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Nanoparticle diffusion in respiratory mucus from humans without lung disease

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ABSTRACT

A major role of respiratory mucus is to trap inhaled particles, including pathogens and environmental particulates, to limit body exposure. Despite the tremendous health implications, how particle size and surface chemistry affect mobility in respiratory mucus from humans without lung disease is not known. We prepared polymeric nanoparticles densely coated with low molecular weight polyethylene glycol (PEG) to minimize muco-adhesion, and compared their transport to that of uncoated particles in human respiratory mucus, which we collected from the endotracheal tubes of surgical patients with no respiratory comorbidities. We found that 100 and 200 nm diameter PEG-coated particles rapidly penetrated respiratory mucus, at rates exceeding their uncoated counterparts by approximately 15- and 35-fold, respectively. In contrast, PEG-coated particles $\geq$500 nm in diameter were sterically immobilized by the mucus mesh. Thus, even though respiratory mucus is a viscoelastic solid at the macroscopic level (as measured using a bulk rheometer), nanoparticles that are sufficiently small and muco-inert can penetrate the mucus as if it were primarily a viscous liquid. These findings help elucidate the barrier properties of respiratory mucus and provide design criteria for therapeutic nanoparticles capable of penetrating mucus to approach the underlying airway epithelium.

1. Introduction

Mucus lines the conducting airways, protecting the respiratory epithelium from the external environment. The respiratory mucus barrier comprises two layers: The gel layer, rich in secreted macromolecules, which is continuously cleared by ciliary beating, and the periciliary layer, consisting of macromolecules tethered to the airway surface, which facilitates ciliary activity [1]. A major role of the respiratory mucus gel is to trap inhaled particles so they can be swept from the airways by mucociliary clearance, thereby defending the lungs against pathogens and toxic materials [2]. However, it is not known what size particles are trapped, or how particle surface chemistry affects mobility, in the airway mucus gel layer of humans without respiratory disease [3]. Airway mucus is difficult to collect and analyze because individuals with healthy respiratory systems generally cannot spontaneously expectorate it, and the mucus gel layer is only a few to a few tens of micrometers thick [2,4]. Yet measuring the permeability of this mucus gel is essential for understanding how effectively it protects the lungs from continuously inhaled pathogens and environmental particulates. Studying the interaction between respiratory mucus and nanoparticles may also elucidate design criteria for inhaled therapeutic nanoparticles to treat diseases such as lung cancer; these particles must penetrate the mucosal barrier to avoid rapid clearance and achieve the pharmacokinetic profile requisite for effective therapeutic outcomes [5]. Finally, characterizing respiratory mucus from humans without pulmonary disease may provide a benchmark against which to compare respiratory secretions from patients with diseases that affect the airways, including asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF).

Nanoparticle diffusion has previously been measured in a variety of human mucus secretions, including cervicovaginal (CV) mucus [6], CF sputum [7,8], and chronic rhinosinusitis mucus [9].

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Conventional, uncoated polymeric particles have hydrophobic surfaces that adhere to hydrophobic domains on mucin fibers – the long, entangled glycoproteins responsible for the mucus gel structure [10]. We discovered that coating polymeric nanoparticles with a dense layer of low molecular weight polyethylene glycol (PEG) minimizes particle adhesion to mucus [6]. PEG minimizes protein adhesion at biomaterial surfaces because the hydrated PEG layer enthalpically resists release of water molecules and entropically resists compression [11]. Using PEG-coated particles, we were able to probe the mucus microstructure [12]. Surprisingly, we found that PEG-coated particles as large as 200 nm in diameter can penetrate sputum freshly expectorated by CF patients [7], and coated particles as large as 500 nm can penetrate CV mucus collected from healthy women [6], whereas comparably sized uncoated particles are immobilized by the mucus mesh. Tracking the motion of individual PEG-coated and uncoated particles in fresh, minimally diluted mucus samples offers several advantages compared to traditional approaches for studying mucus gel structure and permeability. Electron microscopy, for instance, requires fixation methods that can change the mucus structure [10]. Transport studies using only conventional, uncoated polymeric particles cannot distinguish between adhesive and steric contributions to particle trapping. Finally, bulk diffusion measurements do not reveal transport rates at the single-particle level, and thus cannot identify particle subpopulations of interest, such as fast-moving outliers.

Mucus secretions from various wet epithelia are similar in overall properties – they are viscoelastic gels comprised of water and mucins, as well as salts, non-mucin proteins, and lipids – but their exact biochemical compositions, clearance rates, and microbiota differ with anatomical site and disease state [2]. These factors alter the mucus microstructure and permeability, and motivate the analysis of mucus from various tissues and conditions. Here, our aim was to study normal human airway mucus, which we define as airway mucus from individuals without respiratory disease. As our model for normal airway mucus, we collected the mucus from endotracheal tubes of patients without respiratory comorbidities who underwent elective, non-cardiothoracic surgery, as previously described [13]. This collection method is noteworthy because it imposes no additional burden on the patient, and in contrast to bronchiolar lavage or to sputum induction using nebulized hypertonic saline, it minimizes sample dilution and salivary contamination [13,14]. We performed scanning electron microscopy to visualize the mucus samples and conducted biochemical assays to analyze their basic composition. To elucidate the physicochemical properties of nanoparticles that govern their transport rates in normal airway mucus, we measured the mobility of PEG-coated and uncoated 100, 200, and 500 nm polymeric particles in fresh mucus samples using high-resolution fluorescence microscopy and multiple-particle tracking analysis. We further investigated the mucus gel structure and the barrier it poses to nanoparticle diffusion by comparing the bulk rheological properties of respiratory mucus, as measured using a cone-and-plate rheometer, with the microrheological properties, as probed by PEG-coated nanoparticles.

2. Methods

2.1. Mucus sample collection

Human airway mucus samples were collected in accordance with a protocol approved by the Johns Hopkins Medicine Institutional Review Board (study number NA_00038606). Samples were collected by the endotracheal (ET) tube method, as previously described [13,15]. Patients who required intubation as part of general anesthesia for elective, non-cardiothoracic surgery at the Johns Hopkins Hospital were identified. Only patients with no cardiopulmonary or respiratory comorbidities and no smoking history were included in this study. At the end of surgery, the ET tube was removed from the patient, and the distal 10 cm portion, including the balloon cuff, was cut and placed in a 50 mL centrifuge tube. The specimens were then spun at 1000 rpm (220 × g) for 30 s, yielding an average mucus volume of 0.5 mL. Mucus with visible blood contamination was not included in the analysis. Mucus samples were stored at 4 °C and analyzed within 24 h of collection, excluding portions for the mucin and DNA assays, which were frozen at −20 °C until use. The data presented here is from male and female patients; the mean patient age was 56 years, with standard deviation 17 years.

2.2. Rheology of respiratory mucus

Bulk rheological properties of airway mucus were measured using a strain-controlled rheometer (RFS3; TA Instruments) with cone-and-plate geometry (cone diameter 25 mm and angle 0.1 rad). All measurements were conducted at room temperature in a humidified chamber. Oscillatory tests, performed at small strain amplitudes to minimize shear damage to the mucus samples, were used to measure the frequency-dependent elastic modulus, $G'(\omega)$, and viscous modulus, $G''(\omega)$, of the mucus [16]. (The elastic and viscous moduli, respectively, are the in-phase and out-of-phase components of stress induced in the material, divided by the magnitude of the applied strain.) After the mucus sample was loaded onto the rheometer, it was allowed to equilibrate for five minutes. Then, the linear viscoelastic region, for which the viscous and elastic moduli are independent of strain amplitude [17], was determined by conducting strain amplitude sweeps from 0.2 to 10% strain at frequencies of 1, 6.28, and 100 rad/s. Based on the strain sweep tests, 1% strain was determined to be within the linear viscoelastic region, and was thus used for the frequency sweep test (from 0.1 to 100 rad/s).

2.3. Determination of mucin, DNA, and total solids content

Mucin concentration was determined based on the reaction of 2-cyanoacetamide (Sigma–Aldrich) with O-linked glycoproteins, as previously described [8,18]. Mucus aliquots were diluted 20-fold and homogenized by vortexing for at least 15 min. Then, 50 μL of this suspension was mixed with 60 μL of an alkaline solution of 2-cyanoacetamide (200 μL of 0.6 M 2-cyanoacetmaide added to 1 mL of 0.15 M NaOH). The mixture was incubated at 100 °C for 30 min, after which 0.5 mL of 0.6 M borate buffer, pH 8.0, was added to it. Fluorescence intensity was measured at excitation and emission wavelengths of 340 and 420 nm, respectively. Mucin concentrations were calculated by comparing the fluorescence intensity readings to a standard curve generated using known concentrations of mucin from bovine submaxillary gland (Sigma–Aldrich).

A fluorimetric assay was also used to measure DNA concentration, based on the reaction of 3,5-diaminobenzoic acid dihydrochloride (DABA; Sigma–Aldrich) with DNA [8]. Mucus aliquots were diluted 5-fold and homogenized by vortexing for at least 15 min. Then, 30 μL of this suspension was reacted with 30 μL of 20% wt/vol DABA solution. After incubating for 1 h at 60 °C, 1 mL of 1.75 M HCl was then added to stop the reaction. The fluorescence was measured at excitation and emission wavelengths of 400 and 520 nm, respectively. DNA concentrations were calculated with reference to a standard curve generated using known concentrations of DNA from salmon testes (Sigma–Aldrich).

The total solids content of mucus was determined by freeze-drying. Mucus samples were frozen in liquid N_2 and placed in a lyophilizer (FreeZone 4.5 Plus; Labconco) for at least 12 h to extract water from the samples. The ratio of mucus mass before vs. after lyophilization is the total solids content.

2.4. Scanning electron microscopy

Respiratory mucus samples were prepared for scanning electron microscopy (SEM) based on a protocol previously used for CV mucus and CF sputum [8,19]. Mucus samples were fixed for 1 h in 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2, containing 3 mM $CaCl_2$. The samples were then rinsed in buffer and postfixed in 1% OsO_4 in 100 mM sodium cacodylate buffer for 1 h on ice in the dark. Following a brief rinse with distilled water, samples were stained with 2% uranyl acetate for 1 h and then dehydrated through a graded series of ethanol solutions. Upon complete dehydration, samples were soaked in a 50:50 mixture of ethanol and hexamethyldisilazane (HMDS), followed by pure HMDS. Mucus samples were then dessicated under vacuum overnight. The samples were attached to aluminum stub mounts via carbon adhesive tabs (Ted Pella), and then coated with 20 nm of AuPd with a sputter coater (Desk III; Denton Vacuum). The samples were imaged with a field-emission scanning electron microscope (LEO 1530 FESEM; Zeiss) operating at 1 kV.

2.5. Nanoparticle preparation and characterization

Fluorescent, carboxylate-modified polystyrene spheres (PS–COOH) sized 100, 200, and 500 nm in diameter were purchased from Molecular Probes. The surface density of carboxyl groups on the particles, calculated using data provided by the manufacturer, ranged from 3 to 8 COOH/nm^2 depending on the lot. PEG-coated particles were prepared by covalently modifying the PS–COOH particles with 5 kDa methoxy-PEG-amine (Creative PEGWorks) using carbodiimide coupling chemistry [20]. Briefly, 100 μL of PS–COOH particle suspension, supplied by