from each other ($P>0.05$, *t*-test) at all frequency levels. The loss moduli (G'') of the particle-treated AMs showed a similar trend (Fig. 4B). At 1.995 Hz, NaCl and Mannitol particles decreased the G'' of AM by 16% and 22%, respectively, compared to the non-treated AM, and Cipro–NaCl and Cipro–mannitol by 10% and 12%, respectively (Fig. 4B). There was no statistical difference in G'' between AMs treated with Cipro–NaCl and Cipro–mannitol ($P>0.05$, *t*-test). The phase angle δ, calculated from the inverse tangent of G''/G', was below 45° for all samples at all frequencies, indicating that AMs remained dominantly elastic irrespective of the particle treatment. NaCl, Cipro–NaCl, mannitol, and Cipro–mannitol particles induced slight increase of the phase angle.

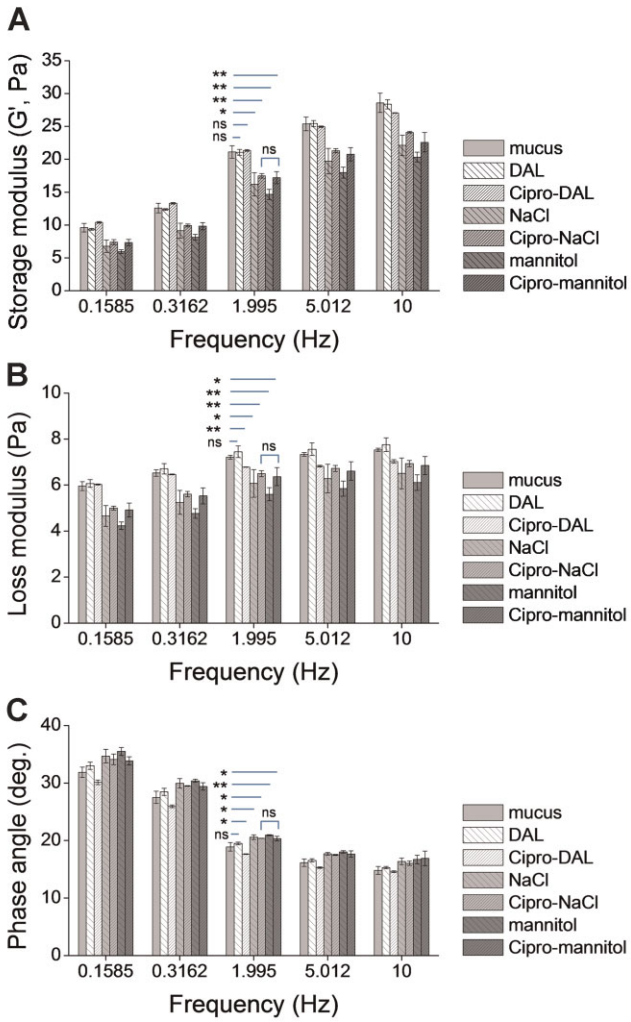

Figure 4. Effect of spray-dried particles on (**A**) storage modulus (G'), (**B**) loss modulus (G''), and (**C**) phase angle (δ) of AM after 1 h incubation. Data are expressed as averages and standard deviations of 3 repeated measurements of a single sample, which is representative of 3 independently and identically produced samples. $^{*}P < 0.05$; $^{**}P < 0.01$ by t-test.

Discussion

Mucus covering a variety of body surfaces, often considered an opportunity for muco-adhesive drug delivery (Serra et al., 2009), has recently gained substantial interest as a critical barrier to inhalational delivery of antibiotics (Yang et al., 2010) and to targeted delivery of nanomedicines to the underlying epithelium (Hanes and Demeester, 2009). Composed of several biopolymers forming physical and chemical networks, mucus forms a gel-like barrier that protects the body from a variety of invading pathogens and environmental ultrafine particles (Cone, 2009). However, abnormally tenacious CF mucus acts as an antibiotic-impenetrable sanctuary for a variety of bacterial pathogens, making the antibiotic therapy inefficient. This delivery challenge becomes even more significant when nanomedicines are involved, not only due to their size but also for the distance they need to travel to reach the underlying epithelium.

In an attempt to address this challenge, Hanes et al. proposed modifying the surface of nanoparticles with low-molecular-weight (~2,000 Da) polyethylene glycol (PEG) to prevent the interactions between nanoparticles and the mucus components (Lai et al., 2007; Suk et al., 2009; Tang et al., 2009). In this approach, densely PEGylated nanoparticles were able to diffuse through cervicovaginal mucus (Lai et al., 2007; Tang et al., 2009) or CF mucus (Suk et al., 2009; Tang et al., 2009) by avoiding adhesive interactions with the mucin network. Here, the molecular weight of PEG was critical in enhancing mucus penetration of nanoparticles. Polystyrene nanoparticles that were densely coated with 2 or 5 kDa PEG penetrated the undiluted cervicovaginal mucus relatively fast, whereas those with 10 kDa PEG did not because the polymer entangled with mucus (Wang et al., 2008).

Alternatively, we aimed to overcome the mucus barrier by influencing the mucus itself and envisioned that the osmotic agents like mannitol and NaCl would be useful for this purpose. Hypertonic saline increases the water flux into the bronchial lumen, thereby increasing the liquid content of the airway surface liquid (Donaldson et al., 2006; Elkins and Bye, 2006; Elkins et al., 2006; Eng et al., 1996; Hirsh, 2002; Nilsson et al., 2007). It may also disrupt ionic bonds between the mucus biopolymers, reducing the degree of networking (King and Rubin, 2002; King et al., 1997). Inhaled dry-powder mannitol, which also has an osmotic effect (Daviskas et al., 1999, 2008; Hirsh, 2002; Jaques et al., 2008; Wills, 2007), is effective in patients with non-CF bronchiectasis (Daviskas et al., 2008) or CF (Jaques et al., 2008; Robinson et al., 1999). Therefore, we anticipated that mannitol and NaCl placed on the mucosal layer of the lung epithelium would induce the influx of water from the epithelial cells, dilute the mucus, and facilitate the drug transport into the mucus.

We further hypothesized that these agents would also create an osmotic gradient within the mucus and induce a local hydration effect. This hypothesis is based on the current understanding of the mucus structure. Biopolymers in mucus are bundled together to form thick cables creating large spaces on the order of hundreds of nanometers, which are filled with low-viscosity fluid (Lai et al., 2010). We expected that the presence of an osmotic agent would increase local influx of water into the microdomains from the neighboring regions, increasing the heterogeneity and macroporosity of the biopolymer networks, and facilitating drug transport through them. We tested this hypothesis using Cipro and the AM model, used in our previous study (Yang et al., 2010). To maximize the concentration gradient, mannitol and NaCl were prepared as dry particles.

Cipro–mannitol particles showed higher antibacterial activity than Cipro–NaCl or Cipro–DAL particles in *Pseudomonas*-laden AM. Mannitol did not have an intrinsic anti-bacterial activity, nor did it decrease the MIC of Cipro, suggesting that the relatively high antibacterial effectiveness

of Cipro–mannitol was not due to mannitol's direct effect on bacteria or on Cipro. Given that Cipro was quickly released from all tested particles upon their dissolution in buffer, the difference between the antibacterial effectiveness of the Cipro–mannitol particles and that of the Cipro–NaCl or Cipro–DAL is likely not due to the differential Cipro release from the particles. Therefore, the unique enhancement of the antibacterial activity of Cipro in AM by mannitol is attributable to mannitol's influence on the AM environment, namely the ability to induce the water flux and penetrate the AM, visualized by transmucus diffusion of CB-mannitol (Fig. 2).

The significant difference between the mannitol and DAL particles is readily explained by the hydrophilicity of mannitol, which facilitated wetting and dissolution of particles in AM. On the other hand, the difference between the mannitol and NaCl particles was unexpected, because both are widely used osmotic agents with good aqueous solubilities. In fact, NaCl has a higher aqueous solubility (400 mg/mL) than mannitol (180 mg/mL) and produces a much higher osmolarity than mannitol at the same mass per volume due to its small molecular weight and ionization. Both the Cipro–NaCl and the Cipro–mannitol particles somewhat decreased the G' of AM, presumably by disrupting ionic and hydrogen bonds, respectively, but the difference between the two was not significant. Therefore, their effects on rheological properties also did not explain the superior antibacterial activity of Cipro–mannitol particles.

Given the lack of difference between NaCl and mannitol in hydrophilicity and their effects on Cipro, bacteria, and rheological properties of AM, the differential antibacterial activities of the two particles in *pseudomonas*-laden AM may be related to the volume of AM that each osmotic agent could diffuse into. The depth to which the particles penetrated the AM, as visualized by CB, suggests that NaCl was localized in a relatively small volume of AM and did not spread as effectively as mannitol. In earlier studies, Edwards et al. reported that the number of exhaled bioaerosol particles was reduced by inhalation of normal saline (Edwards et al., 2004). This phenomenon was attributed to the ability of NaCl to stabilize the air/mucus interface by shielding charges and causing protein gelation on the mucus surface (Watanabe et al., 2007). The mechanism proposed in that study suggests that NaCl is consumed at the air-mucus interface and possibly trapped there, consistent with our observation. It may be for this reason that NaCl was unable to induce the Cipro diffusion as broad as mannitol in AM.

Our results suggest that mannitol is a superior excipient to NaCl for delivery of an active ingredient via the enhanced osmosis. However, we note that the AM model used in this study does not take into account the direct effects of osmotic agents on transepithelial ion transport (Davies et al., 2005; Hebestreit et al., 2007; Middleton et al., 2001), which could result in a substantially different water flux in the patients than in vitro AM model. Moreover, hypertonic NaCl increases tight junction permeability in airway epithelium (Hogman et al., 2002), potentially further influencing the transepithelial transport of ions and water (Reuss, 2001). Therefore, it is possible that NaCl would also enhance the drug transport in vivo, where it may directly influence the ion transport and recruit water from the epithelium.

We also note that the influence on G' or G'' may be greater in CF mucus, where the intermolecular networks of DNA and proteins significantly contribute to rheology. As we previously discussed (Yang et al., 2010), the AM model does not simulate the three-dimensional macromolecular network seen in the CF mucus (King and Rubin, 2002). Mannitol and NaCl may be more influential in the CF mucus, since experimental evidence shows the significant influence of NaCl (King and Rubin, 2002; King et al., 1997) and a non-ionic osmotic agent (Feng et al., 1998; King and Rubin, 2002) on its rheological properties.

Additional advantage of mannitol as an excipient for an inhalable dry particle formulation is that it is non-hygroscopic and easy to handle at relatively high humidity (Daviskas et al., 1999). Spray-dried mannitol forms particles with a reasonable size and FPF on its own. These can be improved by the addition of leucine (Table I), which has the unique ability to form a non-adhesive surface on the particles. The inclusion of leucine does not influence the antibacterial activity of Cipro–mannitol particles (data not shown). This result shows the flexibility of mannitol particles as an inhalable drug carrier.

Inhaled mannitol has been shown to have favorable effects on CF patients in Phase 2 trials (Daviskas et al., 2010; Jaques et al., 2008) and has recently undergone Phase 3 trials (ClinicalTrials.gov identifiers: NCT00446680, NCT00630812). However, one of its disadvantages as a standalone osmotic agent is the large dose requirement (400 mg twice daily), which necessitates the inhalation of several unit doses of mannitol each time (Wills and Greenstone, 2006). On the other hand, the dose requirement of mannitol as an inactive excipient of inhalable dry particles is likely to be smaller. The exact dose will depend on the extent of dose decrease attributable to mannitol and the optimal ratio of Cipro and mannitol, which are yet to be determined. In this study we used 5 wt% Cipro loading, but we may increase the content up to 50 wt% maintaining good aerodynamic properties (Adi et al., 2010). For delivery of 32.5–48.8 mg of Cipro (doses for inhalation in a recent clinical trial with CF patients: ClinicalTrials.gov identifier NCT00645788) at 25–50 wt% loading level, the total required particle dose is 65–195 mg, which translates to 32.5–146.3 mg mannitol, much smaller than the standalone dose. Therefore, mannitol may find more utility as an excipient than as a standalone osmotic agent.

Conclusion

Spray-dried mannitol particles as a carrier of Cipro significantly improved the antibacterial activity of the drug

on *P. aeruginosa* grown in the AM. The high efficacy of Cipro–mannitol is likely due to its ability to increase the local water content in the mucus and enhance the drug transport into it. Mannitol is a promising excipient for inhalable microparticles that aim to facilitate the delivery of medicines into and across the CF mucus.

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