THE INFLAMMATION-ASSOCIATED PROTEIN TSG-6 CROSS-LINKS HYALURONAN VIA HYALURONAN-INDUCED TSG-6 OLIGOMERS

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Tumor necrosis factor-stimulated gene-6 (TSG-6) is a hyaluronan (HA)-binding protein that plays important roles in inflammation and ovulation. TSG-6 mediated cross-linking of HA has been proposed as a functional mechanism (e.g., for regulating leukocyte adhesion), but direct evidence for cross-linking is lacking, and we know very little about its impact on HA ultrastructure. Here we used films of polymeric and oligomeric HA chains, end-grafted to a solid support, and a combination of surface-sensitive biophysical techniques, to quantify the binding of TSG-6 into HA films, and to correlate binding to morphological changes. We find that full length TSG-6 binds with pronounced positive cooperativity, and demonstrate that it can cross-link HA at physiologically relevant concentrations. Our data indicates that cooperative binding of full length TSG-6 arises from HA-induced protein oligomerization, and that the TSG-6 oligomers act as cross-linkers. In contrast, the HA-binding domain of TSG-6 (the Link module) alone binds without positive cooperativity, and weaker than the full-length protein. Both Link module and full length TSG-6 condensed and rigidified HA films, and the degree of condensation scaled with the affinity between the TSG-6 constructs and HA. We propose that condensation is the result of protein-mediated HA cross-linking. Our findings firmly establish that TSG-6 is a potent HA cross-linking agent and might hence have important implications for the mechanistic understanding of the biological function of TSG-6, e.g., in inflammation.

Hyaluronan (HA) is a structurally simple and linear polysaccharide. It is ubiquitous in the extracellular matrix of vertebrates and plays important roles in numerous physiological and pathological processes, such as inflammation, fertilization, embryogenesis, tumor development, osteoarthritis and atherosclerosis (1,2). HA is considered a 'pericellular cue' (3), i.e., it serves as a versatile scaffold within which other molecules are organized and regulated. A number of proteins, called hyaladherins (4), can bind to the flexible HA chains and engender self-assembly into large and hydrated multimolecular complexes (5-7).

The secreted product of tumor necrosis factor-stimulated gene-6 (TSG-6) (8,9), is of particular importance for the formation and remodeling of HA-rich pericellular coats (10,11) and extracellular matrices (12). There is little or no constitutive expression of TSG-6 in most adult tissues (with the exception of bone marrow (13) and epidermis (14)). Expression is elevated in response to stimulation with pro-inflammatory
mediators or certain growth factors (8,9,15-18), and TSG-6 is detected in the context of many inflammatory diseases (19,20) and in inflammation-like processes such as ovulation (21,22).

TSG-6 is composed mainly of two contiguous domains, a Link module and a CUB module (8,17,23,24). The Link module is conserved among members of the hyaladherin family (4), and essential for binding to HA (23). Administration of recombinant human TSG-6 Link module (Link_TSG6) \textit{in vivo} or in cell culture has frequently been found to elicit biological responses comparable to those of endogenously produced TSG-6 (13,25-28), suggesting that Link_TSG6 is a useful model for full length TSG-6. Indeed, most of our current knowledge about the interaction between HA and TSG-6 comes from structural studies and \textit{in vitro} binding assays on Link_TSG6. In contrast, only a few plate-based HA-binding assays have been reported for full length TSG-6 (29-31).

Little is known about the function of the TSG-6 CUB module, although the fact that it is highly conserved between species suggests that it is important for at least some activities of TSG-6 (8,31). A case in point is that the TSG-6 mediated covalent transfer of heavy chains of inter- \( \alpha \) -inhibitor (I\( \alpha \)I) on to HA requires the full-length protein (26,32). Furthermore, we have recently identified that the CUB_C domain of TSG-6 (i.e. the CUB module together with the C-terminal region) mediates the binding of TSG-6 to fibronectin (24).

Some of the functions of TSG-6 are clearly related to its ability to bind to HA. Moreover, TSG-6 and HA were reported to form stable complexes in solution that enhance or induce binding of HA to the cell surface receptor CD44 on lymphocyte cell lines (25). Based on this observation, it was proposed that either Link_TSG6 or full length TSG-6 alone can cross-link HA and that cross-linking is functionally important in the regulation of inflammation (6,25).

TSG-6 has a wide range of binding partners. Apart from HA, the protein interacts with many other glycosaminoglycans, such as chondroitin-4-sulfate, dermatan sulfate, heparin/heparan sulfate, with the G1 domains of the proteoglycans aggrecan and versican, and with a number of other extracellular proteins, including, inter-\( \alpha \)-inhibitor (I\( \alpha \)I), pentraxin 3 (PTX3) and thrombospondin 1 (26,28,33-37) (reviewed in (6,8,9)). Simultaneous binding of TSG-6 to HA and to either of these latter proteins has been suggested as alternative routes for HA cross-linking (6,28).

To obtain direct evidence for and to understand the mechanisms behind TSG-6 mediated cross-linking, it would be desirable to be able to study the interaction between TSG-6 and a well-defined supramolecular assembly of HA. Here, we present a novel experimental approach that realizes this goal. The method is based on the immobilization of either polymeric or oligomeric HA \textit{via} their reducing end to protein-repellent planar solid supports. With a combination of several surface-sensitive biophysical techniques, namely quartz crystal microbalance with dissipation monitoring (QCM-D), ellipsometry, and colloidal probe reflection interference contrast microscopy (RICM), we have quantified the binding kinetics of both recombinant human full-length TSG-6 (rhTSG-6) and Link_TSG6 to HA, and correlated the binding to morphological changes of HA films. Our results provide novel insights into the molecular mechanism of HA cross-linking by TSG-6, and the properties of the ensuing HA supramolecular complexes, which have far-reaching implications for the potential effect of TSG-6 \textit{in vivo}.

\section*{EXPERIMENTAL PROCEDURES}

\textit{Protein and hyaluronan preparations.} Wild type human TSG-6 Link module (Link_TSG6, 10.9 kDa (38)) was expressed in \textit{Escherichia coli} as described previously (38,39). Full length recombinant human TSG-6 (rhTSG-6; 30.1 kDa (31)) was expressed in \textit{Drosophila} Schneider 2 cells and purified as described previously (31). Lyophilized streptavidin (SAv; Sigma) was taken up in ultrapure water. Stock solutions of all proteins, typically at 1 mg/ml, were aliquoted and stored at -20°C.

Lyophilized polymeric HA (pHA), biotinylated by guest on September 16, 2017 http://www.jbc.org/ Downloaded from www.jbc.org by guest on September 16, 2017 save as 20°C. Stock solutions of all proteins, typically at 1 mg/ml, were aliquoted and stored at -20°C.

Lyophilized polymeric HA (pHA), biotinylated at its reducing end and with well-defined molecular weights of 58±3 kDa (Select-HA B50) and 1080±56 kDa (Select-HA B1000) was purchased from Hyalose (Oklahoma City, OK, USA), as well as non-biotinylated HA of
262±13 kDa (Select-HA 250). For reconstitution, HA was taken up in ultrapure water at a stock concentration of 1 mg/ml, gently shaken overnight, aliquoted, and stored at -20°C. HA oligomers (oHA) with D-glucuronic acid at both termini, and with a ~3 nm spacer and a biotin moiety at their reducing end, were prepared as described in the supplemental data.

A ‘Hepes’ buffer (150 mM NaCl, 10 mM HEPES at pH 7.4, 3 mM NaN₃, 2 mM CaCl₂ in ultrapure water) was used throughout all measurements, and protein and HA solutions at their final concentrations were prepared in this buffer, except where otherwise stated.

Preparation of protein repellent, biotin-functionalized surface coatings. Supported lipid bilayers (SLBs) on silica or glass surfaces were formed by the method of vesicle spreading (40), using small unilamellar vesicles that contained dioleoylphosphatidylethanolamine-cap-biotin (DOPE-CAP-biotin) and dioleoylphosphatidylcholine (DOPC) (both Avanti Polar Lipids, Alabaster, AL, USA) at a molar ratio of 1:9. Gold surfaces were functionalized with a dense oligoethylene glycol (OEG) monolayer by incubation in an ethanolic solution of a biotin-OEG disulfide (SS-OEG-biotin) and plain OEG disulfide (SS-OEG; both Polypure, Oslo, Norway) at a molar ratio of 1:99 (see supplemental data for details).

Assembly of HA films on SLBs and OEG layers. HA films were prepared as described previously for SLBs (41). Briefly, a dense SAv monolayer was formed by exposure of 10 μg/ml SAv (30 min) to a biotinylated SLB or OEG layer. Biotinylated HA was then grafted to the SAv monolayer by incubation of 1 to 10 μg/ml HA solution (Figs. S1-2). Samples were kept wet at all times.

Tuning of HA concentration and incubation time allowed for the HA density on the surface to be controlled in a quantitative manner (Figs. S3). The grafting density was set to 65±5 ng/cm² for 58 kDa HA in QCM-D measurements, and to approximately 35 ng/cm² for 1080 kDa HA in RICM measurements (Fig. S3), corresponding to mean anchor distances of 13 and 28 ng/cm², respectively. The surface density of oHA ranged between 13 and 28 ng/cm², corresponding to mean distances of 6.6 to 4.5 nm.

Quartz crystal microbalance with dissipation monitoring. QCM-D measures changes in resonance frequency, Δf, and dissipation, ΔD, of a sensor crystal upon interaction of (soft) matter (e.g. biomolecules) with its surface (42). The QCM-D response is sensitive to the mass (including coupled water) and the viscoelastic properties of the surface adlayer. Adsorption and interfacial processes on silica or gold-coated QCM-D sensors were monitored in situ with sub-second time resolution under continuous flow of sample solution (43) (see supplemental data for details).

In situ ellipsometry. Ellipsometry measures changes in the polarization of light upon reflection at a planar surface. We employed ellipsometry in situ on silica or gold-coated silicon wafers as substrates that were installed in a custom-built open cuvette with a continuously stirred sample solution, to quantify adsorbed biomolecular masses in a time-resolved manner (43) (see supplemental data for details).

Quantification of binding constants. The binding constant, K₀.₅, and the saturation limit, Γₘₐₓ, for the binding of TSG-6 to HA films was obtained from the surface densities at equilibrium, Γₑq, as a function of the TSG-6 bulk concentrations, by numerical fitting to the Hill equation:

\[
\Gamma_{\text{eq}} = \Gamma_{\text{max}} \frac{[\text{TSG-6}^n]}{K_{0.5}^n + [\text{TSG-6}^n]},
\]

with the Hill coefficient n being a measure for the cooperativity of binding (44). K₀.₅ corresponds to the bulk concentration at which half-maximal binding occurs. For non-cooperative binding \((n = 1)\), it equals the dissociation constant K_D. [TSG-6] relates to the concentrations of either Link_TSG6 or rhTSG-6. Dissociation rate constants, k₀ff, were derived from an exponential fit:

\[
\Gamma = \Gamma_{r} e^{-k_{\text{off}} \Delta t} + \Gamma_{ir},
\]

or from a double exponential fit:

\[
\Gamma = \Gamma_{r}^{(1)} e^{-k_{\text{off}}^{(1)} \Delta t} + \Gamma_{r}^{(2)} e^{-k_{\text{off}}^{(2)} \Delta t} + \Gamma_{ir}
\]

to the desorption curve upon rinsing in pure buffer solution. Here, Γ is the TSG-6 surface...
density, and $\Delta t$ is the rinsing time. $\Gamma_r$ and $\Gamma_{ir}$ are the surface density of reversibly and irreversibly bound TSG-6, respectively.

Colloidal probe reflection interference contrast microscopy (RICM). This microinterferometric technique measures the height at which a colloidal probe hovers above a transparent planar substrate with a resolution of a few nanometers. RICM was used to measure the thickness of HA films that were assembled on bare or gold-coated glass cover slips, using custom-built liquid cells (41,45) (see supplemental data for details).

RESULTS

Design of HA model films to study TSG-6 hyaluronan interactions. To analyze the interaction of TSG-6 with HA, we designed surface-confined films that present HA of selected molecular weights in a well-defined supramolecular assembly (Fig. 1). The construction of the films was monitored and controlled by QCM-D (Figs. S1-2).

In a first model system, polymeric hyaluronan (pHA) with a molecular weight of either 58 kDa or 1080 kDa was immobilized via a biotin-tag at its reducing end to solid supports that were previously functionalized with a protein-repellent coating and a dense monolayer of streptavidin (Fig. 1A). The grafting density can be controlled and a highly hydrated film of partly stretched and entangled HA chains with up to several 100 nm in thickness, a so-called HA brush, is formed (41). Assuming a footprint of HA10 per TSG-6 protein (29,46), each pHA chain of 58 and 1080 kDa can accommodate up to 30 and 571 TSG-6 molecules, respectively.

In a second approach, short oligomeric HA chains, comprising only 9 monosaccharides (oHA), were grafted to the surface (Fig. 1B). Grafting was again mediated by a biotin-tag. A linker of about 3 nm in contour length (47) was placed between the biotin and the reducing end of HA to enhance the conformational freedom of the surface-bound oHA chains. Each oHA chain is 4.5 nm long and can interact with only one TSG-6 molecule at a time, where previous studies have shown that ~5 sugar monomers likely fill its HA-binding site (48).

Two different primary surface functionalizations were employed: (i) silica-supported lipid bilayers (SLBs) exposing biotin groups (Fig.S1) and (ii) gold-supported oligoethylene glycol (OEG) films exposing biotin groups (Fig. S2). The properties of the HA films were not affected by the choice of the primary surface-layer (Fig. S4). SLBs were used for all studies related to Link_TSG6. OEG-covered surfaces were used for rhTSG-6 as they were found to exhibit better passivation against nonspecific binding of this protein (Fig. S5A).

Binding of full length TSG-6 to HA films. To obtain quantitative insight into the kinetics of rhTSG-6 binding, we performed titration assays on oHA and pHA films using in situ ellipsometry (Fig. 2). The titration curves (Fig. 2C) exhibited a pronounced sigmoidal shape. The curves could be fitted well by the Hill equation (Eq. 1) with exponents above 2, indicating that binding is cooperative. The exponents for pHA and oHA (2.7±0.8 and 2.8±0.5, respectively) coincided within experimental error (Table 1). The $K_{0.5}$ for pHA was similar to although slightly lower than for oHA (Table 1). On oHA, we found a stoichiometry of 2.7±0.8 rhTSG-6 molecules per oHA chain (Table 1), indicating that more than one rhTSG-6 molecule can on average associate with a single oHA chain.

What is the origin of the cooperative binding? One possibility that has been proposed previously (5,29) is that cooperativity arises from facilitated binding of several proteins to adjacent binding sites on the same HA chain. In light of the similar Hill coefficients and $K_{0.5}$ for pHA (with many binding sites per chain) and oHA (with a single binding site) this scenario is unlikely. Facilitated binding of a second (or more) rhTSG-6 molecules must hence occur either on another HA chain (or distant binding sites on the same pHA chain), or on the HA-bound rhTSG-6, or both. In fact, the supra-stoichiometric binding of rhTSG-6 to oHA provides strong indications that the binding of one rhTSG-6 molecule to HA is sufficient to induce the formation of protein dimers (or even larger oligomers). In this regard, we have observed previously that rhTSG-6 forms elongated end-to-end dimers in the presence of excess HA$_8$, as well as larger species (49).

The dissociation of rhTSG-6 from HA films was generally slow. Only double exponentials (Eq. 2b) provided a good fit to the desorption curves upon rinsing of close-to-saturated HA
films with buffer (Fig. 2A-B; Table 1). The fit did not reveal a significant fraction of irreversibly bound rhTSG-6 ($\Gamma_{ir} = 0$). The first dissociation rate constant was approximately $4 \times 10^{-3} \text{s}^{-1}$ for oHA, and slightly smaller for pHA ($\sim 3 \times 10^{-3} \text{s}^{-1}$). Notably, almost 50% of the protein on oHA but less than 10% on pHA desorbed with the faster dissociation rate. The second dissociation rate constant was at least one order of magnitude smaller than the first, and slightly larger for pHA. One might be tempted to attribute the two apparent rate constants to two discrete unbinding events. The numbers are, however, also consistent with the presence of a spectrum of dissociation rates. The latter would be expected for the formation of a wide range of HA/TSG-6 complexes where the different interactions (e.g. HA/protein and protein/protein) likely have different stabilities.

Given the slow dissociation, not all rhTSG-6 could be unbound within experimentally accessible time scales. However, the remaining fraction could readily be eluted by 8 M of the dissociating agent guanidine hydrochloride (GuHCl; Fig. 3). In contrast, the attachment of HA to our passivation layers via biotin and streptavidin was not disrupted by GuHCl (Fig. S7). rhTSG-6 hence does not form covalent complexes with HA, as had been suggested based on microtiter plate-based HA-binding assays (30).

Full-length TSG-6 oligomers can cross-link HA. To test if rhTSG-6 that is bound to HA films can still bind additional HA, a “sandwich” assay by QCM-D was designed (Fig. 4). rhTSG-6 was first exposed to an oHA film. The total frequency shift for the oHA/rhTSG-6 film was -34 Hz. The corresponding thickness of approximately 6 nm and the minor changes in dissipation are consistent with the formation of a rather dense layer of rhTSG-6; a control measurement on an HA-free surface did not show measurable binding of active rhTSG-6 (i.e. the small amount of non-specifically bound rhTSG-6 was not able to bind to HA; Fig. S5B). This confirms that the rhTSG-6 protein was indeed immobilized via its binding to oHA and that rinsing in buffer induced a slow release of TSG-6 (between 62 and 67 min in Fig. 4A).

Subsequent rapid incubation with pHA (262 kDa) resulted in a two-phase response (Fig. 4A). Initially, the frequency decreased rapidly, together with a pronounced increase in dissipation. Such a response is typical for the formation of a soft layer, as would be expected for the binding of pHA on top of the TSG-6 covered oHA film. For comparison, no pHA binding was observed on a rhTSG-6 free surface (Fig. S5C). Clearly, full length TSG-6 dimers (or higher oligomers) that had already bound to oHA retained the ability to bind additional HA. In the second phase, both the frequency and the dissipation shift increased slowly, suggesting remodeling of the surface-bound film. The rate of frequency increase changed after removal of pHA from the solution phase, indicating that supply of unbound HA must be involved in this process. A plausible explanation for the observed response would be that pHA captures some of the rhTSG-6 that is slowly released from the oHA film, and thanks to the cross-linking activity of rhTSG-6 a multilayer of pHA is formed on top of the oHA film (Fig. 4B-C).

The cooperative binding of rhTSG-6, and its ability to cross-link HA have interesting implications with respect to the stability of rhTSG-6 oligomers in the absence of HA. If rhTSG-6 alone would form stable oligomers, their binding into the HA matrix would correspond to a multivalent interaction. Although multivalent binding would most likely exhibit an increased avidity, as compared to monovalent binding, it would not be cooperative. The observed cooperativity hence implies that the oligomeric state of rhTSG-6 is induced (or stabilized) by HA.

With respect to cross-linking, the binding behavior of rhTSG-6 to oHA films merits detailed consideration. The average spacing between neighboring oHA strands, 4 to 7 nm, is comparable to the size of TSG-6; TSG-6 can be estimated to be ~6-7 nm in length (from the $D_{\text{max}} = 13 \pm 1 \text{nm}$ determined for TSG-6 dimers from small angle X-ray scattering (49); see Fig. 4C), which is consistent with the structures of the Link module (50) and CUB module domains (PDB 2WNO). rhTSG-6 oligomers are hence sufficiently large to interconnect neighboring oHA chains. The immobilization of oHA to a surface, on the other hand, imposes constraints with respect to the exact distance between and orientation of oHA chains, which may hamper cross-linking. These steric constraints might explain, why the binding stoichiometry at
saturation is significantly larger than 1.0, as expected if the surface-confined rhTSG-6 oligomers would mostly connect to only a single oHA chain. This is also consistent with the finding that immobilized TSG-6 is still able to bind pHA from the solution phase (Fig. 4A).

**Full-length TSG-6 induces strong condensation of HA films.** Next, we investigated how the influx of rhTSG-6, and the ensuing cross-linking, affect the overall morphology of pHA films. Exposure of rhTSG-6 to a pHA (58 kDa) film induced a monotonous and strong decrease in the QCM-D frequency response (Fig. 5A). In contrast, the dissipation initially increased and then decreased. The decrease in particular is indicative of a rigidification of the pHA film.

We employed colloidal probe RICM to quantify the variations in film thickness upon rhTSG-6 addition (Fig. 5B). To extend the range of potential thicknesses, we used HA of larger molecular weight (1080 kDa), while maintaining the total mass of HA per surface area comparable to the previously described pHA films (Fig. S3). Titration of rhTSG6 initially resulted in a gradual decrease in film thickness. Significant film condensation was already observed at 0.16 µM rhTSG-6 in the bulk solution, and the film thickness decreased by more than twofold, from 400 to 180 nm, upon exposure to 0.33 µM rhTSG-6 protein. Based on the kinetic data that we had obtained by ellipsometry (Table 1), a bulk concentration of 0.33 µM would result in an average occupancy of one rhTSG-6 molecule per 44 HA2 repeating units (one protein every HA88; Fig. 5B, inset), which is equivalent to one protein every 44 nm of HA contour length, or ~65 TSG-6 molecules per HA chain.

How can this rather low occupancy induce such a strong condensation of the HA films? In the absence of TSG-6, the surface-grafted pHA films form a so-called polymer brush (41). Such a brush can be pictured as a strongly hydrated and highly dynamic meshwork of entangled polymer chains that are weakly stretched in the direction perpendicular to the surface (51,52) (Fig. 6A). The water content in the 1080 kDa pHA films that we used in the RICM measurements, for example, is approximately 99.9%, as can be calculated from the grafting density (35 ng/cm²) and the film thickness (400 nm). Polymer theory (51,52) predicts that the size of the meshes (or ‘holes’) in the pHA meshwork is comparable to the mean distance between the anchor points of neighboring HA chains (~80 nm for 1080 kDa pHA films). The introduction of cross-linkers will force neighboring HA chains closer together and decrease the mesh size (Fig. 6B). As a result, the film’s thickness will decrease while its rigidity will increase. Given that the films have a large mesh-size to start with, a rather small amount of cross-linkers can already have an appreciable effect on the film thickness. The above considerations illustrate that cross-linking is an efficient route for the condensation of HA films, and rationalize the increase in rigidity and the decrease in thickness that we have observed upon introduction of rhTSG-6 in pHA films (Fig. 5).

Maximal film collapse, to about 100 nm, was induced at a protein concentration of 0.7 µM in the bulk solution. This corresponds to an occupancy of one rhTSG-6 per about 10 HA2 repeating units (HA20), and a protein concentration inside the film of around 1.0 mM, or 30 mg/ml. Notably, the film thickness did not decrease further, but instead slightly increased upon further increasing the rhTSG-6 bulk concentration to 2.3 µM. At this point, the rhTSG-6 concentration inside the HA film reached a value of approximately 2.3 mM, or 70 mg/ml, i.e. TSG-6 occupied a significant fraction (about 5%) of the total volume in the HA film. We propose that crowding of rhTSG-6 prevents further film condensation. The thickness of the maximally collapsed film did not change over a period of at least one hour following removal (by rinsing) of the remaining rhTSG-6 in solution, confirming that release of rhTSG-6 is slow and that the protein/HA complexes are rather stable, as previously observed by ellipsometry (Fig. 2A).

**Binding of Link_TSG6 to HA films.** To compare the HA-binding behavior of full-length TSG-6 with that of the Link module, Link_TSG6 was titrated into both oHA and pHA (58 kDa) films, and the binding kinetics were quantified by ellipsometry (Fig. 7). The titration curves (Fig. 7C) could be fitted well with Eq. 1 and a Hill coefficient of ~1, revealing dissociation constants of approximately 5 µM (Table 1). The Hill coefficient of unity, and similar KD values for oHA and pHA, indicate that interactions between adjacent binding sites along a given polymeric
HA chain do not affect binding. Furthermore, Link_TSG6 bound stoichiometrically to oHA at saturation (Table 1). All these findings are consistent with a simple one-site model, i.e. in which all binding sites are identical (as also found in recent confocal-FRAP analysis of the binding of Link_TSG6 to pHA in solution phase (53)), in stark contrast to rhTSG-6.

Overall, the observed simple, non-cooperative binding, and the micromolar affinities for Link_TSG6 are consistent with earlier solution-phase studies by calorimetry on HA oligosaccharides (29,46,50,54) and by confocal fluorescence recovery after photobleaching on polymeric HA (53). However, it should be noted that these measurements were performed at different pH and ionic strength to those used here, and that both parameters have been found to significantly affect the binding affinity (29,34,53,54). A detailed quantitative comparison of the binding affinities is hence far from trivial.

For polymeric HA, we found a stoichiometry of 2.9±0.3 HA disaccharides per Link_TSG6 at saturation (Table 1). This number is similar to the minimum size of HA oligomer that binds with maximal affinity to Link_TSG6 (HA 7 with D-glucuronic acid at both termini (50)), but it is smaller than the decameric footprint that has been reported based on calorimetric binding studies with HA oligosaccharides of different size (29).

The desorption curves upon rinsing of close-to-saturated HA films with buffer could be fitted well by simple exponential fits (Eq. 2a; Fig. 7A-B). The dissociation rate constants (Table 1) for Link_TSG6 were similar for oHA and pHA films, but more than 5-fold larger than the fastest desorption rates observed for rhTSG-6. Most Link_TSG6 could be readily dissociated within experimental time scales, although a proportion of the protein could not be removed by rinsing in Hepes buffer, i.e. ~10% and ~25% for the oHA and pHA films, respectively (Fig. 7A-B). Control measurements with Link_TSG6 on HA-free surfaces (Fig. S6A) revealed minor nonspecific binding that was comparable in magnitude to the irreversibly bound amounts on oHA films. It remains unclear if the significantly larger amount of irreversibly bound Link_TSG6 protein in pHA films is specifically bound to HA. It might also reflect some tendency of the protein to aggregate at the high concentrations reached in the HA film. In this regard, Link_TSG6 has been found to be stable at concentrations in solution phase up to at least 2 mM in the presence of a 1:1 molar ratio of HA (50), although precipitation has been reported under some conditions in the presence of a larger HA oligomer (29). However, the protein fraction that remained bound upon rinsing in buffer could readily be eluted in 8 M GuHCl (Fig. 7A), i.e. Link_TSG6, as rhTSG-6, did not engage in a covalent interaction with HA.

Condensation of HA films by Link_TSG6. Next, we tested how Link_TSG6 affects the morphology of pHA films, as compared to rhTSG-6. Titration of Link_TSG6 at bulk concentrations ranging from 0.05 to 5 μM into pHA (58 kDa) films was monitored by QCM-D (Fig. 8A). A remarkable decrease in dissipation, concomitant with a decrease in frequency, occurred at concentrations above 3 μM, indicating rigidification of the film (43,55,56); in comparison, we had observed a similar decrease in dissipation already at a 8-fold lower concentration of rhTSG-6 (Fig. 5A). The QCM-D responses reversed upon gradually decreasing the Link_TSG6 concentration in solution. Compared to the responses at increasing protein concentrations, a hysteresis was observed, i.e. the process was only partly reversible.

Colloidal probe RICM revealed a gradual decrease in thickness upon titration of close-to-saturated HA films with Link_TSG6 from 427±4 nm in the absence of proteins to 66±16 nm in the presence of 10 µM Link_TSG6. Stepwise elution of Link_TSG6 resulted in a gradual thickness increase. A hysteresis in thickness between adsorption and desorption processes (Fig. 8B) was consistent with QCM-D data (Fig. 8A) and the irreversibly bound protein fraction observed by ellipsometry (Fig. 7A).

In order to compare the potency of rhTSG-6 and Link_TSG6 to condense HA films, it is useful to consider the degree of condensation as a function of occupancy of HA chains with proteins, rather than the bulk protein concentration (Fig. 8C). At low occupancies, full length TSG-6 was considerably more potent in condensing HA than Link_TSG6. To reach a twofold decrease in thickness, for example, only one rhTSG-6 per 44 HA 2 repeating units (HA 88), but more than one Link_TSG6 per 6 HA 2 repeating units (HA 12), were required.
Within experimental error, the film thickness decreased linearly with the occupancy for Link_TSG6. At the maximal experimentally assessed protein uptake, one Link_TSG6 per 4.0 HA₃ repeating units (HA₈) (which is somewhat higher than the stoichiometry at saturation predicted from the Hill equation, Table 1), the film thickness had attained approximately 15% of its original value, and the concentration of Link_TSG6 inside the film was approximately 3.2 mM, or 35 mg/ml.

Several mechanisms might be considered for the rigidification and condensation of pHA films by Link_TSG6. In analogy with our arguments for rhTSG-6 (Fig. 6), cross-linking is one possible driving force. It would be probable that Link_TSG6 dimers (or larger oligomers), rather than monomers, act as cross-linkers: only about 6 carbohydrate monomers are on average available per Link_TSG6 at saturation in pHA films (Table 1); if a monomer was sufficient for cross-linking, then the average footprint per HA chain would need to be an HA trisaccharide unless two Link_TSG6 molecules can simultaneously bind to the same part of the HA chain. Based on the structure of the HA binding site (48,50), this is unlikely. Furthermore, it should be noted that previous studies on Link_TSG6 by analytical ultracentrifugation (46) and NMR spectroscopy (57) indicated that, in solution phase, this protein domain is monomeric in both the absence and presence of HA₈.

An alternative route towards condensation and rigidification of HA films would be via the condensation and/or rigidification of individual HA chains. Recent molecular modelling studies on Link_TSG6 suggest that HA chains bend locally in order to fit into the HA-binding site,³ as is the case for CD44 (58), and such bending might induce an apparent chain shortening. The bending is pronounced, however, it is unclear at present whether alone it could explain the more than 6-fold decrease in film thickness that we have observed. It has also been proposed that the dense coverage of an HA chain with many hyaladherins, like beads on a string, induces or stabilizes distinct HA conformations (5), that are more condensed and/or more rigid than the free HA chain. An increase in chain rigidity would decrease the entropically driven stretching of the individual chains in the HA brush in the direction perpendicular to the surface (51,52), and might thereby induce re-arrangement into a thinner but more densely packed film. The final film density and the linear relationship between film thickness and occupancy would be consistent with such a scenario. Based on the present experimental data, it appears difficult to exclude any of the above mechanisms, which might even act jointly.

Link_TSG6 induces total film collapse at low ionic strength. Previous studies have shown that the affinity of Link_TSG6 increases strongly with decreasing ionic strength, with sub-µM affinities being reached at 5 mM sodium ions (50,54). To test how such an increase in affinity can influence the morphology of HA assemblies, we analyzed the Link_TSG6 induced remodeling of films made from 58 kDa and 1080 kDa HA by QCM-D and by RICM, respectively, at low salt strength (Fig. 9).

Upon addition of 1 µM Link_TSG6 to a pHA film (58 kDa) in 5 mM NaCl, both frequency and dissipation decreased (Fig. 9A). The decrease in dissipation, from 9.3×10⁻⁶ to 1.5×10⁻⁶, was dramatic, and revealed total brush collapse, into a rigid and most likely very dense film. From the frequency response for the collapsed film, we can estimate a thickness of approximately 8 nm. Only minor responses were observed on a pHA free surface (Fig. S6B-C), confirming that the collapse is the result of the interaction between Link_TSG6 and pHA. The dramatic film condensation and rigidification suggests that some form of cross-linking might be at play, although the exact mechanism remains obscure. The film remained fully collapsed after rinsing with 5 mM NaCl buffer. It recovered a swollen conformation at physiological ionic strength, confirming that the strong interaction and film collapse is salt dependent and at least partly reversible.

The results by RICM (Fig. 9B) on pHA (1080 kDa) films correlated with the QCM-D data. Addition of a small concentration (0.1 µM) of Link_TSG6 already decreased the thickness of the pHA (1080 kDa) film by more than 2-fold, and concentrations of 1 µM induced a 15-fold reduction. Again, in 150 mM NaCl the brush thickness was partly restored, to about 65% of the thickness at that salt strength in the absence of protein.

These findings indicate that the affinity of Link_TSG6 for HA at different salt strengths
affects its ability to rigidify and condense HA films. Interestingly, the potency of Link_TSG6 to condense HA at low ionic strength exceeds that of rhTSG-6 at physiological salt concentration. There is hence a notable correlation between the degree of pHA film condensation on the one hand, and the affinity between the TSG-6 constructs and HA on the other.

DISCUSSION

Using a novel experimental platform, based on films of end-grafted HA, in conjunction with a toolbox of surface-sensitive characterization techniques, we have investigated the interaction of TSG-6 with HA in an ultrastructural context. The experimental platform is interesting for several reasons. First, the morphology and quantity of immobilized HA is well-controlled and non-specific binding to the underlying surface is low. These are prerequisites for detailed and quantitative binding studies. Second, the binding behavior on both oligomeric and polymeric HA can be interrogated on the same immobilization platform and directly compared. Third, with a toolbox of surface-sensitive characterization techniques, the amount and kinetics of protein binding can be correlated with changes in the physicochemical properties, such as the dimensions, mechanical properties and morphology, of the resulting HA ultrastructures.

Key results of our study are the direct experimental evidence that full-length TSG-6 alone can cross-link HA (Fig. 4), and that the cross-linking induces condensation of HA at what are likely to be physiologically relevant concentrations (Fig. 5). Furthermore, our study provides novel and quantitative insights into the interaction between TSG-6 and HA. The cooperative binding of rhTSG-6 to both oHA and pHA (Fig. 2), and its supra-stoichiometric binding to oHA (Table 1) provide evidence for HA-induced protein oligomerization.

The HA-binding behavior of Link_TSG6 at physiological ionic strength is distinctly different from that of rhTSG-6; binding of the former is both simpler and weaker. The lack of positive cooperativity might well explain the weaker binding of Link_TSG6. We can though not rule out that, in addition to the Link module, some other part of the TSG-6 protein makes a contribution to HA binding. This could occur in a number of ways, for instance, via a direct interaction of a neighboring region of the protein with the bound HA (e.g. an extension of the Link module binding groove (48,50)) or via an allosteric effect on the affinity of the Link module (e.g. stabilizing the Link module in its bound conformation (50,57)).

Although less potent than rhTSG-6, Link_TSG6 retains the ability to condense and rigidify HA films. At present, the sizes of the HA-induced rhTSG-6 oligomers and the mechanism underlying Link_TSG6-mediated HA-film condensation and rigidification remain unclear. The observed Hill coefficients above 2 on oHA and pHA films, and the maximal binding stoichiometry above 2 on oHA films (Table 1) indicate that rhTSG-6 must form oligomers that are larger than dimers. Small angle X-ray scattering data suggested the formation of rhTSG-6 dimers (Fig. 4C) as well as larger species in the presence of HA octasaccharides in solution (49), and it is possible that a spectrum of oligomer sizes is also present in our HA films.

Our observations that rhTSG-6 binds HA more strongly than Link_TSG6 (Table 1), and that both protein constructs can be fully released from HA by a dissociating agent (Figs. 3 and 7A) contrast an earlier study (30) that had reported similar binding properties and non-dissociable HA-TSG-6 complexes for full length TSG-6 and the TSG-6 Link module in plate binding assays with immobilized HA. These assays, however, were performed at a non-physiological ionic strength of 500 mM NaCl. This, and the limited control on the immobilization of analytes in these plate binding assays, might explain the discrepancies with our study.

Implications for the function of TSG-6 in vivo. For pHA films containing a concentration of approximately 1 mg/ml HA, we observed significant condensation already at 0.16 μM rhTSG-6, and maximal condensation was attained at 0.7 μM (Fig. 5). These concentrations are likely to be physiologically relevant. For example, HA is present in synovial fluid and umbilical cord at >1 mg/ml (59) and is also found at high concentrations in other tissues such as skin (~0.2 mg/ml (59)) and the expanded cumulus matrix surrounding the mature oocyte (~0.2-0.5 mg/ml (60)). While it has been suggested that
TSG-6 is present at up to ~0.6 μM in synovial fluids from patients with inflammatory arthritis (i.e. based on data from Western blots (18)), more recent ELISA analysis suggests a maximum of ~3 nM (61). However, given that the likely source of this protein is synovium and cartilage (62), its local concentration in certain regions of these tissues is likely to be much higher than this (e.g. in the HA-containing pericellular matrix of chondrocytes that are synthesizing TSG-6). Similarly, there is likely to be a high local concentration of TSG-6 in the HA-rich cumulus matrix formed during cumulus-oocyte-complex (COC) expansion, prior to ovulation (21,22). Thus, the effects that we observed in vitro may have physiological relevance. The TSG-6 mediated cross-linking has two major consequences, condensation and rigidification, where both might be functionally important.

The high affinity of TSG-6 for HA and the ensuing cross-linking, might lead to the formation of rather dense and spatially confined HA matrices. For example, Simpson et al. (11) have shown that the coordinated expression of HA and TSG-6 during fibroblast to myofibroblast differentiation is necessary for the formation of a pericellular coat during normal wound healing and that this diminishes with age. Furthermore, it might be envisaged that a TSG-6-mediated contraction of the chondrocyte pericellular matrix during inflammation might serve to promote matrix remodeling that could contribute to its chondroprotective properties (53). Such dense matrices could not only locally sequester TSG-6, but might also enhance the retention of HA and matrix-associated molecules. Since the cross-links formed between TSG-6 and HA are reversible, and the HA chains flexible, it is likely that such matrices can dynamically adopt various shapes as a function of external cues. Upon uniaxial stretching, for example, highly elongated ‘fiber like’ assemblies with particular mechanical properties might form (6). Extracellular matrix rigidity has emerged as an important regulator of cellular behavior (63-65). Thus, TSG-6 induced modulation of the local mechanical properties of the extracellular space might directly affect the phenotype of adjacent cells.

Relevance of the cross-linking of HA-rich pericellular coats for leukocyte homing. Lesley and coworkers have shown that decoration of HA with either Link_TSG6 or rhTSG-6 promotes adhesion and rolling of CD44+ T-lymphocytes, and suggested that cross-linked HA/TSG-6 complexes might be the adhesion-promoting agent (6,25). Our direct evidence for TSG-6 mediated cross-linking and condensation (i.e. the formation of dense HA-networks) supports this hypothesis. Cross-linking likely increases the valency of interactions between HA and the cell surface. For example, rigidifying HA might reduce the entropic cost of cell surface receptor binding (45). It might also trigger a re-distribution/clustering of cell surface receptors (66), or promote their conformational up-regulation (58). Interestingly, condensation occurs gradually with increasing rhTSG-6 concentration, from 0.1 to 0.7 μM (Fig. 5), and local cross-linking of HA in the endothelial matrix by TSG-6 might hence be a dynamic regulator of the inflammatory response (6,25).

It should be noted that other hyaladherins or TSG-6 binding proteins (34,35) might alter the HA cross-linking activity of TSG-6, in subtle ways that remain to be elucidated. A case in point is the catalytic action of TSG-6 in mediating covalent transfer of heavy chains from inter-α-inhibitor (IαI) onto HA, which leads to a different mechanism of HA cross-linking to that described here (6,32,67). In this regard, the heavy chain transfer activity of TSG-6, but not the formation TSG-6/HA complexes, leads to a pro-migratory phenotype of proximal tubular epithelial cells (10). Also, the cross-linking of HA in the COC matrix was shown to involve at least two proteins, IαI and PTX3, in addition to TSG-6 (12,35,60,68-70), in a way that remains only partly understood.

On the other hand, the binding of TSG-6 to other glycosaminoglycans (chondroitin-4-sulfate, heparin/heparan sulfate) and highly hydrated proteoglycans, such as aggrecan and versican (8,27,33,34,37) suggests that TSG-6’s cross-linking activity might not be restricted to HA. In this regard, we showed previously that TSG-6 could promote the interaction of fibronectin with thrombospondin-1, likely by bridging between these two proteins via interactions mediated by its CUB_C domain and Link module, respectively (24). By simultaneously interacting with and cross-linking various components of pericellular and extracellular matrices, TSG-6 might play a central role as a matrix re-organizer. Our finding
that the formation of the HA cross-linking entities (i.e. the TSG-6 oligomers) is induced by the very presence of HA, might be of particular importance in the regulation of such reorganization processes.

The experimental approach that we have applied in this study can in the future be readily extended to study the cross-talk between HA, TSG-6 and their binding partners. Hence it is likely to provide novel insight into the mechanisms behind TSG-6 induced reorganization of pericellular and extracellular matrices.

REFERENCES


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FOOTNOTES

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1 The abbreviations used are: TSG-6, secreted product of tumor necrosis factor-stimulated gene-6; Link_TSG6, recombinant human TSG-6 Link module; rhTSG-6, recombinant human TSG-6; IαI, inter-α-inhibitor; PTX3, pentraxin 3; SAv, streptavidin; HA, hyaluronan; oHA, oligomeric HA (9-mer); pHA, polymeric HA; OEG, oligoethylene glycol; DOPC, dioleoylphosphatidyl-choline; DOPE-CAP-biotin, dioleoylphosphatidylethanolamine-cap-biotin; SLB, supported lipid bilayer; QCM-D, quartz crystal microbalance with dissipation monitoring; RICM, reflection interference contrast microscopy; GuHCl, guanidine hydrochloride; COC, cumulus-oocyte complex.


Supplemental Data. Preparation of biotinylated oHA and functionalized surfaces; details on the implementation of QCM-D, ellipsometry and RICM; assembly of SLB-based and OEG-based HA films, monitored by QCM-D (Figs. S1-S2); quantification of pHA surface densities for QCM-D and RICM measurements (Fig. S3); comparison of the HA-film properties on SLBs and OEG layers (Fig. S4); controls for the specificity of the rhTSG-6 (Fig. S5) and Link_TSG6 (Fig. S6) binding assays; controls for the stability of HA films against the dissociating agent GuHCl (Fig. S7).
FIGURE LEGENDS

Figure 1. Architecture of hyaluronan (HA) films. A dense streptavidin (SAv) monolayer was formed on a biotin-functionalized passivation layer. The passivation layer - either a silica-supported lipid bilayer or a gold-supported OEG layer (Figs. S1-S2) – was designed to inhibit non-specific binding of TSG-6. HA chains were grafted through a biotin functionality at their reducing end to the SAv layer. (A) Polymeric HA (pHA) with molecular weights of 58 or 1080 kDa exposes up to several hundred binding sites for the HA-binding protein TSG-6 on each individual polymer chain. (B) Only one TSG-6 molecule can bind to an HA₉ oligomer (oHA). The size of the proteins, the thickness of the passivation layer and the length of oHA are drawn to scale; the thickness of the pHA films is compressed by up to 20-fold.

Figure 2. Binding of full length TSG-6 into HA films. (A-B) Representative titration curves by ellipsometry for the binding of rhTSG-6 to a pHA (58 kDa) and an oHA (HA₉) film, respectively. The start and duration of the incubation with different samples and buffer is indicated (solid arrows and dashed arrows, respectively). Fits with Eq. 2b to the desorption curves upon rinsing in buffer (thick grey solid lines) provide estimates for the dissociation rate constants $k_{off}^{(1)}$ and $k_{off}^{(2)}$. Binding of rhTSG-6 to HA-free surfaces was negligible (Fig. S5A). (C) Adsorbed amounts of rhTSG-6 at equilibrium in films of pHA (58 kDa; ○) and oHA (HA₉; ●), determined from A and B. Both curves have pronounced sigmoidal shapes and can be fitted well by Eq. 1. Measurements were performed two to three times; determined $K_{0.5}$, $k_{off}$, $n$ and binding stoichiometries are given in Table 1. Hill exponents above $n = 2$ indicate strong positive cooperativity.

Figure 3. Full-length TSG-6 can be fully eluted with dissociating agents. (A) A pHA (58 kDa) film that was previously close-to-saturated with rhTSG-6 and rinsed with Heps buffer was exposed to 8 M guanidine hydrochloride (GuHCl). All of the protein is rapidly diluted in the dissociating agent and, hence, rhTSG-6 does not bind covalently to HA. All processes, except the incubation with GuHCl (shaded in grey), were monitored by ellipsometry. The HA film was stable in GuHCl (Fig. S7A). The measurement was repeated twice, and the displayed data is representative.

Figure 4. Full length TSG-6 cross-links HA. (A) Sandwich assay, monitored by QCM-D. The start and duration of the incubation with different samples and buffer is indicated (solid arrows and dashed arrows, respectively). A film of surface-bound oHA was incubated with rhTSG-6 until equilibrium, reaching a frequency shift of $\Delta f = -34$ Hz and a dissipation shift of $\Delta D = 1.1 \times 10^{-6}$, indicating the formation of a rather dense and rigid film of about 6 nm in thickness. The frequency increase upon subsequent rinsing with buffer indicates slow desorption of some rhTSG-6. Rapid addition of pHA (262 kDa) without biotin linker resulted in a two-phase response that is indicative of two overlapping processes. The initial decrease in frequency indicates binding of pHA to the oHA-bound rhTSG-6. The concomitant strong increase in dissipation is characteristic for the formation of a soft and highly hydrated film. The subsequent increase in frequency is likely a result of desorption of rhTSG-6 and/or migration of rhTSG-6 inside the pHA film. This process continues, albeit at slower pace, after rinsing in buffer. The measurement was performed twice, and the shown data is representative. (B) Schematic illustration of the final sandwich structure. Our data indicates that HA cross-linking is mediated by rhTSG-6 oligomers (here represented by the simplest possible oligomers, i.e. dimers). The Link module and the CUB_C domain of TSG-6 are schematically indicated in light blue and green, respectively. (C) For comparison, a molecular model of a TSG-6 dimer with two bound HA octasaccharides derived from small angle X-ray scattering data (49) is also shown; the Link module structure (blue) in its HA-bound conformation (50), docked HA oligomers (orange; (48,50)) and CUB module model (green (31)) are shown in space filling representations, while the N- and C-terminal regions of TSG-6 (for which no structural data are available) are represented as protein backbone traces predicted from the scattering data (49).
Figure 5. Rigidification and condensation of HA films upon influx of full-length TSG-6. (A) Binding of rhTSG-6 to a pHA (58 kDa) film was monitored by QCM-D. The strong decrease in frequency upon exposure to rhTSG-6 is initially accompanied by an increase, followed by a decrease, in dissipation. The latter indicates rigidification of the HA film. (B) Variations in the thickness of a pHA (1080 kDa) film as a function of increasing rhTSG-6 concentration, quantified by colloidal probe RICM. The film retained its collapsed state upon rinsing in buffer (data not shown). The inset shows the thickness as a function of the occupancy of HA (calculated from B, using Eq. 1 and the data for pHA in Table 1). Error bars are standard deviations for 10 independent measurements on the same surface.

Figure 6. Proposed mechanism for the condensation of pHA brushes upon cross-linking with full length TSG-6. (A) In the absence of TSG-6, HA forms a strongly hydrated meshwork of entangled polymer chains; the size of individual meshes is comparable to the distance between anchor points of HA chains on the surface. (B) The introduction of cross-linkers (TSG-6 oligomers, open circles) forces neighboring HA chains closer together; this leads to smaller mesh sizes, film condensation and rigidification. The mesh sizes, the average distance between anchor points and the size of the cross-linkers are drawn approximately to scale.

Figure 7. Binding of Link_TSG6 into HA films. (A-B) Representative titration curves by ellipsometry for a pHA (58 kDa) and an oHA (HAo) film, respectively. Fits with Eq. 2a to the desorption curves upon rinsing in buffer (thick grey solid lines) provide estimates for the dissociation rate constant \( k_{off} \). The irreversibly bound protein fraction could be fully eluted in GuHCl (A; incubation period shaded in grey), and is hence non-covalently bound. (C) Adsorbed amounts of Link_TSG6 at equilibrium in films of pHA (△) and oHA (▼); minor amounts of nonspecific binding, observed on HA-free surfaces (Fig. S6A), were subtracted from the data in A and B. Fits by Eq. 1 (solid line and dashed line, respectively) provide a Hill exponent close to unity. Measurements were performed two to three times; determined \( K_{0.5}, k_{off}, n \) and binding stoichiometries are given in Table 1.

Figure 8. Rigidification and condensation of pHA films upon influx of Link_TSG6. (A) Titration curve by QCM-D on a pHA (58 kDa) film. Incorporation of Link_TSG6 at bulk concentrations above 3 μM results in a pronounced decrease in dissipation while the frequency continues to decrease, indicating rigidification of the film. A comparison of the responses upon increasing and decreasing bulk concentrations of Link_TSG6, respectively, reveals hysteresis between the adsorption and desorption process. (B) Variations in the thickness of a pHA (1080 kDa) film as a function of Link_TSG6 bulk concentration, determined by colloidal probe RICM. The Link_TSG6 concentration was first increased and then decreased (indicated by arrows). The film thickness decreases with increasing Link_TSG6 concentrations, in a partly reversible manner. Error bars are standard deviations for 10 independent measurements on the same surface. (C) Comparison of the condensation of 1080 kDa pHA films by rhTSG-6 (●; from Fig. 4B inset) and Link_TSG6 (○; calculated from B, using Eq. 1 and the data for pHA in Table 1) as a function of the occupancy of HA. The film thickness scales approximately linearly with the Link_TSG6/HA2-ratio (dashed line). At equal occupancy, rhTSG-6 is a more potent cross-linker than Link_TSG6.

Figure 9. Link_TSG6 induces total pHA film collapse at low ionic strength. (A) Interaction assay by QCM-D on a pHA (58 kDa) film. The dramatic decrease in dissipation upon exposure to 1 μM Link_TSG6 in 5 mM NaCl indicates complete film collapse and rigidification. The film remained collapsed after rinsing in 5 mM NaCl (marked by an asterisk), but recovers partly in 150 mM NaCl. (B) Variations in the thickness of a pHA (1080 kDa) film as a function of the Link_TSG6 concentration in solution at 5 mM NaCl, determined by colloidal probe RICM (■). A more than twofold decrease in film thickness was already observed at Link_TSG6 concentrations of 0.1 μM, and close to complete collapse is attained at 1 μM. Data acquired in 150 mM NaCl under otherwise identical conditions is shown for comparison (○, from Fig. 7B). Note that the decrease in ionic strength induced a swelling of the protein
free HA film, from 450±20 nm at physiological ionic strength to about 1 μm in 5 mM NaCl, as a result of electrostatic repulsion between HA chains. Error bars are standard deviations for 10 independent measurements on the same surface.
Table 1. Binding parameters for the incorporation of Link_TSG6 and rhTSG-6 into oHA and pHA films, determined from titration curves as shown in Figs. 2 and 7.

<table>
<thead>
<tr>
<th></th>
<th>$K_{0.5}$ (μM)</th>
<th>$k_{off}$ ($10^{-3}$ s$^{-1}$)</th>
<th>$f_r$ a)</th>
<th>$k_{on}$ b) ($10^3$ M$^{-1}$ s$^{-1}$)</th>
<th>$N$</th>
<th>stoichiometry at c) maximal binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Link_TSG6</td>
<td>6.1±2.5</td>
<td>28±3 c)</td>
<td>~86±3%</td>
<td>4.6±2.4</td>
<td>0.9±0.2</td>
<td>1.3±0.3 per HA$_9$</td>
</tr>
<tr>
<td>rhTSG-6</td>
<td>1.2±0.4</td>
<td>4.3±1.7 d)</td>
<td>43±9%</td>
<td>0.052±0.018</td>
<td>57±9%</td>
<td>2.7±0.8 per HA$_9$</td>
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<td>in pHA films (58 kDa HA)</td>
<td></td>
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<tr>
<td>Link_TSG6</td>
<td>5.5±0.6</td>
<td>23±8 c)</td>
<td>~79±4%</td>
<td>4.2±1.9</td>
<td>1.2±0.2</td>
<td>1 per 2.9±0.3 HA$_2$</td>
</tr>
<tr>
<td>rhTSG-6</td>
<td>0.79±0.17</td>
<td>2.8±2.0 d)</td>
<td>8±1%</td>
<td>0.14±0.07</td>
<td>92±1%</td>
<td>2.8±0.5 1 per 3.7±1.0 HA$_2$</td>
</tr>
</tbody>
</table>

a) $f_r = \Gamma_r/\Gamma_{total}$ with $\Gamma_{total} = \Gamma_r{(1)} + \Gamma_r{(2)} + \Gamma_{ir}$ is the fraction of TSG-6 that was found to dissociate with the respective $k_{off}$ (see Eqs. 2a-b); b) Determined from $K_{0.5} = K_D = k_{off}/k_{on}$ for non-cooperative binding ($n \approx 1$); c) Determined with Eq. 2a; d) Determined with Eq. 2b for desorption processes of 1 h duration and with $\Gamma_{ir}$ set to 0; e) Determined from $\Gamma_{max}$ in Eq. 1, and the surface density of immobilized HA. Errors correspond to experimental uncertainties and variations across two to three measurements.
Figure 1

A

B

Substrate
Passivation layer

SAv biotin linker HA

HA-binding protein (e.g. rhTSG-6 or Link_TSG6)
Figure 2

(A) Time course of surface density changes for pHA_65kDa and oHA_64kDa in the presence of different concentrations of rhTSG-6 (μM).

(B) Similar time course for pHA_100kDa and oHA_100kDa.

(C) Surface density as a function of rhTSG-6 concentration in bulk solution for pHA and oHA films.
Figure 3

[Graph showing surface density (ng/cm²) over time (min) with pHAb (58kDa) and 2.3 μM rhTSG-6, followed by 8M GuHCl treatment.]
Figure 4

(A) Graph showing changes in $\Delta f$ (Hz) and $\Delta D$ ($10^{-6}$) over time (min) with 0.33 $\mu$M rhTSG-6. The blue line represents $\Delta f$, and the red line represents $\Delta D$.

(B) Molecular structure diagram depicting $\text{HA}_9$ and $\text{CUB}$ with links.

(C) Diagram illustrating $\text{HA}_9$ and its interaction with other components.
Figure 5

A

B

Δf (Hz)

Δf

ΔD

time (min)

rhTSG-6 in bulk solution (μM)

film thickness (nm)

0 20 40 60 120 140

0 20 40 60 80 100 120 140

0 20 40 60 80 100 120 140

0 20 40 60 80 100 120 140

0 20 40 60 80 100 120 140
Figure 6
Figure 8

A. The graph shows the changes in frequency (Δf) and amplitude (ΔD) over time for different concentrations of Link_TSG6 in bulk solution (μM).

B. The plot illustrates the film thickness (nm) as a function of Link_TSG6 in bulk solution (μM).

C. The graph depicts the relationship between TSG-6 per HA and film thickness (nm), indicating a decrease in thickness with increasing TSG-6 concentration.
Figure 9

A

\[ \Delta f (\text{Hz}) \]

\[ \Delta D (10^{-6}) \]

-60
-40
-20
0
20
40
60
-60
-40
-20
0
20
40
60

\[ \text{time (min)} \]

0
20
40
60
80
100
120
140
160

pHA-b (58kDa)
350mM NaCl
5mM NaCl
1μM Link_TSG6
5mM NaCl
150mM NaCl

B

film thickness (nm)

Δf* 0 2 4 6 8 10

ΔD

Link_TSG6 in bulk solution (μM)

0 20 40 60 80 100 120 140 150
The inflammation-associated protein TSG-6 cross-links hyaluronan via hyaluronan-induced TSG-6 oligomers
Natalia S. Baranova, Erik Nileback, F. Michael Haller, David C. Briggs, Sofia Svedhem, Anthony J. Day and Ralf P. Richter

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