Liquid Crystalline Order in Mucus

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Introduction. Mucus plays an exceptionally wide range of important biological roles. It operates as a protective, exchange, and transport medium in the digestive, respiratory, and reproductive systems of humans and other vertebrates (for review see ref 1). It protects fish skin and decreases its hydrodynamic drag,[2] and it also functions as a tegumental dehydration barrier, a surface defense, a navigation aid, and a snare for prey in several invertebrate species.[3] Its rheological properties are of special interest in biomedical contexts, particularly respiratory diseases like cystic fibrosis and asthma in which defective mucus plays a critical pathophysiological role.

Mucus is a polymer hydrogel. It is secreted as discrete packages (secretory granules) by specialized secretory cells. Mucus hydrogel is stored in a condensed state inside the secretory granules. Upon exocytosis, it undergoes dramatic swelling, expanding its volume by a factor of several hundred.[4] Subsequently, the swollen mucus gels released from the individual granules anneal to each other, forming a thin coat on mucosal or skin surfaces.

The topology of the mucin polymer network which forms the macromolecular matrix of the mucus is well established: mucins form an interwoven network held together by random entanglements and noncovalent bonding.[5-8] It has been thought that this three-dimensional macromolecular scaffolding is isotropic at all length scales from molecular to macroscopic, and the rheological behavior of mucus therefore has been modeled accordingly.[9] However, depending upon the architecture of their constituent macromolecules and on the composition of the solvent, polymer gels can form liquid crystalline microstructures,[10] with orientational order being exhibited over optically resolvable distances. Individual mucin molecules consist of alternating rigid segments (heavily glycosylated; hydrophilic) and flexible segments (nonglycosylated; hydrophobic).[11,12] Polymer molecules consisting of rigid units linked by flexible spacers are frequently associated with liquid crystalline behavior,[13] which again raises the possibility that mucus could form anisotropic fluid phases. Suggestions that mucins may be self-associating in dilute solution have previously been challenged on the basis of sedimentation–equilibrium studies performed on mucus in which potential sites of association were competitively blocked with inhibitors.[14] However, the formation of stable liquid crystalline phases does not *depend* on the existence of inter- or intramolecular associations; these phases can form on the basis of steric considerations alone.[15]

Experimental Section. One of the main problems in studying the properties of mucus in vertebrates is the unavoidable contamination of the sampled material. The giant secretory granules of the terrestrial slug *Ariolimax columbianus* provide an excellent model system for investigating the properties of native mucus. Mucin granules can be recovered intact and subsequently lysed and hydrated in a medium of known composition.[16-18] We also studied the trails left by slugs crawling across glass microscope slides.

As-collected giant secretory granules were subjected to one of two possible treatments. In one treatment, secretions were simply agitated in order to encourage lysis of the condensed granules. Varying amounts of distilled water were added to the resulting mucus. (Saline or Ringer's solution was not used, to avoid the deposition of salt crystals in the specimens.) Alternatively, the granules were first allowed to settle, the supernatant liquid was decanted, and granules were gently rinsed twice in excess acetone. Upon addition of varying amounts of distilled water, the condensed mucus network undergoes a characteristic phase transition[18] accompanied by extensive swelling, leading to the formation of a hydrated gel. The acetone rinses are aimed at removing any residual membrane lipids from the material. While the amount of lipid initially present is expected to be too small for the lipid to become organized into a distinguishable component of the microstructure at optical resolutions, it is possible that the presence of lipid molecules could promote order in the decondensed mucin.

Mucus concentration was not measured quantitatively in these experiments, but we distinguish qualitatively between "concentrated" samples (that can be drawn into continuous filaments when a needle or toothpick is immersed in the mucus and then withdrawn) and "dilute" samples (that cannot be drawn into threads). Microstructures were found to depend on the initial concentration as characterized qualitatively in this way and on whether a glass cover slip was placed over specimens used for microscopy but *not* on whether the as-secreted granules had received an acetone rinse.

Microstructures were observed between crossed polars, using a Leitz Laborlux 12 POL microscope, and were recorded on Kodak T_{MAX} 400 film. The textures are diffuse, as is typical of viscous liquid crystalline solutions and gels (for example, solutions of many liquid crystalline cellulose derivatives). While image sharpness can be improved by working with thinner specimens, our studies were limited to thick specimens for two reasons. First, in those cases where a cover slide was used, we did not want to artificially induce molecular alignment in the sample by pushing down on the cover slide. Second, in those cases where no cover slide was used (so that samples dry at rates that are more typical of natural mucus), the sample beads up on the glass substrate.

Results and Discussion. When dilute or concentrated samples are maintained between a glass microscope slide and cover slip, water loss by evaporation occurs sufficiently slowly that the samples can contract from a continuous film into discrete filaments and islands. Anisotropic microstructures (Figure 1) develop in this fluid material over a period of several hours. They are more easily interpreted when formed in initially concentrated samples, because less sample volume is lost before the anisotropic microstructure appears. The anisotropic microstructures resemble the textures of nematic liquid crystals.[19] A continuous network of meandering extinction bands is visible; their positions change continuously as the crossed polars are rotated. The absence of abrupt discontinuities in the microstructure and the lack of discernable contrast when the polars are withdrawn are consistent with the anisotropic sample being liquid crystalline rather than crystalline. We have obtained similar microstructures using *A. columbianus* pedal mucus in the form of slug trails (Figure 2) and also human tracheal mucus. These observations suggest that the formation of a liquid crystalline phase is not an artifact of dissolving mucin in distilled water but can also occur under physiological

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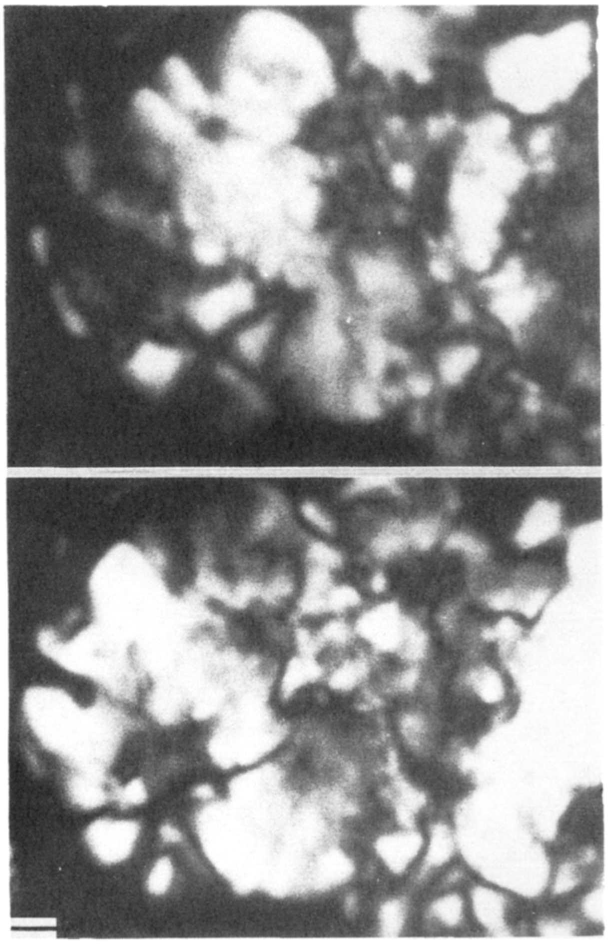

Figure 1. Nematic texture formed in an aqueous solution of *A. columbianus* mucin granules. Mucin granules collected by established methods[16-18] were allowed to lyse, and the mucus was diluted to approximately twice its original volume by adding distilled water. A droplet of the resulting "concentrated" fluid (passing our qualitative test of drawing into continuous filaments when a sharp probe is immersed in the fluid and then withdrawn) was placed on a glass microscope slide, and a glass cover slip was immediately placed over it. Nematic textures began to appear after approximately 2 h. Top: crossed polars in conventional orientation (polarizer E-W; analyzer N-S). Bottom: crossed polars rotated 45° counterclockwise. The bar represents 10 μm.

conditions. For the purpose of initial characterization, however, solutions of *A. columbianus* mucus granules provide a conveniently reproducible system for study.

If a droplet of sample is placed on a glass slide and no cover slip is used, the sample appears to be dry within a few minutes. The area of glass covered by the sample cannot contract significantly within this time, and so a continuous film of material is deposited. When initially *dilute* solutions are placed on a glass slide in this way, the perimeter of the deposited film exhibits distinct nematic textures (Figure 3). It is here that the material will be thinnest, leading to minimal overlap of microstructural features and to maximum drying rates. The nematic textures are retained indefinitely. Although the deposited films are no longer fluid, their molecular order is apparently still characteristic of the nematic liquid crystalline state. There is no evidence of net molecular alignment or preferred radial texture in the microstructure, so we discount surface tension forces as a cause of the observed local anisotropy. The water content of deposited samples can easily be increased by exhaling in their immediate vicinity. Samples can be returned to the isotropic state by this means, and a nematic microstructure then reappears after a few seconds of drying.

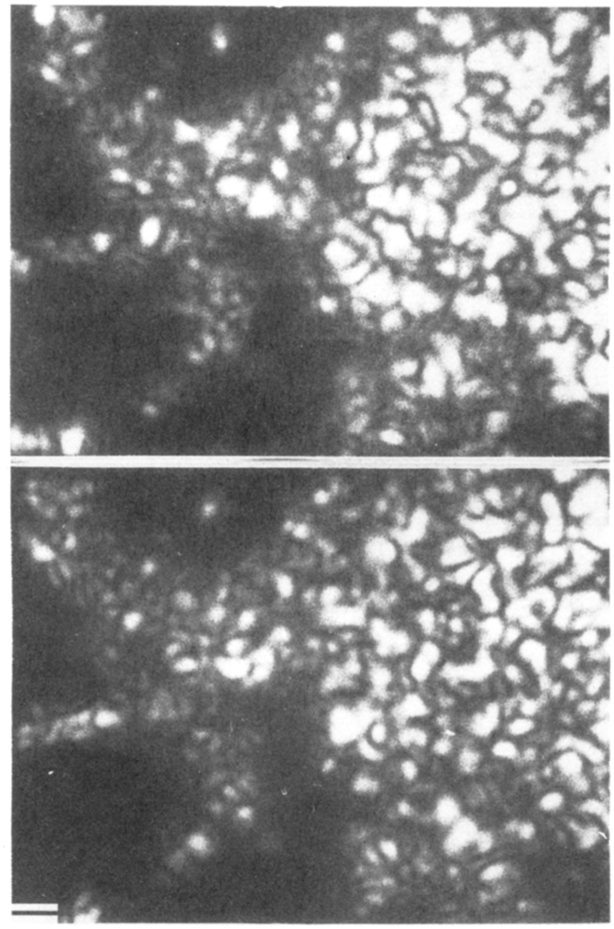

Figure 2. Nematic texture formed in fluid *A. columbianus* pedal mucus. Mucus was deposited on a glass microscope slide by allowing a slug to crawl across it. The deposited fluid was "dilute", in that in could not initially be drawn into continuous filaments. A glass cover slip was placed over a portion of the mucus trail. Nematic textures became discernable after approximately 72 h. Top: crossed polars in conventional orientation (polarizer E-W; analyzer N-S). Bottom: crossed polars rotated 45° counterclockwise. The bar represents 10 μm.

If droplets of initially *concentrated* mucus are allowed to dry on a glass slide without a cover slip, a more highly ordered, fernlike microstructure develops (Figure 4). However, this microstructure will also revert to a less ordered nematic texture (via the isotropic state) if the sample is rehydrated as described above. Our various observations therefore suggest that the slowest and fastest drying times are associated with the formation of liquid crystalline order, while intermediate drying times provide conditions of supersaturation and molecular mobility that can enable a more ordered microstructure to form. To develop a molecular model of these kinetic effects, it will be necessary to couple observations of microstructural evolution with quantitative measurements of concentration as a function of time.

While qualitative microstructural observations by transmitted polarized light microscopy enable the existence of a liquid crystalline phase to be demonstrated, they cannot differentiate between anisotropy arising at the molecular and supermolecular scales in the fluid sample.[20] In other words, the "rods" that are orientationally assembled in the liquid crystalline phase may consist of individual molecules, or they may consist of aggregates of molecules. However, current models of mucus behavior neglect the possible existence of microstructural anisotropy at *any* scale and thus are now seen to be overly simplified.

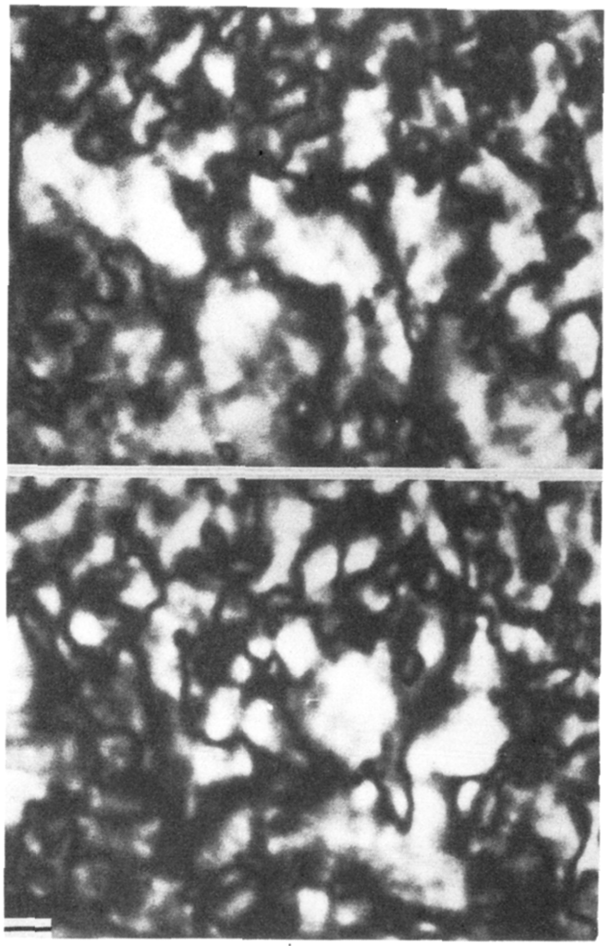

Figure 3. Nematic texture formed by an aqueous solution of *A. columbianus* mucin granules. Mucus was collected as for Figure 1 but was diluted to approximately 5 times its original volume. The resulting fluid could not be drawn into filaments. A droplet of the fluid was placed on a glass microscope slide as before, but no cover slip was used. Free water evaporated under ambient conditions within a few minutes, after which the microstructure was photographed. Nematic textures were most distinct at the perimeter of the residual film and were retained indefinitely. Top: crossed polars in conventional orientation (polarizer E-W; analyzer N-S). Bottom: crossed polars rotated 45° counterclockwise. The bar represents 10 μm.

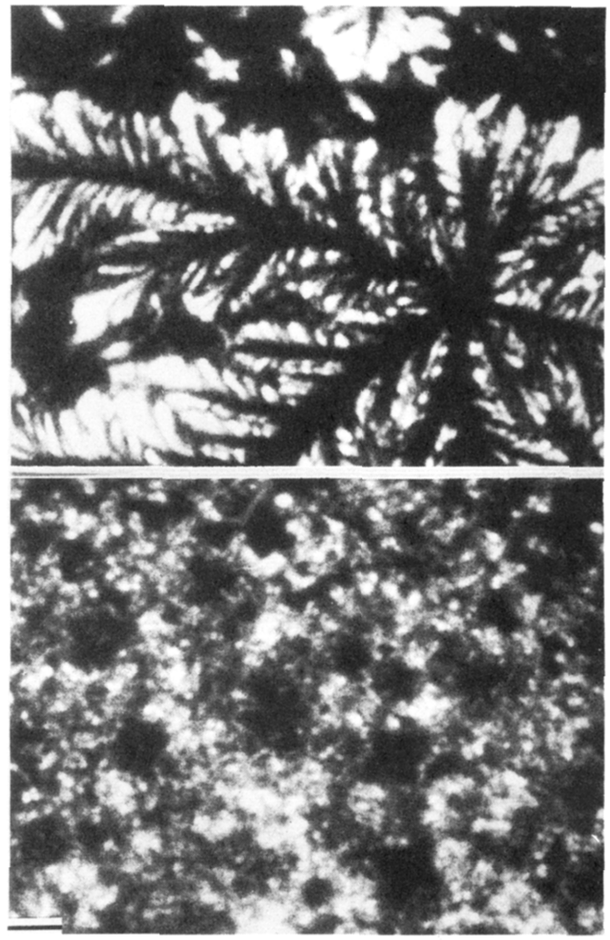

Figure 4. Mucus collected as for Figures 1 and 3. Granules were allowed to settle, and supernatant liquid was decanted. Granules were rinsed twice in excess acetone, which was discarded. Distilled water was added to the granules, to give a fluid volume that was approximately twice that of the originally collected secretion. The resulting "concentrated" fluid could be drawn into continuous filaments. A droplet of the fluid was placed on a glass microscope slide, with no cover slip. Micrographs show a constant region of sample between crossed polars (polarizer E-W; analyzer N-S). Top: fernlike microstructure near the perimeter of the residual film. Bottom: exhaling near the sample allows it to acquire enough moisture to become isotropic, and it then loses moisture to form the texture shown. The bar represents 100 μm.

Conclusions. In addition to its established role in affecting entanglement density,[4,21,22] water is seen to affect the type of microstructural order present in mucus. Both the concentration and the rate of concentration change have a significant effect. From the figures, the direction of preferred orientational order in liquid crystalline mucus is seen to change over distances of a few microns. This microstructural scale may be different on other types of substrate. However, the existence of a finite range of preferred microstructural orientation must limit the ability of mucus to wet smaller particles or particles that have surface topography on a scale smaller than that of the mucus microstructure. Also, even though the net bulk properties of the mucus appear to be isotropic,[23] we now recognize that there are conditions under which mucus can be anisotropic on the local scale of beating cilia or migrating sperm. Local anisotropy might affect reptation of mucin chains, locking the entangled mucin chains into a more stable network. Local ordered microdomains could also facilitate nucleation of the fibrous structures that have been observed[24] in the pedal mucus under a resting slug.

There is another interesting implication of the present results, relating to the condensation and decondensation of mucins respectively during storage inside the secretory granule and upon release in exocytosis. Dynamic laser scattering studies indicate that, in the hydrated mucus gel, mucins form a randomly tangled network. However, upon transition from the condensed phase to the decondensed phase, the mucin network undergoes explosive swelling.[18] Swelling of the mucin network is isotropic, and it is driven by a Donnan potential.[22] These explosive rates of swelling suggest that mucins in the condensed phase must have some folding order, which can explain the quick unobstructed unfolding of the mucin polymer network upon product release in exocytosis.[18] The finding that mucins in a constrained water space can spontaneously adopt liquid crystalline order may reveal a built-in folding property of these polymers, which suggests that condensed mucins might have ordered domains and would be consistent with the high rates of isotropic swelling observed in slug granules.[18]

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