



Gel-Forming Mucin Improves Lubricity Across Model Gemini Epithelial Cell Interfaces

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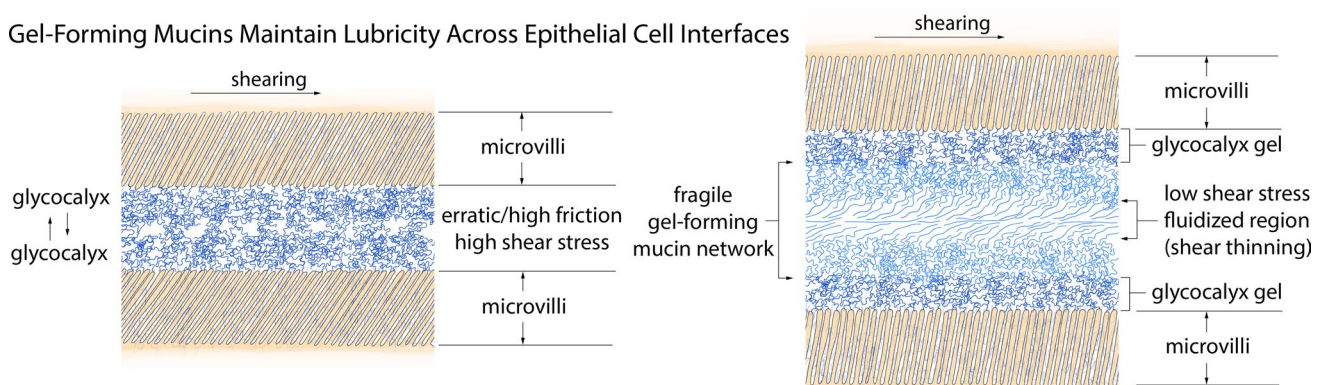
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Abstract

The glycocalyx is a glycosylated protein network gel that protects the underlying epithelial cells. Although the glycocalyx is thought to be lubricious, in Gemini contacts with epithelial cells the glycocalyx is found to have high friction ($\mu \sim 0.20$). The model of the tear film is that of a delicate hierarchical multiscale assembly of mucins that form a network aqueous gel interface between the glycocalyxes of the conjunctival and corneal epithelial layers to provide lubricity and gentle shearing. The integrity of this aqueous gel is maintained through mucin entanglement, and dynamic flickering bonds of disulfide bridges, Ca^{2+} -mediated links, and hydrogen bonding. The tear film has a unique set of rheological properties and behaviors, from a heterogeneous yield stress gel-like fluid at low shear stress to a low-viscosity fluid at high shear rate. In this manuscript, we have demonstrated that the gel-forming mucins are critical to lubricity. A Gemini model of corneal epithelial cells (self-mated and matched) with intact membrane-bound mucins and glycocalyxes (including MUC1, MUC4, and MUC16) was evaluated in the presence and absence of a purified gel-forming purified secretory mucin, MUC2. These experiments were performed under physiological contact pressures of ~ 600 Pa for 300 cycles of reciprocated sliding at 1 mm/s on a micro-biotribometer. With the addition of the MUC2 (5 wt.%), friction reductions from $\mu \sim 0.20$ to $\mu \sim 0.08$ were observed. In addition to the high friction seen in the glycocalyx, Gemini contacts with membrane-bound mucins and glycocalyxes alone showed stick-slip events during sliding and large areas of cell damage after 300 cycles. Micro-rheology experiments using magnetic tweezers showed a yield stress for a MUC2 solution that is below the critical thresholds known to produce proinflammatory cytokines (< 40 Pa) and apoptosis (< 100 Pa). These secreted gel-forming mucins, such as MUC2, are, therefore, important for lubricity in Gemini epithelial interfaces.

Graphic Abstract

Gel-Forming Mucins Maintain Lubricity Across Epithelial Cell Interfaces



Keywords Contact lens · Cell · Hydrogel · Mucin · Glycocalyx

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1 Introduction

Mucin dysfunction is implicated in many infectious and inflammatory diseases of the respiratory tract, the digestive tract, the reproductive tract, cancer, and the ocular surface [1–3]. All moist epithelial surfaces in the body are protected by a secreted layer of mucus, which is a low yield strength, high-water content network composed of mucin glycoproteins [1]. Membrane-bound mucins and secreted mucins work together to structure the mucus gel which protects and lubricates the cell surfaces [4]. As a part of the innate immune system, these epithelial cell surfaces [5] produce and maintain a mucinated barrier gel network which provides hydration and protection, excludes pathogens, maintains homeostasis, and serves as a reservoir to retain antimicrobial molecules [6]. The membrane-bound mucin network that decorates the epithelial cell surfaces can extend hundreds of nanometers, forming anchors for the gel network and establishing the glycocalyx structure [7]; the mucins also contain transmembrane domains that are involved in intracellular signaling events [8].

The diverse set of mucins within the tear film are produced by corneal epithelial cells, conjunctival epithelial cells, and goblet cells located within the conjunctiva [9]. The tear film is a thin gel-spanning mucin network less than 5 μm in thickness that is formed through mucin entanglement and transient weak interactions and flickering bonds of disulfide bridges, Ca^{2+} -mediated links, and hydrogen bonding [10]. While there are a variety of mechanisms by which mucins may intermolecularly crosslink directly with themselves or be bridged by mutual interactions with third-party mucin-binding proteins, arguably the most important characteristic of mucin networks is that their gelation is dynamic: chains involved in entanglements reptate, disulfide bonds are continuously reduced and reoxidized, Ca^{2+} -bridges exchange their carboxylate partners, and hydrogen bonds are repeatedly substituted. These low-affinity supramolecular bonds and reversible covalent bonds are shear-labile; however, unlike conventional hydrogels constructed from permanent covalent crosslinks that undergo irreversible failure upon shear, mucin networks rapidly recover their crosslink density by reassociation of their transient interactions. It is this ability to dynamically restructure that affords mucous the viscoelasticity required to mediate shear stress and adhere to epithelial cells while still be susceptible to efficient clearance [11, 12].

The tear film is a hierarchical multiscale assembly of mucins that results in a unique set of rheological properties, from a heterogeneous yield stress gel-like fluid at low shear stress to a low-viscosity fluid at high shear rate [13]. Although widely described as a “slippery” barrier on epithelial surfaces, the mechanics and network dynamics

associated with gel-forming mucins and their role in epithelial cell friction and shear are not fully understood.

Dry eye is a common chronic problem that has a range of symptoms including redness, pain (stinging, scratching, or burning sensations), sensitivity to light, and blurry vision [14]. There are many different underlying contributors that lead to this condition, which is fundamentally thought to be the result of a loss in lubricity from either poor-quality tears or inadequate amounts of tear film fluid. Patients suffering from dry-eye disease have been found to produce fewer mucins and often have fewer goblet cells, which secrete high molecular weight gel-forming mucins [15]. As the name implies, dry-eye disease is qualitatively described as a condition in which the tears are lacking water, but it is through polymer osmotic pressure that mucins bind and maintain water within the tear film. In our previous work with Gemini hydrogels and aqueous lubrication, relationships were found demonstrating that increasing water content within these gels decreases the friction coefficient, decreases the elastic modulus, and decreases the shear stress [16, 17], and these studies suggest that increasing water within the mucin gel spanning network that makes up the tear film will similarly improve lubricity of the tear film.

In this manuscript, the role of gel-forming mucins on lubricity is explored through the development of a Gemini cell model, which consists of corneal epithelial cells (self-mated and matched) with intact membrane-bound mucins (including MUC1, MUC4, and MUC16 [18–21]) as well as the addition of a gel-forming purified secretory mucin, MUC2. These experiments were performed on Gemini epithelial cell surfaces under physiological contact pressures over 300 cycles of reciprocated sliding on a micro-biotribo-meter [22]. The experiments were performed with and without the addition of MUC2 and reveal dramatic friction reductions coinciding with the addition of gel-forming mucins. In addition, the membrane-bound mucins show high friction and even stick–slip events during sliding in the absence of MUC2, as measured through in situ fast fluorescence microscopy. Micro-rheology experiments using magnetic tweezers [23] demonstrate a yield stress of the MUC2 solution that is below the critical thresholds found to produce proinflammatory cytokines (< 40 Pa) [24] and apoptosis (< 100 Pa) [25], previously determined on these cell models via microtribometry and molecular biology techniques.

2 Methods

2.1 Immortalized Human Corneal Epithelial Cell Model

A human telomerase-immortalized corneal epithelial cell line (hTCEpi) was cultured as described by Pitenis et al. [24]. Cells were grown to 95% confluence on both fibronectin-coated glass-bottom culture dishes and collagen-conjugated polyacrylamide hydrogel membrane probes (described below). To develop mature glycocalyxes, the cells were further matured over 24 h beyond confluence.

2.2 Polyacrylamide Membrane Probe

Performing microtribological measurements on the Gemini interface of cell monolayers require low contact pressures across the cellular interface. To reach pressures below 1 kPa, we combined membrane probes (thin, spherically capped probes that maintain constant pressure under force excursions [26]) made of soft, charge-neutral polyacrylamide (PAAm) gels [27] with a functionalized surface to attach epithelial cells for these experiments. These polyacrylamide membrane probes were polymerized using 12.5 wt.% acrylamide and 0.3 wt.% *N,N*-methylenebisacrylamide following the methods in Urueña et al. [27, 28]. The probes were casted in cyclic olefin copolymer molds made of TOPAS®, which has a combination of low water and oxygen permeability, and processed as described in Marshall et al. [26] using a 2 mm radius of curvature and a shell thickness of ~250 µm. The polymerized probes were then equilibrated in ultrapure water (18.2 MΩ) at least 3 times with > 100:1 volume ratio to remove residual monomer prior to surface functionalization. The important role of oxygen, rather than hydrophobicity, on the surface gel structures of hydrogels was convincingly demonstrated by Simic and Spencer [29], and these probes are expected to have surfaces that are representative of the bulk structure.

2.3 Surface Functionalization of Polyacrylamide

The fully swollen polyacrylamide membrane probes were bio-conjugated with Type I collagen (0.4 mg/ml) following methods described by Wang et al. [30]. In brief, membrane probes were fixed to a nylon probe holder, completely submerged into 2 ml of 1 mM sulfo-SANPAH, and then reacted under a UV lamp for ~5 min. The darkened sulfo-SANPAH solution was subsequently removed, and the probes were washed twice with 50 mM HEPES (pH 8.5) for 15 min on a shaker. This functionalization process was repeated twice. The probes were then placed in a 0.4 mg/ml solution of Type I collagen (COL1) and allowed to react overnight at 4 °C on

a shaker. Prior to exposure to epithelial cells, the collagen-conjugated probes were equilibrated in Keratinocyte Growth Media (KGM Gold™) supplemented with a KGM Gold BulletKit (Lonza, Basel, Switzerland, calcium concentration: 0.1 mM) for at least 24 h at 4 °C on a shaker.

2.4 Biotribometer and Microtribometry

In situ biotribology experiments were performed in a similar manner as previous publications [16, 24, 25, 31]. In brief, confluent monolayers of corneal epithelial cells were carefully imaged, characterized, and confirmed on both surfaces (glass-bottom culture dish and membrane probe) prior to experiments. The culture condition was maintained at 37 ± 0.2 °C, 5% CO₂, and ~100% humidity using a custom-built stage incubator. A normal load of 300 µN was applied between the two epithelial cell surfaces resulting in ~600 Pa mean contact pressure across the interface. The contact pressure was determined by dividing the normal load by the measured contact area of the corneal cells coated membrane probes following the procedure described in Marshall et al. [26]. The corneal cell monolayer on the culture dish, mounted on a reciprocating stage, slid against the cell monolayer counterpart on the membrane probe for 300 cycles (30 min) at a speed of 1 mm/s, with a 3 mm stroke length. The normal and friction force was recorded from the deflection of the cantilever at a data acquisition rate of 100 Hz.

2.5 Purification of Porcine MUC2

Commercially available mucin from porcine stomach Type III partially purified powder was dissolved in DI water and centrifuged at high G forces (4000 G) to remove large impurities. The MUC2 solution was then further purified using size exclusion via a 450 nm membrane filter until a clear fluid was obtained [32]. This clarified MUC2 solution was subsequently collected, lyophilized (LABCONCO, FreeZone 2.5 Plus), and stored at 4–8 °C. The purified protein was then dissolved at the desired concentration in pH 7.4 buffer (0.1 M PBS without calcium and magnesium) for micro-rheology experiments, and buffered cell media for the friction experiments.

2.6 In Situ Scanning Confocal Imaging

The custom-built biotribometer was mounted on and software-integrated with an inverted Nikon A1R confocal microscope, allowing the microscope to image the sliding track with the ability to pause/stop the experiment and remove/apply the normal load for imaging purposes. For every 100 cycles, fast-scanning high resolution confocal microscopy images of the whole wear track were recorded, then digitally

stitched [16]. CellTracker™ Green CMFDA was used to stain the epithelial cells on the glass-bottom surface, and CellTracker™ Deep Red was used for cells plated on hydrogel probes.

2.7 Micro-rheology with Unipolar Magnetic Tweezers

The magnetic tweezers followed the design of Lammerding [23]. A nickel base soft magnetic alloy rod (ASTM A753 Type 4, MIL N-14411C Comp 1) was machined to have a chisel tip of width $\sim 100 \mu\text{m}$. The tip geometry was designed to achieve optimal horizontal magnetic fields at the vicinity of the tip. The rod was then wound with 1200 turns of AWG 22 copper magnet wire over a length of 120 mm. The whole apparatus was mounted on a manual stage manipulator (OptoSigma) alongside an inverted Nikon A1R confocal microscope. Experiments were conducted under temperature-controlled and high humidity conditions to prevent drying or water loss of the sample. A current of 1 A was applied for all measurements. At these settings, electrical resistance heating of the rod is negligible.

Fluorescently tagged and superparamagnetic particles, $5.8 \mu\text{m}$ diameter (COMPEL™ UMDG002/UMC3F, COOH functionalized beads, Bangs Laboratories Inc., Fishers, Indiana, USA) were added at a concentration of ~ 0.02 volume fraction and vortex mixed for 15 s to ensure uniform dispersion. This working concentration has been tested to ensure measurable single-particle trajectories. Data of magnetic probe dynamics were captured using a high-speed electron multiply CCD camera (Andor iXon Life 888, Oxford Instruments, U.K.) at a rate of $100 \text{ frames s}^{-1}$. Horizontal displacements (in the x -direction) are predominant. The movies were analyzed to obtain particle trajectories using the algorithms developed by Crocker and Grier [33].

The force applied on probe particles was calibrated by tracking particle motion in an 80 wt.% solution of glycerol (Sigma-Aldrich) in water, which has dynamic viscosity $\eta = 0.060$ to Pa-s [34], which was confirmed using bulk rheology measurements. Stokes drag is used to model the force on a particle as a function of velocity, which is measured as a function of position through particle tracking. Magnetic tweezers can be used to probe both linear and non-linear micro-rheology, although here we use the magnetic tweezers to confirm a yield behavior in the MUC2 gel, and the yield stress is inferred by measuring the distance from the tip at which particles are not accelerating toward the tip, converting this to an applied force (F_a), and calculating the yield stress as $s = F_a/pR^2$, where R is the radius of the particle.

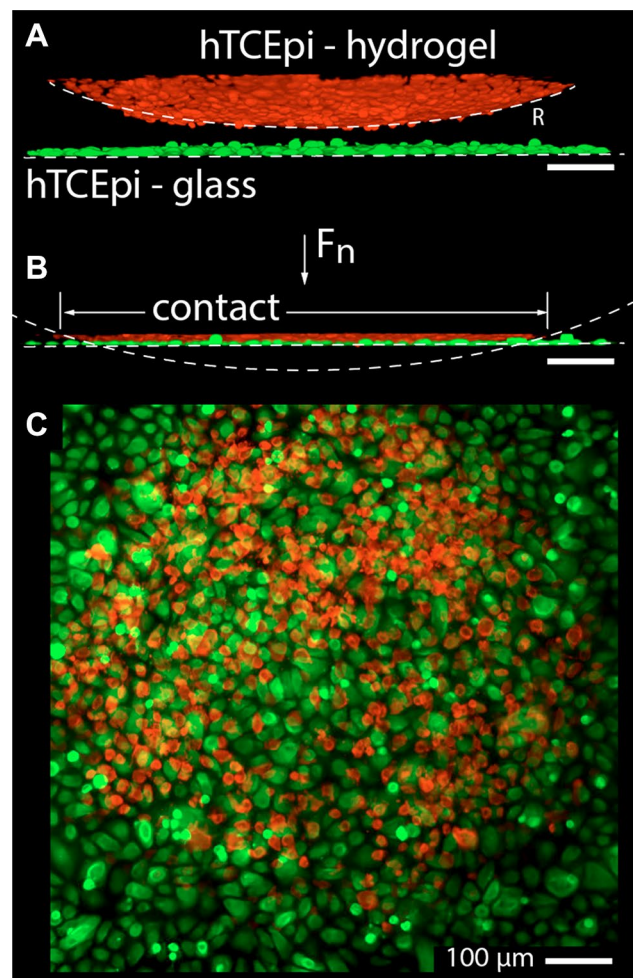


Fig. 1 hTCEPi cell interface: **A** hTCEPi cells grown on PAAM hydrogel probe (red) and on culture dish (green). **B** hTCEPi cells monolayer grown on membrane probe were brought into contact with those cultured on culture dish. The membrane probe had a wall thickness of $\sim 250 \mu\text{m}$ and was functionalized and bio-conjugated with Type I collagen prior to having cells cultured on its surface. **C** Maximum intensity projection showing contact area of the interface (Color figure online)

3 Results and Discussion

Biotribology experiments were conducted on the biotribometer with in situ scanning confocal microscopy; the apparatus and experimental methods have been previously detailed [22]. The greatest challenges in these experiments are the preparation of the cell monolayers on soft, flexible, curved hydrogel membrane probes and the control of the contact forces and pressures to a level low enough to enable extended duration sliding. As shown in Fig. 3, the experiments performed on Gemini cell pairings of mature hTCEPi cell monolayers showed a monotonically increasing friction coefficient over 300 cycles of sliding and damage, including

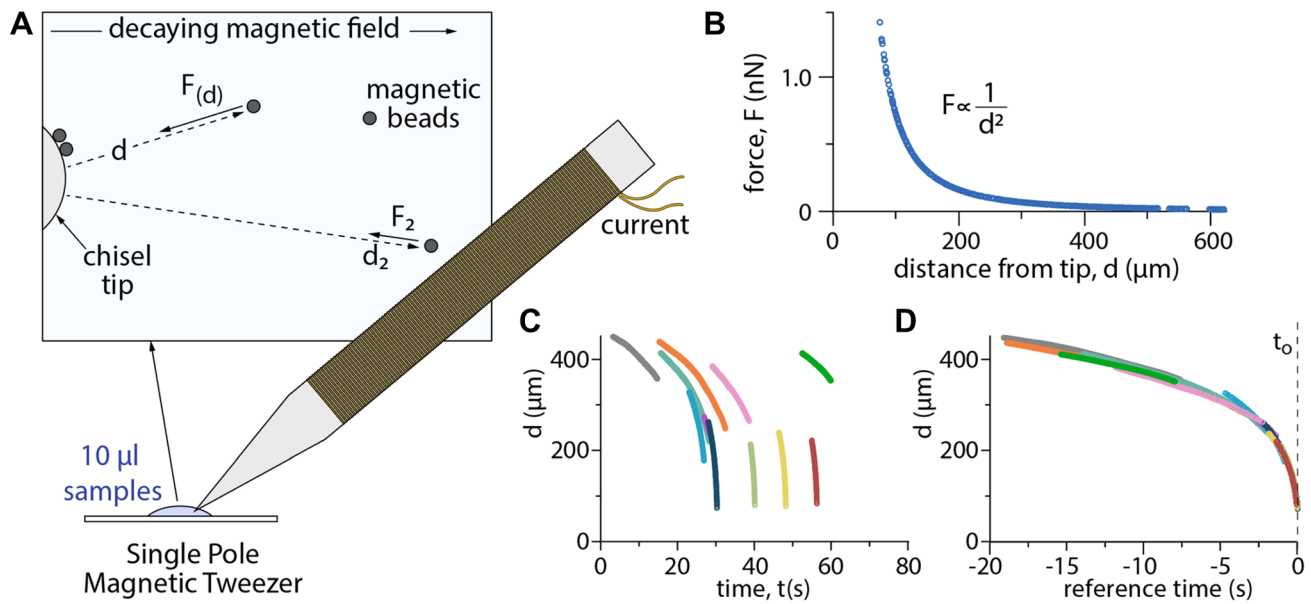


Fig. 2 Magnetic tweezer experiment: **A** schematic of magnetic tweezer experiment. The chisel tip soft magnetic alloy rod, $\sim 100 \mu\text{m}$ width, was positioned directly into the MUC2 solution containing dispersed magnetic beads. A pulse of 1 A current magnetized the core and attracted the superparamagnetic probe particles (diameter: $5.8 \mu\text{m}$) towards the tip with a velocity V . **B** Calibration curves for

the force applied as a function of horizontal distance from the tip. The magnetic force increases as the beads move closer to the tip. **C** Probe trajectory in 80 wt.% glycerol in water, a Newtonian calibration fluid with a known kinematic viscosity. **D** A combined particle trajectory from **C** shows a consistent pattern of acceleration of the particles in the vicinity of the tip

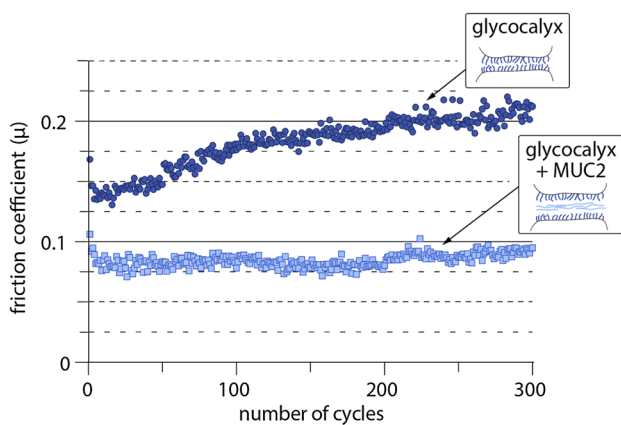


Fig. 3 Friction coefficient measurement of hTCEpi cell interface: Sliding across Gemini hTCEpi cell interfaces with mature glycocalyx surfaces showed monotonically increasing friction coefficients from $\mu < 0.15$ to $\mu > 0.20$ over 300 cycles (top). The addition of a 5 wt.% MUC2-containing cell growth media solution to the Gemini interfaces reduced the friction coefficients to below $\mu = 0.1$, and maintained low shear stress, $\tau \sim 50 \text{ Pa}$ over the duration of the experiment

the removal of epithelial cells in patches preferentially at the end of the sliding tracks, Fig. 4A. Contact area was measured using confocal microscopy imaging, as shown in Fig. 1B, although we have not developed a robust method to evaluate uncertainties associated with these measurements. The normal forces were maintained at a $300 \mu\text{N}$ average for each

cycle based on a cycle-by-cycle feedback approach, which maintained a corresponding contact pressure of 600 Pa ; the shear stress increased from approximately 90 Pa to 120 Pa over the duration of the experiment for the Gemini pairing of hTCEpi cells with the natural glycocalyx. The presence of the glycocalyx is confirmed via staining with Alexa Fluor 488-conjugated wheat germ agglutinin lectin (Invitrogen).

MUC2, a soluble, gel-forming mucin protein secreted from goblet cells, is ubiquitous on the gut epithelia and has also been detected in the tear film. MUC2 and MUC5AC are both secreted from goblet cells and have similar molecular weights (MUC2 $\sim 540 \text{ kDa}$; MUC5AC $\sim 640 \text{ kDa}$). Commercially available porcine gastric mucin MUC2 provides an abundant and low-cost route to purify and study mucins while retaining their gel-forming functionality. The mucins used in these studies are predominantly MUC2 purified through sequential mechanical filtration in an effort to retain the end-group structure and gel-forming functionality (Fig. 2).

The addition of 5 wt.% MUC2-containing cell growth media to the Gemini hTCEpi cell biotribology experiments reduced the friction coefficient to below $\mu = 0.10$ ($\mu \sim 0.08$; $\tau \sim 50 \text{ Pa}$), as shown in Fig. 3. The friction and shear stress remained low throughout the experiment, and there were no measurable areas of removed or damaged cells, Fig. 4B. The patchy removal of the epithelial cells from the fibronectin-coated glass surface, Fig. 4A, is

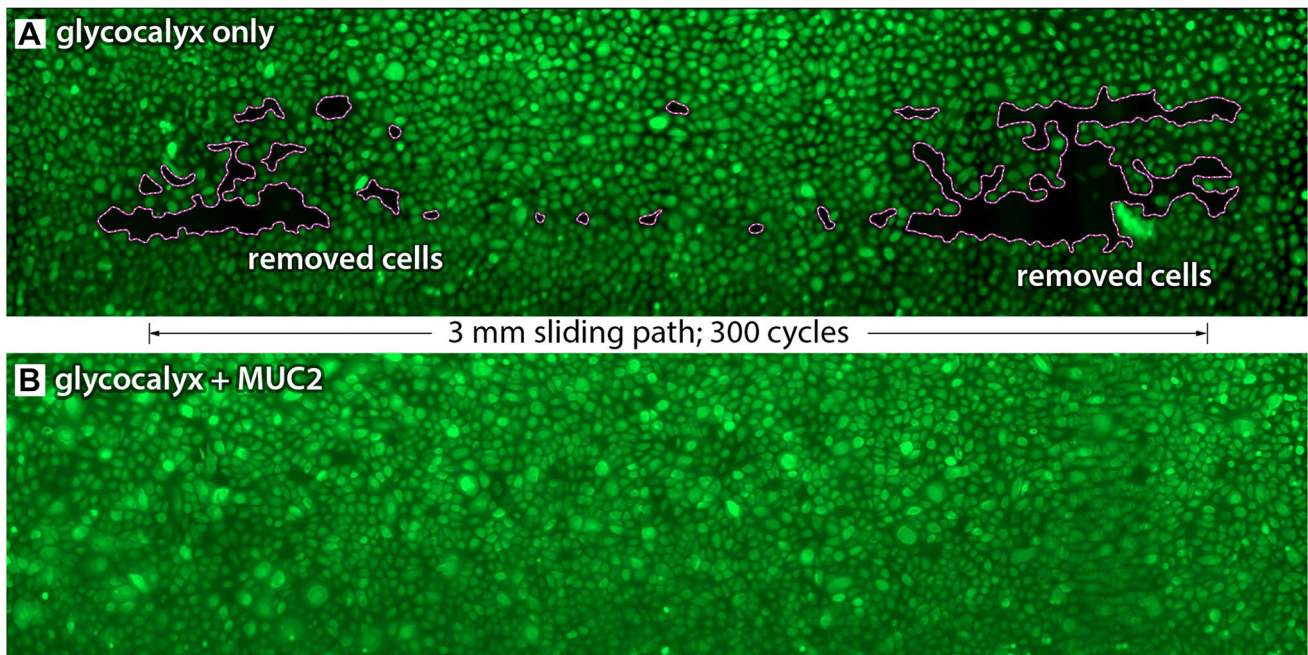


Fig. 4 Reduction of friction-induced damage due to addition of mucin: **A** Friction from cells-on-cells contact caused damage to epithelial cell layers, observable in fluorescence microscopy. Cells from

both surfaces are mechanically sheared and removed. **B** Upon addition of MUC2, the damage was significantly reduced and >90% of cells are still viable after 300 cycles

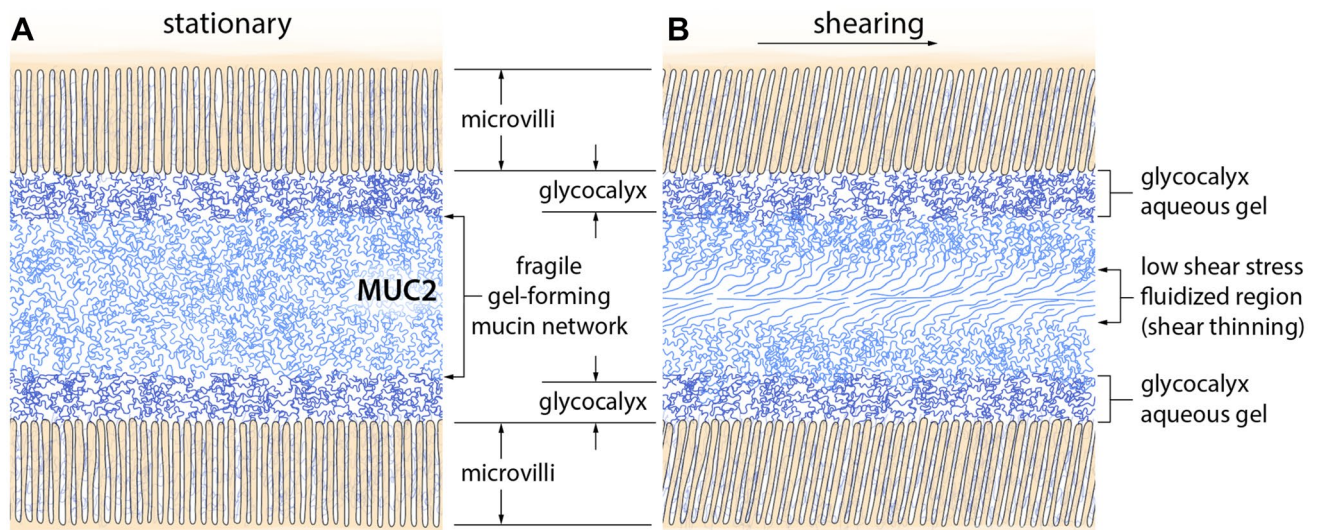


Fig. 5 The gel-forming mucins develop a concentration gradient that decreases in concentration away from the glycocalyx. **A** For stationary contacts, the gel-forming mucins (e.g., MUC2) can integrate with the glycocalyx to form a stable gel that separates the two surfaces. **B**

Under shearing, the fragile mucin network yields at the low concentration region (middle), fluidizes, and then undergoes shear thinning to maintain a low-friction interface during sliding

characteristic of epithelial cell biotribology and is a result of strong inter-cell junctions between neighboring cells that causes groups of cells to detach as a collective [16].

The sliding across Gemini hTCEpi cell surfaces possessing their glycocalyxes but lacking the gel-forming mucin MUC2

produced a significantly higher friction coefficient, shear stress, and damage. Additionally, in situ imaging revealed uncorrelated erratic stick–slip motion that was pervasive during sliding. Fast Fourier transform analysis of the lateral force data from experiments run at 500 $\mu\text{m/s}$ revealed a

dominant peak at 18.5 Hz that corresponds to a lateral spacing of $\sim 27 \mu\text{m}$ (consistent with the diameter of an individual cell), and suggests that the Gemini model of cells possessing only their glycocalyxes are behaving like a commensurate system, effectively jumping between equivalent positions [35]. The addition of MUC2 reduced friction, wear, and the stick–slip behavior; the shearing was graceful, smooth, and steady for the duration of the experiment. In retrospect, the erratic stick slip behavior between the Gemini hTCEpi interfaces is not unexpected, as these glycoproteins are designed to interact strongly with one another to form a resilient protective aqueous gel on the surface of the epithelial cells. The transmembrane glycoproteins that form the glycocalyx are produced on the surface of the microvilli and form a sticky aqueous gel surface on the order of 500 nm, seen in Fig. 5.

The aqueous gel formed by the soluble mucins such as MUC2 is known to be strongly shear thinning [13], approaching a viscosity of $\sim 1 \text{ cP}$ at shear rates, $\dot{\gamma}$, greater than $\dot{\gamma} = 1 \times 10^4 \text{ s}^{-1}$. In these experiments, the sliding speed was 1 mm/s, and the confocal microscopy indicates that the shearing zone is thin ($\ll 1 \mu\text{m}$), although due to vertical scanning limitations, we are unable to reliably quantify the thickness of this zone below this limit. Given a shear stress of 50 Pa and a viscosity of 1 cP, the corresponding shear rate is $\dot{\gamma} = 5 \times 10^4 \text{ s}^{-1}$, which for 1 mm/s sliding speed has a corresponding shearing film thickness of 20 nm. The magnetic tweezers and micro-rheology measurements of the 5 wt.% MUC2 solution revealed a yield stress much less than 10 Pa. The natural tear film may contain as little as 1.8% non-aqueous proteinaceous material such as mucins, and the network arrangements and bonding of these bio-macromolecules are responsible for the rheology of mucin networks. The yield stress of mucus has been most studied in the pulmonary setting and are reported to be in the range of 40–60 Pa [36].

The conceptual framework for this protective mucin network begins with the flickering bonds among the mucin glycoproteins that enable the integration of the gel-forming mucin with the glycocalyx, forming a gel that separates the two epithelial surfaces. We hypothesize that after yielding, this mucin gel network undergoes rapid shear thinning to maintain low shear stress. The mucin gel-spanning networks that form the natural tear film are known to be compositionally graded [37–39], and our modeling reveals that any gradient with increasing water content from the cell surfaces results in an expanding shear zone with increasing sliding speed. Interestingly, weak gradients actually produce nearly constant friction independent of the sliding speed.

This mucin gel-spanning network design of the tear film separates and protects the epithelial surfaces by acting as a mechanical fuse that limits the shear stress to levels below the damage thresholds for the production of proinflammatory cytokines, $\tau \sim 40 \text{ Pa}$ [24]. Earlier experiments with these hTCEpi cell monolayers clearly revealed the upregulation of

genes associated with proinflammatory cytokines, and the production of proinflammatory cytokines including IL6. The glycocalyx is unquestionably designed to protect the underlying microvilli of epithelial cells, but these experiments reveal that it is not itself lubricious; rather, the glycocalyx appears to use the bonding of transmembrane mucins to form a protective gel that is self-adhering [40]. This model of a sticky, self-adhering glycocalyx in Gemini contacts is supported by three observations: (1) a relatively high friction for aqueous gels, ($\mu \sim 0.20$), (2) the stick–slip events that were observed during sliding on Gemini interfaces of the glycocalyx alone, and (3) the gross removal of large patches of epithelial cells after only 300 cycles of sliding.

Within the context of dry-eye disease, these experiments demonstrate the importance of soluble gel-forming mucins such as MUC5AC and MUC2 in developing the weak shearing interface between the two epithelial surfaces in the ocular environment. As shown in Fig. 5, the model that emerges of the tear film is that of a delicate hierarchical multiscale assembly of mucins that form a network aqueous gel interface between the glycocalyxes to provide lubricity and gentle shearing by expanding the shear zone in response to sliding speed. This fragile interface uses transient weak interactions and flickering bonds to gracefully yield and rapidly recover during and after sliding, respectively.

4 Closing Remarks

Although the glycocalyx is thought to be lubricious, in Gemini contacts with epithelial cells, the glycocalyx is found to have a high friction coefficient ($\mu \sim 0.20$). Experiments were performed under physiological contact pressures of $\sim 600 \text{ Pa}$ for 300 cycles in a Gemini model of corneal epithelial cells, with an intact glycocalyx and the addition of a gel-forming purified mucin, MUC2. The addition of the gel-forming mucins at 5 wt.% showed friction coefficient reductions from $\mu \sim 0.20$ to $\mu \sim 0.08$. Gemini contacts with membrane-bound mucins and glycocalyxes without the addition of gel-forming mucins showed stick–slip events during sliding, large areas of cell damage, and cell removal after 300 cycles. Micro-rheology experiments using magnetic tweezers indicated a yield stress of the MUC2 solution below 10 Pa. The reduction in friction coefficient and the protection of the cells to damage over 300 cycles of sliding indicated that the soluble gel-forming mucins are important in epithelial biotribology for both lubricity and protection.

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Declarations

Conflict of interest The authors have no financial or proprietary interests in any material discussed in this article.


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