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Membrane-Grafted Hyaluronan Films: A Well-Defined Model System of Glycoconjugate Cell Coats

Ralf P. Richter,*,† Kai K. Hock,† Jeffrey Burkhartsmeyer,† Heike Boehm,† Pit Bingen,† Guoliang Wang,§ Nicole F. Steinmetz,‡ David J. Evans,‡ and Joachim P. Spatz†

Department of New Materials and Biosystems, Max-Planck-Institute for Metals Research, Heisenbergstrasse 3, 70569 Stuttgart, Germany, Department of Biophysical Chemistry, The University of Heidelberg, INF 253, 69120 Heidelberg, Germany, the Department of Biological Chemistry, John Innes Centre, Colney, Norwich NR4 7UH, United Kingdom, and Q-Sense AB, Redegatan 13, 42677 Västra Frölunda, Sweden

Received December 7, 2006; E-mail: ralf.richter@urz.uni-heidelberg.de

Hyaluronan (HA), ubiquitous in the extracellular space, is intriguing by virtue of its simplicity.[1] With a persistence length of ~4 nm and a contour length of up to a few micrometers, this linear polymer of identical disaccharide units forms an extended random coil in solution.[2] An important biological feature is its attachment to the surface of living cells[3] and the interaction with diverse hyaluronan binding proteins.[1,4] The soft and hydrated pericellular coats thereby created play key roles in the general protection of the cell and in the communication with its environment. Only little is currently known about the supramolecular structure of these assemblies and how it relates to properties and functions. The predominant role of water and the low degree of order in these matrices renders direct structural investigations difficult and, notwithstanding progress in the field,[1,4,5] the in vivo investigation of these coats remains a challenge. For a thorough characterization of the relationship between structure and function, it is desirable to move from living cells with their complex dynamics to model systems with tunable complexity.

Here we present a simple method to create hyaluronan films that are well-defined in their molecular attachment to the substrate, a supported lipid bilayer (SLB). The confinement to a solid support makes these model coats accessible to characterization with a range of surface-sensitive techniques that are not easily applicable on living cells. By employing such techniques, we investigate the films' thickness, grafting density, permeability, and mechanical properties.

SLBs not only provide tunable densities of adhesion sites[6] together with a background of very low unspecific binding,[7] but they also mimic the biological environment of hyaluronan.[3] We incorporated a fraction of 5% biotinylated lipids into small unilamellar vesicles (SUVs). Their exposure to glass or silica results in the formation of SLBs[8] that provide enough functional sites for stable immobilization of a monolayer of streptavidin (SA)[9] (Figure 1A,B). SLB-formation as well as coverage with SA was directly tracked and controlled by quartz crystal microbalance with dissipation monitoring (QCM-D) (Figure 1C).

Exposure of end-biotinylated HA led to immediate binding, as observed by QCM-D. Strong shifts in dissipation, D, accompanied by relatively small shifts in resonance frequency, f, are characteristic of the formation of a very soft and highly hydrated layer such as expected for hyaluronan (Figure 1C). The strong dependence of the QCM-D response on the molecular weight, M_w, of HA is remarkable: much stronger responses in f and D for short-chained hyaluronan (HA50), as compared to long-chained HA (HA1000),

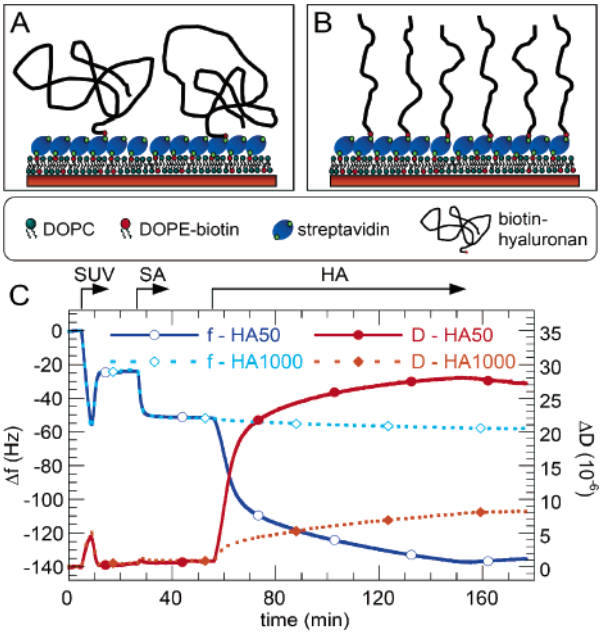

Figure 1. Immobilization of hyaluronan (HA) on a supported lipid bilayer. (A,B) Schematic illustration of the immobilization strategy (not drawn to scale). A solid-supported lipid bilayer (SLB) is formed by the exposure of small unilamellar vesicles (SUVs), containing a fraction of biotinylated lipids, to a silica or glass surface. Hyaluronan, biotinylated at its reducing end, is immobilized on the SLB via streptavidin (SA). The molecular conformation of hyaluronan is dependent on the grafting density, giving rise to a mushroomlike state (A) or a brushlike state (B). (C) Kinetics of film formation, as monitored in real time by QCM-D. All steps in the immobilization scheme can be tracked. The two-phase behavior together with the final changes in frequency and dissipation, $\Delta f = -25$ Hz and $\Delta D < 0.3 \times 10^{-6}$, upon exposure of SUVs are characteristic for the formation of an SLB of good quality.[8] An additional frequency shift of -28 Hz and small changes in dissipation upon addition of SA confirm the formation of a protein monolayer.[9] The strong increase in dissipation upon HA-binding reflects the highly hydrated and viscoelastic state of the forming film.

provide a first indication that the HA-film density increases strongly as M_w decreases. Nonbiotinylated HA did not generate any QCM-D response.

The thickness, h, of the polysaccharide films and the average distance between neighboring HA-anchoring sites, s, were determined by colloidal probe reflection interference contrast microscopy (RICM)[10] and reflectometry, respectively (Table 1, Supporting Information). A comparison of the results with the molecules' radii of gyration,[2] R_g, and contour lengths, L_c, is instructive. The maximal anchoring densities generated with our grafting-to approach were sufficient to induce significant chain stretching for all hyaluronan lengths: while HA1000 stretched to more than five times its radius

† Max-Planck-Institute and Heidelberg University.
‡ John Innes Centre.
§ Q-Sense.

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Table 1. Thickness, Effective Viscoelastic Properties, Mean Distances between Neighboring Anchoring Sites, and Hydration of Hyaluronan Films

	M_w (kDa)	L_c (nm)	R_g (nm)	h (nm)	η (mPa·s)	μ (kPa)	s (nm)	hydration
HA50	58	155	15	140 ± 10	1.26 ± 0.02	2.8 ± 2	7	98%
HA1000	1083	2891	88	530 ± 20	0.97 ± 0.02	1.3 ± 1	60	99.8%

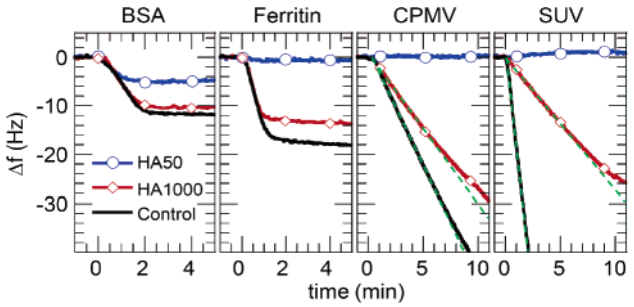

Figure 2. Permeability of hyaluronan films, as tracked by QCM-D, from the adsorption of biotinylated probes to the underlying streptavidin layer. The HA50-film is impermeable to all but the smallest probe (BSA). HA1000-films remain permeable to objects of 30 nm and more in size, although penetration is significantly delayed for CPMV and SUVs, as compared to a control without HA-film. From the constant initial adsorption rates (green dashed lines), mean diffusion constants in the films could be estimated to be >0.1, 0.2 and 0.012 $\mu m^2/s$ for ferritin, CPMV, and SUVs, respectively.

of gyration, HA50 approached the fully stretched state. This is remarkable, given that the activation barrier to adsorption increases as the HA-film builds up.[11] Although the polysaccharide concentration for HA50 is about 10 times higher than for HA1000, the hydration is generally very high (Table 1). The numbers discussed above represent the upper limits in grafting density that can be reached at reasonable time scales. Films with lower grafting densities, including the mushroom regime, can readily be created, by tuning either the number of biotin receptors on the surface or the exposure of HA.

Having established the geometry of the brushlike layers, we proceeded to determine effective viscoelastic properties of the hyaluronan films (Table 1). Fits to QCM-D data with a viscoelastic model[12] (Supporting Information) indicated at most a 40% increase in viscosity, η, compared to the properties of the aqueous solution. The shear modulus, μ, remained small but was not negligible.

To characterize the permeability of the hyaluronan films, we employed biotinylated probes of well-defined sizes. Successful penetration through the film could be detected by QCM-D, upon adsorption of the probes to membrane-bound streptavidin (Figure 2). Positive responses for biotinylated bovine serum albumin (BSA, ~7 nm in size) on all hyaluronan films confirmed that a large fraction of streptavidin binding sites were indeed still available and that the films remained permeable to objects of a few nanometers in size. Larger probes, such as biotinylated ferritin (13 nm in diameter), cowpea mosaic virus (CPMV, 28 nm) or SUVs (>30 nm) were unable to penetrate the HA50-film while permeability was retained for HA1000-films. These results suggest that the permeability of the HA-films is essentially governed by the grafting density.

Adsorption was significantly delayed for the largest probes investigated as compared under identical flow conditions to streptavidin-covered SLBs without HA-films. From the decrease in adsorption rate under laminar flow, a mean diffusion constant of the probes in the HA-film could be estimated (Figure 2, Supporting Information). The diffusion constant for CPMV in the HA1000-film was found to be about 30 times smaller than the diffusion constant in aqueous solution. Suitable biotinylated probes thus allow for a systematic screening of thin film permeability for objects of different sizes ranging from a few to several hundred nanometers.

Taken together, our data provide evidence for the successful formation of end-grafted hyaluronan films that are sufficiently dense in their packing to induce considerable molecular stretching. With the exploited immobilization strategy, a well-defined albeit simple platform is available that allows for a detailed and quantitative investigation of the physicochemical properties of hyaluronan assemblies. A tunable density of anchor points and the use of end-modified hyaluronan molecules of controlled size provide a level of control that distinguishes our approach from those taken earlier.[13]

While the investigation of such films may be interesting in its own right, the underlying principles are expected to have important biological implications. The model platform, together with the outlined characterization techniques, offers the possibility to directly investigate the effect of environmental cues, such as the presence of hyaluronan-binding proteins, on the structure and functional properties of hyaluronan films. Similar approaches may also prove useful for other types of biological molecules that exhibit highly hydrated and extended structures, including mucins, and thus present a new tool for glycobiology.

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Supporting Information Available: Materials and Methods; 3W-RICM measurements; reflectometry; viscoelastic modeling of QCM-D data; determination of diffusion constants. This material is available free of charge via the Internet at http://pubs.acs.org.

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