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Hyaluronan in the pericellular coat: an additional layer of complexity in early cell adhesion events†

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Cell adhesion is a multistage process whereby specific surface receptors interact with the corresponding ligands on the extracellular matrix or on neighboring cells. These complex interactions involve a wide variety of cellular molecules including transmembrane and cytoskeletal components, scaffolding proteins, and a wide variety of signaling enzymes. In this article we discuss recent data characterizing the involvement of the pericellular hyaluronan coat in early stages of cell–matrix adhesion. In particular, we address the mechanisms underlying the transition from hyaluronan- to integrin-mediated adhesion, and the role of the actin cytoskeleton in the “inside-out” regulation and maintenance of the pericellular hyaluronan coat.

Introduction

The role of the hyaluronan pericellular coat in cell adhesion

The interaction of cells with their environment triggers numerous responses that regulate cell behavior and fate, including cell proliferation, differentiation, motility, and survival.^{1,2} In various cell types, the first encounter with the external surface is mediated by a micrometre-thick pericellular coat,^{3,4} consisting of hyaluronan, link proteins and aggregation proteoglycans such as aggrecan,^{3,5} versican,⁶ neurocan⁷ and brevican.⁸ The structural integrity of the pericellular coat is sensitive to hyaluronidase treatment. Hyaluronidase is an endoglycosidase that breaks down hyaluronan into small fragments.⁹ Hyaluronan itself is a linear glycosaminoglycan consisting of 2000–25 000 repeating disaccharide units of β -1,3-glucuronic acid and β -1,4-*N*-acetylglucosamine.¹⁰ The typical molecular weight is 10^6 to 10^7 Dalton, which corresponds to an extended polymer length of approximately 2–25 μm . Hyaluronan molecules are anchored to the cell membrane by transmembranal receptors (*e.g.* CD44, RHAMM, Laylin) or by hyaluronan synthase.¹¹ Despite the very large number of charge bearing carboxylate groups, hyaluronan behaves as a weak polyelectrolyte under physiological conditions.¹² Nevertheless, the pericellular coat is highly hydrated and can reach $\sim 5 \mu\text{m}$ thickness in certain cells (*e.g.* chondrocytes).

Pericellular hyaluronan was shown to mediate initial stages of cell–substrate adhesion, which are followed by integrin-mediated adhesion,^{13–15} leading to the formation of focal

adhesions and related actin-associated adhesions.^{1,16} The latter sites are characterized by membrane-bound adhesion plaques, containing multiple proteins, including vinculin, paxillin, focal adhesion kinase, α -actinin, and more. Over 100 scaffold, cytoskeletal, adhesion receptors and signaling components are associated with these adhesions, and regulate their formation and dynamics.¹⁷

In this article we address the mechanism of the transition between hyaluronan-mediated and integrin-mediated adhesion.

Results and discussion

Hyaluronan organization in the pericellular coat

Hyaluronan is synthesized *de novo* by a family of integral plasma membrane glycosyltransferases known as the hyaluronan synthases (HAS1, 2, and 3).^{18,19} Hyaluronan is produced at the inner aspect of the plasma membrane and is extruded into the extracellular space through the membrane as it is being synthesized.^{19,20} This allows unconstrained polymer growth and the synthesis of hyaluronan chains with thousands of disaccharide repeats and mega-dalton molecular weight. The newly synthesized hyaluronan chain can remain anchored to the plasma membrane *via* the hyaluronan synthase or by binding to one of the hyaluronan receptors. Alternatively, it can be released into the extracellular space and bind to the ECM.¹¹

Examination of the hyaluronan-based pericellular coat reveals two major modes of hyaluronan organization at the cell surface. In chondrocytes (RCJP, rat), which display a particularly thick coat ($\sim 5 \mu\text{m}$) [Fig. 1 a] hyaluronan is apparently organized as an entangled multilayer.^{4,21} This coat mediates “soft” interactions with the substrate, and under flow experiments it can be peeled off the cell surface by a low shear force of 6.5 dyn cm^{-2} ($0.65 \text{ pN } \mu\text{m}^{-2}$).⁴ Around epithelial cells (A6 kidney epithelial cells from *Xenopus Laevis*) a ~ 2 micrometre thick coat was observed by “wet mode” ESEM inspection under highly hydrated conditions [Fig. 1 b]. Under flow experiments, the coat cannot be readily peeled off by shear force, and the cells consequently form “firm”

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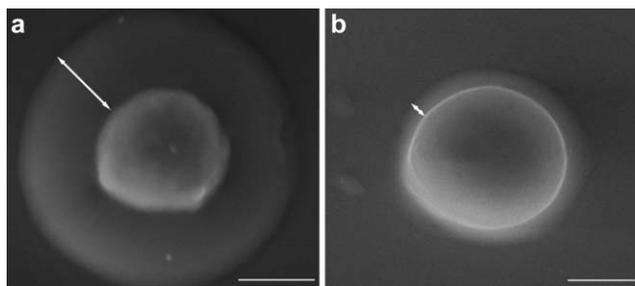


Fig. 1 Electron micrographs of (a) RCJP rat chondrocytes and (b) A6 *Xenopus Laevis* epithelial cells. Cells were labeled with uranyl acetate and visualized under fully hydrated conditions in the ESEM.⁴ Arrows indicate hyaluronan pericellular coats. Scale bar = 5 μm .

interactions with the external surface.⁴ This suggests that hyaluronan is organized in a single layer, where each molecule is tethered to the plasma membrane.²¹ The behavior of the resulting layer might be similar to that of an end-grafted neutral polymer whose conformation—according to the Alexander–De Gennes model—is controlled by the grafting density.²² End-grafted polymer chains at low grafting density exist in a coiled state, assuming a “mushroom” conformation, with a coil radius (radius of gyration R_g) that depends on the molecular weight, all other environmental parameters being equal. However, when the through-space distance between neighboring grafted coils is smaller than their diameter, the osmotic pressure exerted by the tethered polymers may drive the stretching of the chain vertical to the grafted surface, forming a so-called polymer brush.²² A polymer brush may be an order of magnitude thicker than a layer of the same polymer in a coiled mushroom conformation. Thus, hyaluronan molecules with typical molecular weight of 2×10^6 Dalton (5 μm contour length) have a radius of gyration of 180 nm, forming a ~ 350 nm thick mushroom layer. The same molecules, when extended, can in principle form a 2 μm thick layer.[§] There is an energetic price to be paid for the stretching of the molecules from a mushroom to a brush, and consequently a brush is not an energy-minimum structure. Essentially, the same osmotic pressure that drives the extension of the brush also acts on the graft points, and given a chance would drive them apart from each other, thus leading to a collapse of the brush.

In the case of chondrocytes at least, interactions with highly charged proteoglycans in the coat will most certainly influence properties such as visco-elasticity and gelation, including the conditions for formation of a brush. Hyaluronan–aggrecan complexes, for example, are required in order to form thick pericellular coat around these cells.³

Additional evidence for the differences in hyaluronan organization around chondrocytes and epithelial cells comes from labeling of cells with biotinylated hyaluronan binding

[§] We do not have direct information about the size of hyaluronan chains in either of the cells, although we do believe it to be within the mega-Dalton range. Of the three hyaluronan synthases, only HAS3 produces shorter chains ($2\text{--}3 \times 10^5$ Da), whereas HAS1 and HAS2 produce mega-Dalton chains. The coat properties in any case should be dominated by the longer chains.

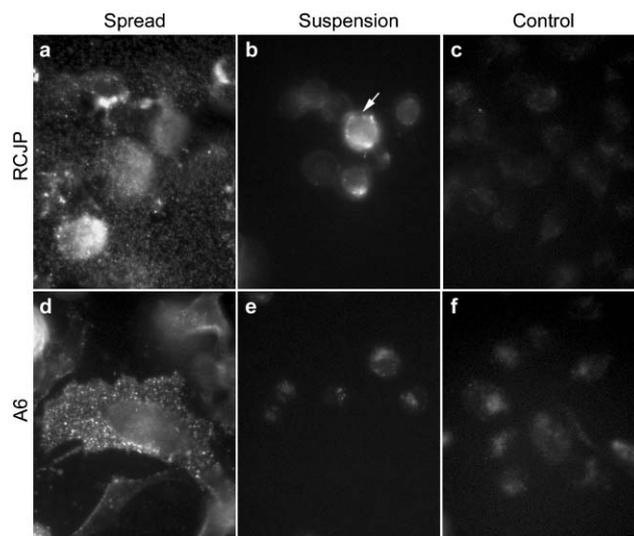


Fig. 2 (a–c) RCJP chondrocytes (d–f) A6 epithelial cells. (a, d) Adherent cells were incubated with bHABP while they were spread in the culture dish, followed by streptavidin-CY3 incubation. Hyaluronan was detected under a fluorescence microscope. (b, e) Labeled cells were suspended using trypsin. A hyaluronan coat is visible around chondrocytes (b, arrow), but the epithelial cells loose their fluorescence (e). (c, f) Control cells were incubated only with streptavidin-CY3, a low level of auto-fluorescence is seen. Scale bar = 5 μm .

proteins (bHABP). The pericellular coat of both chondrocytes and epithelial cells is stained with bHABP when the cells are spread [Fig. 2 a, d respectively]. Following treatment with trypsin, a 5 μm coat is visible around the suspended chondrocytes [Fig. 2 b]; the epithelial cells, however, loose the label upon the same treatment [Fig. 2 e]. One possible scenario is that when the epithelial cells round up as they detach from the surface, the bHABPs, which are not covalently bound, are expelled from the coat due to the high osmotic pressure. In contrast, hyaluronan in the chondrocyte coat is organized in a more relaxed conformation that allows the bHABP to remain bound. Alternatively, we must assume that the coat in the suspended epithelial cells does not have sufficient thickness to allow detection at the resolution of the light microscope. In this latter case, some coat material may have been lost during trypsin treatment.

Transition from hyaluronan- to integrin- mediated adhesion

The considerable thickness of the hyaluronan pericellular coats raises an interesting question regarding the dynamics of the adhesion processes. Initial interactions of hyaluronan with the substrate occur when the cells are separated from the substrate by few micrometres.¹⁵ The following stage involves interaction of integrins with their surface ligands, requiring a membrane-to-surface proximity of 15–20 nm.^{17,23} The pericellular coat thus has to be modified by the cell in order to allow the formation of focal adhesions. We proposed several possible mechanisms by which the pericellular hyaluronan can be removed from the site of adhesion: 1. Lateral displacement of hyaluronan molecules, exposing integrin molecules in the cell

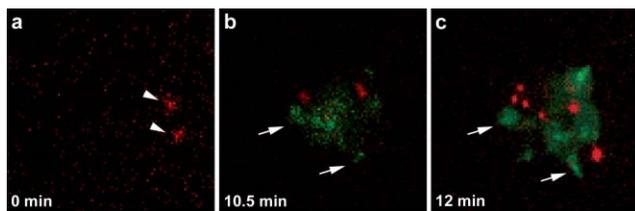


Fig. 3 A stable line of RCJP chondrocytes expressing the chimeric protein paxillin-GFP, was labeled with bHABP followed by streptavidin-quantum dots incubation. The cells were seeded on a glass surface and a time-lapse movie was immediately taken using TIRFM.¹⁴ (a) Hyaluronan-bound quantum dots are visible (red, arrowheads). (b) Paxillin-GFP structures only become visible 10.5 min later (green, arrows). (c) Focal adhesion structures rapidly form thereafter (green, arrows).

membrane to the ECM, 2. enzymatic digestion of the pericellular hyaluronan, 3. “collapse” of the pericellular coat.²¹ We have shown that during cell spreading hyaluronan is reorganized in pockets under the cells as they adhere, rather than being laterally displaced.¹⁴ It is possible that the hyaluronan is partially digested and the hyaluronan fragments are observed under the cells. However the dynamics of the transition process suggests a different mechanism. While hyaluronan–substrate interactions may last 2–10 minutes before the initiation of focal contact formation, once the transition is established, formation of a full-size focal adhesion is very rapid [Fig. 3].¹⁴ This suggests the involvement of a rapid transition mechanism that allows the cell membrane to get into close proximity with the surface within seconds, once it is triggered. It is conceivable that hyaluronan-mediated adhesions may transmit outside-in signals to the cell *via* the receptor hyaluronan is bound to. The major hyaluronan receptor, CD44, is a very potent transmembrane protein that interacts with cytoskeletal components (*e.g.* ezrin, radixin, moesin, ankyrin),²⁴ is involved in the activation of Rho signaling,^{24,25} and was also reported to promote integrin activation.^{26,27} In this scenario, hyaluronan–substrate interactions may trigger a signaling cascade that leads to activation of integrin-mediated adhesion, as well as reorganization of pericellular hyaluronan.

Inside-out regulation of the state of the pericellular matrix by the cytoskeleton

Cell adhesion is known to be a highly regulated process, controlled at multiple levels. In the integrin system, for example, these include the controlled expression of the relevant adhesion receptors and their transport to the plasma membrane, the activation of the receptors, namely their transition to a high-affinity state, and the formation of multi-protein, cytoskeleton-bound adhesion sites such as focal adhesions.^{1,28} The essence of this complex process is that it provides cells with effective tools for switching the cellular adhesion machinery on, or off, at high temporal and spatial resolution. This notion raised the intriguing question whether there are also specific inside-out regulatory mechanisms that affect adhesive interactions mediated by the pericellular hyaluronan coat.

The dynamic analysis outlined above indicated that the transition from the micrometre scale to the 15–20 nm scale

membrane-to-surface distance, characteristic of hyaluronan-mediated and integrin-mediated adhesion, respectively, could be attributed to different mechanisms. These include passive mechanisms, whereby the thick hyaluronan coat collapses due to its interaction with the external surface or is pushed aside by the cell under the influence of gravity, or an active process such as shedding of the coat from the cell surface and its internalization by the cell. Additional mechanisms may involve, as a primary factor, an active “inside-out” modulation of these adhesion systems by the cell. An attractive mechanism for such cell-regulated transition could involve cytoskeletal changes, primarily those affecting the actin-based network. The reorganization of actin, in turn, can affect multiple cellular features including receptor aggregation, endocytosis, and cell surface topology.

To test the possible involvement of the actin cytoskeleton in regulating the organization of the hyaluronan coat, A6 epithelial cells were treated with the actin-disrupting agent latrunculin A (LatA) and then examined by transmission electron microscope (TEM) and environmental scanning electron microscope (ESEM). In suspended untreated A6 cells the pericellular coat is observed by TEM between the microvilli and apparently extends beyond these membrane protrusions [Fig. 4 a, d, g; arrows]. The TEM samples shown here are conventional sections of fixed, dehydrated and polymer-embedded cells, therefore the hyaluronan layer is expected to appear collapsed and shrunk [Fig. 4 d, g]. The dimensions of the pericellular coat in the hydrated state can be seen in the wet mode of the ESEM image as being larger, although there is no proof that this coat is 3-dimensionally homogeneous around the entire cell [Fig. 4 a]. In some cells the induction and maintenance of microvilli-like cell surface protrusions are dependent on ongoing hyaluronan synthesis by hyaluronan synthases, located at the protrusion tips.²⁹ Apparently, hyaluronan is not required for supporting membrane topography of A6 epithelial cells, since removal of the coat by hydrolysis with hyaluronidase does not affect gross cell morphology [Fig. 4 b, e, h]. In fact the force exerted by the osmotic pressure of hyaluronan polymers is expected to be ~ 2 orders of magnitude smaller than the force exerted by the cortical cytoskeleton. On the other hand, treatment with LatA—a drug that sequesters G-actin, effectively causing deterioration of F-actin-based structures—induces loss of microvilli and overall membrane flattening. Interestingly, this treatment also leads to the disappearance of the micrometre-thick coat [Fig. 4 c, f, i] seen in the ESEM or in the TEM micrographs. The effect of latrunculin on actin is readily reversible³⁰ and indeed the microvilli are partially recovered after removal of the drug (data not shown). Unfortunately, quantification of the concomitant recovery of the hydrated coat proved to be inconclusive. To rule out the possibility that latrunculin directly affected the pericellular coat integrity, cells were fixed and then incubated with the drug. Fixation is not expected to play a major role in this process, since hyaluronan does not react with aldehyde fixatives, although associated proteins do. The fixed cells displayed a hyaluronan coat that was indistinguishable from that of untreated cells (data not shown).

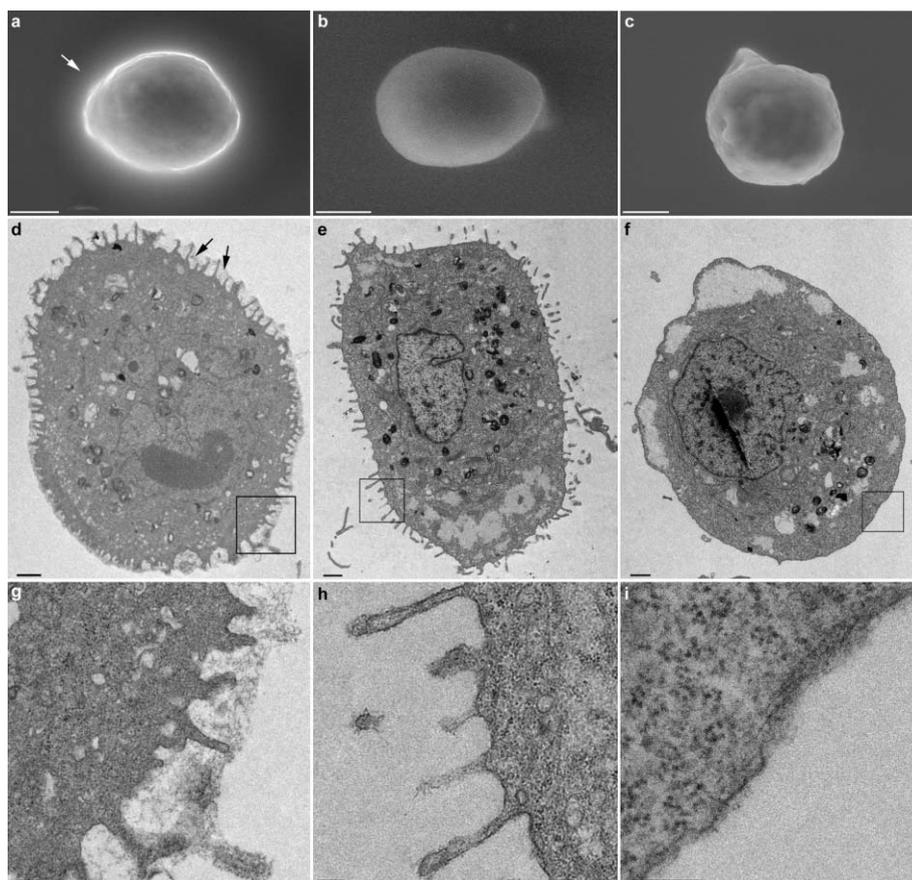


Fig. 4 (a–c) ESEM micrographs, (d–i) TEM micrographs of A6 epithelial cells. (a, d) Untreated cells have a micrometres-thick hyaluronan pericellular coat (arrows). The hyaluronan coat is visible between the membrane protrusions and is extended at least 500 nm. (b, e) Hyaluronidase treatment removes the pericellular hyaluronan, but does not affect the membrane protrusions. (c, f) Latrunculin A treatment abolishes the hyaluronan coat and results in flattening of the cell membrane. (g–i) Higher magnification of the areas marked with a box in d–f, respectively. Scale bar = 5 μm (a–c), 1 μm (d–f), 200 nm (g–i).

The mechanism underlying this cytoskeleton-regulated loss of the pericellular coat is not clear. It is possible that latrunculin treatment leads to partial or full shedding of hyaluronan from the surface, its degradation *in situ*, altering its synthesis, or alternatively to its internalization by affecting CD44 expression or signaling. Each of these processes is expected to lead to a decrease in the local concentration of hyaluronan at the cell–surface interface, thereby allowing cell surface integrin to access their RGD-containing ECM ligand. Another possibility is that the hyaluronan molecules change their conformation from a more extended to a less extended conformation due to an increase in the space available to each hyaluronan molecule, following loss of microvilli. This hypothesis is based on the concept mentioned above, *i.e.* that the lateral spacing between neighboring polymers determines the conformation of grafted polyelectrolytes.²² Whichever is the explanation, it appears that by changing surface topography, even locally, cells can modify effective hyaluronan density at the cell periphery, hence altering the coat thickness.

Conclusions

The stepwise nature to cell adhesion, including the interplay between the two hyaluronan and integrin adhesion-promoting

mechanisms, appears to play an important role in the regulation of the cell's interaction with its environment. We illustrated here some basic features of this complex process, which is regulated by a dynamic two-way crosstalk between the cell and the surface. We suggest that hyaluronan mediates an outside-in signaling *via* its receptors, triggering the transition to integrin-mediated adhesion. *Vice-versa*, reorganization of the actin cytoskeleton, leading to topographical changes of the cell membrane, may serve as an inside-out modulator of the pericellular coat thickness. Based on these observations we have outlined here the main open questions concerning the fate of the hyaluronan coat during the transition from the initial surface recognition event to the establishment of mature, integrin- and cytoskeleton-mediated focal adhesion.

Experimental

Cell culture

RCJ-P chondrocytes (rat chondrocytes from fetal calvaria, batch 15.01.98; Prochon Biotech, Rehovot, Israel) were cultured at 37 $^{\circ}\text{C}$ in a humidified atmosphere of 5% CO_2 in air in α -minimum essential medium (Biological Services, The

Weizmann Institute) supplemented with 15% fetal calf serum (Biolab Ltd., Jerusalem, Israel).

A6 cells (kidney epithelial cells from *Xenopus laevis*, ATCC.CCL 102) were cultured at 27 °C in a humidified atmosphere of 5% CO₂ in air in Dulbecco's minimum essential medium (Biological Services) diluted to 85% with water and supplemented with 10% fetal calf serum (Biolab Ltd.).

Latrunculin A treatment

Cells from a confluent culture were suspended using trypsin-EDTA (Biological Services, The Weizmann Institute), centrifuged, and gently re-suspended in fresh medium. Latrunculin A (Molecular probes, Oregon, USA) was added to the suspended cells to a final concentration of 15 μM, and the cells were incubated for 5 min at 27 °C. Some of the cells were then washed and allowed to recover from the drug for 30 min at 27 °C. An equal volume of DMSO was added to control cells instead of the drug. After treatment the cells were fixed with the appropriate fixative for ESEM or TEM preparations (see below).

Hyaluronan labeling

Cells were seeded sparsely on tissue culture dishes and allowed to adhere for 1 h, then 5 mg ml⁻¹ biotinylated hyaluronan binding protein (bHABP, Seikagaku, Japan) was added for 2 h. The cells were washed 3 times with medium and incubated for 30 min with 20 nM streptavidin conjugated Qdots 655 nm (Quantum Dot Corporation, Hayward, CA), or with 1 : 200 streptavidin-CY3. Labeled cells were examined with a digital microscope system (Delta Vision, Applied Precision, Inc., Issaquah, WA) or with TIRFM (see below).

Sample preparation for ESEM

Cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, 5 mM CaCl₂, pH 7.2, for 30 min. After rinsing with 0.1 M cacodylate buffer for 5 min, and three times with water (5 min each), the cells were incubated for 45 min with 2% uranyl acetate in water at pH 3.5, washed, re-suspended in water, and seeded on serum-coated glass coverslips at 4 °C for 16 h. The samples were examined in the environmental scanning electron microscope, XL 30 ESEM FEG (Philips/FEI) at 10 kV, using the wet mode at 5 °C, 6.4 Torr (867 Pascal), 7.8 mm working distance.

Sample preparation for TEM

Cells were fixed for 2 h in 2% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer containing 5 mM CaCl₂. The cells were embedded in 3.4% agarose, and postfixed for 1 h with 1% osmium tetroxide, 0.5% potassium ferrocyanide and 0.5% potassium dichromate in 0.1 M cacodylate buffer, pH 7.2. The cells were stained *en bloc* with 2% aqueous uranyl acetate, followed by ethanol dehydration and embedded in t-Epon 812 (Tuosimis, Maryland, USA). Sections of 500–700 Å were cut using a diamond knife (Diatome, Switzerland), and examined using a Philips CM-12 transmission electron microscope at an accelerating voltage of 100 kV.

Sample preparation for TIRFM

Suspended cells were washed twice and re-suspended in Dulbecco's minimum essential medium without riboflavin and phenol red (Biological industries, Beit Haemek, Israel) supplemented with 30 mM HEPES (Sigma) and 15% or 0.5% fetal calf serum. The cells were then seeded on 35 mm culture plates with glass bottoms (Mattec, Ashland, MA) that were pre-incubated for 1 h with 15% or 100% fetal calf serum or with 10 μg ml⁻¹ fibronectin (Sigma), and examined in an Olympus IX81 inverted microscope equipped with IX2-RFAEVA TIRF attachment (Olympus, Tokyo, Japan) as described.¹⁴

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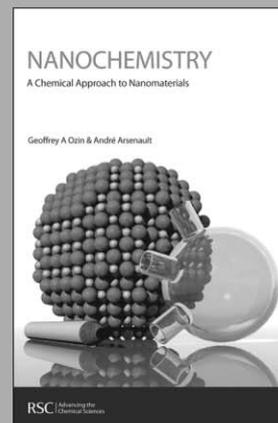
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