Films of End-Grafted Hyaluronan Are a Prototype of a Brush of a Strongly Charged, Semiflexible Polyelectrolyte with Intrinsic Excluded Volume

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ABSTRACT: Hyaluronan (HA) is a naturally occurring linear, negatively charged polysaccharide that plays a vital role in the organization and function of pericellular coats and extracellular matrices in vertebrates, and that is becoming increasingly popular in biomedical applications. To gain insight into the physical phenomena that govern the mechanical behavior of HA assemblies, we have studied the response of films of end-grafted HA to compression over a large range of ionic strength. Compression forces were measured as a function of the absolute distance between a colloidal probe and the planar surface on which the HA film was constructed, using a combined atomic force microscopy and reflection interference contrast microscopy setup. The HA films were well-defined in the sense that they are made of chains with a narrow size distribution that are grafted at controlled density to a solid support. Detailed comparison of the experimental data with analytical expressions derived from polymer and polyelectrolyte brush theory reveals that films of end-grafted HA behave as strongly charged polyelectrolyte brushes. To quantitatively reproduce the experimental data, intrinsic excluded volume interactions and chain stiffness of the polymer backbone must be taken into account. At low ionic strength, chains become almost fully stretched. In our experimental system, several micrometer thick films are formed that reach a hydration of up to 99.98%, and the brush thickness decreases by more than 5-fold with increasing ionic strength. More generally, the study provides quantitative theoretical predictions for the film thickness and compressive response as a function of HA length, grafting density and ionic strength.

INTRODUCTION

The polysaccharide hyaluronan (HA) is ubiquitous in the pericellular and extracellular space of vertebrates. It is also becoming increasingly popular in biomedical applications as surface coating and in tissue engineering. HA is a linear polymer of disaccharides, made of glucuronic acid and N-acetylgalactosamine, that are linked via alternating β-1,4 and β-1,3 glycosidic bonds. Each disaccharide has a length of 1 nm and carries one chargeable group in the form of a carboxylic acid. For polymeric HA, the pKₐ is approximately 3, and HA is, hence, negatively charged above pH ≈ 3. In vivo, HA is expressed by HA synthases at the cell membrane and extruded into the extracellular space. These HA molecules have a molecular mass of typically a few million Daltons or a contour length of several micrometers. Once produced, they can stay grafted to the synthases and/or bind to other HA receptors at the cell surface, such as CD44 and form so-called pericellular coats (PCCs), or they can be released into solution to serve other functions in the extracellular space. Numerous biological functions of PCCs and extracellular assemblies of HA in general (e.g., in cartilage) have been related to their mechanical properties. To better understand how the mechanical properties of PCCs are connected to their supramolecular structure and dynamics, measurements on well-defined model systems that recapitulate the two-dimensional confinement and the self-assembly properties of the PCC are desirable.

To this end, we developed a method to graft HA chains with one of their ends (the reducing end) to a planar solid support. These HA films are well-defined in the sense that they are made of polymer chains of known contour length and charge density and with a small size distribution (polydispersity index ≤ 1.03) and that the grafting density can be estimated well. With such a model system, the physicochemical properties of the HA films as a function of external cues become accessible to investigation by surface-sensitive analysis techniques. For example, we recently reported the mechanical properties of HA films in a buffered aqueous solution reproducing physiological ionic strength and pH. The HA molecular weight and grafting density were chosen such that the mean HA density in the film is around 1 mg/mL, that is, in a biologically relevant range. Under these conditions, the response of HA films to compression by a colloidal probe could be described well by...
a simple theoretical model of a polymer brush. In that study, however, the polyelectrolyte nature of HA was not explicitly considered.

In the present work, we systematically examine the influence of ionic strength, over a range from 50 μM to 500 mM NaCl, on the thickness and compressive mechanics of end-grafted HA films. To be able to access low ionic strengths, the measurements were performed in the absence of buffer. We compare experimental results with several theoretical models of polymer and polyelectrolyte brushes. By doing so, we confirm that films of end-grafted HA exhibit polyelectrolyte brush behavior and we are able to quantitatively predict the thickness and force response as a function of ionic strength, HA length, and grafting density. Using the HA films as a well-defined experimental model, we also test how well the different theoretical approximations can reproduce the real data quantitatively.

For the experiment, we employed a setup that combines colloidal probe atomic force microscopy (AFM) and reflection interference contrast microscopy (RICM). With this setup, interaction forces can be quantified at the same time as the absolute distance between the colloidal probe and the substrate on which the brush was built. The latter is not readily available by AFM alone because the distance at closest approach remains unknown when analyzing polymer films. The combined setup provides the level of control on the absolute distance that is needed for a rigorous comparison of experiment and theory.

The employed theoretical models are based on different mean-field and self-consistent mean-field, as well as scaling approaches, as they have been developed throughout the past three decades. They provide a description of the morphology and force response of films of end-grafted polymers or polyelectrolytes as a function of general properties of the individual chains, that is, contour length, charge density, stiffness and excluded volume, and the grafting density. Intentionally, the models are kept simple enough such that analytical expressions for the film thickness and for the compression forces as a function of intersurface distance can be derived.

The particularity of HA is that it is a rather stiff polymer. From solution-based studies, persistence lengths between 4 and 8 nm have been reported, exceeding the size of the disaccharide repeat unit by 4- to 8-fold. In comparison, the intrinsic persistence length tends to be close to the monomer unit size for most water-soluble synthetic polymers and polyelectrolytes and for biopolymers such as single-stranded DNA or natively unfolded peptide chains. Hydrogen bonds between adjacent saccharide units and water caging of the glycosidic linkages have been proposed to be responsible for the increased stiffness of HA.

As we will see, the intrinsic excluded volume (i.e., the volume from which a segment of a macromolecule effectively excludes all other segments of the same macromolecule or other macromolecules in solution) and the intrinsic stiffness of the polymer backbone are crucial for a satisfactory quantitative description of the properties of HA brushes. Therefore, and in contrast to earlier work by others and us on the compression of planar polymer or polyelectrolyte brushes, we consider both parameters explicitly in our theoretical models. From fits to the experimental data, we can extract a refined value of the persistence length and estimate the intrinsic excluded volume of HA.

**Materials and Methods**

**Substrate Preparation.** Glass coverslips (1.0, 24 × 24 mm²; Menzel-Glaser, Thermo Scientific, Germany) were cleaned by rubbing with a lint-free tissue paper and immersion in freshly prepared piranha solution (3:1 (v/v) mixture of concentrated H₂SO₄ and 50% H₂O₂) for 1 h, rinsed thoroughly with ultrapure water and gently blow-dried with a stream of N₂ gas. Substrates were stored in sealed Petri dishes. Prior to use, the glass coverslips were treated with UV/ozone (UV/Ozone ProCleaner; Bioforce Nanoscience, Ames, IA, U.S.A.) for 30 min.

**Preparation of Colloidal AFM Probes.** Polystyrene microspheres of 25 ± 3 μm diameter (Polysciences, Eppelheim, Germany) were attached to tipless V-shaped Si₃N₄ cantilevers with a nominal spring constant of 0.06 N m⁻¹ (Veeco Probes, CA, U.S.A.), as described previously. The real cantilever spring constant was determined before attaching the microsphere using the thermal noise method.

**Preparation of the Liquid Chamber.** A glass coverslip was attached to a custom-made holder using a two-component glue (Twinsil; Picodent, Wipperfurt, Germany), and the desired biomolecular film was prepared on the glass substrate. The holder was designed to accommodate an AFM liquid cell (SmallVolumeCell; JPK, Berlin, Germany) on the top and a light microscope objective on the bottom. The liquid cell was equipped with silicone tubings and a silicone rubber. All parts were cleaned and assembled to form a closed liquid cell, as described previously. Liquid was exchanged with a peristaltic pump (Ismatec, Glattbrugg, Switzerland) at flow rates of typically 0.5 mL/min.

**Preparation of Lipid Vesicles, Streptavidin, and Hylauronan.** Lyophilized dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine-CAP-biotin (DOPE-CAP-biotin; Avanti Polar Lipids, Alabaster, AL, U.S.A.) were dissolved in chloroform, mixed in a molar ratio of 9:1, dried, resuspended in buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 3 mM NaHCO₃), and homogenized, as described earlier. Small unilamellar vesicles (SUVs) of 2 mg/mL concentration were obtained by sonication, as described earlier and stored at 4°C. Lyophilized streptavidin (Sigma) was dissolved in ultrapure water at concentrations of 1 mg/mL and stored at −20°C.

Lyophilized hylauronan (HA), biotinylated at its reducing end and with a well-defined molecular weight of 1083 ± 53 kDa, was purchased from Hyalose (Select-HA B1000; Oklahoma City, OK, U.S.A.). HA was dissolved in ultrapure water at a concentration of 1 mg/mL and gently shaken for 2 h. The stock solution was aliquoted and stored at −20°C.

**Preparation of Films of End-Grafted HA.** HA films were prepared within a 250 μL droplet of buffer solution (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM NaHCO₃) on the glass coverslip, as described in detail previously. Briefly, a supported lipid bilayer (SLB) was formed from biotinylated vesicles by the method of vesicle spreading. Second, streptavidin was incubated at 20 μg/mL for 30 min to form a protein monolayer on the SLB. Subsequent incubation of biotinylated HA at 20 μg/mL for 8 h eventually led to the assembly of a grafted HA film.

**Combination of Colloidal Probe AFM and RICM.** We used a NanoWizard II with TAO module (JPK), installed on an inverted optical microscope (Axio Observer D1; Zeiss, Oberkochen, Germany) to combine colloidal probe atomic force microscopy (AFM) with reflection interference contrast microscopy (RICM), as described previously. AFM force curves were acquired in closed-loop mode at an approach speed of v₀ = 100 nm/s. RICM interferographs were acquired simultaneously at two different wavelengths, 438 and 543 nm, using exposure times of typically 100 ms.

Custom-developed algorithms implemented in Matlab were used to quantify the absolute distance between the colloidal probe and the glass substrate from the RICM images. The method, which is described in detail elsewhere, is based on the analysis of radially averaged intensity profiles with a simple optical model, the parallel plate approximation with incident light parallel to the surface normal. The correlation of multiple solutions generated by this model provides...
RESULTS

Experimental Data. HA was grafted to a streptavidin-coated glass-supported lipid bilayer via a biotin anchor at one of the ends of the HA molecules. The formation of the HA brushes proceeded in 150 mM NaCl. Compression forces as a function of absolute distance were measured with a setup that combines colloidal probe AFM and RICM in situ. The force-distance curve in Figure 1A, acquired in the presence of 100 mM NaCl, is representative in its qualitative features for all acquired force curves. First, the interaction was purely repulsive. Only in rare cases did we observe minor adhesion forces upon retraction (data not shown), indicating that adhesion of HA to the polystyrene did not affect the force curves appreciably. This is not surprising, given that both HA and polystyrene are negatively charged. Second, the approach and retract curves superposed very well, indicating that compression was purely elastic. Third, the force curves upon repeated compression of the same spot and upon compression of different spots on the same sample were highly reproducible: variations between curves were on the order of the line thickness of the curve in Figure 1A.

To study the influence of ionic strength on the conformation and compressive response, the HA brush was first incubated in ultrapure water and force curves were then acquired in water with gradually increasing NaCl concentrations, between 0.05 and 500 mM. After each addition step, the system was left for 8–10 min to equilibrate in the new ionic strength environment. Figure 1B shows selected approach curves. The interaction range and the forces required to compress the film to a given thickness decreased gradually with increasing salt concentration. To assess reversibility of the HA film’s response to ionic strength, force curves were taken on the same spot by switching the solution from 500 mM NaCl to ultrapure water and vice versa. The force curves at 500 mM NaCl before and after exposure to ultrapure water were similar, indicating that the salt-dependent collapse and swelling of HA films was fully reversible (data not shown). In the following, we will analyze the ensemble of force curves in the context of polymer and polyelectrolyte brush theories.

Brush Thickness. To rationalize the response of HA brushes to compression, we start by examining the dependence of the interaction range on ionic strength. To quantify the interaction range \( L \) in a simple way, we determined the distance \( d \) at which the repulsive force increased beyond a threshold level of \( F_{th} = 50 \text{ pN} \). The choice of this threshold is somewhat arbitrary. It is dictated by the force resolution of our experimental data, that is, slightly above noise level. \( L \) is also a first measure for the thickness of the HA brush. With increasing salt concentration, \( L \) decreased monotonically (Figure 2). This is not unexpected: the hyaluronan chains are charged, and with increasing ionic strength, the electrostatic interactions are increasingly screened, that is, interchain repulsion decreases. Close inspection of the data reveals three regimes. At low ionic strength (\( \leq 0.5 \text{ mM NaCl} \)), the brush thickness is approximately constant. The measured thickness, about 2.5 \( \mu \text{m} \), was remarkably close to the contour length of HA (2.86 \( \pm 0.15 \mu \text{m} \)), indicating that at least a part of the chains became very strongly stretched. At higher ionic strength (\( \geq 1 \text{ mM NaCl} \)) the thickness decreased gradually. Up to 150 mM NaCl, the decrease in the log–log plot in Figure 2A is well-approximated by a straight line with slope \(-0.26 \) (not shown), that is, there is an apparent power-law dependence between brush thickness and ionic strength. Above 150 mM, the salt-dependence of thickness becomes progressively weaker.

\[ F/R = \frac{1}{R} = \text{constant} \]

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Qualitatively, these three regimes match with theoretical predictions for polyelectrolyte brushes. For brushes of sufficiently high charge and grafting density, essentially all counterions can be considered trapped inside the brush. At low added salt, the brush thickness is then determined by the balance between the osmotic pressure of the trapped counterions and the entropic losses upon stretching of the HA chains. In this “osmotic brush” regime, the brush thickness is virtually unaffected by ionic strength. Added salt will affect the brush thickness when the ionic strength of the solution becomes larger than the concentration of mobile counterions inside the brush. In this “salted brush” regime, the thickness decreases with increasing ionic strength, as the electrostatic interactions between chain segments become partially screened. Here, theory predicts a power law dependence on the external salt concentration with an exponent of 1/3 as long as intrinsic excluded volume interactions can be neglected. Toward high salt concentrations, when the electrostatic interactions become increasingly screened, the excluded volume of the polymer backbone will increasingly determine the brush behavior. A positive excluded volume leads to an increased thickness and toward very high salt concentrations, the “quasi-neutral brush” regime, a plateau would be expected. Even though this plateau is not attained experimentally, the progressive deviation from the power law between 150 and 500 mM NaCl (Figure 2) provides a first indication that HA exhibits a positive intrinsic excluded volume.

For a quantitative fit of the experimental results for the brush thickness and compression force profiles as a function of added salt concentration, we apply a mean-field theory of polyelectrolyte brushes. The approach is based on representing the (Helmholtz) free energy of the brush confined between two planar surfaces as

$$E(d) = E_{\text{iou}}(d) + E_{\text{ev}}(d) + E_{\text{elastic}}(d)$$  

where $E_{\text{iou}}$, $E_{\text{ev}}$, and $E_{\text{elastic}}$ are the contributions of ionic and excluded volume interactions and of the conformational entropy of the chains, respectively, and $d$ is the distance between the surfaces. Below, we use mostly an approximation that assumes equal and uniform stretching of the chains, implying a constant monomer density within the brush (“box profile”). However, in our discussion of the compression force profiles we shall also refer to results of the more advanced mean-field theory of polymer brushes and polyelectrolyte brushes.

Within the box-profile approximation, the brush is characterized by its thickness $d$ or, equivalently, by the concentration $c = l_c/(b^2d)$ of monomer units in the brush. Here, $l_c$ and $b$ are the contour length and the monomer unit length, respectively. $s$ is the characteristic spacing between adjacent grafting points, and $\nu$ is the surface area per chain. The thickness $L$ of the free (uncompressed) brush is found from minimization $\partial E(d)/\partial d |_{L_s} = 0$ of the free energy given in eq 1. The box-profile approximation for polyelectrolyte brushes is usually coupled to the local electroneutrality approximation (LEA), introduced in ref 30. The LEA assumes that the charge density created by monomer units of grafted polyelectrolyte chains is locally compensated by the (excess) concentration of mobile counterions. It is assumed that the brush is in equilibrium with the reservoir of solvent with fixed concentration $c_0$ of monovalent salt ($2c_0$ is the number of positively and negatively charged ions per unit volume).

The ionic contribution to the free energy (per unit surface area) is presented as

$$E_{\text{iou}}(d)/kT = \frac{a_l}{bs^2} \left( 1 - \sqrt{1 + \frac{a_l}{2c_0}} \right) + \text{arcsinh} \frac{a_l}{2c_0}$$  

with $\alpha$ the fractional charge per monomer unit length. $kT = 4.1 \times 10^{-21}$ J is the thermal energy (at 25 °C). The free energy of excluded volume interactions is presented as:

$$E_{\text{ev}}(d)/kT = \frac{l_c}{bs^2} \nu c$$  

if we consider binary interactions but neglect higher order interactions. $\nu = A_v b^3$ is the “bare” excluded volume (second virial coefficient) of a monomer unit. $A_v$ is a numerical prefactor of order unity that depends on the monomer unit shape and the solvent quality. Gaussian chain elasticity,

$$E_{\text{elastic}}(d)/kT \approx 3d^2/(2l_p b^2)$$

provides a good approximation for the elastic contribution at moderate stretching. Here, the ratio $p = a/b \geq 1$ characterizes the intrinsic chain stiffness, or the anisotropy of the intrinsic statistical (Kuhn) segment of the uncharged chain, where $a$ is the segment length. Because our HA chains become close-to-fully stretched toward low ionic strength, finite chain extensibility needs to be considered. To this end, we follow an approach suggested by Chen et al. and use the

Figure 2. Brush thickness as function of bulk NaCl concentration. HA brush thicknesses $L$ were obtained from the force–distance curve in Figure 1B and additional curves on the same HA film (not shown), using a force threshold of $F_{th} = 50$ pN (black lozenges). Error bars relate to noise of the data. The solid line is a fit with eq 3A for $s = 57$ nm and $p = 14$, yielding $A_1 = 1.1$ and $A_2b_0 = 1.8$ nm$^3$. The dashed line was simulated using the same parameters but assuming Gaussian chain extensibility (eq 3B). The dotted line was simulated using eq 3A with $\nu = 0$. Finite chain extensibility and intrinsic excluded volume significantly affect the data at low and high salt concentrations, respectively. The best fit with eq 3B (gray dash-dotted line, obtained with $A_1 = 0.2$ and $A_2b_0 = 2.6$ nm$^3$) clearly cannot reproduce the experimental data.
elastic term in the form of a finitely extendible nonlinear elastic (FENE) potential, which we chose as

\[ E_{\text{elastic}}(d)/kT = -3l_r/(2pbs^2) \times \ln[1 - (d/l_r)^2] \]  

(2D)

At weak chain extension \((d \ll l_r)\) it is equivalent to eq 2C, and its derivative describes linear Hook’s law for elastic chain elongation. However, the elastic term given by eq 2D and its derivative diverge at \(d \to l_r\). Hence, the corresponding elastic force tends to infinity and prevents chain stretching beyond the contour length. We remark that eq 2D, as well as a similar expression used by Chen et al., \(^{33}\) \(E_{\text{elastic}}(d)/kT = -l_r/(pbs^2) \times [(d/l_r)^2/2 - \ln(1 - (d/l_r)^2)]\) capture the effect of finite extensibility, but lead to a stronger increase in the elastic force upon approaching the \(d \to l_r\) limit, \(\partial E_{\text{elastic}}/\partial d \propto (1 - d/l_r)^{-1}\), than rigorous treatments in the freely jointed chain (where \(\partial E_{\text{elastic}}/\partial d \propto L^{-1}(d/l_r)\), with the inverse Langevin function \(L^{-1}(x)\)) or the worm-like chain models.

Minimization of the free energy defined by eqs 1 and 2A, 2B, and 2D leads to an expression for the thickness \(L\) of the uncompressed brush

\[
\left\{2A_1\epsilon^c \left[1 + \left(\frac{\alpha c}{2c_i}\right)^2 - 1\right] + A_2\nu c^2 - \frac{3}{pbs^2} \frac{d}{l_r^2}\right\}_{d=L} = 0
\]

(3A)
in which we have also introduced two numerical prefactors, \(A_1\) and \(A_2\). At this point, it is worth reminding that the local electroneutrality and box-profile approximations enable reproducing correctly the trends in terms of the dependence of brush thickness on brush architectural parameters and salt concentration. However, the internal structure of the brush (i.e., the distribution of the polymer density) as well as the distribution of co- and counterions between the interior of the brush and the adjacent solution are not taken properly into account. Therefore, one should not expect that these approximations provide an exact quantitative description of any experimentally measurable brush thickness. The empirical adjustable parameters \(A_1\) and \(A_2\) were introduced to account for these limitations. They ensure matching of the measured brush thickness at high \((A_1)\) and at low \((A_2)\) salt concentrations, when the brush swells predominantly due to excluded volume repulsions or osmotic pressure of counterions, respectively. A similar approach has been taken by Chen et al. \(^{33}\) for fitting results from molecular dynamics simulations, with the difference that \(\nu_0\) was used as an adjustable parameter instead of \(A_1\).

For the HA brushes, we employed the following values. \(b = 1.0\) nm is the length of a disaccharide unit. \(^4\) \(l_r = 2860 \pm 150\) nm can be calculated from the polymer molecular weight \((1083 \pm 53\) kDa) and the length and molecular weight \((378\) Da) of the disaccharide repeat unit. Each disaccharide has one chargeable group, a carboxyl group on the glucuronic acid, which is likely to be fully ionized under the experimental conditions. Counterion condensation \(^{44}\) is unlikely because the distance between chargeable groups is larger than the Bjerrum length \((0.7\) nm in aqueous solution), that is, \(\alpha = 1\). \(p = a/b\) is expected to lie between 8 and 16, based on the equivalence of \(a\) in the freely jointed chain and \(2l_r\) in the worm-like chain models, and reported persistence lengths \(l_p\) between 4 and 8 nm. \(^{15-18}\) In earlier work, \(^{12}\) we had estimated \(s \approx 56\) nm by reflectometry.

Based on results that will be exposed further below, we fix \(p = 14\) and \(s = 57\) nm. This leaves the product \(A_1\nu_0\) and \(A_2\) as the only fitting parameters. We obtained an excellent fit over the entire range of ionic strengths (Figure 2, solid line), with \(A_1\nu_0 = 1.8\) nm\(^3\) and \(A_2 = 1.1\). To illustrate the impact of finite chain extensibility and excluded volume interactions, two simulated curves that neglect either one effect or the other were included in Figure 2 (dashed and dotted line, respectively). For Gaussian chain elasticity, eq 3A simplifies to

\[
2A_1\epsilon^c \left[1 + \left(\frac{\alpha c}{2c_i}\right)^2 - 1\right] + A_2\nu c^2 - \frac{3}{pbs^2} \frac{d}{l_r^2} = 0
\]

(3B)

Excluded volume interactions were neglected by setting \(\nu_0 = 0\) in eq 3A. The effect of finite chain extensibility is noticeable below 5 mM NaCl or above 50% stretching. Above 30 mM NaCl, on the other hand, excluded volume interactions have an appreciable impact on the brush thickness. Comparison of simulated and fitted curves suggests that the apparent power law behavior with exponent \(-0.26\) that we observed experimentally is the result of the gradual increasing impact of finite chain extensibility toward low ionic strength and excluded volume interactions toward high ionic strength.

To assess the discriminative power of the fit method, we did also attempt to fit the experimental data with eq 3B. In this case, no satisfactory fit could be obtained: theory and experiment could be matched at the lowest and highest salt concentrations, by adjusting \(A_1\nu_0\) to 2.6 nm\(^3\) and \(A_2\) to 0.2, but the theory then underestimated the thickness at intermediate salt concentrations by up to 35% (Figure 2, gray dash-dotted line). It is noticeable that the transition to the osmotic brush plateau is sharper when finite chain extensibility is taken into account. With \(L = 2.5\) μm and \(s = 57\) nm, the counterion concentration inside the brush would be 0.5 mM. This number is in good agreement with the salt concentration at which the osmotic brush plateau is seen to start experimentally and in the fit with eq 3A (but not eq 3B). The remarkable quantitative agreement between experiment and theory therefore provides strong evidence that finite chain stretching and excluded volume repulsion are important determinants in the behavior of HA brushes.

**Compression Force Profiles.** For a brush on a planar surface, the compression force \(F(d)/R\) by a sphere of radius \(R \gg L\) is related to the energy of compression \(E(d) = E(L)\) by a plane via the so-called Derjaguin approximation:\(^{21}\)

\[
F(d)/R = 2\pi\left[E(d) - E(L)\right]
\]

(4)

**Forces Profiles at High Ionic Strength (Salted Brush).** At high salt concentrations \((ac \ll c)\), eq 2A reduces to \(E_{\text{os}}(d)/kT = l_r/(bs^2)\alpha^2/(4c_i)c\). Consequently, \(E_{\text{os}}(d) + E_{\text{ev}}(d)/kT = l_r/(bs^2)\) \((\nu_0 + \alpha^2/(4c_i)c)\), and one can introduce the “effective” excluded volume \(\nu = \nu_0 + \alpha^2/(4c_i)c\), which accounts (within the accuracy of the mean-field approximation) for superposition of the “bare” excluded volume interactions and partially screened Coulomb repulsion between the charges on the HA chains. It follows that

\[
\nu^2 = \nu_0^2 + \alpha^2/(4c_i)^2 = \alpha^2 \left(A_0 b + \alpha^2/(4b_c c_i)\right)
\]

(5)

Furthermore, the chains are only moderately stretched at high ionic strength (Figure 2), and elastic stretching is hence well-described by Gaussian chain elasticity (eq 2C). With the simple
box-profile (BP) approximation, we obtain from eqs 1, 2B, and 2C:

\[ F = \frac{C_{\text{MFT-BP}}}{R} \left[ \frac{L}{d} + \left( \frac{d}{L} \right)^2 - \frac{3}{2} \right] \]

with

\[ C_{\text{MFT-BP}} = 6 \pi \frac{L^2}{4} \frac{\text{MFT-BP} kT}{\text{bps}^2 l_c} \]  

(6A)

and

\[ L_{\text{MFT-BP}} = \left( \frac{1}{3} \right)^{1/3} \frac{\text{MFT-BP}}{\text{bps}^2 l_c} \]  

(6B)

More advanced self-consistent mean-field theory for neutral brushes predicts a monomer density profile that, in good solvent, has the form of an inverted parabola with its maximum at the grafting surface ("parabolic profile", PP). This approach, developed independently by Zhulina et al.\(^{41}\) and by Milner et al.\(^{38,39}\) gives

\[ F = \frac{C_{\text{MFT-PP}}}{R} \left[ \frac{L}{d} + \left( \frac{d}{L} \right)^2 - \frac{1}{5} \left( \frac{d}{L} \right)^5 - \frac{9}{5} \right] \]

with

\[ C_{\text{MFT-PP}} = \frac{\pi^2}{4} \frac{L^2}{4} \frac{\text{MFT-PP} kT}{\text{bps}^2 l_c} \]  

(7A)

and

\[ L_{\text{MFT-PP}} = \left( \frac{8}{\pi^2} \right)^{1/3} \frac{\text{MFT-PP}}{\text{bps}^2 l_c} \]  

(7B)

From the above equations, it is clear that the uncompressed brush thicknesses in the MFT-PP and MFT-BP models are not identical, but related by \( L_{\text{MFT-PP}} = 1.34 L_{\text{MFT-BP}} \).

Another approximation for neutral brushes, based on scaling theory (ST) and historically the first approach, is the one by Alexander and de Gennes.\(^{45}\) This model also assumes a box profile. The contributions to the free energy are \( E_{\text{os}}(d)/kT \approx 4/5(s - 1)/b(pv/b)^{3/2}(ch^3)^{1/4} \) and \( E_{\text{cl,os}}(d)/kT \approx 4/7s^{-2}(l_c/b)^{-1}(d/b)^{3/2}(ch^3/pv)^{1/4} \), and \(^{21,28}\)

\[ F = \frac{C_{\text{ST}}}{R} \left[ \frac{7}{5} \frac{L}{d} + S \left( \frac{d}{L} \right)^{7/4} - 12 \right] \]

with

\[ C_{\text{ST}} = A_F \frac{L_{\text{ST}} kT}{s} \]  

and

\[ L_{\text{ST}} = A_s \frac{pv}{b} \]  

(8A)

\[ A_F \] and \( A_s \) are numerical prefactors of order unity that are a priori unknown, that is, they cannot be determined by the theory alone.\(^{73}\) Eq 8A is similar to 6A, though the exponents of the power law dependences of different terms on the ratio \( d/L \) are slightly different. This is because eq 8A accounts for local correlations in polymer density inside the brush, which are essential under good solvent conditions for flexible chains, but are disregarded by the mean field theories. The mean-field approximation, however, applies at marginally good solvent for semiflexible chains.\(^{46}\)

The full self-consistent field Poisson–Boltzmann theory describing the structure of a polyelectrolyte brush at arbitrary salt concentration without any preassumption concerning ion distribution was developed in ref 32. However, this theory does not allow for a closed analytical expression for the free energy of the compressed polyelectrolyte brush (in the presence of salt). Therefore, for fitting the experimental results, we used expressions obtained for a neutral brush under good solvent conditions, replacing the excluded volume parameter by the effective salt-dependent second virial coefficient, eq 5. This approach is expected to be accurate at high salt concentrations, when the "electrostatic excluded volume" becomes comparable to (or smaller than) the bare excluded volume. However, we expect that its extrapolation to moderate salt concentrations does not lead to major quantitative discrepancies.

To compare experiment and theory, we fitted our force curves with eqs 7A and 8A over the range of weak to moderate compressions. The approximation of ionic interactions by an effective excluded volume is expected to become increasingly inaccurate with increasing compression and decreasing ionic strength. Data below \( d/L = 0.5 \) (with \( L \) determined through the force threshold \( F_{\text{th}} \)) and force curves at ionic strengths \( < 1 \text{mM} \) NaCl were therefore not included in the fit. Experimental approach curves at all tested ionic strengths \( \geq 1 \text{mM} \) NaCl with the fits are displayed in Figure 3. To facilitate comparison, \( F/R \) was normalized by the prefactors, \( C_{\text{MFT-PP}} \) and \( C_{\text{ST}} \), respectively, and \( d \) was normalized by \( L_{\text{MFT-PP}} \) and \( L_{\text{ST}} \), respectively. The normalized force curves revealed a curve shape independent of ionic strength. The MFT-PP model fitted the data at weak and moderate compression very well, but systematically underestimated the data at strong compression. The ST model fitted the data at moderate compression well, but systematically underestimated the data at weak and strong compression. To illustrate the importance of the parabolic density profile in the mean-field theory, an additional curve that was simulated with eq 6 and the results from the fit to eq 7A is displayed as a dotted line in Figure 3A. The simulated MFT-BP curve agrees well with the fitted MFT-PP curve at strong compression, but deviates significantly at weak and moderate compressions. Also, we found the fits with the MFT-BP model to reproduce the experimental data less well than the MFT-PP or ST models (not shown) and, therefore, do not further consider the MFT-PP model.\(^{72}\)

Figure 4A,B displays the results of the fits. With \( l_c \) being known, the parameters \( L_{\text{MFT-PP}} \) and \( \text{bps}^2 \) could be extracted via eq 7A and \( L_{\text{ST}} \) and \( s^3/A_F \) via eq 8A. The brush thicknesses (Figure 4A) determined with the MFT-PP model were typically about 20% larger than the values extracted via \( F_{\text{th}} = 50 \text{ pN} \), suggesting that forces as low as 50 pN do already induce appreciable compression. This is also readily apparent in Figure 3A, where the range of the experimental data is essentially confined to \( d < 0.85 L_{\text{MFT-PP}} \). The agreement between the values derived from the ST model and via \( F_{\text{th}} \), respectively, was excellent. This is likely to be coincidental, given that the choice of \( F_{\text{th}} \) is arbitrary. Notably, the ratio between \( L_{\text{MFT-PP}} \) and \( L_{\text{ST}} \) did not depend on ionic strength, indicating that \( A_s \) is indeed constant as expected. With \( L_{\text{MFT-PP}}/L_{\text{ST}} = 1.20 \pm 0.01 \), and by comparing eqs 7B and 8B, we find \( A_L = 0.78 \), which is close to unity as expected.

\( \text{bps}^2 \) and \( s^3/A_F \) (Figure 4B; cubic roots of both values are displayed to facilitate operation with numbers of unit length) scattered appreciably, but exhibited only a weak (if any) systematic dependence on ionic strength. Within the limits of validity of the effective excluded volume approximation, this is indeed expected because both \( p, b, s \), and \( A_F \) are constant in the experiment.
Figure 4C displays (the cubic root of) \( p^2v \), which was determined via eq 7B (MFT-PP model; A) and 8A (ST model; B) over the range 0.5 < \( d/L \) < 1 (with \( L \) estimated through the force threshold \( F_{th} \)). Distances \( d \) were normalized by the brush thicknesses \( L_{MFT-PP} \) and \( L_{ST} \), and forces \( F/R \) were normalized by the prefactors \( C_{MFT-PP} \) and \( C_{ST} \) respectively, as obtained from the fitting of each individual force-distance curve. Symbols represent experimental data >50 pN at different salt concentrations (listed in B); solid black lines are extrapolated fits. In these normalized presentations, all experimental data fall on a single curve. At low and medium compression, the MFT-PP model fits the data very well; while the ST model shows systematic deviations at low compression (\( d/L > 0.9 \)). Both models underestimate the data at high compression (\( d/L < 0.4 \)). The dotted line in A is simulated data, based on eq 6A (MFT-BP model) and the results of the MFT-PP model.

Figure 4. Parameters extracted from the fits of theoretical models to salted and osmotic HA brushes. (A) Film thickness, obtained with the ST (green circles) and MFT-PP (red squares) models for salted brushes (SB), and with the MFT-BP (blue triangles) and MFT-GP (orange triangles) models for osmotic brushes (OB). Confidence intervals were smaller than the symbol size. Brush thicknesses obtained via \( F_{th} = 50 \) pN (black lozenges, from Figure 2) are shown for comparison. (B) Cube roots of \( s^3/A_F \) (green circles) and \( pbe^2 \) (red squares, blue triangles), extracted through the three different models, respectively. Mean values (solid lines) were determined from data at \( \geq 50 \) mM NaCl for salted brushes and \( \leq 0.5 \) mM NaCl for osmotic brushes (fitting range indicated by thick solid lines). (C) Cube root of \( p^2v \) (red squares), extracted with the MFT-PP model for salted brushes. Equation 5 was fitted to data at \( \geq 50 \) mM NaCl (thick solid line) and extrapolated (thin solid line), giving \( p = 13.9 \) nm and \( A_{v_0} = 0.9 \). Extrapolated fits with \( A_{v_0} \) fixed to 0 and 2 (dotted lines) give \( p = 15.0 \) and 12.8, respectively.
reported in the literature. Molecular dynamics simulations predict a cross-section of about 1.0 nm for the HA chain, which happens to coincide with the HA disaccharide contour length \( b \). The result for \( A_{v_0} \) is very close to unity and hence consistent with aqueous solution being a good solvent for HA. We note, however, that the uncertainty on \( A_{v_0} \) is rather large, as further illustrated by fits in which \( A_{v_0} \) was fixed to 0 and 2 (Figure 4C, dotted lines). Equation 5 could reproduce the experimental data reasonably well down to at least 3 mM NaCl (Figure 4C, thin solid line), confirming that the treatment of ionic interactions as an effective excluded volume provides reasonable results even at rather low ionic strengths.

From the above estimates for \( a \) and \( b \), we find \( p = 13.9 \) and \( v_0 = A_{v_0}b^3 \approx 0.9 \) nm\(^3\). The mean value of \((pbs^2)^{1/3}\) for \( \geq 50 \) mM NaCl was 36 nm (Figure 4B), leading to \( s = 57 \) nm. This value is in good agreement with results from independent analysis of the surface density and permeability of HA films.2 We recall that the mean value of \( s/A_{v_0}^{1/3} \) for \( \geq 50 \) mM NaCl was 49 nm (Figure 4B). Based on the estimate of \( s \approx 57 \) nm, we would obtain \( A_{v_0} \approx 1.6 \), which is again on the order of unity, as expected.

Taken together, self-consistent mean-field theory and scaling theory could reproduce the experiment well. The quality of the fits, and the extracted quantitative parameters suggest that grafted HA films can be treated as polymer brushes, in which ionic interactions are treated as an added excluded volume, over a large range of ionic strengths.

**Force Profiles at Low Salt Concentrations (Osmotic Brush).** In the limit of low salt concentrations, we can neglect excluded volume interactions of the polymer backbone. In a first approximation, we assume a box profile, local electroneutrality and Gaussian chain elasticity. For \( \alpha \gg 1 \) eq 2A simplifies to \( E_{osm}(d)/kT = \alpha l/(bs^2) \ln(d/L) \). With eqs 1, 2C, and 4, we obtain

\[
\frac{F}{R} = C_{MFT-\text{BP}} \left[ \frac{1}{2} \left( \frac{d}{L} \right)^2 - \ln \left( \frac{d}{L} \right) - \frac{1}{2} \right] \quad \text{with}
\]

\[
C_{MFT-\text{BP}} = 6\pi \frac{L_{MFT-\text{BP}}^2 kT}{pbs^2 l_c^2} \tag{9A}
\]

and

\[
L_{MFT-\text{BP}} = \left( \frac{ap}{3} \right)^{1/2} l_c \tag{9B}
\]

We fitted force curves at all tested ionic strengths \( \leq 0.5 \) mM NaCl to eq 9A over the range \( 0.5 < d/L < 1.0 \) (with \( L \) estimated through the force threshold \( F_{th} \)). Forces \( F/R \) were normalized by the prefactors \( C_{MFT-\text{BP}} \) and \( C_{MFT-\text{GP}} \), respectively, as obtained from the fitting of each individual force–distance curve. Distances \( d \) were normalized by the brush thicknesses \( L_{MFT-\text{BP}} \) in (A), and by the decay length \( H \) in (B). Symbols represent experimental data >50 pN at different NaCl concentrations, as listed; the black solid line in (A) represents the extrapolated fit for all data sets, while the colored solid lines in (B) are extrapolated fits to individual data sets.
As follows from eq 10B, the chains may reach the limit of the extension (d → L) provided \(ap \gg 1\), that is, for strongly charged (\(P \approx 1\)) and intrinsically rigid (\(p \ll 1\)) chains. In contrast, for weakly charged and relatively flexible chains (\(ap \ll 3\)), the well-known power-law dependence (eq 9B) is recovered. Equation 10A generates a steeper increase in the forces at low compression than eq 9A. Consequently, the fit with eq 10A was always worse (not shown). Clearly, the rather weak increase in the forces at low compression that we observe experimentally cannot be explained by finite chain extensibility.

Alternatively, the weak force increase may result from a nonconstant monomer density profile. The box profile assumption can be relaxed in the framework of self-consistent mean-field theory. Assuming again local electroneutrality and Gaussian chain elasticity, a Gaussian density profile would be expected for osmotic brushes (MFT-GP). In this case, the compression force can be derived from eq 15 in ref 30 and eq 4

\[
\frac{F}{R} = C_{\text{MFT-GP}} \ln \left[ \frac{\text{erf}(L_{\text{MFT-GP}}/H)}{\text{erf}(d/H)} \right] \quad \text{with}
\]

\[
C_{\text{MFT-GP}} = \frac{2\pi}{\alpha kT} \frac{a}{bs^2}
\]

where \(\text{erf}(\xi) = 2/\sqrt{\pi} \int_{0}^{\xi} \exp(-x^2) \, dx\) is the error function. The decay length of the Gaussian profile \(H\) (which should not be confused with the brush thickness) is given by

\[
H = \left( \frac{8}{3\pi^2} \right)^{1/2} \left( ap \right)^{1/2} l
\]

In order to derive eq 11A, we have assumed that a cutoff distance \(L_{\text{MFT-GP}}\) exists at which \(\partial E(d)/\partial d|_{d=L} = 0\). \(L_{\text{MFT-GP}}\) is hence an empirical measure for the uncompressed brush thickness. Using \(s\), \(L\), and \(H\) as adjustable parameters, this approach significantly improved the fit (Figure 5B) compared to the box-profile model (Figure 5A), even though the theory still underestimated the forces at very weak compression. \(L_{\text{MFT-GP}}\) agreed well with the brush thickness determined via \(F_{\text{th}}\) (Figure 4A). In contrast, \(s\) was approximately 2-fold larger than expected (mean 107 nm). By comparing eqs 9A and 9B with eq 11A, it can easily be seen that \(C_{\text{MFT-GP}} = C_{\text{MFT-BP}}\) and that an error of comparable magnitude would occur when deriving \(s\) through eq 9A using \(C_{\text{MFT-BP}} = \frac{2\pi \alpha kT}{bs^2}\). The overestimation of \(s\) is hence an indirect consequence of the underestimation of \(p\), due to the neglect of finite chain extensibility. \(H\) was 2.0 ± 0.1 fold smaller than \(L_{\text{MFT-GP}}\). \(H\) was also 1.9-fold smaller than \(L_{\text{MFT-BP}}\), that is, 1.7-fold larger than what would be expected from the comparison of eqs 9B and 11B. We suspect that this discrepancy may also originate from ignoring finite chain extensibility in our model.

Taken together, the experimental data are overall consistent with the behavior expected for the osmotic brush regime. The best fit is obtained with the MFT-BP model, yet \(s\) remains overestimated in this case possibly because of the neglect of finite chain extensibility. A more realistic self-consistent model that accounts for finite chain extensibility is likely to lead to results that cannot be expressed by a simple analytical expression, and is outside the scope of this work. In the light of the results with eqs 10 and 11, the reasonably good quality of the fit with the MFT-BP model (eq 9) appears to be coincidental, that is, the result of a fortunate cancellation of errors due to the Gaussian chain elasticity approximation on the one hand and the box profile approximation on the other.

**DISCUSSION**

Employing a recently developed method that combines AFM and RICM, we have systematically quantified the thickness and the compressive response of HA brushes over a broad range of NaCl concentrations. With regard to polymer physics, our HA brushes are interesting as a well-defined model system for end-grafted polyelectrolyte chains. The molecular weight distribution of HA is narrow, and no bulky groups for attachment to the surface are required, such as it is the case for diblock copolymers. Essentially, HA interacts exclusively via a biotin moity at its reducing end with the surface. The grafting-to-approach produces surface densities that are large enough to induce chain stretching into the brush regime, even at high ionic strengths.

Our study provides evidence that grafted HA films behave very much like brushes of strong polyelectrolytes with an intrinsically stiff backbone (i.e., \(P \gg 1\)). Strong polyelectrolyte brushes that have so far been reported in the literature were predominantly made of a few different molecules: poly(styrene sulfonate) (PSS),\(^{20,50–53}\) poly(vinyl methyl pyridinium) (PVP),\(^{54}\) and double-stranded DNA.\(^{35,56}\) When compared to these brushes, HA brushes reveal particular features.

At high salt concentrations, HA films show an attenuation in the dependence of thickness on ionic strength (Figure 2). The excellent fit with eq 3A to the brush thickness data provides strong evidence that intrinsic interchain repulsion (i.e., positive excluded volume, \(v_0 > 0\)) is responsible for this behavior. Such a behavior is not commonly observed for water-soluble synthetic polyelectrolytes such as PSS or PVP: they typically display interchain attraction once charges are sufficiently screened. Several earlier reports\(^{57,58}\) had proposed HA self-association through interchain hydrophobic interactions and hydrogen bonds as a determinant of HA behavior, but this view has been challenged by more recent solution-based studies\(^{59,60}\) (see ref 61 for a detailed discussion). In this regard, our study provides further and independent experimental evidence that attractive interchain interactions do not play an important role in the physicochemical properties of polymeric HA assemblies.

At low ionic strength, on the other hand, HA becomes very strongly stretched: \(L/L_s\) reached values up to 0.87, which is larger than the largest values that we found in the literature; Balastre et al.\(^{20}\) reported \(L/L_s \leq 0.8\) for PSS brushes. Equation 10B can rationalize why this is. Relative chain stretching increases monotonously with \(ap = aa/b\). The statistical segment length \(a\) of HA (14 nm) exceeds that of PSS (0.25 nm) by more than 50-fold. In addition, the fraction of charged monomers \(a\) is likely to be unity in HA, while values of PSS are about 3-fold lower, due to incomplete sulfonation and counterion condensation.\(^{34}\) The latter gain is though offset by the fact that the disaccharide repeat unit in HA is about 4-fold larger than the monomers in PSS. Hence, the main reason for the stretching of HA is its intrinsic stiffness (\(P \approx 1\)), while the charges are distributed such that charge density is maximal yet counterion condensation is avoided. From theoretical considerations, we can expect that the grafting density affects the degree of stretching in the osmotic brush regime only weakly, although we have not tested this experimentally. Even though the intrinsic stiffness of HA is considerable, it should be pointed out that the statistical segment length \(a = 14\) nm (or the
corresponding persistence length $l_p = 7$ nm) remains by several orders of magnitude below the contour length. Therefore, mechanical effects of chain bending under confinement, which may dominate the force response for even stiffer chains, such as double-stranded DNA, remain negligible.

The overall quality of the fits to eqs 7, 8, 9, and 11 confirms that the brush compression data can be reasonably well approximated by simple scaling and mean-field models. Self-consistent mean-field theories reproduced the data best, for both salted (MFT-PP) and osmotic (MFT-GP) brushes. Since these models account for a distribution of the chain ends in the brush, it is not surprising that they captured the soft initial repulsion particularly well. For salted brushes, scaling theory (ST) reproduced the data better than the simple mean-field theory with constant density profile (MFT-BP).

We recall, however, that none of the models should be expected to reproduce the data perfectly. First, the applied theoretical approaches neglect Gaussian fluctuations of the free chain ends. Such fluctuations would be expected to lead to an onset of repulsion at larger distances. Second, for a polyelectrolyte brush, the assumption of all counterions being confined to the brush is an approximation. Residual counterions above the brush, and the cloud of counterions from the colloidal probe surface, will produce another soft repulsion at $d \gtrsim L$. Third, the polydispersity of the polyelectrolyte chains, even if it is minor in our case, is likely to have a sizable effect on the distribution of chain ends and might, hence, also affect the forces at low compression. In the case of salted brushes, the interaction distances measured at $F_{th} = 50$ pN were already about 20% smaller than the brush thicknesses determined with the self-consistent mean-field approach (Figure 4A). We therefore speculate that these subtle effects are too small to be picked up with the current force resolution of our experimental setup. It is also likely that the ST model reproduced the force profiles better than the MFT-BP model, because it has a softer profile at weak compression. For osmotic brushes, the counterion-cloud between the brush and the charged sphere, as well as the effect of polydispersity, would be longer ranged and more pronounced due to the lack of screening by additional salt. This could explain the weaker rise in the force at low compression than predicted by our theoretical models. We note, however, that the assumption of local electroneutrality is a very good approximation for our experimental system: according to ref 42, the LEA is valid if $H/\Lambda \gg 1$, with $\Lambda = b^2/(2\pi \sigma_0 a L)$ being the Guy-Chapman length of an equivalent surface that carries all the negative charges of the HA brush; from $H = 1.2 \mu m$ and $s = 57$ nm, we find $H/\Lambda = 4700$. Moreover, the surface density of counterions outside the brush can be estimated by $Q_{outside} = 3\pi L/(16\sigma^2 L d^2)$. With $L_{MFT-GP} = 2.4 \mu m$, we find a very small value of one charge per 4000 nm$^2$. This value is likely to be several orders of magnitude below the surface charge density of the colloidal probe surface, suggesting that the counterion cloud between brush and probe will be dominated by counterions from the probe. Last, but not least, as the film is compressed, the local monomer density increases, and higher-order interactions would be expected to increasingly affect the forces. Such interactions could explain why our theoretical models underestimated the forces above 50% compression systematically. Under good solvent conditions, ternary (and higher order) interactions typically contribute to a similar extent as binary interactions to the free energy, when the volume fraction of polymers inside the brush reaches a few tens of percent. In our experiments, the HA volume fraction did not exceed 1%, and the effect of higher order interactions should hence remain small.

The elastic response of pure HA brushes to compressive forces over a wide range of ionic strengths indicates that the chains can rearrange rapidly, within seconds, in the direction normal to the surface, and readily regain their original conformation if external stress is released. The longest segmental relaxation time in the system pertains to the relaxation of meshwork strands. To first approximations, the mesh size in the HA brush equals the characteristic spacing between grafting points, and the meshwork strand relaxation time is $\tau_s \approx \eta s^3/(kT)$. With the viscosity of water $\eta = 0.9 \mu Pa s$ and $s = 57$ nm, we find $\tau_s \approx 41 \mu s$, that is, segmental relaxations are indeed much faster than the experiment, consistent with the elastic response. At the same time, concomitant liquid flow in and out of the compressed area must occur with relative ease, that is, without significant dissipative losses. The hydrodynamic forces due to liquid drainage can be estimated as $F_{hl} \approx R^2 \eta s^{5/2} s^{-3} v_s d^{-3/2}$ (eq 2.13 in ref 67). With an approach speed of $v_s = 100$ nm/s and smallest approach distances of $d_{min} = 0.1 \mu m$ in our assays, the maximal force would be $F_{hl} \approx 16$ pN, which is indeed close to the detection limit. On the other hand, we note that the purely elastic response, and the good agreement of our data with polymer and polyelectrolyte brush models, suggest that lateral displacement of HA out of the compression zone is negligible in our case. This finding is not self-evident, given that the HA brushes were formed on fluid supported lipid bilayers, and that lateral displacement had previously been reported for films of HA molecules that were either physisorbed to surfaces or covalently bound to SLBs via multiple attachment points along the chain. A likely explanation is that the streptavidin molecules that anchor HA to the SLB are forming two-dimensional crystalline domains, thereby rendering HA laterally immobile. In addition, the approach speed in our study was rather high, compared to previous studies with the surface force apparatus. Future studies that systematically vary the grafting density, the molecular weight, and the lateral mobility of HA, and the approach speed should allow to further refine our understanding of the behavior of HA assemblies and the dynamics of reorganization of HA films under mechanical stimuli.

**CONCLUSIONS**

Pure HA films showed a pronounced salt-dependence in the uncompressed thickness that is characteristic of strong polyelectrolyte brushes. The brush thickness could be modeled quantitatively, if finite chain stretching, intrinsic chain stiffness ($p \gg 1$), and intrinsic excluded volume ($v_0 > 0$) interactions were taken into account. Simple theoretical polyelectrolyte brush models could reproduce the response upon brush compression well. From the biological perspective, the generated data represent a valuable reference for future quantitative studies of more complex HA-rich films, such as pericellular coats. The level of control in their preparation should also make HA brushes a useful experimental model system to further test and refine the theories of brushes of strongly charged and intrinsically stiff polyelectrolytes.

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Notes
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(71) The equivalent of eq 8A is often presented with a prefactor of $8\pi/35$, or $16\pi/35$ if both interacting surfaces are coated with a brush, in addition to an unknown numerical prefactor of order unity. For simplicity, and given that $8\pi/35 \approx 0.72$ is close to unity, we have lumped this prefactor into $A_E$. Also, it can be seen by comparing eqs 6B and 8B that the relationship between the unperturbed brush thicknesses in the MFT-PP and ST models cannot be quantitatively predicted from theory alone because $A_E$ is unknown. In the literature, a factor of $(24/\pi^2)^{1/3} \approx 1.34$ has repeatedly been proposed, presumably due to confusion between the MFT-BP and ST approaches.
(72) The quality of the fit improves when introducing additional numerical prefactors, analogous to what we did in eq 3A. This approach has been taken by others to fit polyelectrolyte brush compression data.