Microrheology of the pericellular matrix: gels, cells and organ: an optical tweezers study

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Microrheology of hyaluronan solutions: implications for the endothelial glycocalyx

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Abstract

The endothelial glycocalyx (EG) is a complex biopolymer network produced by vascular endothelial cells that forms a layer with multiple functions at the luminal side of blood vessels. The EG acts as an anti-adhesive protection layer, as a molecular sieve and chemical sensor site, and as a mechanotransducer of fluid shear stress to the underlying cell layer. A major component involved in these processes is the highly hydrated glycosaminoglycan (GAG) hyaluronan (HA). Here we used laser interferometry to measure the broad-band mechanical response of reconstituted HA solutions at close to physiological conditions. HA showed rheological behavior consistent with that of a flexible polymer. The elastic behavior observed for entangled HA networks showed reptational relaxation with a large distribution of time scales, which disappeared quickly (15 min) with the addition of hyaluronidase (HAase). We conclude that the broad-band mechanical probing of model systems (HA solutions) provides quantitative data that are crucial to understand the mechanical response of the EG in vivo and its role in mechanosensing.
Introduction

The luminal side of blood vessels is covered with a protective, chemically and size selective, and mechanosensing polymer layer, called the endothelial glycocalyx (EG) or endothelial surface layer [1, 2]. The EG forms a highly negatively charged hydrated network consisting of membrane-bound proteoglycans, glycoproteins, glycosaminoglycans (GAGs), and adsorbed plasma proteins. Damage of the EG triggers an increased adhesion of platelets [3] and leukocytes [4], leakage of fluid through the vessel wall [5], and causes a diminished sensitivity to shear stress (diminished NO-production by endothelial cells) [6, 7]. These multiple patho-physiological consequences of damage underline the important role of the EG for the proper functioning of the vasculature.

The three most prominent GAGs in the EG are heparan sulfate (HS), chondroitin sulfate (CS) and hyaluronan (HA). Both HS and CS are produced via the Golgi pathway, sulfated and attached to core proteins. HA, in contrast, is not sulfated and is produced at the inner side of the plasma membrane [8, 9] and extruded through the plasma membrane into the EG. It is constructed as a repeating structure of disaccharide units (D-glucuronic acid and N-acetyl-D-glucosamine) linked by $\beta$(1-3) and $\beta$(1-4) glycosidic bonds [10] (Figure 3.1). HA can far exceed the other GAGs in length, with a molecular weight ranging from $2 \times 10^5$ to $2 \times 10^6$ Da [11], compared to 20 kDa and 36 kDa for HS, and 20 kDa for CS [12]. It was found, by scattering experiments, to behave like a wormlike chain molecule with an intrinsic persistence length of ~ 80 Å [13]. HA is not only found in the EG, but can be produced by many other cell types. It plays an important physiological role in the extracellular matrix, synovial fluid and the vitreous humor of the eye [14].

Figure 3.1: Microrheology in an entangled HA solution. Sketch of the experimental geometry.
Endothelial cells express three vertebrate HA synth ases: HAS1, HAS2, and HAS3 [15, 16]. The molecular weight of a single HA chain seems to depend on the HAS involved in its synthesis, with HAS2 responsible for the largest chains (> 2x10^6 Da) [11]. Endothelial cells also express hyaluronidase (HAase) [17]. HAase randomly hydrolyzes the 1→4 linkages between N-acetyl-D-glucosamine and D-glucuronate, reducing the polymer size. Both HAase and plasma HA levels are elevated in type 1 diabetes patients, who show a reduction in glyocalyx volume compared to healthy volunteers [18]. It is thus likely that the balance between HAS and HAase influences the length and amounts of HA in the EG.

The thickness of the EG is in the range of 0.5 - 3 µm [3, 5, 19, 20, 21], which is much larger than the mesh size of the HA network [22]. Thus it is reasonable to study the mechanics of HA-bulk solutions as a first approach. In a high-salt buffer, HA is expected to behave as a flexible hydrophilic polyelectrolyte [23, 24]. Above the entanglement concentration \( c_e \), which is determined by the molecular weight of its chains, it forms an highly viscoelastic network [25, 26]. In the EG, if arranged similarly, HA can thus withstand externally applied stress and can also act as a molecular sieve, permeable for small molecules, but not for larger ones. In support of this model, in vivo studies have shown a decrease in mechanotransduction and an increase in permeability of the EG when treated with HAase [7, 27].

Prior studies, using conventional rheometers, have shown that an entangled HA solution is viscoelastic, with a shear modulus depending on concentration and molecular weight [25, 26]. Macroscopic conventional rheometers, however, can not measure the viscoelasticity of the microscopic EG layer, and furthermore have a severely limited frequency range. In recent years, new techniques, collectively called microrheology, have been developed to measure viscoelastic properties on microscopic length scales by observing the motion of micrometer-sized probe particles [28]. Good agreement between conventional rheology and video-based microrheology at low frequencies has been demonstrated with synthetic polyelectrolytes [29]. We here use laser interferometry to measure the position of an optically trapped probe particle precisely in a wide frequency range [30, 31]. Earlier work has shown that there is good agreement between macro- and this type of microrheology in flexible polymer solutions for frequencies of at least up to 10 kHz [32].

We have probed the viscoelastic behavior of HA solutions at conditions (concentration and molecular weight) resembling the physiological EG in order to elucidate its possible role as a mechanotransducer. We show that HA in a high salt buffer can be regarded as a flexible polymer [24]. The entangled HA network has an elastic plateau displaying a weak power-law dependence caused by the distribution of reptational relaxation times [33]. Addition of HAase to an entangled HA network has a drastic effect and leads to a strong decrease of the elastic properties of the network. This is likely to be relevant for understanding endothelial function in the presence of glyocalyx degrading enzymes.

**Materials and methods**

**Sample preparation**

Sodium hyaluronate in powder form, with a weight-average molecular weight \( (M_w) \) of 231 kDa \( (\text{HA}_{2000}) \), 1024 kDa \( (\text{HA}_{1000}) \) and 1844 kDa \( (\text{HA}_{2000}) \), was obtained from LifeCore Biomedical. Hyaluronidase, extracted from bovine testes (fraction IV-S), was purchased from Sigma. Phosphate-buffered saline (PBS) was obtained from Fresenius Kabi, and spherical silica particles \( (0.8 \mu m, 50 \text{ mg/l}) \) from G. Kisker GbR. Samples were prepared at different HA concentrations by diluting with PBS (pH 7.4): \( \text{HA}_{2000}: 10 \text{ mg/ml}, \text{HA}_{1000}: 10 \text{ mg/ml}, \text{HA}_{2000}: 0.5 \text{ to } 10 \text{ mg/ml} \). For the microrheology measurements, silica particles with a diameter of 0.8 µm - larger than the average hyaluronan mesh size (approximately
0.1 µm for 0.5 mg/ml, MW 680 kDa) [22] - were added. The mixture was put into a disposable sample chamber, with the inner dimensions of 26 mm × 5 mm × 100 µm (height), consisting of a coverslip and a microscope slide separated from each other by double-stick tape. The chamber was sealed with vacuum grease. The measurements were carried out at room temperature (21.4 °C). HAase was mixed with the silica particles and the 10 mg/ml HA$_{2000}$ solution to a final concentration of 46.8 U/ml. The mixture was incubated at 37 °C for 15, 30, 60, or 120 min before being inserted into the sample chamber for measurements at room temperature. The solutions were heated to 37 °C prior to incubation.

**Experimental setup**

The microrheology measurements were carried out with a custom-built inverted microscope equipped with optical tweezers as described previously [30]. In short, a near infrared laser (Nd:YVO$_4$, cw, λ = 1064 nm, maximum power = 4 W, Compass, Coherent) was focused to a diffraction-limited spot with a high-NA oil-immersion objective lens (Zeiss Neofluar 100x, NA = 1.3). The focused beam is able to trap a probe particle inside the sample chamber. The outgoing laser light, interfering with the light scattered by the particle, was collected with a condenser lens (NA = 1.4) and imaged onto a quadrant photodiode placed conjugate to the condenser back-focal plane. The output signals from the quadrant photodiode, which are proportional to the displacements of the probe particle in two axes normal to the laser beam [34], were digitized and sampled at 195 kHz (AD16 board on a ChicoPlus PC-card, Innovative Integration). The power spectral density (PSD) was calculated from ~4 × 10$^6$ data points recorded for each axis. We calibrated both trap stiffness and particle displacement by measuring the PSD of identical beads in water with identical instrument settings. The spatial resolution of the probe particle position obtained with this method (back-focal plane laser interferometry) is better than 1 nm [35]. All measurements were carried out ~20 µm away from the bottom surface of the chamber in order to avoid an influence of the surface [36].

**Shear modulus determination**

Details of the method used to derive the shear modulus of the surrounding material from the PSDs of probe particles embedded in the material are given elsewhere [30]. The accessible range of shear moduli that can be probed with this method lies between ~ 10$^{-3}$ to ~ 10$^3$ Pa. The fluctuation-dissipation theorem relates the calculated PSD $S(\omega)$ of the position fluctuations of the probe particles to the imaginary part of the complex response function $\alpha(\omega) = \alpha'(\omega) + i\alpha''(\omega)$ as $\alpha''(\omega) = (1/4k_B T)\omega S(\omega)$. Here, $k_B$ is the Boltzmann constant, and $T$ the temperature of the solution. Provided that $\alpha'(\omega)$ is obtained over a wide frequency range, the real part $\alpha'(\omega)$ can be calculated via a Kramers-Kronig integral relation as $\alpha'(\omega) = \frac{2}{\pi} \int_0^\infty dt \cos(\omega t) \int_0^\infty d\xi \alpha''(\xi) \sin(\omega t).$ As reported in ref [37], the obtained complex response function $\alpha(\omega)$ includes the influence of the optical trap. Therefore, $\alpha(\omega)$ is corrected to the bare material response function $\alpha_{cor}(\omega)$ without the presence of the optical trap as $\alpha_{cor} = \alpha / (1 - k_t\alpha)$, where $k_t$ is the stiffness of the optical trap [37]. The complex shear modulus of the HA solution $G(\omega) = G'(\omega) + iG''(\omega)$, with $G'$ the elastic and $G''$ the viscous component, is given via the generalized Stokes-Einstein relation (GSER),
\[ G(\omega) = \frac{1}{6\pi \alpha_0 \omega} \], with \( a \) the bead radius. For a purely viscous medium of viscosity \( \eta \), the Stokes relation is recovered by using \( G(\omega) = -i\omega\eta \).

**Results and Discussion**

**High band-width mechanical response of HA.**

First, we measured the molecular weight dependence of the shear modulus for a fixed HA concentration of 10 mg/ml. PSDs were taken for at least ten different probe particles for each molecular weight and averaged as shown in Figure 3.2 (b). PSDs, for each probe particle, were analyzed to derive \( G' \) and \( G'' \) and then averaged for each molecular weight as shown in Figure 3.2 (c) and (d) (absolute value of \( G'' \)) respectively. \( G'' \) for the solvent is shown as a dotted line in Figure 3.2 (d).

**Figure 3.2:** (a) Power spectral densities (PSD) (units V\(^2\)/Hz) of thermal motion in \( x \) and \( y \)-direction for beads in 10 mg/ml hyaluronan solutions of molecular weights: 231 kDa (11 different beads), 1024 kDa (17 beads) and 1844 kDa (21 beads). (b) Averaged PSDs calibrated to nm\(^2\)/Hz for 231, 1024, and 1844 kDa. Averages were taken over all beads and the \( x \)- and \( y \)-directions. (c) Average storage moduli \( G' \) for 231, 1024 and 1844 kDa calculated from the data in (a). Averages were taken over all beads and the \( x \)- and \( y \)-directions. (d) Average loss moduli \( G'' \) (absolute value of the intrinsically negative numbers) for 231, 1024, 1844 kDa and solvent. Averages were taken over all beads and the \( x \)- and \( y \)-directions.
Results and discussion

Figure 3.3: (a) Average storage $G'$ and loss modulus $G''$ (absolute values) superimposed for a 10 mg/ml 1844 kDa HA solution, (b) for a 10 mg/ml 1024 kDa HA solution, (c) for a 10 mg/ml 231 kDa HA solution, (d) $G''_{1844 \text{kDa}}$ - $G''_{\text{solvent}}$. Line indicates a slope of 0.63. Dotted and solid lines in (a), (b) and (c) show the fit with Equation 3.1 for $G'$ and $G''$ respectively.

The frequency dependence of $G'$ and $G''$ for different molecular weights ($M_w = 1844$ kDa, 1024 kDa, 231 kDa) is depicted in Figure 3.3 (a)-(c), each in its own panel.

Individual chains in a polymer solution interact sterically when their concentration becomes larger than $c^* (\sim 1/R_g^3)$, where $R_g$ is the radius of gyration of a chain. At concentrations higher than the entanglement concentration $c_e (\gg c^*)$, interactions between polymer chains, due to strong entanglement, affect the rheological behavior of the solution and lead to an elastic plateau region at intermediate frequencies [33]. Stress applied to the entangled polymer solution relaxes through the thermal disentanglement of the network (reptation). Thus, the elastic modulus decreases at frequencies below the inverse of the characteristic time for disentanglement. For the HA solution with the highest molecular weight (1844 kDa), the plateau and long-time relaxation are clearly visible (Figure 3.3 (a)). The smallest HA tested (231 kDa) at the same concentration (10 mg/ml), in contrast, is hardly entangled and shows no plateau (Figure 3.3 (c)).

At the high-frequency end, the frequency dependence of $G(\omega)$ has a power-law form, which typically reflects the relaxation process of the polymer chain within a mesh [30, 32, 38]. At even higher frequencies (or in very dilute solutions), a purely viscous response (power law slope 1) is expected due to solvent drag on the probe. The exponent for the power law depends on the flexibility of the single polymer chain and on hydrodynamic...
interactions: it is about 1/2 for flexible polymers in the Rouse limit, from 5/9 to 2/3 in the Zimm limit [33], and about 3/4 for semiflexible polymers [30].

We assume that the frequency dependence of $G$ over the whole frequency regime can be described with an empirical relaxation function as:

$$G(\omega) = \frac{-i \omega \eta_0}{1 + (-i \omega)\tau} + B(-i \omega)\gamma - i \omega \eta_{sol}.$$  \hspace{1cm} (3.1)

The minus signs in this equation follow from the physical fact that the viscous response must follow the force in time, and from our choice of Fourier transform convention ($f(t) = \int \frac{d\omega}{2\pi} f(\omega)e^{-i\omega t}$). The first term on the right hand side of Equation 3.1, called Cole-Cole relaxation function, represents both the elastic plateau region and the low frequency behavior due to reptation, with $\beta(\leq 1)$ empirically indicating the broadness of the low frequency relaxation, $\eta_0$ the steady state viscosity, and $\tau$ the average relaxation time. The second term expresses the above mentioned power law behavior at high frequencies. The third term includes the solvent contribution with $\eta_{sol}$ the viscosity of the solvent. The fits are shown in Figure 3.3 (a)-(c). In our experiments, $G$ does not properly follow the high frequency power law due to the cut-off effects in the Kramers-Kronig integration [30]. We did find, however, an exponent of 0.63\footnote{A similar exponent was found in prior work on wormlike micelle solutions [32]. Both results are consistent with Zimm-like dynamics where hydrodynamic interactions play an important role.} for the high frequency scaling of $G''$ (HA$_{2000}$) after removing the contribution of the solvent viscosity to the total loss modulus $G''$; $G''_{HA} = G''_{\text{tot}} - G''_{\text{solvent}}$ (Figure 3.3 (d)). Thus we kept $\gamma$ fixed at 0.63 to obtain a stable fit. The parameters obtained from the fits are listed in Table 3.1. Both the relaxation time $\tau$ and the specific viscosity $\eta_{sp}$ ($\equiv \eta_0 / \eta_{sol}$, with $\eta_0 = -\lim_{\omega \to 0} G''(\omega) / i \omega$) scale with molecular weight as $M_w^3$ (solid lines in Figure 3.6 (a)-(b)). This is characteristic for an entangled network composed of flexible polymers [33].

Table 3.1: Parameters obtained from a fit with Equation 3.1 of the shear modulus $G$ for different HA molecular weights. Characteristic frequency $f_{rep} = 1/2 \pi \tau$ obtained from the average relaxation time $\tau$.

<table>
<thead>
<tr>
<th>$M_w$ (kDa)</th>
<th>$\eta_p$</th>
<th>$\tau$ (s)</th>
<th>$f_{rep}$ (Hz)</th>
<th>$\beta$</th>
<th>$B$ (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1844</td>
<td>3224</td>
<td>0.16</td>
<td>0.99</td>
<td>0.84</td>
<td>0.12</td>
</tr>
<tr>
<td>1024</td>
<td>274</td>
<td>0.02</td>
<td>7.96</td>
<td>0.71</td>
<td>0.13</td>
</tr>
<tr>
<td>231</td>
<td>24</td>
<td>0.0003</td>
<td>530</td>
<td>0.52</td>
<td>0</td>
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</tbody>
</table>

Next, we measured the concentration dependence of the shear modulus for a fixed HA molecular weight of 1844 kDa. For each concentration, PSDs were taken for at least ten different particles, and averaged as shown in Figure 3.4 (b). PSDs taken for each probe particle were first analyzed to derive $G'$ and $G''$ as a function of frequency and then averaged for each concentration. Figure 3.4 (c) and (d) depicts the averaged $G'$ and $G''$ respectively. $G''$ for the solvent is shown as a dotted line in Figure 3.4 (d).
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Figure 3.4: (a) Power spectral densities (PSD) (units V^2/Hz) in x- and y-direction for beads in 1844 kDa hyaluronan solutions of different concentrations: 0.5 mg/ml (21 different beads), 1 mg/ml (17 beads), 2.5 mg/ml (16 beads), 5 mg/ml (17 beads) and 10 mg/ml (20 beads). (b) Averaged calibrated PSDs (nm^2/Hz) for 0.5, 1, 2.5, 5 and 10 mg/ml. Averages were taken over all beads and the x- and y-directions (c) Average storage modulus G' for 0.5, 1, 2.5, 5 and 10 mg/ml. Averages were taken over all beads and the x- and y-directions. (d) Averaged loss moduli G'' (absolute values) for 0.5, 1, 2.5, 5 and 10 mg/ml HA, and solvent. Averages were taken over all beads and the x- and y-directions.

As expected an elastic response (G') emerges with increasing concentration. Nishimura et al. probed a similar HA sample (c = 5 mg/ml, M_w = 1900 kDa), with oscillating shear between ~ 0.01 –1 Hz in a plate–and-cone rheometer and our microrheology results are in good agreement with their macrorheology results [39]. The frequency dependence of G' and G'' for each concentration is depicted in Figure 3.5, each in its own panel. Lines are the fit with Equation 3.1, carried out as explained above. Parameters are listed in Table 3.2.

In Figure 3.6 (e) the concentration dependence of the broadness factor β is shown, which rapidly decays to smaller values for semidilute unentangled solutions and asymptotically approaches a single relaxation (β ~ 1) at higher concentration. The narrow distributions of reptational relaxation times are observed in concentrated flexible polymer solutions or polymer melts. These materials then have elastic plateaus which are practically frequency independent. Our semidilute solutions, on the other hand, showed approximate power law dependence \( \omega^{-\beta} = \omega^{0.15-0.5} \) in the same region, which indicates that the distribution of reptational relaxation times is broad (Figure 3.6 (e)). The reason
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Figure 3.5: Averaged storage $G'$ and loss moduli $G''$ (absolute values) superimposed for a 1844 kDa HA solution at different concentrations. Dotted and solid lines show the fit with Equation 3.1 for $G'$ and $G''$ respectively.

Table 3.2: Parameters obtained from a fit with Equation 3.1 of the shear modulus $G$ for different HA concentrations. Characteristic frequency $f_{rep} = 1/2\pi \tau$ obtained from the average relaxation time $\tau$.

<table>
<thead>
<tr>
<th>$c$ (mg/ml)</th>
<th>$\eta_{sp}$</th>
<th>$\tau$ (s)</th>
<th>$f_{rep}$ (Hz)</th>
<th>$\beta$</th>
<th>$B$ (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3224</td>
<td>0.16</td>
<td>0.99</td>
<td>0.84</td>
<td>0.12</td>
</tr>
<tr>
<td>5</td>
<td>428</td>
<td>0.09</td>
<td>1.77</td>
<td>0.78</td>
<td>0.06</td>
</tr>
<tr>
<td>2.5</td>
<td>42</td>
<td>0.04</td>
<td>3.98</td>
<td>0.68</td>
<td>0.02</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>0.02</td>
<td>7.96</td>
<td>0.56</td>
<td>0.002</td>
</tr>
<tr>
<td>0.5</td>
<td>4</td>
<td>0.01</td>
<td>15.92</td>
<td>0.53</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

most likely is a gradual transition between elastic plateau and high frequency response and could also be affected by polydispersity in $M_w$. In our case the polydispersity was low: $PDI = 1.1$. It should be noted that it is necessary to use a high-bandwidth technique to analyze broad relaxation processes quantitatively and obtain an average relaxation time $\tau$. 
Figure 3.6: Molecular weight dependence of (a) the specific viscosity $\eta_s$ and (b) the relaxation time $\tau$. Concentration dependence of (c) the specific viscosity $\eta_s$, (d) the relaxation time $\tau$ and (e) the broadness factor $\beta$. Solid lines in (a) and (b) show the $M_w^{3/2}$-dependence of the scaling. Solid lines in (c) and (d) show the fits with Equations 3.3 and 3.2 respectively.

(Table 3.1 and Table 3.2). Here high-bandwidth microrheology has a definite advantage over conventional techniques.

The viscoelasticity of polyelectrolyte networks in the high salt limit, as we used in this study, is qualitatively expected to follow the same scaling law as that of a flexible polymer network, with $\tau \propto c^{1/4}$, $\eta_s \propto c^{5/4}$ for the semidilute unentangled and $\tau \propto c^{3/2}$, $\eta_s \propto c^{15/4}$ for the semidilute entangled [24]. Solid lines in Figure 3.6 (c) and Figure 3.6
(d) are fits with an interpolating function of the semidilute unentangled and semidilute entangled regimes as,

\[ \tau = A_1 c^{4/3} + A_2 c^{3/2}, \]  

(3.2)

\[ \eta_{sp} = B_1 c^{5/4} + B_2 c^{15/4}. \]  

(3.3)

The cross-over behavior from the semidilute unentangled to the semidilute entangled state was observed at similar concentrations: 1.9 mg/ml for the \( \tau \) - fit and 2.9 mg/ml for the \( \eta_{sp} \) - fit. These values are in a similar range as the previously reported value of 2.4 mg/ml which was obtained with a DC viscometer for a slightly smaller HA-chain (1500 kDa) [23]. Furthermore, at low concentrations, we find reasonably good agreement between our specific viscosity values and those found by Krause et al. [23], when the difference in molecular weight is taken into account.

Our experiments show that the empirical expression Equation 3.1 can describe the rheological behavior of a HA solution in the semidilute regime, and that the concentration and molecular weight dependence of the obtained parameters follow the expected scaling laws. More specifically, we confirmed that not only the prediction for the specific viscosity (Equation 3.3) but also the prediction for the relaxation time (Equation 3.2) is consistent with the data.

Effect of HAase activity on the mechanical response of HA.

We followed the effect of HAase on the power spectra and shear moduli of the 1844 kDa solution (10 mg/ml) as a function of the incubation time (at 37 °C) with the enzyme. Figure 3.7 depicts PSDs obtained, at room temperature, from at least six beads, taken within 15 minutes per time point. Except for the 30 min time point for which half of the measurements were taken after 15 min. The average of these PSDs for each time point is shown in Figure 3.7 (b). The initial point is taken without incubating with HAase. PSDs, taken for each probe particle, were analyzed to derive \( G' \) and \( G'' \). The time dependences of \( G' \) and \( G'' \), after treatment with HAase, are displayed in Figure 3.7 (c) and (d) respectively. \( G' \) for the solvent is shown as a dotted line in the Figure 3.7 (d).

The averaged moduli, measured 15 min after HAase treatment, are compared with those for the 231 kDa solution (10 mg/ml) in Figure 3.8 (a) and (b). \( G' \) and \( G'' \), measured at 15 min, are shown in Figure 3.8 (c), with \( G' \) dominating over \( G'' \) for the whole frequency range. Figure 3.8 (d) shows the effect of HAase on the specific viscosity \( \eta_{sp} \) as a function of time.

The enzyme HAase cuts HA and reduces its length. Prior to incubation with HAase, the high molecular weight entangled HA solution (1844 kDa, 10mg/ml) was predominantly elastic over a wide frequency range (2 - 3 kHz) with an elastic modulus of about 100 Pa. When incubated with HAase, we observed within minutes a drastic change in \( G' \) and \( G'' \), with a rapid loss of the elastic component due to the disentanglement of the solution. After 15 minutes of incubation with HAase, the moduli closely resembled those of the small molecular weight 231 kDa solution of the same concentration (Figure 3.8). The specific viscosity was similar: 13 (15 min HAase) compared to 24 (231 kDa). This observation is thus consistent with an approximately ten-fold reduction in chain length of the original

\[ c^* \] is measured to be 0.59 mg/ml for 1500kDa HA in PBS and the specific viscosity at \( c^* \) was found to be 2.0 in ref [23]. Following their result and extrapolating our data to \( \eta_{sp}(c^*) = 2 \), gives us an estimate of \( c^* \sim 0.3 \) mg/ml for our 2000kDa HA in PBS, which is consistent with ref [23].
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The polymer length is further reduced with longer incubation times. The solution after 2 hours of incubation was almost purely viscous ($G'' \sim \omega$ at high frequencies) with negligible elastic moduli $G'$. The specific viscosity had been decreased to 2.85.

![Figure 3.7: Effect of hyaluronidase on a 10 mg/ml 1844 kDa HA solution.](image)

The effect of HAase on the in vivo EG layer has been studied by Henry et al. [27]. They used bright-field and fluorescence microscopy to measure the permeability of the EG for FITC labeled dextrans of different sizes as a function of time. The addition of HAase at a similar concentration as in our experiments induced an increase in permeability of the EG for the smaller FITC-dextrans (70 and 145 kDa), which is consistent to our in vitro observation (Figure 3.7). The permeability for the larger FITC-dextrans (580 and 2000 kDa), however, was hardly influenced by the dose of HAase regardless of incubation time, indicating an unaltered apparent glycocalyx thickness. Thus it is likely that HAase penetrates into the glycocalyx and opens up the network such that smaller dextrans can enter more easily. Under this condition, weak cross-linking of the EG which possibly exists in vivo [40], prevents the total breakup and decomposition of the EG layer.
Figure 3.8: (a) Storage modulus $G'$ for a 10 mg/ml 1844 kDa hyaluronan solution after 15 min of hyaluronidase treatment, compared to a 10 mg/ml 231 kDa hyaluronan solution without hyaluronidase treatment. (b) Loss modulus $G''$ (absolute value) for a 10 mg/ml 1844 kDa hyaluronan solution after 15 min of hyaluronidase treatment compared to a 10 mg/ml 231 kDa hyaluronan solution without hyaluronidase treatment. (c) $G'$ and $G''$ (absolute values) for a 10 mg/ml 1844 kDa hyaluronan solution after 15 min of hyaluronidase treatment. (d) Effect of incubation with hyaluronidase on the specific viscosity $\eta_s$. The viscosity $\eta_s$ at various time points was obtained from the fits of $G$ with Equation 3.1.

Estimation of the in vivo molecular weight and concentration of HA.

HA is expected to be an important factor for the rheological behavior of the EG. To gain a better understanding of the EG's mechanical property, it is necessary to know the concentration and molecular weight of the HA-polymers existing in the in vivo EG. Exclusion and staining measurements so far provided evidence for a physiological glyocalyx thickness between 0.5 and 3 µm [19, 5, 3, 20, 21]. The maximal thickness of the EG is likely to be limited by the length of a single HA chain. The contour length of the 1844 kDa HA chain is about 4.6 µm [41], and thus we can assume that the molecular weight of the HA polymers used in this study is within a realistic range compared to the endothelial glyocalyx.

An estimate of the concentration can be made using an isolated cell study in which after 24 hours of flow an amount of $46 \times 10^{-4}$ ng HA/cell was found [42]. When we take an endothelial cell surface area between 1200 µm$^2$ and 300 µm$^2$, we find that the estimated
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HA concentration for cells exposed to 24 hours of flow is about 8-31 mg/ml for a glycocalyx thickness of 0.5 µm, and 1-5 mg/ml for a glycocalyx thickness of 3.0 µm. Therefore, our choice for the concentration of the in vitro HA solutions, is physiologically relevant.

Physiological relevance of the HA mechanical response

The EG is hardly deformed by red blood cells (RBC) when their velocity is higher than 20 µm/s [19]. The characteristic time for the impact of a high velocity RBC on the glycocalyx is estimated by dividing the cell velocity by the thickness of the EG. When we take, for the sake of argument, a RBC velocity of 200 µm/s and a thickness of 0.5 µm, we find a characteristic time of about 5/200 = 0.0025 s. This corresponds to a frequency of $1/(2\pi \cdot 0.0025)$ Hz, which lies within the frequency region in which the high molecular weight solutions are more elastic (10~100 Pa) than viscous. A predominantly elastic response implies storage rather than dissipation of energy. Thus stress applied to the EG can be transmitted to the plasma membrane as well as to the cytoskeleton, which is likely the initial process for mechanotransduction.

When the motion of a RBC is arrested, the EG layer is, however, almost completely crushed by the RBC. In order to compress the EG completely, most of the solvent molecules have to be squeezed out from the EG. This compression process takes much longer than the time estimated above due to the strong friction between the polymer network and the solvent. Indeed, in vivo, it has been observed that the endothelial glycocalyx, compressed by a white blood cell, restores its shape/volume in about 0.5-1 sec [43], which means that even at such long times the response is still elastic.

This behavior is different from the predominantly viscous response we measured in our HA solutions at low frequencies (< 1 Hz). For our entangled HA solution, the original integrity of the network structure is lost due to the reptation of polymer strands and is never elastically restored at these low frequencies. The reason for the different behavior in vivo is likely due to interaction with other EG components which could inhibit reptation by cross-linking. In a prior study [39] it was shown that chondroitin sulfate has a small effect on the shear modulus, while the HA binding proteoglycan aggrecan has a considerably larger effect. The latter, however, has not been found in the EG. Further studies, taking cross-linking and compressive deformation modes into consideration, will be particularly helpful for modeling the passage of RBCs and leukocytes through blood vessels.

Conclusions

We have quantitatively probed the rheological behavior of model systems for the endothelial glycocalyx using microrheology, a technique with which one can access small sample volumes and characterize the response over a large frequency range, reaching frequencies that are expected to be relevant for the endothelial glycocalyx. We confirmed that HA in a high-salt buffer is a flexible polymer. We found that the rheological behavior of the HA network can be described by the broad distribution of reptational relaxation times. We also found that the elasticity characteristic of the entangled HA network disappears quickly (15 min) with HAase addition, while the remaining viscosity after two hours of HAase activity is still about three times higher than that of water.

The HA model networks were investigated in an approximately physiologically range of molecular weights and concentrations. The physiological events occurring at the luminal side of a blood vessel have various time-scales. Thus, in conclusion, our broad band
Microrheology of hyaluronan study is a step towards a better understanding of the physiological role of a mechanical HA layer at the luminal side of blood vessels.

References


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