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Optical assessment of gel-like mechanical and structural properties of surface layers: single particle tracking and molecular rotors

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Abstract Thin gel-like layers form at many surfaces of natural or artificial origin. Important properties of such layers include thickness, viscosity and density. Here we discuss two optical approaches which allow assessment of these properties with high resolution.
The first approach relies on centroid calculation and defocus imaging based 3D tracking of fluorescent tracer particles, which is based on standard fluorescent microscopy and allows a precision of particle detection in the range of 10nm. The size of the particle and its surface charge and polarity will determine the particle invasion into the layer. Thus simultaneous application of different colored beads with different size and properties can reveal the thickness and nature of the layer. Via tracking the thermal vibration of particles invading the layer the bulk viscosity of the layer can be calculated.
The second approach uses "molecular rotor" fluorophores (MR). Due to their molecular structure, the MR's fluorescence quantum yield increases as their internal rotation is hampered by e.g. high viscosity of the embedding medium. The MRs are several orders of magnitude smaller than the structural (macro) molecules of a gel-like layer and therefore the MRs are not necessarily directly sensitive toward the bulk viscosity of the layer. In contrast, the MRs internal rotation will be attenuated by the MRs interaction with the structural elements of the layer or the solvent included in it. Depending on their molecular structure MRs exhibit different sensitivity to the mechanical properties of the large macromolecules or the solvent in a layer. Thus, they may be used to assess the microdomain's viscosity or density in a surface layer. Using a ratiometric imaging approach, they can be used for continuous measurements in very different experimental settings.

Keywords: Surface layer, Microrheology, Fluorescent microscopy

Several kinds of gel-like surface layers exist in the body with close relation to human health. Bacteria form biofilms e.g. on tooth surface (dental plaques) or on catheters inserted for longer period into the blood stream or the urinary tract via surrounding themselves with a gel-like layer which increases their resistance against antibiotic treatment (Stickler 2008; Donlan 2011; Marsh 2011). The gastrointestinal and the respiratory system are covered by a 10-50 micron thick mucus layer secreted by the cells covering these tracts (Fahy 2010; Johansson 2011). The mucus layer, amongst its plenty of other effects, shields the organ from invading bacteria. Every eukaryotic cell presents a surface layer (also called pericellular coat or glycocalyx) which regulates the interaction between the cell and its environment. For example in case of cartilage cells (chondrocytes) the mechanical load, of the egg cells (oocytes) the interaction with sperm cells, of the innermost cells (endothelial cells) of the vessel wall the interaction with leukocytes or vessel wall permeability toward blood plasma molecules is known to be influenced by the surface layer (Pries 2000; Papi 2010; Wilusz 2012). Very similar layers may be generated by microorganisms or biophysical effects in technical equipment outside the human body, e.g. in 'lab on a chip' devices or in sensors interacting with complex fluids containing macromolecules.

Gel-like layers are composed of a mesh-like network formed by structural (macro)molecules (glycoproteins, proteoglycans, glycans, protein,

DNA) and of solvent (e.g. water containing small solutes) filling up the pores of the network. Thickness, viscosity, charge and density are essential physical parameters of the layer which are determined by the physical properties, amount and cross-linking of the structural elements and the ionic strength of the solvent.

The thickness of a surface layer may be assessed via labeling based imaging techniques (e.g. electron microscopy, confocal microscopy). However, often the applicability of these techniques is limited due to necessity of sample fixation or due to the resolution limit of light microscopy. Additionally, fixation and even the binding of label molecules may already change the thickness of the surface layer.
Atomic force microscopy (AFM) was introduced to measure the surface layer thickness and mechanical stability via detecting the indentation of a cantilever compressing a surface layer (Peters 2012). While AFM has the advantage of a purely physical way of thickness measurement, it requires an expensive instrumentation and a special setting. Another similarly physical probing tool was introduced where a bead was hold and moved with a holographic optical trap in the surface layer (McLane 2013). The relation between the applied force and the observed displacement of the bead reports about the presence and resistance of the surface layer at the probed distance from the cell. Also, this method requires highly specialized instrumentation.
Some measurement approaches to probe the thickness of surface layers are based on its particle exclusion property, i.e. the lower permeability of the layer toward particles larger than its intrinsic pore size. Red blood cells are widely used to demarcate the surface layer of cultured cells ***(Fig.1.)***. This easily performable method allows the assessment of horizontal dimensions of the surface layer and its sensitivity is limited by the size and weight of red blood cells. It has a spatial resolution in the range of about 500 nm - 1000 nm.
Colloidal probe reflection interference contrast microscopy (RICM) was used to measure the vertical thickness of a synthetic model surface layer (Wolny 2004). For that a 25 µm large passivated polystyrene bead was loaded onto the model surface layer and the distance between the bottom surface and the bead was calculated from the RICM image of the bead. This method provides a nanometer range spatial resolution, but it requires special optics and its application is mainly limited to flat synthetic model surface layers because of the size of the colloidal probe.
An exclusion assay was also performed with fluorescent microparticles, where the position of particles loaded on cultured chondrocytes was tracked with spinning disc confocal microscopy or confocal microscopy (Boehm 2009; McLane 2013). Again, the surface layer thickness and density can be estimated via measuring the dimension of the particle exclusion zone for different size particles around a fluorescently labeled cell.
For particles which invade the layer different options may be used. Via quantifying the thermal vibration of the particles within the layer, the viscosity of the layer can be assessed. According to the Stokes-Einstein law, the mean square displacement of a particle's thermal vibration is inversely related to the viscosity of the embedding medium. These approaches are limited by the resolution limit of confocal microscopy (about 200 nm lateral and 600 nm vertical resolution) and by the necessity of a confocal microscope. The same measurement principle was leveraged to measure the thickness and viscosity of surface layer via tracking the lateral motion of gold microparticles in dark-field microscopy (Zhou 2011). While this approach has low technical requirements, its resolution is limited by the limits of bright field microscopy (about 400 nm lateral and above 1 µm vertical resolution).

To combine 1) the advantages of microparticle exclusion assays, 2) high resolution 3D single particle tracking, 3) applicability on cellular samples with 4) requirements of standard fluorescent microscopy we have developed a

fluorescent defocus imaging based single particle tracking tool (Marki 2013). In defocus imaging the fluorescent particle's relative vertical position i.e. its distance from the focus plain is calculated from the particles defocus image, which appears as a central spot surrounded by concentric rings. The defocus image is best visible in wide-field fluorescent microscopy, compatible with wide-spread microscope equipment. The number and diameter of the rings increases as the defocus distance increases ***(Fig.2.)***.
For defocus distance decoding firstly a calibration set of images is necessary where the same type of particle at known defocus distances is captured. Secondly an image analysis tool is used which correlates the captured image to the calibration set of images. The comparison can happen via comparing the outermost ring diameter or the whole pattern of the defocus images.

We have developed an image analysis software for this purpose (Marki 2013) which also calculates the horizontal position of the imaged particle via centroid calculation or spatial correlation. During the centroid calculation, the center point of the particle's image is determined via fitting to the central part of the particle's image a rotationally symmetric 2D parabola. We have tested the performance of the application via tracking fluorescent particles displacements generated by a piezo device. According to this calibration, the approach allows 3D tracking with about 10 nm precision along each axis. Via tracking the thermal vibration and convective displacement of fluorescent particles in stationary and flowing solvents of different viscosity we could estimate the local viscosity and flow profile.

A second approach to estimate viscosity or density of surface layers is the application of molecular rotors (MR). An advantage of MRs is their easy applicability under many experimental conditions, the disadvantage of MRs is their limited range of sensitivity. MRs are fluorescent molecules capable of energy dissipation via radiation (fluorescence) or via non-radiating internal rotation. Higher surrounding viscosity can hamper the internal rotation and increase the quantum yield of MRs. Thus MRs can be applied as fluorescent viscosity sensors, where a higher viscosity is indicated by a higher MR fluorescent signal (Kuimova 2012).
Due to their several order of magnitude smaller size compared to the structural elements of a surface layer, MRs are not directly reporting the bulk viscosity of a surface layer. The internal rotation of MRs invading a gel-like layer will be determined by the viscosity and mobility of the solvent filling up the 'pores' of the layer and by interactions between the MR and the structural elements of the layer. While the solvent viscosity inside and outside the layer is the same, the mobility of the solvent may be restricted in the close vicinity to larger structural molecules and the structural elements may directly hamper MR mobility. Both effects will occur more often in a denser layer with smaller 'pores'. The reduction of MR mobility also depends on their molecular structure and interaction with the structural elements of the layer. Accordingly different sensitivity of MR fluorescence in response to bulk concentration or viscosity for dextran and starch solutions with varying molecular size or for blood plasma was shown for different MR-types (Haidekker 2002).
For the practical use of this approach, it is to be considered, that an uneven spatial distribution of MRs inside and outside of a surface layer would result in uneven fluorescence intensity and thus interfere with the mobility/viscosity measurements. This potential problem can be overcome via applying life time imaging (which requires complex illumination and recording techniques) or by the simpler ratiometric imaging approach. Here this is possible by using MRs which provide a viscosity sensitive and a viscosity insensitive fluorescence with different spectral properties. The viscosity independent signal is then used to normalize by the signal of the viscosity sensitive component (Kuimova 2012). Using ratiometric imaging, this approach

can be used for many experimental settings, allowing determining fluorescence intensity at the surfaces of interest after MR application. In addition, different sets of MRs with different molecular properties may provide information on density or 'pore' size for the tested surface layers. Since MR approaches can not directly asses layer thickness and bulk viscosity, they may well be complemented by a combination with micro particle tracking allowing these measurements.

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Figure legends

Figure 1 Surface layer of a cultured primary human chondrocyte detected with a red blood cell exclusion assay. Left: The bright-field image shows the chondrocyte surrounded by red blood cells. Middle: A fluorescent image shows the WGA-Alexa488 lectin labeled chondrocyte cell membrane. Right: Merged image show the several micron thick chondrocyte surface layer between the red blood cells and the cell membrane. Scale bar: 10 µm.

Figure 2 Ring patterns generated by fluorescent particles imaged at different defocus distances. Left: 5000 nm, right: 8000 nm defocus distance.

Figures

Figure 1

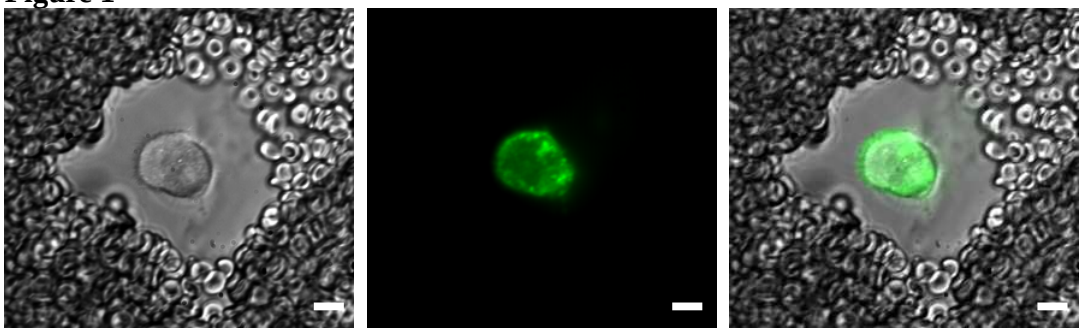

Figure 2