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Self-regenerating giant hyaluronan polymer brushes

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Tailoring interfaces with polymer brushes is a commonly used strategy to create functional materials for numerous applications. Existing methods are limited in brush thickness, the ability to generate high-density brushes of biopolymers, and the potential for regeneration. Here we introduce a scheme to synthesize ultra-thick regenerating hyaluronan polymer brushes using hyaluronan synthase. The platform provides a dynamic interface with tunable brush heights that extend up to 20 microns – two orders of magnitude thicker than standard brushes. The brushes are easily sculpted into micropatterned landscapes by photo-deactivation of the enzyme. Further, they provide a continuous source of megadalton hyaluronan or they can be covalently-stabilized to the surface. Stabilized brushes exhibit superb resistance to biofilms, yet are locally digested by fibroblasts. This brush technology provides opportunities in a range of arenas including regenerating tailorable biointerfaces for implants, wound healing or lubrication as well as fundamental studies of the glycocalyx and polymer physics.

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The design of functional biomaterials requires exquisite control over interfaces. Integrating physical and chemical features that can be dynamically tuned such as self-healing, stimulus responsiveness, stiffness, crosslinking, or hydrophobicity is a common goal in many modern biomaterials applications. One popular strategy to engineer such sophisticated biointerfaces is the polymer brush. Polymer brushes have been used to optimize a range of applications including sensors, antibiofouling coatings, protein separations, drug delivery, tissue engineering, and implants. The brushes used in these applications are typically high-density, and therefore require assembly using a grafting-from strategy rather than a grafting-to approach. This constraint is unfortunate since natural biopolymers are often desirable for biomaterials applications, yet few methodologies exist for the synthetic catalysis of biopolymers from surfaces. Further, many synthetic polymer brushes carry the disadvantage that they cannot be dynamically grown in vivo or from surfaces. Further, many synthetic polymer brushes carry the disadvantage that they cannot be dynamically grown in vivo or regenerated after wear. This work overcomes these issues by introducing a versatile platform to grow ultra-thick, dense hyaluronan polymer brushes, whose biocompatibility and possibility for regeneration provides a route for controlling dynamic biointerfaces.

The biopolymer hyaluronan (HA), also called hyaluronic acid, is widely used in an array of biomedical and clinical applications. Ubiquitous throughout human tissues and widely used in an array of biomedical and clinical biointerfaces is the polymer brush. Polymer brushes have been used to optimize a range of applications including sensors, antibiofouling coatings, protein separations, drug delivery, tissue engineering, and implants. The brushes used in these applications are typically high-density, and therefore require assembly using a grafting-from strategy rather than a grafting-to approach. This constraint is unfortunate since natural biopolymers are often desirable for biomaterials applications, yet few methodologies exist for the synthetic catalysis of biopolymers from surfaces. Further, many synthetic polymer brushes carry the disadvantage that they cannot be dynamically grown in vivo or regenerated after wear. This work overcomes these issues by introducing a versatile platform to grow ultra-thick, dense hyaluronan polymer brushes, whose biocompatibility and possibility for regeneration provides a route for controlling dynamic biointerfaces.

Hyaluronan synthase activity is robustly preserved in fragments of bacterial membrane. The activity of the HA synthase within the membranes can be dynamically switched on and off by controlling the availability of Mg$^{2+}$ or the UDP-sugars used by the enzyme to make HA. Previous studies used HA synthase-rich membrane fragments to investigate the function and kinetics of HA synthase, including the Group C Streptococcus equisimilis HA synthase used in this work. The work also extensively characterized the molecular weight distribution produced by the HAS fragments using SEC-MALLS (size exclusion chromatography—multi angle laser light scattering). Those studies show the HAS fragments produce a wide distribution of HA sizes, whose average molecular weight increases with time and plateaus at ~4.8 h with an average size of ~6 MDa ($M_w$).

Membrane fragment immobilization onto surfaces is achieved by first coating glass surfaces with polyethyleneimine (PEI) and then activating with glutaraldehyde (GA). The primed surface is used to crosslink with the numerous proteins in the membrane fragments. One popular strategy to engineer such sophisticated biointerfaces is the polymer brush. Polymer brushes have been used to optimize a range of applications including sensors, antibiofouling coatings, protein separations, drug delivery, tissue engineering, and implants. The brushes used in these applications are typically high-density, and therefore require assembly using a grafting-from strategy rather than a grafting-to approach. This constraint is unfortunate since natural biopolymers are often desirable for biomaterials applications, yet few methodologies exist for the synthetic catalysis of biopolymers from surfaces. Further, many synthetic polymer brushes carry the disadvantage that they cannot be dynamically grown in vivo or regenerated after wear. This work overcomes these issues by introducing a versatile platform to grow ultra-thick, dense hyaluronan polymer brushes, whose biocompatibility and possibility for regeneration provides a route for controlling dynamic biointerfaces.

In this work, we leverage the capacity of engineered bacteria to express high densities of HA synthase in their membranes, as well as the ability of HA synthase to generate extraordinarily long polymers. Immobilizing HA synthase-rich bacterial membrane fragments on interfaces provides a methodology to generate tunable, regenerative, giant HA brushes driven by enzymatic HA synthesis. With HA brush heights in the micron range fabricated in less than an hour, the brushes have the distinct advantage that they can be directly visualized with fluorescence microscopy. This platform is an original technology for growing brushes, using neither the traditional grafting-to nor grafting-from methodology. The outcome is one of the thickest (~22 μm, Fig. 1), if not the thickest polymer brush ever reported for synthetic or natural polymers. Here we introduce how to make the active enzymatic interfaces. We then characterize the resultant brush structure, demonstrate its regenerative properties, engineer the brush interface to expand performance range (stabilization, micro-patterning), and explore a range of potential applications in fundamental and applied materials science.

**Results**

**Immobilization of hyaluronan synthase membrane fragments.** Hyaluronan synthase activity is robustly preserved in fragments of bacterial membrane. The activity of the HA synthase within the membranes can be dynamically switched on and off by controlling the availability of Mg$^{2+}$ or the UDP-sugars used by the enzyme to make HA. Previous studies used HA synthase-rich membrane fragments to investigate the function and kinetics of HA synthase, including the Group C Streptococcus equisimilis HA synthase used in this work. The work also extensively characterized the molecular weight distribution produced by the HAS fragments using SEC-MALLS (size exclusion chromatography—multi angle laser light scattering). Those studies show the HAS fragments produce a wide distribution of HA sizes, whose average molecular weight increases with time and plateaus at ~4.8 h with an average size of ~6 MDa ($M_w$).

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The disaccharide unit of HA is comprised of \(\alpha\)-glucuronic acid (GlcUA) and N-acetyl-\(\alpha\)-glucosamine (GlcNAc). b HA synthase (blue) embedded in a bacterial membrane (red) polymerizes and extrudes the growing HA polymer through the pore. The sugar substrates UDP-GlcUA (circle) and UDP-GlcNAc (square) are alternatively bound at the intracellular glycotransferase sites where processive assembly takes place. HA is extruded at an average rate of ~1 nm/s corresponding to 1 disaccharide/s.

To estimate the monomer number, \(N\), for a polydisperse system at \(t_{\text{growth}} = 4\) h, we can use the number-averaged molecular weight \(M_n\), 2.42 MDa as measured by the solid-state nanopore sensor technology, which yields \(N = 6050\); or we can use the weight-averaged molecular weight \(M_w\), 6.76 MDa, which yields \(N = 16,900\). Hence, examining a range of 6050 < \(N < 16,900\) and \(H = 12.5\) nm, we arrive at a grafting density range of 0.0074—0.0021 chains per nm\(^2\), or equivalently, 740–2100 chains per \(\mu\)m\(^2\). Notably, these values are two orders of magnitude smaller than those for a typical synthetic brush generated via controlled radical polymerization (typically ~0.1 to 1 chain per \(n m^2\)). However, the density is still effectively high for this system, considering the micrometer lengths of the grafted polymers. Since we expect that about 50% of the fragments are upside down and only 30% of the surface is covered by HAS fragments, an approximate grafting density of 740 chains per \(\mu\)m\(^2\) as compared to a maximum 2100 HAS density in the fragments (predicted from protein analysis) seems reasonable, as it is 35% of the estimated enzyme density.

The HA synthase brush. The ultra-thick HA synthase-generated brushes afford unusual access to spatial characterization via high-resolution confocal microscopy. Figure 2a,b shows cross-sections of the fluorescently labeled HA brushes grown on a planar glass surface and of spherical brushes grown on 8 µm glass microspheres. For both geometries, the growth took place for 4 h at 30 °C \(t_{\text{growth}} = 4\) h). The concentration profiles of both planar and spherical brushes are well fit by an exponential (see the distribution of fitting parameters shown in Supplementary Note 1 and Supplementary Fig. 4). A monodisperse planar brush is predicted to have a parabolic concentration profile while polydisperse brushes, both planar and spherical, have a profile that depends on their length distribution. Hence the nonparabolic profile is consistent with the polydispersity associated with HA synthase (Fig. 1f, Supplementary Table 1).

That HA polymers are indeed produced in this unusual configuration was verified in several ways. First, we demonstrated that the enzyme Streptomyces hyaluronidase, which is highly specific for HA, quickly and thoroughly degrades the HA polymers. This result was further confirmed by the specific binding of HA-binding proteins tagged with GFP fluorophores used to make the fluorescent profiles of the brush shown in Fig. 2a,b.45,46 Last, we performed X-ray photoelectron spectroscopy (XPS) measurements of the dried brushes. Although the resultant spectra were a convolution from the underlying PEI/
HA-rich glycocalyx on cells

assays (Figs. 1h, 2c

ene nanoparticles >100 nm stop roughly at the interface of the

spectra consistent with making them more like pure HA

that increasing HA deposition leads to systematic changes in the

GA/membrane fragments and HA brush, we were able to show

experiments, nanoparticle sieving and growth dynamics. a Fluorescent profile of a planar HA brush and its intensity profile. b Fluorescent profile of spherical brush and its intensity profile. In both a, b the brush growth time is 4 h at 30 °C and the ionic strength 150 mM. c – e Cross-section of brush grown for 4 h on 8 μm microsphere (left) and the particle penetration intensity profile (right) for c 20 nm, 150 mM, d 200 nm, 150 mM and e 200 nm, 1.5 mM. Twenty nanometer particles show a distinct gradient within the brush while 200 nm particles remain excluded. Dextran (10 kDa, ~5 nm) is present in all cases (cyan) and shows an enhanced gradient in the low ionic strength swollen brush. f Dynamic growth of hyaluronan brushes generated by HA synthase (150 mM). The brush height reaches 2.62 ± 0.2 μm (st. dev.) in just 1 h (planar) in high ionic strength conditions. After 16 h the brush is 10.6 ± 1.0 μm (st. dev.). Measurements from individual brushes are shown in gray, averages and standard deviations are indicated in black. N_planar = 3 brushes, 12 regions per sample, except 16 h brush which is just one brush. The spherical brush plateaus at much earlier times (~5 h) at a final height of 4.3 ± 0.4 μm (st. dev.). N_spherical > 120 for spherical brush height measurements. All scale bars are 5 μm.

The dynamic generation of HA brushes by HA synthase (Fig. 2f) was characterized next. Measurements indicate that the brush height ranges from a few microns at early times up to 10.6 ± 1.0 μm after 16 h in physiological conditions (high ionic strength, 130 mM, ± is st. dev.) on planar surfaces (Supplementary Note 3 and Supplementary Fig. 6). On the microspheres (always 8 μm diameter in this manuscript), the maximal spherical brush height is significantly lower, plateauing at 4.3 ± 0.4 μm after 8 h (130 mM, ± is st. dev.). The height difference between the planar and spherical surfaces likely arises from geometrical effects, which on positively curved surfaces like spheres leads to more accessible free volume as the polymers extend radially. The HA brush height was determined using particle exclusion assays (Figs. 1h, 2c–e), a common approach to characterizing HA-rich glycocalyx on cells. Fluorescently labeled polystyrene nanoparticles >100 nm stop roughly at the interface of the brush (Supplementary Fig. 7). Comparison of estimates of brush thickness by fluorescence labeling and 200 nm particles showed statistically equivalent results.

HA synthase-generated HA films thicker than H ~ 300 nm should lie well within the brush regime, which is defined by the requirement that the average distance between grafting points is less than the diameter of the polymer in solution. The hydrodynamic radius for HA is RH ~ Mw 0.6, where 0.6 < v < 0.8 for high MW HA. Using v ~ 0.7, we find that at 1 h, RH ~ 324 nm. Since the brush height increases linearly in early times, at 10 min the brush height should be approximately ~333 nm. Correspondingly, the HA Mw increases linearly at early times as well, yielding 0.72 MDa or RH ~ 100 nm. The grafting density is 740 chains per μm2 or 1 chain every 37 nm, much less than a chain diameter of 200 nm. Hence at 10 min, with a 300 nm brush, the typical chain will have a diameter five times the average distance between chains, putting the system well into the brush regime.

Examination of the size-dependent penetration of the brushes by nanoparticles reveals that under physiological conditions, 200 nm and larger diameter particles are repelled from entry into
the brush, while 100 nm and smaller penetrate to varying extents (Fig. 2c–e, Supplementary Fig. 7). A concentration gradient throughout the spherical brush is clearly visible for the 20 nm particles (Fig. 2c, \( I_{\text{growth}} = 4 \text{ h}, 130 \text{ mM} \)). The 20 nm particles penetrate the brush, with approximately 30% of the bulk concentration value reaching the surface. Fluorescent negatively charged dextran (~10 kDa, ~5 nm diameter\(^2\)) shows a 30–50% approximately linear decrease in intensity in the presence of 200 and 20 nm particles at high ionic strength. When exposed to highly extended brushes at low ionic strength (~1 mM), the dextran gradient is pronounced enough to be visible by eye and drops by 50% (Fig. 2e). The observed gradient may arise from spatially varying density of dextran arising from excluded volume and/or charge effects; further the brush may act as a sieve to size separate the polydisperse dextran. While traditional dense synthetic brushes exclude molecules\(^2\), the partial penetration into these spherical HA brushes is not unexpected given the low grafting densities, the spherical geometry, and the broad polydispersity—all of which are expected to enhance particle penetration into brushes\(^5\). These studies show the potential to use HA brushes to sieve objects and molecules.

Hyaluronan is a weak polyelectrolyte and therefore sensitive to ionic strength and changes in pH. Figure 3 illustrates the stimulus responsiveness and reversibility of the HA brushes to changes in ionic strength. The planar brushes nearly double in height upon reduction of the ionic strength from 130 to 1.3 mM. For brushes grown for 16 h, the height increases from 12.1 ± 0.2 to 22.2 ± 2.5 \( \mu \text{m} \) (± is st. dev.), and it can be reversibly collapsed and stretched by exchange of the solvent, as shown in Fig. 3. Similar results were found for brushes grown for 4 h which increase more than 200% from ~7 to ~15 \( \mu \text{m} \) (see Supplementary Fig. 8). In both cases, there is a small decrease in height with each solvent exchange, with more HA loss from the inherently thicker brushes. We studied the impact of brush loss from pipetting effects on a 5.5 h growth brush, and found that the height change is consistent with loss from solvent exchange (shear-induced desorption), rather than the natural desorption of HA from HA synthase over short time periods (Supplementary Note 4 and Supplementary Fig. 9).

**Brush regeneration and on-demand synthesis.** The HA synthase maintains the ability to generate multiple polymers sequentially after natural release or external degradation of the HA\(^3\). Hence, the HA synthase interfaces should be able to regenerate or continuously replenish an HA reservoir as needed. To demonstrate this, we grew the brush for 1 h on planar interfaces, removed it by enzymatic degradation (hyaluronidase) and then regrew it three times—each time, after enzymatic removal (Fig. 4a, b). Little deviation in final brush height from the initial height is detectable with each subsequent regeneration. The small decay in brush height after each regeneration step may be due to the hyaluronidase sticking to the surface. We found that we had to minimize hyaluronidase adsorption to the surface using bovine serum albumin (BSA), or no brush would grow. It is possible that small amounts of hyaluronidase still bind to the surface, increasing with each exposure. These experiments demonstrate the potential for HA synthase brushes to be employed as regenerative interfaces.

The system also has capability for on-demand synthesis. Figure 4c shows how the HA polymerization and brush growth can be halted at a desirable height and then later, reintiated by controlling the availability of Mg\(^{2+}\) ions and sugar substrates. In this experiment, the brush growth was halted after 2 h, paused for 30 min and then reintitated for 2 h. Despite the pause, the final brush height was in the expected range of brush heights after 4 h uninterrupted growth.

Brush engineering: stabilization and micropatterning. Some applications like lubrication or antifouling interfaces or fundamental polymer physics studies will require a permanently stabilized brush. This is not possible with HA synthase binding alone, as the polymers release from the enzymes over a few days (Fig. 4d). Thus, we developed a strategy to generate stabilized brushes by crosslinking the HA to the underlying PEI surface chemistry with EDC-NHS chemistry. The EDC activates the carboxyl groups on HA for conjugation to secondary amine groups available from the PEI of the underlying grafting surface.

The surface-reinforced brushes are slightly reduced in height after processing, but remain stable for up to a year with weak decay in thickness over long times (Fig. 5a, Supplementary Fig. 10). Reinforced spherical brushes have similar exponential-like decay concentration profiles as untreated spherical brushes (Supplementary Fig. 11) and when exposed to hyaluronidase the brush is quickly eliminated (Supplementary Fig. 12). For unreinforced HA brushes, exposure to SDS quickly eliminates the HA structure by destroying the membrane fragments (Fig. 5b). For reinforced HA brushes, SDS does not eliminate the brush, confirming that the processing achieves covalent linkage of HA to the underlying surface (PEI) rather than the membrane fragments or the HA to itself (Fig. 5c).

Patterning polymer brushes is an important component of brush engineering for applications\(^5\). We therefore established a method to photopattern the HA brushes by deactivating HA synthase function with UV irradiation. Using a confocal microscope equipped with a 405 nm laser, we found that energy densities above 2.13E-04 W s per \( \mu \text{m}^2 \) are sufficient to destroy HA synthase enzyme activity and prevent HA growth in
micropatterned regions (Fig. 5d, e). Figure 5e shows a checkerboard pattern of the brush comprised regions with active HA synthase and other regions with no active HA synthase ($t_{\text{growth}} = 4\ h, 130\ mM$). This simple, scalable method enables the binary patterning of HA polymer brushes, and could be extended to mask aligners to rapidly pattern larger areas. Such engineered interfaces should have applications in various fields including controlling cell adhesion, tuning wettability, and indirectly modifying the height of brushes. Further, future work focused on tuning the power density delivered to the HA synthase interfaces may enable a simple approach to controlling its density, and consequently the brush grafting density.

**Cell-brush and biofilm interactions for biomaterials applications.** Motivated by the possibility of biomaterials applications such as implants and treatment of chronic wounds, we explored the interaction of mammalian cells and bacteria with the HA brushes. Mouse embryonic fibroblasts (MEF) seeded on top of reinforced HA synthase brushes accessed the underlying glass substrate within 1–3 h ($t_{\text{growth}} = 8\ h$; see Movie 1, Fig. 6a–e). Particle exclusion assays reveal that the MEFs express their own endogenous membrane-bound hyaluronidase in MEF cells. Reinforced brushes on spherical particles resist detergent treatment (SDS) despite the removal of membrane fragments. This confirms the HA is stably bound to the underlying glass substrate. UV micropatterning of HA synthase activity with a confocal microscope ($\lambda = 405\ nm$). Checkerboard pattern illustrating binary patterning of the brush. Red regions indicate areas where no brush grew; cyan regions indicate regions where brush expelled red nanoparticles. All scale bars are 10 $\mu$m.

Fluorescent labeling of the brush after 12 h of cell interaction showed visible black tracks at the surface, consistent with partial digestion of the brush (Fig. 6a). The spatial range of the brush digestion was relatively localized, consistent with the activity of endogenous membrane-bound hyaluronidase in MEF cells. Furthermore, particle exclusion assays of the brush-cell samples revealed that the brush had become unusually inhomogeneous in...
Fig. 6 Fibroblast and biofilm interactions with HA brush. a Black tracks in the GFPn-labeled HA brush reveal areas of brush digestion by MEF cells. Scale bar 50 µm. b Fluorescent dextran (cyan) highlights space under adherent cells (to the brush). Black regions correspond to cell area in close contact with surface. Red fluorescent particles fill areas of brush elimination due to digestion by cells. Scale bar 50 µm. c Fibroblasts attach to the interface underlying the HA brush, expressing mature focal adhesions (green, vinculin). Dextran is apparent underneath the spread cell. Scale bar 20 µm. d z-slice of the same adherent fibroblast from (c) sitting on a cushion of HA. Scale bar 10 µm. e Particle exclusion assay reveals MEFs express a thick glycocalyx. All MEF images were taken after 12 h exposure to the HA reinforced brush. Scale bar 10 µm. f–h Confocal micrographs of GFP-producing Pseudomonas aeruginosa (PAO1) interacting with a glass interface (f), an HA film (g), and a reinforced HA brush (h). All images were taken at the glass interface. Left: biofilm growth before washing (1 day). Right: biofilm growth after washing (1 day). Dextran was used to identify the glass interface beneath the brush. XZ side views of the biofilms are presented below each respective XY top view of the samples. Scale bars, 10 µm in (f–h). i Comparison of the number of bacteria retained after washing different surfaces. Data were taken in triplicates and averaged over five regions per sample. Error is SEM. Data are available in Table 3 in the Supplementary Information.
height. When focusing at the surface of the sample (defined here as the base of the brush), no beads should be visible if the brush remains intact (outside of the cell areas). Yet frequently, the beads were clearly visible at the surface, indicating that the brush had been compromised or removed (Fig. 6b and Supplementary Fig. 15 for zx confocal images). The destruction of the brush must be cell driven, since the reinforced brushes are homogenous and stable over months.

Previous studies have shown that in order for cells to adhere to high molecular weight HA films, membrane-bound hyaluronidase removes the HA film under the cell. Indeed, in the areas around the focal adhesions, we found fluorescent dextran (10 kDa) was excluded from under the cell, indicating the very confined distance between the cell and the surface. Yet, despite the presence of focal adhesions and localized HA removal, well-spread MEFs frequently maintained a significant HA-rich gap with the substrate (2.7 ± 1.5 μm, N = 21 at maximum separation, see Fig. 6c, d, ± is st. dev.). The voluminous space beneath the cells is evident when examining fluorescent dextran (10 kDa) penetration under the cell, which is only present for cells adherent to HA brush interfaces (Fig. 6c, d). The same cells when bound to glass (or to membrane fragment-coated glass with no HA growth) display no measurable gap and no penetration of dextran under the cell (see Supplementary Fig. 13c). The variability in the dextran intensity (cyan) the cell—brush interface (see Fig. 6b, c and Supplementary Fig. 13c) is consistent with the interpretation that some regions beneath the cell have more or less digested HA.

How this final cell—brush configuration is realized is unclear. Without some method to compress or digest the underlying brush, the cells could not reach the underlying surface. Gravitational force plays a minimal role. For example, 8 μm silica spheres remain suspended above the brush with little brush deformation (Supplementary Fig. S16). An (inactive) cell of lesser density (ρ_{cell} ∼ 1.1 g per cm^3 < ρ_{silica} ∼ 2.65 g per cm^3) but of slightly larger size would also remain suspended. Hence, two possible mechanisms could enable cell penetration of the brush and adhesion to the surface. The first is membrane-bound hyaluronidase digestion of the brush, a hypothesis for which some supporting evidence has been provided. Second, the cells may be able to manipulate the brush through adhesive interactions via HA-binding proteins like CD44, and/or extend protrusions through the HA brush to reach the surface and then exert contractile forces to compress the brushes as the cells approach the interface.

In summary, MEF cells flourish within the brush environment. These preliminary findings demonstrate that the HA brushes will be a useful platform to systematically study the interplay of sugar-rich hydrogels and glycocalyx in cell adhesion, an area of growing relevance and interest.

Discussion
The enzyme-generated giant polymer brush introduced here is distinct from traditional polymer brushes in several ways. Notably, rather than grafting-from or grafting-to the surface, the HA brush is generated by a transmembrane protein that extrudes the HA through a protein pore. The enzyme is able to polymerize much higher molecular weight molecules than most other techniques such as free radical polymerization with rare exception. Further, the selective biointeractivity, i.e. inhibition of pathogenic microbial adhesion but enhancement of beneficial host cell responses, establishes the HA brush interface an interesting option for coating implants, bandages or other materials with similar requirements.

We consider factors that may affect future applications. Biomedical tools that could benefit from this technology such as implants or bandages may require large surface areas. It is possible to scale up the surface area covered. We estimate that 1 L of (E. coli) bacterial culture can be used to cover ~2 m^2 of area. Glass surfaces prepared with HA synthe fragments can be stored for up to at least 9 months at −80 °C with similar performance (spherical brushes made from fresh surfaces have 3.8 ± 0.3 μm, N = 146, i_{growth}= 4 μh, N = 146; 9-month-old have 3.9 ± 0.2 μm, N = 32, ± is st. dev.). Indeed, longer functionality is expected since the fragments can be stored for at least 12 years and maintain similar functionality (spherical brushes made with these fragments have brush heights of 3.0 ± 0.3 μm, N = 147, ± is st. dev.). Binding the membrane fragments to other materials such as plastic or titanium, i.e. for catheters or implants, should be achievable using modified surface functionalization schemes.

Two concerns arise with the system in the context of biomaterials applications and regeneration. The first is that the bacterial membranes likely contain lipopolysaccharide (LPS), which could stimulate an immune response. One approach to bypass the LPS trigger is to work with endotoxin-free bacteria that are nonimmunogenic. Another strategy is to use genetically improved PAO1 bacteria for 1 and/or 5 days, rinsing, and then imaging remnant bacteria attached to or embedded within the brush. Each sample was randomly imaged at five locations and the total volume occupied by bacteria assessed to estimate the number of bacteria (Fig. 6f–i). The results were compared with PAO1 biofilm formation on glass and HA thin films. The images in the left column correspond to the three surfaces (glass, HA film, HA brush) prior to washing. The right column shows the same samples after washing. Quantification of the total number of bacteria under each condition is summarized in Fig. 6i (see Supplementary Table 3). The percentage of bacteria relative to the glass slide was dramatically decreased on both the HA film and even more so on the HA brush at 1 and 5 days (Fig. 6i, d, and Supplementary Table 4). The glass surfaces retain 99% more bacteria than the HA brushes after 1 day. As expected, the HA film shows improved resistance to fouling, yet it still retains six times more bacteria than the HA brush (Fig. 6h) after both 1- and 5-day exposures. On the HA brushes, only 1–2% of the remnant adhered bacteria were found at the underlying brush—glass interface. Addressing the brush uniformity, not yet optimized, could further improve the outcomes.

In future work, the antifouling properties of the brush could be complemented with doped the brush with antimicrobial compounds using straightforward and accessible chemistry associated with HA. Further, the selective biointeractivity, i.e. inhibition of pathogenic microbial adhesion but enhancement of beneficial host cell responses, establishes the HA brush interface an interesting option for coating implants, bandages or other materials with similar requirements.
engineered mammalian cells as the source of HA synthesize, possibly even from the future host of the biomaterial. The second concern is the stability of the HA synthesize and its ability to continually synthesize HA. HA synthesize activity decays over time. This timescale would limit the window of opportunity for regeneration of interfaces or use of an interface to continuously release HA while maintaining a protective HA brush. If there is sufficient interest, protein engineering may enable the development of a HA synthesize that is stable for longer periods of time.

The HA brush platform also presents a myriad of opportunities for fundamental polymer physics studies, where many open questions remain in the arena of polyelectrolyte brushes. The system enables the direct visualization of the detailed spatial structure of brushes and ultimately, possibly even dynamics of individual strands. For example, both polymer concentration profiles with height and particle penetration or protein absorption can be studied directly and compared with theory. Schemes to probe the structure of brushes and ultimately, possibly even dynamics of individual strands. In another example, the curvature-dependent brush height data shown in Fig. 2f deserves further systematic study and comparison with theoretical predictions. Studies like these, combined with full characterization of the polymer length distribution similar to that shown in Fig. 1f, will be a very exciting and powerful tool to make progress in polymer physics.

Engineered biomaterials based on HA are abundant in the literature and medical practice. The regenerative HA brush interface introduced here represents a platform for creating a distinct class of interfacial HA biomaterials. The straightforward and established protocols to chemically modify and cross-link HA present numerous opportunities for exploration and expansion of traditional approaches. The regenerative ability of the platform is a useful tool to explore, as are the distinct properties of a gigantically thick brush. From lubrication to cell glycocalyx, which plays an important but poorly understood role in the biological role of the glycocalyx, and for the ends of the polymers. In another example, the curvature-dependent brush height data shown in Fig. 2f deserves further systematic study and comparison with theoretical predictions. Studies like these, combined with full characterization of the polymer length distribution similar to that shown in Fig. 1f, will be a very exciting and powerful tool to make progress in polymer physics.

**Methods**

**Dynamic light scattering.** The membrane fragment suspension was removed from the −80 °C freezer and defrosted. Then, it was diluted from 1 mg/mL to a final concentration of 0.02 mg/mL with phosphate buffer (pH 7.3, 75 mM NaKPO4, Na2HPO4 (I.T. Baker 3827-01), KH2PO4 (Sigma-Aldrich P8655)) together with 0.1 mM EDTA (Mallinkrodt Chemicals 3590-12), 50 mM NaCl, 2% glycerol, 5 mM DTT (Sigma-Aldrich D9779). Dynamic light scattering was performed and analyzed using a Malvern Zetasizer Nano ZS. The autocorrelation function was acquired every 10 s with ten measurements for each of three runs. The intensity of light was collected at a scattering angle of 90°. Analysis yielded an average membrane fragment diameter of ~145 nm (before HA growth).

**Activating HA synthesis.** The storage buffer was exchanged with activation buffer (pH 7.3, 75 mM NaKPO4, Na2HPO4, 50 mM NaCl, 20 mM MgCl2, 0.1 mM EDTA). After warming the sample for 45 min in a 30 °C incubator, uridine 5-diphosphoglucuronic acid trisodium salt (UDP-GlcUA, Sigma-Aldrich U6751) and uridine 5-diphospho-N-acetylgalactosamine sodium salt (UDP-GlcNAc, Sigma-Aldrich U4375) were added to a final concentration of 5 mM.

**Quenching HA synthesis.** The activation buffer was thoroughly exchanged with quenching buffer (pH 7.3, 75 mM NaKPO4, 50 mM NaCl, 20 mM MgCl2, 0.1 mM EDTA) via pipetting and mixed by gently pipetting the liquid up and down a few times (repeat buffer exchange seven times).

**HA purification for solid-state nanopore analysis.** HA grown from bacterial membrane fragments in suspension was purified for later quantification by solid-state nanopore. After HA production, EDTA was added to a final concentration of 40 mM to quench the growth and the solution was put on ice for 10 min. The solution was then placed on a 1 mL heat block to 80 °C for 2 min and put on again for 1–2 min to inactive the HA synthesize. To dissociate HA from the synthesize, the solution was mixed in a 3:1 ratio of Folch to HA solution and allowed to shake for 15 min. The Folch/HA solution was centrifuged for 7 min at 7168 × g (8000 rpm) and then the supernatant containing HA was removed and speed vacuumed until the solution volume was reduced to one-third.

**Solid-state nanopore determination of HA molecular weight distribution.** Enzymatically generated HA samples were mixed with measurement buffer (6 M LiCl, 10 mM Tris, 1 mM EDTA, pH 8.0) to a final concentration of 30 ng/μL and stored at −20 °C until measurement. Solid-state nanopore analysis was performed on 10 μL aliquots of the samples. A single pore (6–8 nm diameter) was fabricated in a 19 nm thin, free standing silicon nitride membrane supported by a silicon chip and the reservoir of clean measurement buffer and one reservoir of sample mixture. Ag/AgCl electrodes (Sigma-Aldrich, St. Louis MO) were placed in each reservoir and an Ag/AgCl 200b patch clamp amplifier (Axon Instruments, Union City, CA) was used to apply both a voltage of 200 mV and record trans- pore current and resistive pulses caused by HA translac- tation through the pore. Data were collected at a rate of 200 kHz with a four-pole Bessel filter and an additional 5 kHz low-pass filter was applied using custom software. Resistive pulses (“events”) in the current signal were identified as transient interruptions in the ionic current >5σ in amplitude from the baseline and with a time duration range of 25 μs–25 s. The event charge deficit (ECD) was calculated as the area under the event determined for each pore. Time constants were determined for each sample with these values and used for subsequent analyses.

**Immobilizing bacterial membrane fragments on glass slides.** Coverslips (VWR 48366 246 or VWR 48366 067) were sonicated in ultrapure water for 15 min and cleaned in reagent grade acetone in a sonicking water bath for 15 min. The coverslips were sonicated in ultrapure water and dried with nitrogen. This timescale would limit the window of opportunity for regeneration of interfaces or use of an interface to continuously release HA while maintaining a protective HA brush. If there is sufficient interest, protein engineering may enable the development of a HA synthesize that is stable for longer periods of time.

**Immobilizing bacterial membrane fragments on silica microphreses.** In a 90 °C water bath on a stirring hot plate, 100 mg monodisperse silica beads (Cospheric LLC, 1.8 g/cm³, 7.75 μm diameter, CV = 3.7%, <1% doublets) were added to 1 mL 30% H2O2 in a 1.5 mL centrifuge tube. The beads were brieﬂy vortexed to suspend them and then the tube was put in a sonicking water bath for 15 min to disperse the beads. A stir bar was added along with 3 mL sulfuric acid (BDH Aristar Plus, average Mw 0.98 kDa) in a disposable glass vial (22 mL, VWR 470206-384). The 1 mL beads suspended in 30% H2O2 were gently pipetted into the vial while allowing slow stirring. The vial was sealed with a clamp in the stirring hot water bath. The piranha cleaning was allowed to proceed for 2 h during which the level of the solution was maintained. Finally, 10 mL ultrapure water was added to the vial, allowing the water to fully mix. Heating and stirring was turned off and the vial was allowed to sit overnight to let the microphreses to settle. On the next day, most of the piranha solution was removed using a glass pipette tip. The beads were resuspended with stirring and then transferred to a glass centrifuge tube. Using a swing bucket centrifuge, the solution was exchanged with ultrapure water seven times and checked to ensure that the pH of the solution was above six. After supernatant removal, the beads were suspended in 2.5% w/v PEI and then stirred for 1 h before washing seven times with ultrapure water. Glutaraldehyde was added to a final concentration of 2.5% w/v with PBS to a clean glass vial. The washed beads were slowly added and the suspension was allowed to stir for 1 h before washing seven times with ultrapure water. To prepare the membrane fragments for deposition on glutaraldehyde-modified beads, exchange the Tris-based membrane fragment storage buffer into a phosphate buffer using a Slide-A-Lyzer Mini Dialysis Device (ThermoScientific 69570) in an ice box (4 °C). The phosphate buffer was refreshed twice in 45 min intervals. The final concentration of buffer was put on again for 1–2 min to inactive the HA synthesize. To dissociate HA from the synthesize, the solution was mixed in a 3:1 ratio of Folch to HA solution and allowed to shake for 15 min. The Folch/HA solution was centrifuged for 7 min at 7168 × g (8000 rpm) and then the supernatant containing HA was removed and speed vacuumed until the solution volume was reduced to one-third.
4 °C. The beads were allowed to settle and then the supernatant was removed. The beads were exchanged into the Tris storage buffer by adding the buffer, allowing the beads to settle before pouring the supernatant off. Finally, the beads were aliquoted in storage buffer and stored in −80 °C freezer.

**Confocal imaging.** Fluorescent characterization of the HA brush was made using a scanning laser confocal microscope (FV1000, Olympus, Tokyo, Japan; Objective: PlanApo, N.A. = 1.42 NA oil). When imaging planar polymer brushes, a 100 nm vertical step was used and a 20-μm-thick z-stack was taken of the planar brush. When imaging the polymer brushes on microspheres, a 20–60 nm horizontal pixel size and 470 nm vertical step was used. A 4-μm-thick z-stack was taken for each microsphere. The vertical range was selected to, at minimum, measure to slightly above the bead center. Imaging was completed within 1 h after quenching HA synthesis in order to avoid significant desorption of the HA polymers.

**HA brush concentration profile analysis.** The concentration profile of the HA brush is estimated by looking at the fluorescent intensity generated by GFPPs, a protein that binds to HA. The GFPp is comprised of green fluorescent protein (GFP) with the HA-linking domain of neurocan connection, specifically binds to HA. GFPp was expressed by HEK 293 EBNA cells purified according published protocols. In experiments where HA-GFPp profiles were assayed, we incubated with 8 mg/mL BSA for 40 min to backfill the surface before HA synthesis. This reduces the adhesion of GFPp to the underlying substrate. Before starting the HA synthesis, the BSA solution was removed. After quenching HA synthesis, we added −44 μM GFPp to a final concentration of 3 μM GFP. For planar brushes, the z-fluorescence profile was extracted from confocal microscopy z-stacks. For spherical brushes, the centers of the microspheres are identified by segmenting either the image of the dextran channel or the GFPn channel using Otsu’s method. An azimuthally averaged profile was extracted from 30° cones from the center of a microsphere, only analyzing the cones that are clear of neighboring brushes. The mean background (and its standard deviation) was determined from the last 50 data points at the edge of the image (away from the microsphere). The edge of the brush was defined where the intensity is greater than or equal to twice the standard deviation above the mean background. The peak in the GFPp was identified as the surface of the microsphere. The thickness of the brush measured using 200 nm particle exclusion assays is 3.75 ± 0.30 μm (Nbeads = 146) is consistent with the value measured using GFPp of 3.70 ± 0.25 μm (Nbeads = 112) for nonreinforced brushes on 8 μm diameter particles (tgrowth = 4 h, high salt condition).

**Particle exclusion and nanoparticle penetration assays.** Particle exclusion assays were performed by adding red 200 nm FluoSpheres (carboxylate-modified Molecular Probes, Inc., catalog number: F8810) to a final concentration of 0.7% w/v along with fluorescent dextran (Molecular Probes, Inc. Alexa Fluor 647, 10 kDa) to a final concentration of 33 μg/mL. Nanoparticle penetration was investigated using the red, 200 nm FluoSpheres, green 100 nm FluoSpheres (Catalog number: F8803), and green 20 nm FluoSpheres (Catalog number: F8787). When the grafting surface needed to be labeled, 0.007% w/v of the green, 20 nm nanoparticles were added. For details of planar brush height analysis using 200 nm particles, see Supplementary Note 3 and Supplementary Fig. 6.

**Stimulus response and reversibility to ion strength.** The sample was first imaged to establish a baseline height for the brush (using 200 nm particles) in the standard imaging buffer (40 μL activation buffer and 14 μL quenching buffer), which has an estimated total ionic strength of ~130 mM. The media was then exchanged three times with a 1% activation buffer diluted with ultrapure water, removing all liquid with each final wash. To image again, the 200 nm FluoSpheres were added to the sample, along with the typical volumes of activation buffer (40 μL) and quenching buffer (14 μL), but at 1% concentration diluted with ultrapure water to make the ion strength 1.3 mM. To switch back to the normal growth media for activation buffer, in order to define the surface, a low concentration of 8 μm silica microspheres were added to the sample and brought into focus with diffraction interference contrast (DIC) microscopy on the confocal microscope (within a stage-top temperature incubator at 30 °C incubator and a wet sponge to maintain humidity). Then, the focus was adjusted to be below the center of the microspheres an amount equal to the radius such that the focus should now be on the surface of the sample, rather than at the center of the spheres. This method allows one to focus at the plane of the membrane fragments containing HA without introducing other chemistries or fluoroscantime three times. After exchanging the Tris storage buffer, we imaged the polymer brushes on microspheres, a 30° laser was scanned in a predetermined area using optimized settings to eliminate HA synthe activity. We found that treating the surface with an energy density greater than 2.13E-4 J/µm² was sufficient. When applying the laser to an area, we first refocus and surface using the microscope to a reference. After all desired areas were laser treated, we activated HA synthe activity and allowed the sample to grow for the desired time.

**Mouse embryonic fibroblast interaction with reinforced planar brushes.** Mouse embryonic fibroblasts (MEF) were used to express fluorescent (green) vinculin. Mouse embryonic fibroblasts (MEF) were used to express fluorescent (green) vinculin. Vinculin-null MEFS were used to study how cells interact with reinforced HA brushes generated by HA synthe. The cells were cultivated in Dulbecco’s modified Eagle’s medium (D-MEM) supplemented by 10% FBS (fetal bovine serum, Corning CellGro 35-010-CV), 1% penicillin and 4 mM l-glutamine. Brushes were grown for 8 h and then reinforced to avoid decay over the length of the 12 h experiment. The reinforced brushes were covered with cell culture media and then seeded with MEF cells. Time lapse videos were made using DIC imaging for 12 h using a ×40 Nikon Plan Fluor oil lens on a Nikon TE2000. Fluorescent images of the vinculin-rich nonreinforced area, we first refer to the surface using the microscope to a reference. After all desired areas were laser treated, we activated HA synthe activity and allowed the sample to grow for the desired time.

**HA film preparation.** Rosette coil HA (Thomas Scientific H5388, MW range 5.0 × 10⁵–1.2 × 10⁶ Da) was fluorescently labeled with Alexa 546 (ThermoFisher Scientific A20002) using established protocols. Briefly, the HA was dissolved in water and mixed with a 20–40-fold molar excess of dihydroazide and pH balanced to 4.75. A 4 M excess of EDC was dissolved in water and added to the HA solution and the pH was maintained at 4.75 by addition of HCl over the course of 2 h. After exchanging the Tris storage buffer, the pH was balanced to 7 and the HA was precipitated with chilled ethanol. To achieve 1 dye molecule per 50 nm HA, we added dye at a final concentration of 0.1 mg/mL with 2 mg/mL of the dihydroazide-modified HA.
remove unbound dye, the solution was dialyzed with ultrapure water for 1 week, refreshing the water every day. The HA film was deposited on glass coverslips that were washed in ultrapure water and acetone, then ethanol dried. The film was then covered with 50 µg/mL of the fluorescent HA and allowed to sit in the 4 °C refrigerator overnight with wet sponges to minimize evaporation. In the morning, the slides were rinsed with DI water, dried with nitrogen, and mounted in teflon sample holders.

**Pseudomonas aeruginosa bacterial culture.** A GFP-producing strain of *Pseudomonas aeruginosa* PA01 was streaked on a lysogeny broth (LB) (Teknova L9130) agar plate incubated at 37 °C. A single bacterial colony from the agar plate was inoculated into 5 mL of fresh LB broth under similar conditions until the optical density (OD) had a value of 1. This bacterial culture was then diluted to a value of OD 0.02 and used for inoculating the hyaluronan film and brush samples.

**Biofilm growth on HA brush.** Post hyaluronan growth, the synthesis was quenched and the brushes were reinforced to create stable structures. The EDC/sulfo-NHS buffer that was used to reinforce the brush overnight was replaced with borate buffer for 2 h, which was then replaced with fresh borate buffer and allowed to sit overnight at 4 °C. This was followed by two PBS buffer exchanges, each for 1 h at room temperature. One hundred microliters of the OD 0.02 culture was then added to the sample and incubated at 37 °C. For the 1-day experiments, the samples were imaged with confocal microscopy before bacteria removal. After imaging, the sample was washed in a manner where the solution was pulled up and down into the pipette three times, and for two rounds, and this procedure was repeated twice with fresh LB broth both times. The washed samples were topped with fresh LB and imaged again to check for bacterial adherence and biofilm formation. Twenty nanometers fluorescent beads were gently added and imaged to locate the coverslip surface. For the 5-day experiments, the samples were replenished with fresh LB broth every 24 h and on the fifth day, the brushes were imaged using the procedure described above.

**Bacteria growth on HA films and glass substrates.** Post HA film preparation, 100 µL of the OD 0.02 culture was added to the sample and incubated at 37 °C. The sample area of the HA brush, HA film and glass was the same size for all cases. Imaging pre- and post-wash and the washing steps are identical to that for the biofilm.

**Bacteria quantification.** The total volume of living bacteria stuck to the surfaces in PA01 biofilms after washing was quantified. Confocal microscopy was used to image a 3D volume. The total number of bacteria was determined for all samples by counting all voxels in the confocal z-stacks that had a signal that was above the noise threshold, indicating bacteria was present. Briefly, the 3D z-stacks were exported as .vols files in order to preserve the metadata and processed with Matlab through custom codes. The BioFormats package was used for reading the images and defining the stack structure for further processing. Noise reduction was performed using high-pass filtering with a Gaussian filter and the images were further binarized using a suitable thresholding algorithm. The total volumes of live bacteria on the brushes, films and glass substrates were calculated by counting the nonzero voxels in the binarized image stack. The average live bacterial volume in the region with a depth of 5 µm above the brush, film, and glass surface was used in calculations.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Raw data for all figures in the manuscript and the supplemental materials are provided online at https://github.com/wwb203/HyaluronanNatureCommunication. All data are available from the corresponding authors upon reasonable request.

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W.W. was responsible for concept and research design, spherical brush experiments, fibroblast experiments, data interpretation, and paper preparation. J.L.F. was responsible for research design, planar brush experiments, micropatterning experiments, data interpretation, and paper preparation. H.S. was responsible for the biofilm experiments concept, research design, and data interpretation. D.T.K. was responsible for brush development and paper preparation. J.T. was responsible for the SEM experiments and data interpretation. F.R., E.R. and A.R.H. were responsible for the solid-state nanopore experiments and data interpretation. A.T.M. and M.A.F. were responsible for the AFM experiments and data interpretation. M.K. was responsible for the SEM experiments and data interpretation. J.L.W. and P.H.W. were responsible for the concept and research design, data interpretation, and paper preparation. J.E.C. was responsible for concept and research design, data interpretation, supervision, and paper preparation. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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