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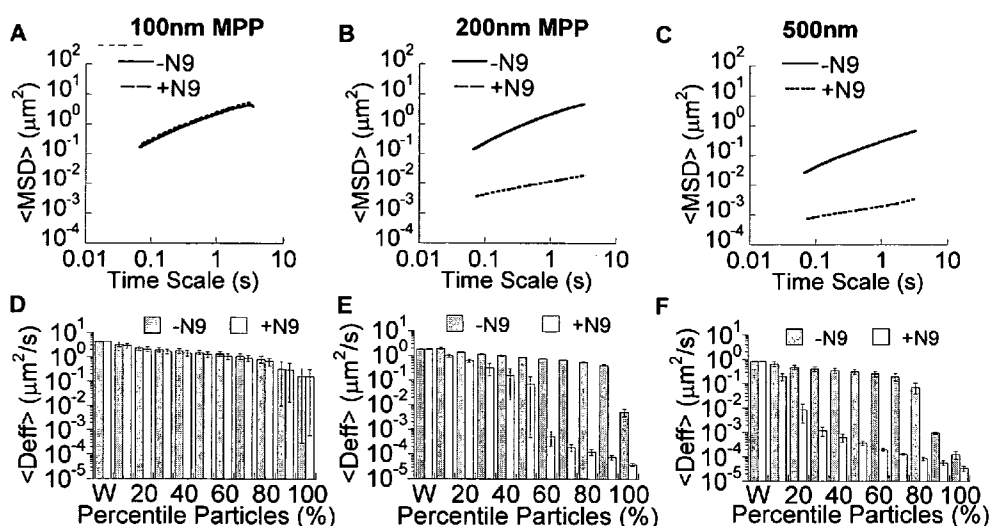


Figure 1

(57) Abstract: Compositions and methods for reducing or preventing particles such as pathogens and toxic substances from penetrating mucus, and that are useful for preventing or treating infection or toxicity in a subject.

Compositions and Methods for Reducing Particle Penetration through Mucus

FIELD OF THE INVENTION

Compositions and methods for reducing particle penetration through mucus by reducing the mesh spacing of the mucus gel.

BACKGROUND

Human mucus forms a protective barrier on all mucosal surfaces of the body, such as the lung airways, gastrointestinal tract, nose, eye, female reproductive tract, and more. Mucus possesses a mesh-like structure. We have recently found that the mesh spacing in mucus is much larger than previously expected based on electron microscopy studies and biochemical predictions (average spacing $\sim 340 \pm 70$ nm, with 80% of pores larger than 200 nm). This suggests that (i) the gel-forming mucins are strongly bundled together in native physiological mucus, and (ii) mucus does not pose a physical barrier to objects that are sufficiently small and do not adhere to mucus. Evidence that underscores the ability of various foreign particulates, such as pathogens and synthetic particles, to penetrate mucus include:

- (1) Effective diffusivities in mucus (D_m) for Norwalk virus and human papilloma virus (HPV) that are roughly equivalent to their effective diffusivities in water (D_w) (i.e. $D_m/D_w \sim 1$) [1];
- (2) The ability for human immunodeficiency virus (HIV) and influenza, as well as many other pathogens, to be transmitted at mucosal surfaces [2]; and,
- (3) The ability for synthetic polymeric particles 500 nm or larger, with surfaces mimicking viral particles, to diffuse in mucus at rates as little as 4-fold reduced compared to their transport rates in water [3].

Accordingly, viruses and other pathogens that do not adhere to mucus can diffuse through mucus at rates similar to the rates at which they diffuse through water, thus eluding the protective barrier actions of mucus.

Therefore, new compositions and methods that alter the properties of the mucus to slow or block the pathogen from reaching target tissues or cells are desirable.

This application claims priority to U.S. provisional application no. 61/010,659, filed January 10, 2008, which is hereby incorporated by reference.

SUMMARY

Mucus can play a vital role in the protection of the human body if it traps and removes pathogens and foreign particles from the lungs, eyes, gastrointestinal and female reproductive tracts. The mesh structure of mucus determines in large part the ability of mucus to trap foreign objects. To measure the structure of fresh undiluted human mucus, we engineered synthetic particles that can readily penetrate mucus as non-perturbing nanoprobe. We discovered that the mesh spacing of mucus (average pore size 340 ± 70 nm, with 80% of pores larger than 200 nm) is large enough to

allow rapid penetration by bacteria, even the largest viruses and a wide variety of particles if they are not slowed by adhesive interactions with mucus. Consequently, the mucus barrier cannot physically obstruct these particulates from reaching the epithelial cells. We further discovered that the unexpectedly large average mesh spacing is markedly reduced in the presence of a nonionic detergent.

Administration of surfactants that can shrink the mesh spacing in mucus may provide a simple method to reduce the harmful effects of pathogens and bioterrorist agents by reducing their mucus permeability.

Accordingly, it is an object of the invention to provide compositions and methods for preventing or reducing penetration of particulate matter, including infectious pathogens and foreign particles, through the mucus barrier that protects various mucosal surfaces in a subject, in particular a nonhuman mammal or a human subject.

The compositions and methods act, for example, by decreasing the mesh size in the mucus, thereby preventing or slowing the diffusion or transport of pathogens or foreign particles through the mucus to target cells.

In one aspect, the invention provides a composition and method to reversibly reduce the mesh ("pore") size of human mucus. Reduction of the mesh size greatly slows the rate at which pathogens and/or foreign particles move through (penetrate) the mucus gel, thereby effectively reducing the fraction that can cross the barrier and infect or exert injurious actions to the underlying cells. The method involves administering an effective amount of a composition that reduces the mesh spacing in said mucus barrier, e.g., a surface active agent, for example a detergent. A pharmaceutical composition comprising, e.g., a detergent, may be administered by direct (e.g., topical) application to the surface to be protected, in a manner that produces effective protection.

The ability to chemically alter mucus architecture, in particular to reduce its mesh size, has many important implications for the prevention of pathogen penetration at mucosal surfaces. First, reducing the permeability of pathogens through mucus may prevent pathogens from reaching the underlying epithelia altogether. Second, by reducing the extent of mucin aggregation, the surface area available for mucin-pathogen interaction can be greatly increased, further hindering pathogen transport. Third, pathogens that are efficiently entrapped will then be removed via mucus clearance, either by gastric sterilization or direct clearance from the body, thus avoiding the use of potentially toxic chemicals and microbicides to inactivate pathogens. Fourth, this approach may provide more general protection against a variety of pathogens, since molecularly-targeted recognition is not needed, and may prove particularly useful for viruses that can be rapidly mutated, such as HIV. This is markedly different from current microbicides, anti-viral compounds and vaccines, the large majority of which are pathogen-specific.

Particulate matter to be impeded or blocked from crossing the mucus barrier includes pathogens, such as viruses, bacteria, and fungus; and undesirable particulates such as environmental pollutants and toxic substances. For example, the methods described herein should be effective in

blocking viruses, such as herpes simplex virus (HSV), HPV, HIV, rotavirus, Norwalk virus, echovirus, Epstein-Barr virus, rhinovirus, adenovirus, influenza virus, coronavirus, measles virus, poliovirus, variola virus (small pox), and varicella zoster virus (chicken pox); bacteria, such as chlamydia (*Chlamydia*), *Helicobacter*, gonorrhea (*Neisseria gonorrhoeae*), syphilis (*Treponema pallidum*), pseudomonas ; and other particulates such as environmental pollutants, e.g., particulate matter from diesel exhaust, and ultrafine particles, e.g., nanometal oxides, silica.

As used herein, the term "environmental pollutant" or "toxic substance" is intended to cover particles in the indicated size range to which a subject may be exposed that are potentially toxic or produce unwanted effects, such as particulates from combustion processes or industrial exhaust, pollen or other plant material, e.g. from harvesting or processing activity, and the like.

Particle size as described herein is defined as the hydrodynamic diameter of the particle. The methods described herein should be effective in slowing or blocking particles having a hydrodynamic diameter between 25 nm and 1000 nm, e.g., between 50 nm and 1000 nm, 100 nm and 1000 nm, or 200 nm and 1000 nm.

The hydrodynamic diameter of a particle, d_{particle} , is the diameter of a hard sphere that diffuses through water at the same rate as the particle diffuses through water. It is defined by the Stokes Einstein relationship:

$$d_{\text{particle}} = kT / (3\pi\eta D_{\text{particle}})$$

where k is Boltzmann's constant, T is temperature, η is the viscosity of water, and D_{particle} is the diffusion constant for the particle diffusing through water.

For spherical virus and bacterial particles, the hydrodynamic diameter is essentially the same as the structural diameter. For long rod-shaped particles the hydrodynamic diameter is approximately equal to the long dimension of the rod.

Agents that are expected to be effective in reducing mesh pore size include but are not limited to:

- (1) Nonionic surfactants, including amphiphilic polymers, such as alkyl or alkylphenol poly(ethylene oxide) (e.g., nonoxynols, such as nonoxynol-9), poloxamers (e.g., Pluronic F127), polysorbates (e.g., Tween or Span 20, 40, 60, or 80), alkyl glycosides (e.g., octyl glucoside) and Vitamin E-TPGS; and fatty alcohols, such as cetyl alcohol or oleyl alcohol;
- (2) Anionic surfactants, such as soaps, detergents, or fatty acid salts, sodium cholate, and sodium laureth sulfate or sodium lauryl sulfate;
- (3) Zwitterionic surfactants, such as cocamidopropyl betaine; and,
- (4) Lipid or lipid-derived amphiphilic small molecules, such as fatty acyls, fatty acids, glycerolipids (e.g., triglycerides), phospholipids, sphingolipids, prenol lipids, glycolipids, bile salts, cholesterol, and the like.

These agents may be used alone or in combination, as will be evident to those of skill in the art, at concentrations that are effective, and that are below any toxic levels that may exist for specific agents.

Pharmaceutical compositions in accordance with the invention are useful for prophylaxis or treatment of a condition. Accordingly, compositions in accordance with the invention are useful as a drug or as information for structural modification of existing compounds, e.g., by rational drug design.

For therapeutic or prophylactic uses, the compositions or agents identified using the methods disclosed herein may be administered by a variety of methods and routes, as will be evident to those of skill in the art. Suitable carriers and their formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin.

The amount of the therapeutic agent to be administered varies depending upon the manner of administration, the age and body weight of the subject/patient, and with the subject's symptoms and condition. A compound is administered at a dosage that best achieves medical goals with the fewest corresponding side effects.

Administration

The pharmaceutical compositions of this invention, including biologically active variants, or analogs thereof, can be administered by any suitable routes to reach mucosal surfaces, including oral, topical, rectal, intranasal, vaginal, ophthalmic, intra-uterine, and the like.

A variety of biocompatible polymers (including hydrogels), both biodegradable and non-degradable, can be used to form a device for the sustained release of a bioactive factor at a particular target site.

Generally, the amount of administered agent of the invention (dosage) will be empirically determined in accordance with information and protocols known in the art. Typically agents are administered in the range of about 10 to 1000 $\mu\text{g/kg}$ of the recipient. For peptide agents, the concentration will generally be in the range of about 50 to 500 $\mu\text{g/ml}$ in the dose administered. Other additives may be included, such as stabilizers, bactericides, and anti-fungals. These additives will be present in conventional amounts

The administration of a compound of the invention may be by any suitable means that results in a concentration of the therapeutic that, combined with other components, is effective in having the desired result. The compound may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The pharmaceutical compositions may be formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, 2000, Lippincott Williams & Wilkins, and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

Suitable dosage/administration forms can be formulated for, but are not limited to, oral, rectal, sub-lingual, mucosal, nasal, ophthalmic, bronchial, vaginal and intra-uterine administration, and other dosage/administration forms for delivery of active agents to a mucosal surface.

5 In some applications, it may be advantageous to utilize the active agent in a "vectorized" form, such as by encapsulation of the active agent in a liposome or other encapsulant medium.

Methods in accordance with the present invention using formulations suitable for, e.g., oral or vaginal administration, may be presented as discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the active ingredient as a powder or granules. Optionally, a suspension in an aqueous or non-aqueous liquid may be employed, such as a syrup, an
10 elixir, an emulsion, or a draught.

Nasal and other mucosal spray formulations (e.g., inhalable forms) can comprise purified aqueous solutions of the active compounds with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal or other mucous membranes. Alternatively, they can be in the form of finely divided solid powders suspended
15 in a gas carrier. Such formulations may be delivered by any suitable means or method, e.g., by nebulizer, atomizer, metered dose inhaler, or the like.

Formulations for rectal administration may be presented as a suppository with a suitable carrier such as cocoa butter, hydrogenated fats, or hydrogenated fatty carboxylic acids.

In addition to the aforementioned ingredients, formulations of the invention may further
20 include one or more accessory ingredient(s) selected from diluents, buffers, flavoring agents, binders, disintegrants, surface active agents, thickeners, lubricants, preservatives (including antioxidants), and the like.

A formulation of the present invention can have immediate release, sustained release, delayed-onset release or any other release profile known to one skilled in the art.

25 Pharmaceutical compositions according to the invention may be formulated to release the active compound substantially immediately upon administration or at any predetermined time or time period after administration. The latter types of compositions are generally known as controlled release formulations, which include (i) formulations that create a substantially constant concentration of the drug at the surface to be treated over an extended period of time; (ii) formulations that after a
30 predetermined lag time create a substantially constant concentration of the drug at the surface to be treated over an extended period of time; (iii) formulations that sustain action during a predetermined time period by maintaining a relatively, constant, effective level at a mucosal surface with concomitant minimization of undesirable side effects associated with fluctuations in the level of the active substance (sawtooth kinetic pattern); (iv) formulations that localize action by, e.g., spatial
35 placement of a controlled release composition adjacent to or on the surface to be treated; (v) formulations that allow for convenient dosing, such that doses are administered, for example, once

every one or two weeks. For some applications, controlled release formulations obviate the need for frequent dosing to sustain the administered agent at a therapeutic level.

Any of a number of strategies can be pursued in order to obtain controlled release in which the rate of release outweighs the rate of metabolism of the compound in question. In one example, controlled release is obtained by appropriate selection of various formulation parameters and ingredients, including, e.g., various types of controlled release compositions and coatings. Thus, the therapeutic is formulated with appropriate excipients into a pharmaceutical composition that, upon administration, releases the therapeutic in a controlled manner. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions, emulsions, microcapsules, microspheres, molecular complexes, nanoparticles, patches, and liposomes.

The compositions of the invention can be incorporated into microspheres, microcapsules, nanoparticles, liposomes, or the like for controlled release. Furthermore, the composition may include suspending, solubilizing, stabilizing, pH-adjusting agents, tonicity adjusting agents, and/or dispersing agents. Alternatively, the active compound may be incorporated in biocompatible carriers, implants, or infusion devices.

Materials for use in the preparation of microspheres and/or microcapsules include, e.g., biodegradable/bioerodible polymers such as polygalactin, poly-(isobutyl cyanoacrylate), poly(2-hydroxyethyl-L-glutamine), poly(lactic acid), poly(lactide-co-glycolide), polyanhydrides (e.g. poly(sebacic acid)), and biocompatible polymers (e.g. poly(ethylene glycol) or combinations thereof). Materials for use in implants or insertable devices (such as those for vaginal delivery) can be non-biodegradable (e.g., polydimethyl siloxane) or biodegradable (e.g., poly(caprolactone), poly(lactic acid), poly(glycolic acid) or poly(ortho esters) or combinations thereof).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Transport rates of PEG-modified polystyrene (PEG-PS) particles in human cervicovaginal mucus before and after exposure to nonoxynol-9 (N9). (A-C) Ensemble-averaged geometric mean square displacements ($\langle \text{MSD} \rangle$) as a function of time scale with and without N9 for (A) 100 nm, (B) 200 nm, and (C) 500 nm particles. (D-F) Comparison of average D_{eff} at a time scale of 1s in water (W) vs. mucus for subfractions of (D) 100 nm, (E) 200 nm, and (F) 500 nm particles before and after exposure to N9, from fastest to slowest. Theoretical D_{eff} for same sized particles in water is shown as W. Data represent ensemble average of three experiments, with $n \geq 120$ particles for each experiment.

Figure 2. (A) Fractions of PEG-PS immobilized in mucus before and after exposure to N9. (C) Fractions of PEG-PS undergoing diffusive transport before and after exposure to N9. Classification of immobilized and diffusive fractions is as described in [3].

DETAILED DESCRIPTION

Compositions and methods for reducing mesh spacing

In one aspect, the invention provides compositions and methods to reversibly reduce the mesh (“pore”) size of human mucus. Reduction of the mesh size greatly slows the rate at which viruses and other pathogens or particles move through the mucus gel, thereby effectively reducing the amount of pathogen or particulate that can cross the barrier and infect or injure underlying cells.

We have found that we can alter the mucus architecture such that the transport of model particulates in mucus is slowed significantly compared to untreated mucus. In particular, a model nonionic detergent (Nonoxynol-9, or N9) was used to reduce mucin self-bundling and thus significantly decrease the average interfiber spacing of the mucus gel (to ~130 nm). Correspondingly, the transport of 200 and 500 nm synthetic particles with viral-mimicking surfaces was reduced 160- and 140- fold, respectively. This provides direct evidence that the mesh size in mucus can be chemically altered to slow down the mucus penetration of pathogen-like particles. Based on the biochemical properties and typical concentrations of mucin fibers in mucus secretions [4, 5], the mesh size for a flexible mucin network [6] may be reduced to as small as ~25 nm, which makes this method potentially highly useful for preventing the transmission of a wide range of pathogens, since most virus and bacterial particles are larger than this. Chemicals that can lead to the desired changes in mucus architecture can be inhaled, ingested or topically-applied to mucosal surfaces prior to or after exposure to pathogens and other particles. The change in mucus mesh size is expected to be transient and reversible since mucus is continuously secreted and renewed.

The ability to chemically alter mucus architecture, in particular to reduce its mesh size, has many important implications for the prevention of pathogen and other particulate penetration at mucosal surfaces. First, reducing the permeability of pathogens and other particles through mucus may prevent pathogens/particles from reaching the underlying epithelia altogether. Second, by reducing the extent of mucin aggregation, the surface area available for mucin-pathogen (or other particle) interaction can be greatly increased, further hindering pathogen transport. Third, pathogens that are efficiently entrapped will then be removed via mucus clearance, either by gastric sterilization or direct clearance from the body, thus avoiding the use of potentially toxic chemicals and microbicides to inactivate pathogens. Fourth, this approach may provide more general protection against a variety of pathogens and other particles, since no molecular-targeted recognition is needed, and may prove particularly useful for viruses that can be rapidly mutated, such as human immunodeficiency virus (HIV). This is markedly different from current microbicides, anti-viral compounds and vaccines, the large majority of which are pathogen-specific.

Many pathogens, such as viruses and bacteria, toxins and other particulates can exert infectious or injurious actions at mucosal surfaces, such as the respiratory, gastrointestinal, and cervicovaginal tracts. For infection or toxicity to occur, pathogens and toxins typically must traverse the mesh-like mucus gel and enter underlying epithelial cells. Currently, methods are lacking that can efficiently prevent mucosal transmission of a wide range of pathogens.

This approach is fundamentally different from the use of specific microbicide, anti-viral compounds or vaccines, most of which are pathogen-specific, as it may be capable of stopping the penetration of a wide range of pathogens, including ones where the molecular entities remain poorly understood. For example, new influenza vaccines are produced every year since the antibodies
5 produced by the previous year's vaccine are no longer effective in neutralizing the virus. The present approach has the potential to prevent or mitigate infection by any strain of influenza, current or future.

The compositions and methods described herein have the potential for the prevention and treatment of a broad spectrum of pathogens or pathogen-related diseases at mucosal surfaces. They are expected to be especially useful for the following mucosal sites: female reproductive tract, lungs,
10 gastrointestinal tract, nose, eye and bladder. They may be used, for example, to prevent or mitigate infection by influenza virus, adenovirus (common cold), sexually-transmitted pathogens, and more.

Topical pharmaceutical compositions (e.g., comprising surfactants) may be administered, e.g., to mucosal surfaces, either via inhalation (airway), ingestion (gastrointestinal) or topical application (cervicovaginal), to alter mucus architecture to prevent pathogen transmission. Dosages can be
15 determined by those of skill in the art without undue experimentation. Suitable pharmaceutical compositions can also be administered, e.g., in aerosols. For example, they may be inhaled on a daily basis during peak flu season by patients or healthcare workers, or by soldiers who are at higher risk of exposure to bioterrorist agents. The pharmaceutical compositions may also be administered prior to intercourse to help reduce sexually transmitted diseases and may serve as a measure to enhance
20 contraception.

Materials and methods

Cervicovaginal mucus collection and preparation

The cervico-vaginal mucus collection procedure was performed as published previously [7]. Briefly, undiluted cervicovaginal secretions from women with normal vaginal flora were obtained
25 using a self-sampling menstrual collection device following protocols approved by the IRB of the Johns Hopkins University. The device was inserted into the vagina for approximately 30 s, removed, and placed into a 50 mL centrifuge tube. Samples were centrifuged at 1500 rpm for 2 min to collect the secretions. Collected mucus was used for microscopy within 4 h.

Preparation and characterization of mucus-penetrating particles (MPP)

Fluorescent, carboxyl-modified polystyrene (PS) particles sized 100 nm, 200 nm, 500 nm and
30 1 μ m (Molecular Probes, Eugene, OR) were covalently modified with either 3.4kDa diamine PEG or 2kDa methoxy-PEG-amine (Nektar Therapeutics, San Carlos, CA) via a carboxyl-amine reaction, as published previously [3]. Unconjugated PEG was removed by 3 rounds of washing and centrifugation, and MPP were stored at 4°C until use. The size and ξ -potential were determined by
35 dynamic light scattering and laser Doppler anemometry, respectively, using a Nanosizer ZS90 (Malvern Instruments, Southborough, MA). Size measurements were performed at 25°C at a

scattering angle of 90°. Samples were diluted in 10 mM NaCl and measurements performed according to instrument instructions.

Multiple particle tracking (MPT) in cervicovaginal mucus

Particle transport rates were measured by analyzing trajectories of fluorescent MPP, recorded using a silicon-intensified target camera (VE-1000, Dage-MTI, Michigan, IN) mounted on an inverted epifluorescence microscope equipped with 100X oil-immersion objective (numerical aperture 1.3). Experiments were carried out in 8-well glass chambers (LabTek, Campbell, CA) where diluted MPP solutions were added to 250-500 µL of fresh mucus to a final concentration of less than 2% v/v and incubated for 2 h prior to microscopy. Immediately after imaging of MPP in native mucus, nonoxynol-9 (N9) was added at 1% v/v to a final concentration of 0.1%, gently stirred, and incubated for 2 h prior to a second round of microscopy. Trajectories of $n > 120$ particles were analyzed for each experiment and three experiments in cervicovaginal mucus from different donors were performed for each condition. Movies were captured with Metamorph software (Universal Imaging Corp.) at a temporal resolution of 66.7 ms for 20 s. The tracking resolution was 10 nm, determined by tracking displacements of particles immobilized with a strong adhesive [8]. The coordinates of nanoparticle centroids were transformed into time-averaged mean squared displacements (MSD), $\langle \Delta r^2(\tau) \rangle = [x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2$ (τ = time scale or time lag), from which distributions of MSDs and effective diffusivities were calculated, as previously demonstrated [3, 9]. Additional information for measuring 3D transport by 2D particle tracking is described in a recent review [10].

Particle transport mode classification

The mechanism of particle transport over short and long time scales was classified based on the concept of relative change (RC) of effective diffusivity (D_{eff}), as discussed previously [3, 11, 12]. In brief, RC values of particles at short and long time scales were calculated by dividing the D_{eff} of a particle at a probed time scale by the D_{eff} at an earlier reference time scale. By calculating RC values for two time regimes (i.e. short and long time scales), one can obtain the transport mode that describes the particle transport properties over different length and temporal scales. RC_{short} was defined at $\tau_{\text{ref}} = 0.2$ s and $\tau_{\text{probe}} = 1$ s, whereas RC_{long} was found at reference $\tau_{\text{ref}} = 1$ s and $\tau_{\text{probe}} = 2$ s. The rigor of the transport modes classification was confirmed previously.

Probing the structural arrangement of the mucus gel mesh

The interfiber spacings of human cervicovaginal mucus were estimated based on an obstruction-scaling model pioneered by Amsden and coworkers that was originally developed for covalently cross-linked hydrogels but is equally applicable to gels with physical entanglement cross-links [1, 13-15]. The model is valid in the case where there is no interaction between solute particles and the gel mesh, and particles traveling through the pores of gel experience the viscous drag of water. The model describes the ratio of diffusion in a gel and diffusion in water as $D_g/D_o = \exp\{(-\pi/4)[(r_s + r_f)/(r_g + r_f)]^2\}$, where D_g is the diffusion coefficient of particle in polymer gel, D_o is the diffusion

coefficient of particle in water, r_s is the radius of particle, r_f is the gel fiber radius, and r_g is the effective radius of mesh spacing. An r_f of 3.5 nm was used as that represents the current best estimate from biochemical, EM, and AFM observations of individual mucin fibers and images of mucus gels [1, 15]. Effective mesh spacing $2r_g$ was obtained by fitting measured transport data via the maximum likelihood estimation method.

Example 1

The barrier properties of mucus at nano-scales relevant to pathogens and nanoparticles are intrinsically related to the structural arrangements of the mucus mesh. The molecular structure of physiological mucus has been poorly characterized, due in part to dehydration and other fixation artifacts needed for electron microscopy (EM) preparations. We reasoned that tracking the diffusion of nanoparticle probes in minimally perturbed mucus at high spatiotemporal resolution might reveal the effective mesh spacing, provided that nanoprobe could be engineered that do not adhere to mucus, a widely noted phenomenon of nanoparticles in mucus. We recently discovered that a dense surface coating with low MW polyethylene glycol (PEG) vastly reduced nanoparticle adhesion to mucus and enabled engineering of mucus-penetrating particles (MPP) [3]. We used high speed multiple particle tracking (MPT) to obtain detailed analysis of the diffusional motions of MPP in fresh undiluted human cervicovaginal mucus. MPP with diameters of 100-500 nm rapidly penetrated mucus with rates, measured by geometric ensemble mean-squared displacements ($\langle \text{MSD} \rangle$), that were 6000-, 600- and 400- fold faster than uncoated particles of the same size [3]. In contrast, the diffusion of 1 μm MPP was markedly hindered by mucus.

Example 2

To infer the effective mesh spacing of mucus, we next fitted an empirically-derived obstruction-scaling model (OSM), initially developed to model diffusion of non-interacting solutes in hydrogels [13, 14] but equally applicable to entangled and cross-linked gels such as mucus [1, 15]. Using maximum likelihood estimation fitting, the average mesh spacing of physiological human mucus was estimated to be $\sim 340 \pm 70$ nm, significantly larger than previous estimates of ~ 100 nm based on EM and on transport of viruses in mucus secretions [1]. MPT/OSM also revealed considerable variations in the distribution of mesh spacings. The largest 10% of interfiber spacings, estimated from transport of the fastest 10% of MPP experiencing the least obstruction to diffusion, were in excess of 750 nm. The smallest mesh spacings were no larger than roughly 100 nm. Overall, more than 80% of the mesh spacings experienced by these MPP in mucus were larger than 200 nm.

The large mesh spacing obtained from the MPT/OSM method contrasts sharply to the size predicted by the diameters and concentrations of mucin fibers. Both the biochemical structure of mucin fibers and electron microscopy of individual mucin fibers indicate their diameter to be 7 to 10 nm wide. At physiological mucin concentrations (~ 1 -5%), this predicts an average mesh spacing of 40-100 nm based on a cubic lattice model. By further assuming that the mucin mesh is a flexible polymer network [6], the average mesh spacing is estimated to be ~ 25 nm, based on published values

of mucin glycoprotein molecular weight and radius of gyration [5] and a mucin concentration of 2.5%. The markedly larger mesh spacings found here strongly suggests physiological mucus to comprise large fractions of aggregated and/or self-bundled mucins to create enlarged distances between mesh elements. Biochemically, mucins are characterized by “naked” (non-glycosylated)

protein globules interspersed between long negatively charged glycosylated fibers. Since the negatively charged fibers are expected to repel each other, mucin-mucin bundling likely reflects adhesive interactions between multiple hydrophobic domains. The adhesive interactions can lead to mucin-mucin bundling via intra-mucin (longitudinal) or inter-mucin condensations.

Example 3

To reduce pathogen and other particle penetration across the mucus barrier, we sought to reduce the mesh spacing of native mucus, since the physical obstruction to pathogen diffusion and the likelihood for mucus adhesion and immobilization may be enhanced with smaller mesh spacing. We hypothesized that, if mucin fibers are condensed or bundled by hydrophobic interactions, then smaller mesh spacings can be achieved by disrupting hydrophobic interactions between mucin fibers, since unbundled mucins would then occupy low viscosity pores. To test this hypothesis, we characterized the dynamics of MPP in the presence of a nonionic detergent, nonoxynol-9 (N9), which was added to the same mucus samples. N9 had a size-dependent influence on MPP transport; addition of N9 greatly reduced transport rates of 200 and 500 nm MPP, evident by the 160- and 140-fold lower ensemble MSDs, respectively, while the transport rates of 100 nm MPP were reduced by only 10% (Figure 1A-C). In part reflecting the vastly reduced transport, 25% and 29% of 200 and 500 nm MPP were immobilized upon N9 treatment (Figure 2A). The diffusive fractions were also reduced significantly, from 54% to 22% for 200 nm MPP and from 56% to 8% for 500 nm MPP (Figure 2B). In contrast, for 100 nm MPP, immobile fraction was less than 1% without N9 and 0% with N9, and the fractions of diffusive particles were only reduced from 53% to 49%. The distributions of effective diffusivities for 100 nm MPP were also highly similar regardless of N9 content, whereas the distributions for 200 and 500 nm MPP were both uniformly lower (Figure 1D-F). The addition of the same volume of phosphate buffered saline (1% v/v) instead of N9 did not alter the diffusive motions of MPP in mucus, underscoring that the observed difference in transport was dependent on N9.

Example 4

To evaluate the corresponding change in mucus structure, OSM was fitted to the transport data of various sized MPP in mucus treated with N9. As expected from the lower transport rates, the average mesh spacing upon N9 treatment was sharply reduced to $\sim 130 \pm 45$ nm. The variations in the average fiber spacing were also markedly reduced, with 86% of the mesh spacings falling between 40-200 nm. The significantly smaller mucus mesh spacings underscore the critical role of hydrophobic condensation or bundling in the assembly and structure of physiological human mucus. This implies hydrophobic interaction is an important mechanism controlling mucin-mucin interactions, in addition to calcium crosslinking, pH, and disulphide linkages.

We showed here that by using surfactants that can alter mucin-mucin interactions, changes to the mucus mesh structure can be induced. As mucus relies on its dense and highly adhesive network of fibers to trap and prevent foreign particulates from reaching the underlying epithelia, smaller mesh spacings are expected to greatly improve its barrier property. Based on the molecular weight, radius of gyration, and concentrations of mucins in physiological mucus, the average mesh spacing can be reduced to as small as 25 nm, assuming a flexible mucin network. This is expected to greatly increase the steric obstruction to diffusion for larger pathogens, such as Ebola and poxviridae, or bioterrorist agents delivered via large nanoparticle systems. Moreover, by increasing exposed surface area of mucins, small mesh spacing may also increase the polyvalency of adhesive interactions between particulates and mucus, leading to even greater immobilization. The effects of N9 on the barrier properties of mucus are expected to be transient, as mucus is continuously secreted and replaced. Together with an earlier finding that inhalation of isotonic saline mitigates exhaled bioaerosols and thus potentially the spread of airborne infectious disease [16], it may be possible to develop a simple means where airborne pathogen transmission, independent of the identity of the pathogen, is reduced at both the infected individual (preventing pathogen-containing bioaerosol generation) and uninfected individual (preventing mucus-penetration of pathogen) levels.

Given the similar biochemistry and rheological properties between human cervicovaginal mucus and other mucosal secretions [3, 4], the large interfiber spacing estimate obtained with cervicovaginal secretions may be characteristic of other entry sites into the body. In particular, the mucin glycoform and the mucin content are similar among cervical, eye, nasal, and lung mucus. The composition of water in the aforementioned mucus types all falls within the range of 90–98%, resulting in similar rheology among most human mucus secretions characterized by log-linear shear thinning of viscosity. It is interesting to note that the large interfiber spacing observed here is consistent with the only electron microscopic investigation in which the mucus gel was prepared by freeze substitution, suggesting freeze substitution may cause minimal disturbance of the native distribution of mucin fibers [17].

Mucus gels have long been considered a diffusional barrier protecting mucosal organs due to the highly viscoelastic bulk rheology. However, our new knowledge of the interfiber spacing of mucus shifts the attention of the mucus barrier properties from its bulk rheology to its highly adhesive nature. At length scales relevant to small particles, viruses and macromolecules, the biophysical properties of mucus greatly deviate from those of the bulk fluid, and fluids in pores between the fiber elements of the dense mesh possess a viscosity similar to that of water. Thus, foreign pathogens can rapidly penetrate physiological human mucus if they can evade adhesion to mucus.

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WE CLAIM:

1. A method of reducing or blocking penetration of a particle through a mucus barrier, said method
5 comprising administering to the mucus barrier an effective amount of a composition that reduces the
mesh spacing in said mucus barrier.
2. The method of claim 1 wherein the composition comprises a surface active agent.
- 10 3. The method of claim 2 wherein the surface active agent is a soap or detergent.
4. The method of claim 3 wherein the surface active agent is a nonionic detergent.
5. The method of one of the previous claims wherein the particle is a pathogen.
15 6. The method of claim 5 wherein the pathogen is selected from the group consisting of a bacterium,
a virus, a yeast, and a fungus.
7. The method of claim 6 wherein the pathogen is a virus.
20 8. The method of claim 7 wherein the virus is selected from the group consisting of HIV, HSV, HPV
and influenza virus.
9. The method of one of claims 1-4 wherein the particle is a toxic particulate.
25 10. The method of one of the preceding claims wherein the particle has a hydrodynamic diameter of
between 25 nm and 1000 nm.
11. The method of claim 10 wherein the particle has a hydrodynamic diameter of between 50 nm and
30 1000 nm.
12. The method of claim 11 wherein the particle has a hydrodynamic diameter of between 100 nm
and 1000 nm.
- 35 13. The method of claim 12 wherein the particle has a hydrodynamic diameter of between 200 nm
and 1000 nm.

14. A method of preventing, reducing or treating infection by a pathogen or injurious action by a foreign particle in a subject, said method comprising administering to said subject an effective amount of a composition that reduces the mesh spacing in a mucus barrier of the subject.

5 15. The method of claim 14 wherein the mucus barrier is selected from the group consisting of a mucus barrier in the ocular tract, nasopharyngeal system, respiratory system, the digestive system, and the genito-urinary system.

16. The method of claim 15 wherein the mucus barrier is in the female genito-urinary system.

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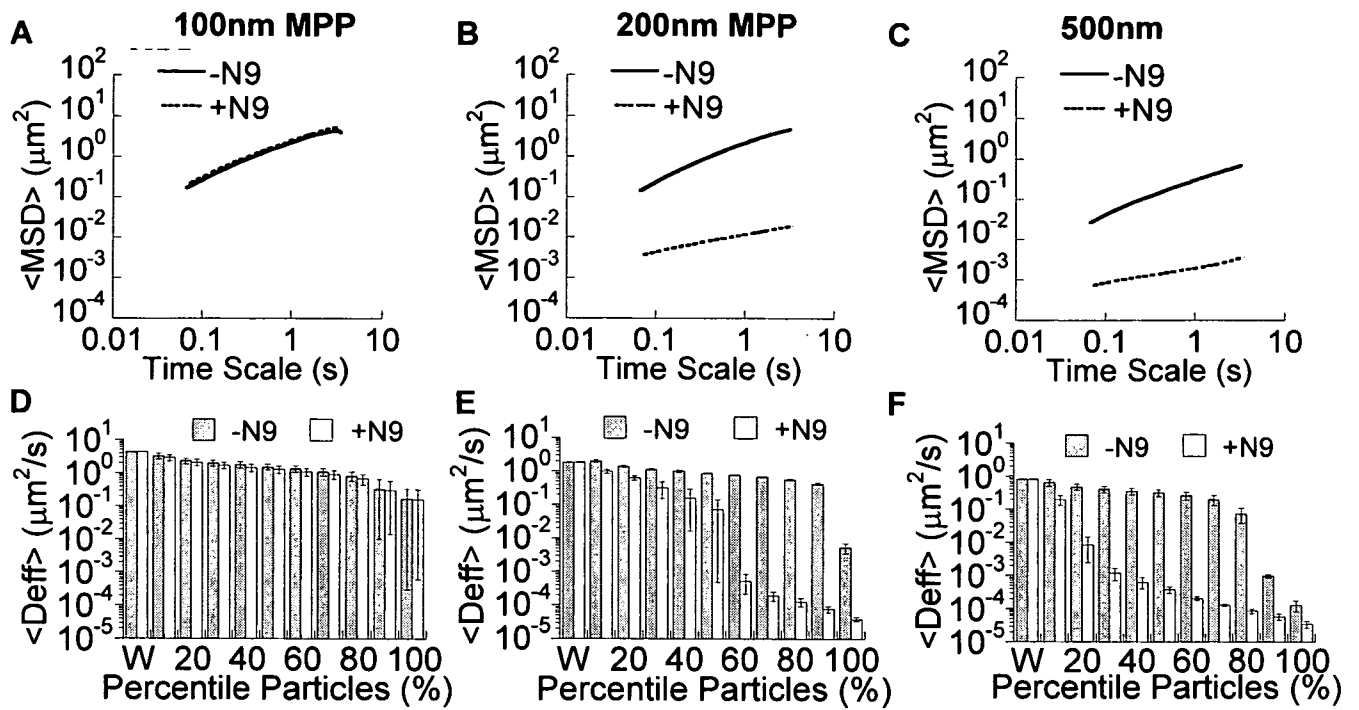


Figure 1

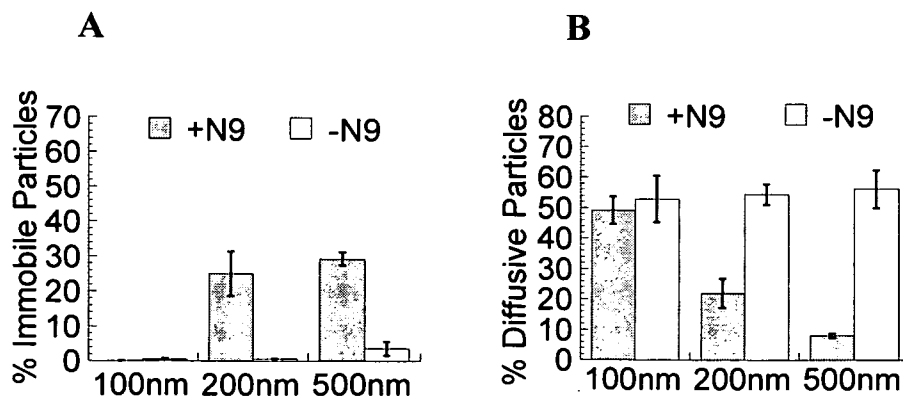


Figure 2