Microrheology of the pericellular matrix: gels, cells and organ: an optical tweezers study
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Citation for published version (APA):

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Scanning microrheometry: high resolution mechanical probing of the pericellular matrix

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Abstract

Many cells express on their outside a membrane-coupled mechanical layer, the pericellular matrix (PCM). The PCM can be micrometers thick and is amongst others associated with cell proliferation and migration. It has the glycosaminoglycan hyaluronan (HA) as its backbone, to which proteoglycans like aggrecan or versican can bind. The latter, like HA, is associated with cancer progression and metastasis. Exploring the local viscoelasticity of the PCM is essential for understanding its mechanical design and functioning. Here we introduce the application of a micro-rheometric technique, based on the optical trapping of < \( \mu \)m sized colloids, with which we obtained a mechanical cross-section of the PCM of cultured prostate cancer cells (PC3 cells), with 500nm spatial resolution. In the presence of exogenously aggrecan we not only found an increase in thickness of the layer but also the expression of long microvillus-like protrusions (<\~ 10 \( \mu \)m) embedded in the soft HA-aggrecan coat.
Introduction

Many cells express on their outside a mechanical layer, the pericellular matrix (PCM), which forms the interface between the cell and the extracellular matrix and is thus likely to be of significant importance for cellular functioning [1, 2, 3, 4, 5, 6]. The PCM has been shown to play an important mechanical role in various cellular processes like proliferation, migration, and mechanosensing [7, 8, 1, 3]. It is rapidly formed during mitotic cell rounding and observed mostly at the trailing edge of migrating cells, facilitating their detachment [1, 3]. A particular PCM, the endothelial glyocalyx (EG) is expressed by vascular endothelial cells, forming the interface between the flowing blood and the underlying cell layer, acting as a protective barrier and mechanotransducer of shear stress [5, 9, 10]. Although there are differences in composition between PCMs depending on the cell type they enclose, for instance chondrocytes have collagen incorporated in their PCM [7], while this protein is absent in the EG [11], the PCMs share the dependence on a common denominator the polymer hyaluronan (HA). HA is the mechanical backbone of the PCM: replacing native high molecular weight HA by HA oligosaccharides through a competitive mechanism results in inhibition of cell migration and proliferation [1]. The motility and invasive nature of osteosarcoma cells are abolished by the same treatment [12], which suggests the involvement of a mechanical PCM in cancer cell metastasis. Endothelial cells lose their ability to respond to fluid flow when HA is enzymatically removed [10].

In order to express a natively extending PCM, not only HA but also HA binding proteoglycans like aggrecan or versican are necessary [3, 13]. High molecular weight HA, however, is expected to be the main mechanical component (chapter 4). Here we mechanically probed the PCM expressed on HA-synthesizing prostate cancer epithelial cells (PC3 cells) [3] using an optical tweezers setup equipped with a far-field interferometer.

We employed an upright microscope, instead of the inverted configuration which is common for optical trapping. This ensures a better laser focusing without being disturbed by inhomogeneous cell constituents, which enabled us to efficiently manipulate and precisely measure the position of the probe particle. Furthermore, in the upright configuration, it is possible to push the probe particle down to the cell surface with a weaker optical trap, which is essential when measuring soft and fragile materials [14]. We used a water immersion objective to eliminate the dependence of lateral trap stiffness on the distance to the cell surface [15]. With this setup we obtained a mechanical cross-section of the PC3-PCM and quantitatively resolved the local viscoelasticity and its sensitive response to aggrecan and hyaluronidase (HAase) with sub-micrometer resolution.

Material and methods

Sample preparation

The culture medium D-MEM, as well as antibiotic-antimycotic, non-essential amino acid solution, trypsin and inactivated fetal bovine serum (FBS) were obtained from GIBCO-Invitogen. Phosphate Buffered Saline (PBS, pH 7.4) was purchased from Fresenius Kabi, and the silica spheres (0.8 µm, 50 mg/l) from G. Kisker GbR. Aggrecan and hyaluronidase (bovine testis, fraction IV-S) were obtained from Sigma Aldrich. Fibronectin was a kind gift from the Sanquin Research Foundation (Amsterdam, The Netherlands). PC3 cells (human prostate adenocarcinoma cell line) were grown in cell culture flasks coated with 10 mg/ml fibronectin at 37 °C in 5 % CO₂. Cells were cultured in D-MEM supplemented with 10 % FBS, 1 % non-essential amino acid solution and 1 % antibiotic-antimycotic solution. For each microrheology measurement, cells were trypsinized from a culture flask and seeded
on a glass coverslip, located in a re-usable glass chamber (Figure 5.1). The bottom of the chamber was made of a slide glass. The spacers at the four lateral sides were made of glass with a height of 0.39 mm. The spacers were glued to the slide glass with silicon resin, forming an inner volume of $22 \times 14 \times 0.39 \text{ mm}^3$. After seeding, cells were incubated for 90 minutes before the culture medium was replaced by either new culture medium without any extra additives, or with culture medium containing aggrecan (1 mg/ml) with or without HAase (40 units/ml). After 90 min, 0.8 µm silica beads were added and the chamber was sealed by a second coverslip, placed on top of the spacers. Measurements were done within an hour after the sample was taken out of the incubator at room temperature (21.8 °C).

**Figure 5.1:** Schematic of the sample chamber assembled from (slide) glass and sealed with a coverslip (not to scale).

**Immunofluorescence confocal imaging**

For the immuno-fluorescence staining of actin, PC3 cells were seeded on glass coverslips and incubated for 90 min with normal culture media, in a protocol identical to that used in the microrheology measurements. After further 90 min incubation with aggrecan (1 mg/ml), the cells were fixed with paraformaldehyde (4 %), without intermediate washing of the cells. After 10 min the paraformaldehyde was removed and the cells were washed with PBS (2×). The cells were then stained with Alexa Fluor® 555 phalloidin (5 U/ml, Molecular Probes) for 60 min. After a final wash step with PBS, the cells were mounted on a slide glass, and sealed. 50% glycerol in PBS was used as mounting medium. Fluorescent images of actin containing microvilli were obtained with a confocal microscope (Leica TCS SP2). 3-dimensional scans were made with a stepsize of 120 nm in the z-direction. Data were analyzed and projected on to cross-sections using WCIF ImageJ software.
The microrheology setup

The microrheology measurements were performed with a custom-built optical tweezers setup. The setup was built around, and attached to an upright microscope equipped with differential interference contrast (Olympus BX51WI). Its water immersion objective (UPlanApo, 60x, NA=1.2, Olympus) was used to focus an 830 nm laser beam (diode laser, cw, 140 mW, 1Q1C140 G5, Laser 2000) in the sample chamber. The thermal motion of a trapped 0.8 µm silica bead inside the chamber was detected with a quadrant photodiode (diameter = 10 mm, SPOT-9DMI, UDT Sensors, Hawthorne, CA, USA) using back-focal-plane interferometry. For optimal trapping, the laser was expanded (3×) by a beam expander (LINOS Photonics, Germany). The laser power was controlled by a combination of a half wave plate and a polarizer. A 1:1 telescope system was used for fine positioning and steering of the beam focus. To detect the position of the trapped bead, the out-going laser light interfering with the bead-scattered light, was collected by a condenser lens (NA = 0.8, WI-UCP, Olympus), and projected onto a quadrant photodiode (diameter = 10 mm, SPOT-9DMI, UDT Sensors, Hawthorne, CA, USA), positioned at the image plane of the back focal plane of the condenser lens [16]. The four output signals of the quadrant photodiode were processed with analog amplifiers to get the x-, y- voltages, corresponding to the position of the trapped bead in two (x, y)- directions parallel to the sample plane. These voltages were recorded using a data acquisition board (PCI-4451, National Instruments, Austin, TX, USA) operated with LabView software (National Instruments, Austin, TX, USA). Data was taken with a sampling rate of 195 kHz ($2 \times 10^6$ data points). The precise z-positioning of the trapped bead was achieved by mounting the objective lens on a piezo-driven nano-focussing device (P-720, Physik Instrumente) which was computer-controlled. The microscope was operated in high contrast DIC-mode.

Measurements were started at a height of about 10 µm above the cell surface, going down with initial steps of 1 µm and ending with steps of 0.5 µm for the last 2 µm range. The z-position of the trapped particle was manipulated with a PC-controlled piezo stage. When the focus of the laser beam crosses over the cell membrane surface, the bead is displaced from the center of the trap, leading to a loss of quadrant photodiode signal. 0.5 µm higher than this point was set as the origin of the height coordinate ($h = 0$ µm). After scanning across the PCM along the height direction, we brought the probe bead upwards and measured again at $h = 25$ µm.

Shear modulus determination

The imaginary part of the complex response function $\alpha(\omega) = \alpha'(\omega) + i\alpha''(\omega)$ ($\alpha = x / F$, with $x$ the displacement of the probe particle, and $F$ the force exerted on the probe particle) was obtained via the fluctuation-dissipation theorem from the power spectral density $S(\omega)$ [17]: $\alpha''(\omega) = (1 / 4k_B T)\omega S(\omega)$, with $k_B$ the Boltzmann constant, and $T$ the temperature of the solution. Under the condition that $\alpha''(\omega)$ is known over a wide frequency range, a Kramers-Kronig integral can be used to obtain

$$\alpha'(\omega) = \frac{2}{\pi} \int_0^\infty dt \cos(\omega t) \int_0^\infty d\xi \alpha''(\xi) \sin(\xi t) .$$

In the case of a spherical particle, as in our experiments, a generalized Stokes-Einstein equation can furthermore be used to finally derive the complex shear modulus: $G(\omega) = 1 / (6\pi a \alpha(\omega))$, with $a$ the bead radius. Before this final step is taken, however, the complex response function should be corrected for the contribution of the optical trap to the measured response: $\alpha_{cor} = \alpha / (1 - k_I \alpha)$ where $k_I$ is the trap stiffness and $\alpha$ the uncorrected response [14].
Position and force calibration

The voltage output of the quadrant photodiode used for laser interferometry is proportional to the trapped bead’s position [16, 18]. In order to get the actual displacements from the trap center in (nano-) meter units, probe particles, identical to the ones used in the cell experiments, were measured in water using the same technique [19, 20, 21]. The power spectra of the fluctuations of beads in water have a Lorentzian form: \( S_0 f_c^2 / (f_c^2 + f^2) \), where \( S_0 \) is the value for the low frequency plateau and \( f_c \) the corner frequency. The calibration factor \( R (m/V) \) relates the power spectrum of the output signal (\( V^2/Hz \)) to that of the bead’s fluctuations (\( m^2/Hz \)) as \( S(m^2/Hz) = R^2 S(V^2/Hz) \). By fitting the power spectra of the output signal \( S(V^2/Hz) \) with a Lorentzian, values for \( S_0 f_c^2 (V^2/Hz) \) and \( f_c^2 \) are obtained.

The Brownian motion of a trapped particle in a purely viscous solvent is described by \( 2 B_0 / 4 \kappa \gamma T K \), and \( \pi \gamma \kappa / c_f = f \), where \( \gamma \) is the drag coefficient and \( \kappa \) is the trap stiffness. Using the drag coefficient for a spherical particle given by Stoke’s law (\( \gamma = 6 \pi \eta a \), \( \eta \) is the viscosity of the medium, \( a \) = the particle’s radius), we get \( S_0 f_c^2 = k_B T / 6 \pi^3 \eta a \) in \( m^2/Hz \).

Comparing the measured value of \( S_0 f_c^2 \) (in \( V^2/Hz \)) with the calculated value \( = k_B T / 6 \pi^3 \eta a \) gives \( R \). From the measurement of \( f_c \) the trap stiffness follows:

\[ \kappa = 12 \pi^2 \eta a f_c. \]

Correction for surface effect (Faxen’s law)

In prior works [22, 23, 24, 25], experiments as well as calibrations were carried out at around 20 \( \mu \)m away from the glass surface. This was necessary to avoid artifacts such as spherical aberrations of the objective lens, which give rise to a height dependence in calibration and trap stiffness [15]. By using a water-immersion lens in this study instead of an oil-immersion lens we could avoid the effect of spherical aberrations on the trap stiffness and calibration. For a water-immersion lens used for imaging in an aqueous solution the trap stiffness is independent of height [15]. The corner frequency, however, is still dependent on height due to the hydrodynamic interaction between the particle and the glass surface, at distances from the surface comparable to the particle size. This hydrodynamic interaction between the particle and the surface results in an apparent higher viscosity \( \eta_{app} \), due to an increase in the viscous drag coefficient \( \gamma \) also known as Faxen’s law [26]. For scanning microrheometry, therefore, the viscoelasticity obtained using the same calibration procedure, increases on approach to the cell membrane, in the proximity of the surface even if there is no viscoelastic pericellular layer present. The data were thus corrected for this effect by measuring the apparent viscous modulus as a function of height above the glass surface, where no cells were present. The in this way obtained Faxen curve is shown in Figure 5.2, and is an average over 8 height scans. From the experimental curve, correction factors for each height point were obtained by normalizing the curve by the value at the 25 \( \mu \)m-point where calibrations in this study were taken. Finally by dividing the apparent \( G' \) and \( G'' \) with the height dependent correction factors, the true shear moduli were found. These were analyzed to find the mechanical parameters of the PCM.
Figure 5.2: Faxen curve: apparent increase in viscosity due to surface proximity. Average curve experimentally obtained from 8 height-scans of $G'$ (1676 Hz), and normalized by $G'$ at 25 μm.

Analysis of $G'$ and $G''$-scan

$G'$ at low frequencies is influenced by the existence of a mechanically stabilized HA network (e.g. cross-linked or entangled). $G''$ at high frequencies, approximately expressed as $G'' \sim -i\omega\eta$, however, indicates the viscosity of the PCM, which is an approximate measure for the concentration of the viscoelastic components in the PCM regardless of the presence or absence of stable network formation. Thus we focused here on the height dependence of $G'$ at low frequencies (15 Hz, taken as an average between 7 and 22 Hz), and of $G''$ at high frequencies (1700 Hz, taken as an average between 1100 and 2200 Hz) of the Faxen corrected curves.

Results

The existence of a PCM on PC3 cell surfaces incubated with aggrecan is tested in Figure 5.3 (a), using a particle exclusion assay. Free particles identical to the probe particles used for the microrheometry experiments could not enter towards the peripherals of the PC3 cells. Cells co-incubated with HAase, on the other hand, lost their PCM as can be seen in Figure 5.3 (b). The hindrance on the approach of the probe particles to the cell membrane when HA is not diminished indicates the presence of a mechanical layer. That the layer is viscoelastic can be seen in Figure 5.4 where the PCM of a PC3 cell is mechanically probed in the presence of aggrecan. The elastic (Figure 5.4 (a)) and viscous (Figure 5.4 (b)) modulus spectra are shown at different heights above the cell surface. The elastic modulus $G'$ at 15 Hz and the viscous modulus $G''$ at 1700 Hz are shown as a function of height ($h$) in Figure 5.4 (c) and (d) respectively. As can be seen from the figure, the increase in viscoelasticity near the cell membrane is predominantly due to the
mechanical properties of the PCM and not to the presence of the surface since correcting the data for the influence of the surface (Faxen) had hardly an effect. For the intact PCM, both $G'$ and $G''$ have the largest value at the cell surface and decay to a smaller value in the culture medium. The half width at half maximum (HWHM) value of the height-curve is taken as the characteristic thickness $\lambda$ of the PCM. In the case of the example in Figure 5.4, both the $G'$- and $G''$-scan give $\lambda = 4 \mu m$.

When cells were incubated with aggrecan, some cells were found to express microvillus-like protrusions projecting straight out from the cell surface. The protrusions were visible with high contrast DIC during the microrheology experiment (Figure 5.5 (d) for a cross-section of the protrusions) and their lengths, sometimes longer than 10 $\mu m$, were measured directly. The actin filaments inside the protrusions were visualized with AlexaFluor®555 phalloidin using confocal microscopy (Figure 5.5 (a)). An example of a mechanical scan in between the protrusions is shown in Figure 5.5 in which the height dependence of the elastic (b) and viscous (c) modulus is shown.

A plateau region in the $G'$-scan is distinguishable between 2.5-15 $\mu m$. $\lambda = 14 \mu m$ is found, taking $G_{\text{plateau}}$ instead of $G_0'$ while the HWHM of the $G''$-scan gives $\lambda = 11 \mu m$. A strong correlation between the thickness of the PCM and the length of the protrusions was found as shown in Figure 5.5 (e). The Pearson correlation coefficient $r$ was 0.77 and 0.85 for the thickness obtained from the $G'$-scan (circles) and the $G''$-scan (triangles), respectively. $r$ is given by

$$r = \frac{\sum_n (x_n - \bar{x})(y_n - \bar{y})}{\sqrt{\sum_n (x_n - \bar{x})^2} \sqrt{\sum_n (y_n - \bar{y})^2}},$$

with $\bar{x}$ the mean protrusion length and $\bar{y}$ the mean PCM thickness $\lambda$.

Figure 5.3: Pericellular matrix (PCM) of PC3 cells, incubated with aggrecan in the culture medium, indirectly visualized using a particle exclusion assay with 0.8 $\mu m$ silica particles. Particle depleted zone indicates an expression of thick PCM (a), which disappears upon co-incubation with hyaluronidase (b). Scale bars 10 $\mu m$. 

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Scanning microrheometry
Figure 5.4: Pericellular matrix (PCM) of PC3 cells mechanically probed with a 0.8 µm silica bead. (a,b) Elastic modulus ($G'$) (a) and viscous modulus ($G''$) (b) at different heights above PC3 cell surface incubated with aggrecan. (c,d) Vertical cross-section of $G'(15\text{Hz})$ (c) and $G'' (1700 \text{ Hz})$ (d) over the pericellular region. Data between vertical dashed lines (7-22 Hz) in (a) and between vertical dashed lines (1100-2200 Hz) in (b) are averaged for each height, and results are shown in (c) and (d) respectively. Horizontal lines indicate the PCM thickness as obtained from the $G'$-scan (c) and $G''$-scan (d).

Enzymatic removal of HA disrupted the PCM, and no mechanical layer or microvillus-like protrusions remained. An example of a scan is shown in Figure 5.6. The increase in viscous modulus near the cell surface is predominately due to proximity of the cell surface as can be seen in Figure 5.6 (d).
Figure 5.5: Mechanical properties (thickness $\lambda$ and elastic modulus $G_0'$ at the surface) of the pericellular matrix (PCM). (a) Long microvilli visualized by staining actin with Alexa Fluor®-555 phalloidin. Vertical projection obtained from a z-stack of horizontal confocal images. (b,c) Vertical cross-section of $G'(15\text{Hz})$ (b) and $G'(1700\text{Hz})$ (c) over the pericellular region of the cell pictured in (d), expressing straight long microvilli in the presence of aggrecan, corrected (closed circles) and not corrected (open circles) for the surface effect. (d) DIC-cross section of similar protrusions as in (a). The arrow points at a 0.8 µm silica bead trapped in between the protrusions (smaller dots). (e) Correlation diagram between PCM thickness $\lambda$ and length of microvillus-like protrusions obtained from $G'$ (circles) or $G''$ (triangles) height profiles ($n=12$). The Pearson correlation coefficient of 0.77 ($G'$) and 0.85 ($G''$) indicates strong correlation between microvilli and PCM. Also shown is the line of identity.
Results

Figure 5.6: Disruption of the pericellular matrix by hyaluronidase (HAase). (a,b) Elastic (a) and viscous (b) modulus at different heights above PC3 cell incubated with both aggrecan and HAase. (c,d) Vertical cross-section of $G'(15\text{Hz})$ (c) and $G''(1700\text{ Hz})$ (d) over the pericellular region in the presence of aggrecan+HAase, corrected (closed circles) and not corrected (open circles) for the surface effect. Data between vertical dashed lines (7-22 Hz) in (a) and 1100-2200 Hz in (b) are averaged for each height, and results are shown in (c) and (d) respectively. Horizontal line in (d) indicates the PCM thickness as obtained from the $G''$-scan.

The distributions of $G'_0$ and $\lambda$ for all experimental conditions ( +/- aggrecan, +/- microvilli, +/- HAase) are shown in Figure 5.7 and the median values are shown in Table 5.1. $\lambda$ is obtained from the $G'$- and $G''$-scan respectively, both give about 4 $\mu$m as median value for the PCM thickness when aggrecan was present, and no protrusions were observed (Table 5.1). Cells expressing protrusions had a median thickness of 6.5 $\mu$m. When cells were not co-incubated with aggrecan the thickness was about 1 $\mu$m. No PCM was left after enzymatic removal of HA by HAase ($\lambda = 0 \mu$m ($G'$), and $\lambda = 0.25 \mu$m ($G''$)).
Figure 5.7: (a) Distribution of the elastic modulus at the cell surface $G_0'$ with aggrecan present in the culture medium. Further distinction is made between cells expressing microvilli ($n=11$) and cells not expressing microvilli ($n=11$). (b) Distribution of $G_0'$ (with both aggrecan and hyaluronidase ($n=17$) and without aggrecan and hyaluronidase ($n=10$) present in the culture medium. (c,d) Distribution of the PCM thickness $\lambda$ with aggrecan present in the culture medium without HAase (c) and with HAase (d), and without the presence of both aggrecan and HAase (d). Values were derived from the $G'$-scans. (e,f) Same as (c,d), except values were derived from the $G''$-scans. N: number of data points per bin, $N_{av}$: total of data points/number of bins.
Table 5.1: Mechanical properties of the pericellular matrix (PCM). Median values of the elastic modulus at the cell surface $G'_0$ and the PCM thickness $\lambda$. The latter was obtained from both the $G'$- and $G''$-scans respectively.

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<td>0.38</td>
<td>0.43</td>
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Discussion

With our micro-mechanical technique we successfully measured the viscoelasticity of the PCM of prostate cancer cells. Even though the elastic modulus increased on approach to the cell membrane, the elasticity at the surface ($G'_0$) was still less than 1 Pa. The presence of exogenous aggrecan had only a significant effect on the thickness, which was increased, but not on the viscoelasticity of the PCM. Furthermore the presence of aggrecan could induce the formation of long microvillus-like protrusions, which were coupled to a further extension of the PCM to many micrometers. The thickness of the PCM was found to strongly correlate with the length of the protrusions.

Hyaluronan structuring the PCM

As expected, hyaluronan (HA) was found to play a dominant role in the mechanical functioning of the PCM. After cleavage of the chains by the HA reducing enzyme hyaluronidase (HAase) no mechanical layer remained. Furthermore the presence of aggrecan in this study did not have a significant effect on $G'_0$, which corresponds to the mechanical properties of model PCM solutions where added aggrecan had only moderate effects as demonstrated in chapter 4. The thickness of the PCM, however, remarkably increased in the cellular system. This can be explained by extension of the HA-chains from the cell surface due to attachment of the aggrecan proteoglycans to the HA-chains [27]. The HA-aggrecan brush extends from the cell surface with an extension limited by the HA chain length. The average PCM thickness of 4 $\mu$m found here indicates a molecular weight of at least 1600 kDa for the HA-chains [28]. HA synthases are capable of synthesizing HA chains $> 1000$ kDa [29]. Without aggrecan the HA chains are thought to be collapsed, in a so-called mushroom-like configuration, when the distance between HA cell surface attachment points (e.g. the synthase itself [30] or a HA receptor like CD44 [31]) is larger than the radius of gyration of the chains [32]. The thickness of the layer is then equal to the Flory radius of a coil in good solvent: $R_c = a \cdot N^{3/5}$, with $N$ the number of monomers and $a$ the monomer length (= 1 nm for HA [28]) [32]. Thus for a 4 $\mu$m long chain the thickness is about 145 nm. In our microrheology study we find without aggrecan on average a thickness of $\sim 1$ $\mu$m, which suggests that the grafting density is already high.
enough for the HA chains to be partly extended without aggrecan. With aggrecan attached to the chains the extension is increased. Furthermore when cells express microvillus-like protrusions, the thickness of the PCM is even higher (6.5 µm on average). The existence of similar protrusions has been reported for several HA producing cell types [33]. In this study, our scanning microrheometer could probe the mechanical properties of the PCM in between the protrusions and a strong correlation was found between protrusion length and PCM thickness. Possibly the protrusions need to be supported by the PCM, and/or the PCM is, at least partly produced and tethered on the protrusions where HA synthase is expressed [33]. The formation of microvillus-like protrusions suggests the involvement of cellular signaling processes and an active remodeling of the PCM induced by aggrecan. The trigger for microvillus formation might be mechanical since in another study similar protrusions were observed after transfection of HA synthesizing cells with the HA synthase HAS3 [34]. Upregulation of HAS3 increases the number of HA binding sites on the cell surface and can thus lead to the extension of the HA chains. In our case, aggrecan binding induces extension of the chains. The induced force on the cell membrane due to extension of the chains (osmotic pressure) might be responsible for the formation of the protrusions through bending of the membrane. This is a hypothesis and the molecular details, however, of the findings in this study (thickening of PCM, long microvilli, and correlation of these) are yet to be known.

Study limitations

In this study we used prostate cancer cells as a model for an HA producing cell type, and studied the effect of the proteoglycan aggrecan on the viscoelastic properties of the PCM of these cells. The question is how relevant the findings are resulting from adding aggrecan to a PCM that has natively no aggrecan incorporated. Prostate cancer cells, however, have been shown to upregulate the synthesis of versican by fibroblasts in the peritumoral stroma [3]. Aggrecan and versican are similar, belonging to the same hyalectin family, and expressing the same HA binding domain, although versican has less chondroitin sulfate side chains [35, 36]. Furthermore, versican containing medium extracted from fibroblasts culture induces similar thick pericellular matrixes as found here [3]. Thus, aggrecan is used here as a substitute for versican for studying the pathophysiological function of the thick PCM to which the malignant activity of cancer cells seems to be linked [3, 12].

Many cells, with thick pericellular matrices, express similar microvillus-like protrusions as found here and hence these protrusions may be considered as an integral part of the PCM [33]. It is thus likely that they are also expressed by prostate cancers cells when versican is integrated in their PCM and not only when aggrecan, as used in these experiments, is present. The PCM of prostate cancer cells is thus a good model system to study, with the possibility of discriminating between the contributions of different components, in our case HA and aggrecan, to the viscoelastic properties of the PCM.

Mechanical probing of soft PCM

The measuring technique used in this study is ideal for probing the PCM as expressed by prostate cancer cells, which is too soft to detect with other techniques. Atomic force microscopy (AFM), for instance, needs at least sub-nN forces to deform the indentation tip [37]. By manipulating the location of a probe particle in 3D directions with an optical trap, it is possible to measure the local mechanical response of the surrounding material in a less invasive way by microrheology [37]. The mechanical property of the PCM probed in this study is about a factor 1000 softer than the cell body, which gives rise to an intriguing question, namely how such a soft structure influences the mechanical functions of the
whole cell system and of the mechanical interaction between the cell and the extracellular matrix. Furthermore the question remains what role the microvillus-like protrusions fulfill.

**Conclusion**

By using a non-invasive micromechanical technique we were able to make a high resolution scan of the HA-based pericellular matrix of prostate cancer cells, finding a soft layer with a maximum thickness in between microvillus-like protrusions. Our micromechanical study thus revealed the unique possibility to explore the biological reasoning for the existence of such soft pericellular matrices by measuring the mechanical design on the microscopic scale.

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