Reviews

Engineering of Biomaterials Surfaces by Hyaluronan

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This review addresses the area of study that defines the field of surface modification of biomedical materials and devices by hyaluronan (HA), as related to the exploitation of HA biological properties. To provide a comprehensive view of the subject matter, initial sections give a quick introduction to basic information on HA−protein and HA−cell interactions, together with some discussion on the bioactive role of HA in wound healing and related phenomena. This is followed by a description of current theories that correlate HA properties to its molecular structure in aqueous media, underlying how HA molecular details are crucial for its biological interaction and role. Finally, existing approaches to surface modification by HA are reviewed, stressing the need for HA-surface engineering founded on the knowledge and control of the surface-linked HA molecular conformation at the solid/aqueous interface.

Introduction

The interest of the biomedical community in hyaluronan (HA) is constantly rising, as shown by the increasing number of papers and communications on this topic. HA is now the subject of periodic meetings that host an ever-growing number of contributions (meeting proceedings are collected in refs 1−4; besides the cited meetings, Hyaluronan 2003 was held in Cleveland, OH, in October 2003, the proceedings of which are available online at http://matrixbiologyinstitute.org/ha03/toc.htm; also, an excellent review of HA science and properties is available online at http://www.glycoforum.gr.jp/science/hyaluronan/hyaluronanE.html). This continuous growth is fueled by the exceedingly interesting properties of the HA molecule: Despite its apparent simplicity, a long linear polymer based on a disaccharide repeating unit (Figure 1), without branching or ramification, completely conserved along the evolutionary path, HA is emerging more and more as a key molecule in the regulation of many cellular and biological processes, as extensively described in refs 1−4. While most of the current applications of HA in the biomedical field are based on its physical properties (hydration, viscosity, space filling), it is now clear that even more opportunities lie in the exploitation of its specific biological and bioactive properties; a sentence from an interesting work of Chen nicely states this point: "Currently HA-based medical products are mostly classified as medical devices, utilising the physical attributes of HA to achieve their intended functions. No doubt when the biology of HA becomes better known, applications will also be developed to utilise its biological functions." [5] See also ref 6.

The shifting of the focus from physicochemical properties to specific biological activity is a matter of great interest
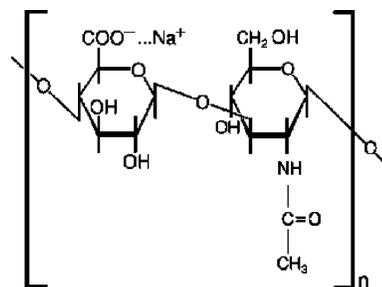

Figure 1. Hyaluronan repeating unit (sodium hyaluronate).

also in the equally growing field of surface modification of implant materials and devices. Actually, the trend of research in this field is aiming exactly in the same direction, moving away from biopassive surfaces, "walled off" by encapsulation, toward bioactive biomolecular surfaces that can communicate with the host tissue and respond in a physiological manner.[7−9] The exploitation of the biospecific properties offered by HA fits well within this line of development, and surface-linking of HA to improve existing and to develop new implant devices is a central theme of and our and other work.

The aim of this paper is to try to define some common basis for this endeavor, by gathering existing information on the surface modification by HA. In the spirit of present-day biomolecular surface science, the conscious approach to surface modification by HA (or any other biomolecule) is based on the impressive body of information that medical and molecular science is building up: The biomolecular surface scientist is no longer (simply) asked to perform a surface modification reaction and to confirm, by some form of surface spectroscopy, that the intended molecule is indeed on the surface.[10] Rather, surface modification schemes must take into full account that biomolecules are molecular

machines built to exploit the unique interactions occurring in water, and that biomolecular interactions are often cooperative, finely orchestrated sequences of low-energy events.[11] Shape and function, and space and time dependence of molecular conformations are keywords of biomolecular interactions in tissue, and they must be translated to biomolecules immobilized to device surfaces, to keep the desired biological activity. For this reason, in this review it is felt that surface modification by HA must be discussed within the broader area of HA structure and properties, to completely disclose the opportunities related to HA immobilization and the complexity involved in capturing and predicting the surface-linked HA behavior. Thus, the outline of this paper is the following: The first section, after some preliminary classification and definition, will deal with some of the most important biological properties of HA, introducing its interactions with proteins, cells, and tissue. Then, since the molecular structure of HA is at the basis of its properties, a paragraph will address present information on HA molecular behavior in aqueous solutions, trying to identify which are the molecular features that impart to HA its biological properties. After that, existing approaches to the surface immobilization of HA will be reviewed, trying to understand, when possible, which are the implications for the biological activity of HA. The final section will then present some reflections and conclusions.

HA: Some Properties

Chemical Classification. HA is a linear polysaccharide consisting of the repeating disaccharide unit shown in Figure 1 (MW = 401.1; including Na as the counterion, the length of the unit is ~1 nm). It is produced by HA synthase enzymes, as a linear polymer; the number of repeat disaccharides units can reach 10000 or more, yielding a molecular mass of about 4 million Da, or a chain with an end-to-end distance of about 10 μm length.[1]

From a general point of view, it belongs to the class of anionic glycosaminoglycans (GAGs); HA is actually the simplest GAG. It shares with the other components of this class of polysaccharides a disaccharide repeating unit based on *N*-acetylhexosamine linked to either hexuronate or galactose. In HA, the two components of the repeating unit are *N*-acetyl-D-glucosamine and D-glucuronate, linked by $\beta1-4$ and $\beta1-3$ linkages. Contrary to more complex GAGs, HA is not sulfated, and it does not occur as part of a proteoglycan, linked to a protein carrier. Contrary to other GAGs, it does not owe its properties to minor intrachain structures. In other GAGs, particularly the heparins/heparan sulfates, these structures are made by sulfation, epimerization, or deactelyation of the already formed glycan chain. As compared to other connective tissue GAGs, HA is unique in that it does not undergo postpolymerization modifications: It looks like it is perfect as it is. It is synthesized at the inner face of the plasma membranes, contrary to other GAGs that are synthesized by the resident Golgi enzymes and covalently attached to a protein core.[12] HA is translocated by dedicated enzymes out of the cell into the extracellular matrix as a free linear polymer.

From this quick picture, it can be concluded that HA is a comparatively simple polymer, without significant sophistication as biopolymers go. The fascination, interest, and opportunities of HA science lie exactly in the sharp contrast between the apparent simplicity of the primary molecular structure and the multiplicity of the roles HA plays in cell and tissue biology. Learning how nature can store so much information in such a simple structure can be of valuable help in the design of polymers for biology. Learning how to link this molecule to biomaterial surfaces to exploit its information content can lead to significant advancements in the engineering of biomolecular surfaces.

Hyaluronan−Protein Interactions. The first few decades of studies on HA (since its discovery by Karl Meyer at Columbia in 1934[13]) yielded the picture of a highly hydrophilic biomolecule, behaving in aqueous solution as an expanded random coil of considerable intrinsic stiffness. The molecular domain includes a large amount of solvent, and the chains entangle already at a concentration of 0.1% (1 g/L). Results based on light-scattering were in agreement with sedimentation, diffusion, and rheological data.[14−20] Ogston and co-workers concluded that the molecule behaved like a large hydrated sphere, which is compatible with a random coil configuration.

Studies and speculations on the role of HA in tissues were then focused on properties arising from its chemicophysical characteristics. Among them, entanglement and the ensuing formation of three-dimensional chain networks were considered as the basis of the HA role in tissues. In particular, the obvious role played by the very high viscosity of HA concentrated solutions in joint lubrication, the effect of osmotic pressure on water homeostasis in tissues, and the barrier to diffusion of macromolecules were among the main examples of HA activity.[14−20] The effects of HA on cell behavior were, according to prevailing views, entirely through physical interactions. The implication of an active role of HA in biological processes was mainly based on the presence or absence of HA in histological investigations. The findings from Toole and co-workers went a significant step ahead, by showing that different developmental processes (regeneration of the newt limb and development of chick cornea) shared a common time sequence of HA synthesis and subsequent removal by hyaluronidase, with a clear indication that accumulation of HA coincided with periods of cellular migration in the tissues.[21,22] Later studies confirmed that this is a common pattern, and it happens also in wound healing.

Coming back to the chemicophysical view engendered by the application of classic techniques of polymer chemistry to the study of HA, both the apparent randomness of the HA molecule (hence its high entropy content) and the evidence of extensive intramolecular hydrogen bonding were in tune with a general view of HA as an inert biomolecule.[23] This was considered an important property for the suggested role of HA as a space-filling molecule: According to the proposed structure, almost every polar group is involved in intramolecular interactions, making potential interactions with other species less likely. As a component of communication channels in the pericellular matrix, HA will then not interact

with molecules passing through them, thus avoiding clogging. Quoting Scott, "HA did indeed seem to be an entropy rich, perfect, non interactive stuffing" (p 8 of ref 23). While more recent approaches to HA structure in aqueous solutions will be discussed later on, in this section it is important to remark that this view came to an end by 1972, with the discovery of proteins that can specifically interact with HA. In that year, Hardingham and Muir showed that HA can aggregate cartilage proteoglycans.[24,25] Hascall and Heinegård demonstrated the existence of specific binding among HA, the N-terminal globular part of the proteoglycan, and a link protein.[26-28] Many aggrecan (the large proteoglycan that binds HA) molecules bind to the same HA chain, making up one of the most characteristic building blocks of cartilage and showing that HA can be involved in supramolecular structures. Almost at the same time, it was shown that an "aggregation factor" (AF) was present in the culture media of cancerous cells, promoting adhesion between malignant cells and leading to cell aggregates much larger than usual. Pressac and Defendi were able to show that the AF was indeed HA and that the interaction involved an HA-specific cell surface receptor bearing at least a protein moiety.[29] In a short time frame, the placid, silent, and inert giant molecule of light scattering and sedimentation found itself fully involved in the burgeoning world of cell and molecular biology.[30] The shift of the paradigm of HA as an inert material to a biologically active molecule was then completed by the first demonstration that HA activates signaling pathways.[31]

Proteins interacting with HA were named hyaladherins. The biochemical purification of the first cellular HA binding protein (HABP), presently known as RHAMM (receptor for hyaluronan-mediated motility), was obtained by Turley in 1982.[32] Work by Underhill and Toole led to the purification of another cell surface receptor in the 1980s, which was then identified as the same HA binding protein, the CD44 receptor, involved in lymphocyte homing.[33-35] Since then, a number of other cell surface proteins recognizing HA have been described, and literature in this field is booming.

While a complete review of hyaladherins and HA receptors is outside the scope of this paper and of its author's knowledge, it is of interest to evaluate some general features that are of direct relevance for the present discussion (excellent starting points for readers interested in this topic are the chapters by A. J. Day and by E. Turley and R. Harrison at the Web site cited at the beginning of this review). It is now clear that hyaladherins show significant differences in their tissue expression, cellular localization, specificity, affinity regulation, and mechanisms of interaction with HA. Among them, however, a few families can be defined. For instance, several hyaladherins share the same HA binding domain, called the link module (also referred to as a proteoglycan tandem repeat).[36] The link module contains about 100 amino acids with four cysteines.[37] Within this general framework, significant differences can exist in the structure of link domains of different hyaladherins. For instance, the HA binding domain of the HA cell receptor CD44 contains about 160 amino acids, and it is made up by a single link module with additional N- and C-terminal

Table 1. Some Examples of Hyaldherins and of the Minimum Sequence Length of the HA Disaccharide Repeating Unit Required for Recognition[a]

protein	no. of HA disaccharides required	features of the HA binding domain
TSG-6	3	link module, ~90 amino acids
stabilin-1	3	link module, ~90 amino acids
CAB61358	3	link module, ~90 amino acids
CD44	3-5	link module + extensions, ~160 amino acids
lyve-1	3-5	link module + extensions, ~160 amino acids
link protein	5	two link modules, ~200 amino acids
aggrecan	5	two link modules, ~200 amino acids
versican	5	two link modules, ~200 amino acids

[a] All proteins belong to the link module superfamily.

extensions, which are required for proper folding and functional activity.[38,39] Aggrecan and aggrecan-like proteoglycans use two link modules, both of them participating in HA binding.[40,41] Of great signficance for the scope of this paper, the details of the HA binding domain of a given hyaladherin affect the minimum sequence required for HA binding and recognition, as shown in Table 1. HA repeating units involved range from three to five disaccharide repeats; that is, the interaction involves continuous HA segments of ~1200-2000 Da.

As for the molecular basis of the link module–HA interaction, existing evidence shows that it involves ionic interaction between HA carboxylates and basic amino acids. In the case of CD44, two arginines and two tyrosines play a critical role.[38,42] Goetnick and co-workers provided evidence of the effect of ionic strength on the link protein–HA interactions, confirming that it is largely of ionic nature and showing that the percent of protein bound is a decreasing function of the ionic strength.[43] On the other hand, important hyaldherins such as CD44 and aggrecan are, as a whole, highly negatively charged, and increasing the ionic strength of the medium signficantly increases the binding of HA,[35] probably due to the neutralization of the ionic repulsion. Their specific interaction with HA is, however, driven and controlled by local ionic interaction between HA carboxylate and cationic amino acids, even though both involved molecules can be depicted as highly negatively charged colloidal particles. Actually, an extensive network of interactions maintains the association of receptors with HA, and the overall energetics of the interactions is finely balanced, to the point that the loss of a single hydrogen bond or ionic interaction can be enough to abolish binding.[36]

A significant finding arising since the first studies on hyaladherins in general, and of CD44 in particular, is that the HA receptor interaction is of a cooperative nature. Already in the pioneering work by Underhill and Toole it was suggested that a single molecule of HA could bind to multiple cell surface receptors, as suggested by molecular-weight-dependent binding inhibition studies.[35] A corollary to this view is that a single HA–CD44 interaction may have a low binding affinity, yet the combined effect of multiple HA–CD44 bindings can yield strong interactions (even if,

as stated by Underhill,[44] and important to note, the HA-mediated binding is relatively weak in comparison with other cell adhesion mechanisms, namely, those involving integrins or cadherins). Lesley and co-workers have provided many fundamental insights into the CD44−HA interactions.[45−47] Among them, of particular relevance for the scope of the present paper, is the relationship between HA length and CD44 binding. Confirming previous suggestions, it is stated that the CD44−HA interaction is of a cooperative nature, involving multiple closely arrayed CD44 receptor molecules at the cell surface interacting with the highly multivalent repeating disaccharide chain of HA.[45] There is much evidence that points to the cooperativity of the HA−CD44 interaction; namely, a "threshold" level of CD44 on the cell surface is required for HA binding. Clustering of CD44 on the cell surface increases the HA binding activity without an increase in the number of CD44 molecules. Using fluorescein-conjugated HA and flow cytometry to detect the ability of small unlabeled HA fragments to block binding, Lesley and co-workers were able to draw clear relationships between several binding parameters and HA length:[45] HA_6, which is three HA disaccharide units (MW $\approx$ 1200), is the minimum size of HA chain required to occupy the CD44 binding site, and HA_{10} or greater is the optimal length. A significant increase in blocking activity was observed for $HA_{\sim 20}$ (MW $\approx$ 4000), suggesting that this chain length can yield divalent binding, that is, interaction of the same HA chain with two CD44 receptors. Multiple binding, that is, binding of HA with more than two CD44 receptors, was detected only in the case of cells pretreated with an antibody that induces CD44 expression. It was suggested that the antibody could promote larger aggregation of CD44 and/or orient existing CD44 to optimize divalent binding (or induce multivalent binding). Also displacement studies show some unusual features: In particular, cell-surface-bound high-molecular-weight HA is displaced by competing free HA (about 25 min to decrease by 50% the amount of cell-bound HA); lower molecular weight ($\sim$30000 or about 75 repeating units) HA is displaced from the cell surface in a few minutes. In summary, from this fine characterization work, the following picture of the CD44−HA interaction emerges: Cooperativity is the primary feature, resulting from multiple binding sites on the polysaccharide ligand and multiple closely arrayed receptors on the cell surface. The length of the carbohydrate chain that determines the number of physically connected binding sites is the main factor as far as the ligand is concerned. Each HA−CD44 interaction may be very weak and transient, yet many binding interactions along the length of the HA chain can keep the molecule bound to the cell surface and increase the probability of rebinding and binding at adjacent sites, in agreement with earlier suggestions from Underhill.[35] The low avidity of an individual CD44 receptor for its ligand and the reversible and multivalent nature of the HA−CD44 interaction may be essential features for the biological function of HA in mediating cell migration.[44,45]

The findings presented by Lesley and co-workers address some key points of the mechanism of the HA−CD44 interaction. They are of great relevance also when it comes to another noticeable feature of the behavior of HA, namely, its molecular-weight-dependent biological activity. Actually, as discussed in the next section, HA oligomers, produced by the degradation of high-MW HA, can have significant biological activity, often opposed to that shown by high-MW HA in the same system, suggesting that cells can "distinguish" between high- and low-MW HA. Since CD44 is the main cell surface receptor, many of these effects will involve CD44, which has just been shown to require high-MW HA for stable binding. HA oligomers play a role, for instance, by interfering with high-molecular-weight HA binding.[45] Moreover, physiological or pathological mechanisms could lower the threshold for stable binding of HA. For instance, Lesley and co-workers have recently reported that another hyaladherin, TSG-6 (tumor necrosis factor-stimulated gene 6), enhances the binding of HA to CD44.[47] In a very interesting study involving interaction between HA and a CD44 chimera containing the link module of TSG-6, it was shown that the TSG-6 link module strongly enhances CD44−HA interactions, to the point that the link module of TSG-6 confers integrin-like characteristics to CD44: Cells expressing CD44/TSG-6 chimeric receptors become firmly adherent or permanently tethered to HA.[47] Even if complete details of the interaction mechanisms are still to be unraveled, the dependence of the binding stability on the number of HA repeating units is a first step toward the understanding of how cells might discriminate between HA chains of different lengths and respond differentially.

In closing this section, it is important to underline that even if CD44 is recognized as the main cell surface receptor for HA, it is by no means the only one. The already cited RHAMM is associated with cell locomotion, and its expression is often associated with migrating cells.[48,49] It does not contain the link module, so it belongs to a different family of HA binding proteins, whose binding properties are possibly controlled by basic amino acid sequences.[48] The number of identified HA receptors on the cell surface is constantly growing.[1−4,6,50]

HA Behavior in Tissues. From the quick review presented in the previous section, it is clear that HA can and does directly communicate with proteins and cells present in tissues. The interaction can be orchestrated by hyaldherins resident on the cell surface or can be induced in response to a given stimulus (e.g., TGS-6). What are the instructions imparted by HA to cells, and how do they affect tissue behavior? Being present in almost every tissue, HA is actually involved in a huge number of different processes, and it would be impossible to review them all. Here, some discussion will be presented on the role of HA in tissue repair processes. Studies in this field have benefited from inputs coming from different areas of active studies on HA such as oncology, morphogenesis, and embryogenesis.[1−4] This is in tune also with the scope of this paper, that is, to highlight the opportunities for HA in the surface modification of implant materials. As already evidenced by the studies from Toole in the 1970s,[21,22] HA accumulates in repair or morphogenesis in periods corresponding to cell migration. Actually a common pattern of the time dependence of HA content is seen in many biological phenomena involving

tissue remodeling: A transient HA-rich matrix develops during, e.g., embryogenesis, or adult tissue remodeling, coincident with rapid cell proliferation and migration. This is temporarily followed by a HA decrease in the tissue, concomitantly with tissue differentiation, vasculogenesis, and angiogenesis. Accumulation of HA is often associated with tissue damage, inflammatory disease, and tumors. The localization and time-dependent properties of HA in the mentioned tissues suggest that HA plays an important regulatory role in these processes.

HA's unique hygroscopy and its rheologic and viscoelastic properties undoubtedly can play a role, by creating a swollen macro- and microenvironment very conducive for cell migration.[6] The highly hydrated matrix provided by HA can result in weakening of cell anchorage to the extracellular matrix, allowing temporary detachment to facilitate cell migration and division. Besides these general features, HA plays an active role in every step of wound healing (reviewed as well and fully referenced in refs 5 and 6): The initial stage of wound healing involves inflammation, a necessary step to generate many of the factors required in later steps leading to complete wound healing. It has been shown that the wound tissue in the early inflammatory phase is rich in HA, which, at this stage, can show a proinflammatory action. Also, production of proinflammatory cytokines such as TNF-α (tumor necrosis factor-α) and IL-1β (interleukin-1β) is related in a dose-dependent manner to HA concentration. Microvascular endothelial cells increase their HA production in response to these cytokines, and this facilitates adhesion of lymphocyte to the cells via HA by a CD44-mediated mechanism.[51]

Also the granulation tissue, which is formed in the subsequent stage of wound healing, is rich in HA.[52] This is essential to facilitate cell migration and organization into the provisional wound matrix, and as already discussed, HA is always present in areas of extensive cell migration. In particular, previously mentioned RHAMM forms links with several protein kinases, and many cell types show enhanced cell movement in response to HA.[48,49] During granulation tissue formation HA can act as a moderator of inflammation, in a way somehow contradictory to its role in inflammation stimulation as described before. While inflammation is an integral part of wound healing, it must be moderated to allow stabilization of the newly formed tissue matrix. Here HA shares with other large polyelectrolytes a free radical scavenging effect, protecting cells against free radical damage.[6,53] Besides this general mechanism, HA can contribute to moderation of inflammation through specific interactions: The previously mentioned hyaladherin TSG-6, which is expressed by inflammatory cells, can form a stable complex with IαI (inter-α-inhibitor), a serum proteinase inhibitor, inducing a synergistic effect on IαI plasmin-inhibitory activity.[6] Plasmin, in turn, plays an important role in activation of proteolytic cascades that lead to inflammatory tissue damage. Thus, the TSG-6/IαI complex, linked to HA in the extracellular matrix of granulation tissue, may act to down-regulate plasmin activation and can exert a negative feedback loop to moderate inflammation.[54] Interestingly, in the same in vivo model where HA has been shown to have

proinflammatory properties, the administration of TSG-6 resulted in marked reduction of inflammation.[6]

Angiogenesis, or new capillary formation, is an important step in wound healing definitely required in the proliferation phase involving matrix synthesis and organization. Here HA shows one of its most intriguing features, namely, the completely different biological effect induced by oligomers vs high-molecular-weight species. In short, high-molecular-weight HA in the extracellular matrix has been shown to inhibit angiogenesis; on the other hand, many in vitro and in vivo experiments have shown that HA oligomers (OHAs) have proangiogenetic behavior.[55−61] This behavior is also supported by the observation of angiogenesis coinciding with an increase of hyaluronidase activity and consequent degradation of matrix HA, to the point that HA degradation is seen as a prerequisite for wound angiogenesis. In a related process, it has been claimed that hyaluronidase produced by tumor cells could induce angiogenesis and be used by tumor cells as a "molecular saboteur" to depolymerize HA to facilitate tumor invasion through angiogenesis.[62]

The first evidence of a molecular-weight-dependent effect of HA was presented by West and co-workers.[55,56] In particular, experiments with the chick chorioallantoic membrane showed that partial degradation products of sodium hyaluronate produced by the action of testicular hyaluronidase induced an angiogenic response, while neither macromolecular HA nor exhaustively digested material had any angiogenic potential. Later on it was shown that OHAs between 3 and 16 disaccharides in length (that is 1200− 6400 Da), produced by hyaluronidase digestion of high-MW HA, stimulated angiogenesis in vivo and the proliferation of tissue-cultured endothelial cells in vitro, while high-molecular-weight HA both inhibited endothelial cell proliferation and disrupted newly formed monolayers.[56]

How does HA size affect angiogenesis? It has been reported that OHAs induce expression of angiogenic proteins in cultured endothelial cells.[63] Slevin and co-workers, Savani and co-workers, and Lokeshwar and co-workers have published detailed studies on the mechanisms that control the angiogenic activity of OHAs,[60−62,64,65] providing evidence that both CD44 and RHAMM regulate key functions during angiogenesis. From these studies, it is concluded that OHA interaction with HA cell surface receptors induces multiple signaling pathways affecting vascular endothelial cell mitogenic and wound-healing responses, while native, or high-molecular-weight, HA fails to show direct signaling activity or synergistic effects with vascular endothelium growth factors. Atherosclerosis and restenosis, key problems of contemporary Western world societies, are fully involved with HA and its MW-dependent effects: HA accumulates at different stages in atherosclerosis and restenosis, even if it is not clear whether it accumulates at an early or late stage and if it contributes to the formation of atherosclerotic lesions.[66] HA is produced by cells of the arterial wall, among them endothelial cells and smooth muscle cells. HA synthesis is up-regulated, as in other cell types, when smooth muscle cells leave their quiescent state and are stimulated to multiply and migrate, leading to neointima formation and restenosis. Wight and co-workers demonstrated that an HA and versican-

rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells.[67] By a particle-exclusion assay they were able to show that an HA-rich coat exists around the cells as they migrate and proliferate, the coat being formed by the interaction of HA and associated molecules with receptors on the surface of smooth muscle cells. Interestingly, while OHAs stimulate endothelial cells to proliferate and migrate, together, as previously discussed, with angiogenesis, blocking of HA receptors on vascular smooth muscle cells by OHAs (MW < 3000) prevents the formation of the pericellular coat and the proliferation and migration of smooth muscle cells. Slightly larger HA fragments (MW > 3000) were not able to inhibit cell growth. The different effects exerted by HA on endothelial and smooth muscle cells are obviously of great relevance in the hot field of atherosclerosis, interventional cardiology, and coronary stenting.[68−71]

Besides endothelial cells and angiogenesis, other biological processes show a dependence on the HA molecular weight, among them the expression of heat shock protein 72[72] and the activation of dendritic cells.[73] Huang and co-workers have presented molecular-weight-dependent effects of HA on osteoblast proliferation and differentiation.[74] Recent results allow investigators to fully appreciate the growing importance of this arm of HA science: Taylor and co-workers have shown that OHAs can stimulate recognition of injury by endothelial cells, triggering in this way the initial stages of the wound defense and repair response.[75] Fieber and co-workers have shown that OHAs induce transcription of matrix metalloproteases (MMPs) in tumor cells, suggesting that the metastasis-associated HA degradation in tumors might promote invasion by inducing MMP expression.[76] Nasreen and co-workers have shown that low-molecular-weight HA induces malignant mesothelioma cell (MMC) proliferation and haptotaxis, providing evidence that the interactions mediated by CD44 transmit regulatory signals for mediating the locomotion and proliferation of MMCs.[77] On the other hand, evidence exists that OHAs (MW ≈ 2500) inhibit growth of several types of tumors in vivo.[78]

HA: Molecular Structure in Aqueous Solutions

Results reviewed in the previous sections show that, despite the apparent simplicity of the primary structure of HA, this molecule can exert multiple, often molecular-weight-dependent, biological effects not only through its physicochemical properties, but also through direct interactions with proteins and cell surface receptors. What are the molecular bases that control communications between HA and other biomolecules? To try to answer this fundamental question, this section reviews some of the models of HA in aqueous solutions, since biomolecular function is emergent from the interplay with water molecules.

It has already been mentioned that the first picture of HA suggested by studies performed by classical methods of polymer solution physical chemistry was that of a comparatively stiff random coil. As aptly underlined by Scott, "it can be asked whether, in principle, the available methodology could give any other kind of picture",[79] since the data were extrapolated to infinite dilution, implicitly removing inter-

molecular contacts and specific interactions. More in general, and even if this matter is still highly debated, the reading of the papers by Scott and co-workers is of great interest, since they often tackle the debate between "statistical-mechanical" and "atomistic" views of biomolecules and biomolecular interactions, a subject of enormous importance in present-day biomolecular biomaterial surface science.[79,80] See also the contibutions by Scott in refs 1−4 and on the mentioned Web site. In short, Scott makes the point that biology does not like entropy, but rather biological function needs shape. A few indications suggesting that aqueous HA does indeed have a shape were gathered by Scott.[1−4,79,80] Namely, the reactivity of the glycol group in the uronate of the repeating disaccharide unit, as probed by periodate oxidation, is much lower in HA and chondroitins as compared to other GAGs and model monomers. Through a series of studies, it was shown that the reactivity of the glycol glucuronate was hindered by the polymer environment around it; in particular, it was concluded that a hydrogen bond exists between carboxylate and acetamido groups and between glycol glucuronate and acetamido. It was then suggested that, in water, the direct NH → COO$^-$ hydrogen bond is replaced by a water bridge, and the picture was completed by two more hydrogen bonds, as shown in Figure 2A (in this figure, the water bridge is not shown). Computer simulations were compatible with the proposed structure. Extensive intraresidue hydrogen bonding, repeated throughout the polymer, forces the molecule into a 2-fold helix structure and accounts for the stiffness and resistance to periodate oxidation. A notable consequence of the proposed structure is that it yields large clusters of eight contiguous −CH groups, forming regularly spaced patches of hydrophobic character. Moreover, the two sides of the tapelike 2-fold helix are antiparallel; that is, both of them possess hydrophobic patches present on alternate sides of the tapelike helices. In short, these studies suggest that the formerly proposed random coil HA has a secondary structure; that is, it has a shape, and it is decorated by patches conducive for hydrophobic interaction. This feature opens the door to interaction with other biological molecules and with other HA molecules, possibly leading to supramolecular or tertiary structures. The tertiary structure of aqueous HA has been discussed by Scott and Heatley on the basis of ^{13}C NMR data[80] (Figure 2). In particular, comparing NMR spectra of high-molecular-mass (∼10^6 Da) and hyaluronidase-digested (probably mostly tetrasaccharides) HA, it was observed that the C═O peak from the undigested sample was much broader than any other peak, from both the undigested and the digested HA. By comparison with the NMR spectrum of partially methylated HA, it was concluded that it was the acetamido C═O, and not the carboxylate one, that shows the anomalous broadening. These data suggest that motion of the acetamido carbonyl is severely restricted in the high-molecular-mass sample, but not in the digested one. Detailed analysis of the spectra leads to the conclusion that the restricted motion can be accounted for by a hydrogen bond from a neighboring molecule. This could be provided by a carboxylate that, according to modeling, was shown to be suitably placed in antiparallel HA duplexes. In summary, these data suggest that tertiary
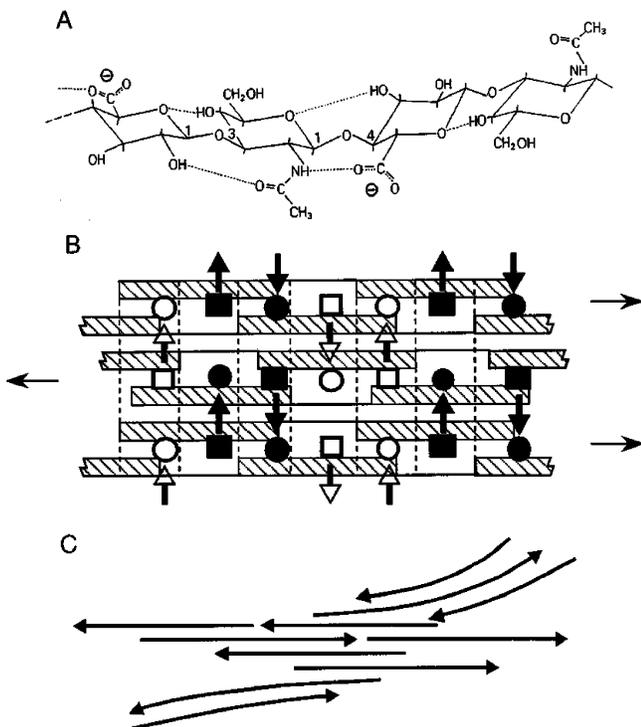
Figure 2. Scott model of primary, secondary, and tertiary structures in HA solutions.[80] (A) A tetrasaccharide fragment of HA comprising two repeating disaccharides, showing five H bonds that help to maintain the 2-fold helix. The direct H bond between the acetamido NH and carboxylate seen in dimethyl sulfoxide solution is largely replaced in aqueous solution by a water bridge. (B) Three HA chains in a proposed tertiary structure, i.e., 2-fold helices with gentle curves in the polymer backbone in two planes at right angles and hydrophobic patches (hatched) on alternate sides of the polymer. The vertical dotted lines delineate the sugar units, and the arrows at the right and left sides indicate the reducing terminal direction. Only in antiparallel orientation do the gentle curves in the backbones of the participating molecules complement each other so that interactions are optimal. In antiparallel arrays, the acetamido (■, □) and carboxylate (●, ○) groups are positioned so that H bonds are possible between them as indicated by arrows pointing from the donor to the acceptor groups. The open symbols refer to groups on the proximal side of the tapelike molecule and the closed symbols to groups on the distal side. Hydrophobic patches (hatched) on neighboring molecules are contiguous, and hydrophobic bonding between them can occur. (C) Scheme of overlapping HA molecules that would allow infinite meshworks to form from HA of high molecular mass. Neighboring molecules are antiparallel, interacting at the atomic level as shown in (B). Reprinted with permission from ref 80. Copyright 1999 National Academy of Sciences.

structure of HA in aqueous solutions exists, involving 2-fold helices with gentle curves in the polymer backbone in two planes at right angles and hydrophobic patches on alternate sides of the polymer. Only in antiparallel orientation do the gentle curves in the backbones of the participating molecule complement each other so that the interactions are optimal. Within these antiparallel arrays, the acetamido and carboxylate groups are positioned so that hydrogen bonds are possible between them. The suggested tertiary structure of HA is based on the weak, cooperative forces typical of biomolecules: hydrophobic interaction and hydrogen bonding. Only multiple interactions provided by high-molecular-mass HA can stabilize the supramolecular arrangement of HA helices against electrostatic repulsion of the negatively charged colloid. Low-molecular-weight, or digested, HA is not able to assemble in tertiary structure, as shown by the unrestricted

mobility, according to the cited work, of the acetamido carbonyl of digested HA. It is suggested, on the basis of light scattering data, that aggregation of HA oligosaccharides might be possible between oligomers of >20 disaccharides (MW ≈ 8000), while above 300 disaccharides (MW ≈ 120000) HA chains are clearly aggregated.

Taking this model a step further, and considering the previously mentioned molecular-weight-dependent properties of HA, Scott and Heatley proposed the fascinating idea that biological properties of HA are sequestered and controlled by its reversible tertiary structure.[79] According to this view, "bioactivity" is an intrinsic characteristic of HA chains, which disappears whenever assembly in tertiary structure masks direct communication between HA 2-fold helices and the surrounding milieu and is made available whenever the tertiary structure denatures, releasing, in this way, HA chains. Thus, HA biological properties are controlled by molecular-weight-dependent transitions between tertiary structures and 2-fold helices. Interestingly, Arrhenius plots show that HA tertiary structures are on the edge of instability under physiological conditions, ready to switch toward bioactivity or bioinertness as a response to external stimuli. A number of transitions between structures, obtained by warming and alkalinization (both of them reversible) and methylation and hyaluronidase digestion (obviously both irreversible), were followed by NMR. Structures in agreement with the supramolecular organization proposed by Scott have been observed by the electron microscope, as reviewed in the Scott papers, and also by atomic force microscopy (AFM).[81]

The model proposed by Scott and Heatley suggests a possible answer to the initial question of how nature can store so much information in such a simple molecule: Here part of the trick is a facile transition between secondary and tertiary structures under physiological conditions, which actually switches intrinsic bioactivity off and on. Even if the proposed model is criticized by findings of other researchers, as discussed in the following, the work by Scott and co-workers is of high significance, as is their effort to get rid of constraints imposed by existing schools of thought: This work was instrumental in bringing the HA of classic polymer science, mostly viewed as a mechanical segment discussed by statistical approaches, within the realm of molecular/atomistic interactions that make up the stuff of biological interactions.[82]

Gribbon, Heng, and Hardingham have provided experimental studies of HA in physiological aqueous solutions that do not support the model proposed by Scott.[83,84] According to these authors, no evidence exists of stable or cooperative HA intermolecular associations in physiological solutions; rather their experimental results show that even at high concentrations a simple hydrodynamic model with chains stiffened by hydrogen bonds accounts for the major solution properties of HA. Incidentally, this picture is supported by early studies on inert protein–HA interactions: Inert proteins are excluded from HA solutions and gels as expected from a random chain network of single rigid polysaccharide chains.[85]

The work of the Hardingham group[83,84] is based on the study of the solution properties of HA by confocal fluores-

cence recovery after photobleaching (confocal FRAP). In particular, to understand how electrostatic interactions and hydrogen bonding of HA contribute to its properties in solution, the self-diffusion coefficient of HA was determined over a range of macromolecular concentrations and counterion concentrations and at neutral and alkaline pH. The characteristics of HA networks formed under different conditions were also investigated by measuring the permeability to an uncharged dextran macromolecular probe. The properties of HA in urea (up to 6 M) were investigated to test for hydrophobic interactions. Data show that, at physiological ionic strength, the major contribution to the large hydrodynamic volume of HA, and hence its other important solution properties, is due to intramolecular hydrogen bonding between adjacent saccharides: Hydrogen bonding restricts rotation and flexion at the glycosidic bonds and creates a stiffened polymer chain. It is concluded that, even at very high concentrations, under physiological conditions, individual HA chains remain mobile. Studies performed in urea indicate reduced hydrodynamic size and increased permeability to fluorescein-conjugated dextran, suggesting increased chain flexibility, but without the changes predicted if chain−chain associations were disrupted by urea. Also, oligosaccharides of HA had no effect on the self-diffusion of high-molecular-mass fluorescein-labeled HA (830 kDa) solutions, or on dextran tracer diffusion, showing, according to the authors, that there were no chain−chain interactions open to competition by short-chain segments.

An exceedingly interesting series of studies, based on molecular dynamics simulation, has added a further dimension to the just-described pictures of HA in water, that of time.[86−88] Almond, Brass, and Sheehan have modeled the dynamic interaction between HA oligomers and explicit water molecules. Since the accepted persistence length of HA is about 5 nm, and since the accepted length of the HA disaccharide repeating unit is about 1 nm, the shortest oligomer that is expected to exhibit polymer-like behavior is the decasaccharide (five repeating units). A 5 ns molecular dynamics simulation of an HA decasaccharide shows indeed extensive intramolecular hydrogen bonding, the acetamido group being, as in all GAGs, a key player.[88] Significantly, all intramolecular hydrogen bonds are rapidly rearranging and exchanging with water, and a high proportion of all possible hydrogen-bonding interactions were observed over a period of 200 ps. This finding brings significant value also from the experimental point of view: Predicted hydrogen bonding may not be persistent enough to be captured by NMR, possibly explaining why hydrogen bonds observed in dimethyl sulfoxide (DMSO) are not always confirmed by observation in aqueous solutions. The picture of HA that arises from this simulation is somehow in tune with the classical model of a 2-fold structure that is stabilized by intramolecular hydrogen bonds between neighboring sugars, to the point that the decasaccharide is consistently close to the maximum molecular end-to-end distance. However, it is suggested that to capture the HA behavior in aqueous solutions a more dynamic view is needed: HA can be seen as a stiff, flexing coil that, although almost fully extended most of the time, can undergo rapid rearrangement to a more

contracted state, as highlighted by the ephemeral observation of strong conformational fluctuations on the subnanosecond time scale. Importantly, these fluctuations are emergent by combined polymer−water interactions, which stabilize or destabilize specific intramolecular interactions. Long-lived intramolecular interactions, such as those involving the acetamido moiety, are probably highly favored because they cause the least disturbance to the surrounding water organization. Instantaneous destabilization of the hydrogen bond array involves large rearrangement of the surrounding water structure, introducing dynamic flexing into polymer molecules.[88]

This dynamic model has been discussed in relation to HA−protein interactions in a delicious paper by Day and Sheehan.[89] The starting point is the just presented picture of HA in solution as a highly organized extended dynamic coil that can undergo sharp kinks, bends, and folds while maintaining distinctive hydrogen bond conformation, within the frame of a chain reorganization time on the pico- to nanosecond time scale.[88] According to this picture, HA in aqueous solutions may be viewed as a stiffened highly dynamic ensemble of chaotically interchanging semiordered states, which can be redistributed by any field of force, but which can resist the force and are readily restored upon force removal. Ligand−receptor interactions between HA and hyaladherins are among the forces acting upon the dynamic HA chain. Taking advantage of recently published crystal structures of hyaluronate-digesting enzymes complexed with short HA oligosaccharides,[90−92] the authors speculate that interacting HA can be stabilized in a wide variety of conformations by binding to proteins. Growing knowledge of hyaladherins is actually showing that there is great diversity in the way they can interact with HA, and a reasonable explanation for the diversity in HA−protein interactions is that hyaladherins have evolved specific binding sites to capture different HA conformations. Thus, the primary suggestion of the authors is that the specificity of the protein−HA interaction may drive the formation of different quaternary structures depending on the conformation of bound HA and the nature of the stabilizing protein−protein interaction. According to this view, the information content of HA lies not just in HA itself, but in the conjugation of HA and proteins, which moves the HA extended dynamic coil "from polysaccharide chaos to protein organisation".[89]

Surface Modification by HA

Previous sections set the stage for a proper discussion of strategies for surface immobilization of HA. If the aim of surface modification by HA is to exploit its biological potential, then it must be taken into account that HA interactions with proteins, including cell surface receptors, requires minimum sequences of HA repeating units.[35,45−47] Applying this principle to surface-bonded HA, interactions require minimum consecutive sequences of non-surface-bonded HA between two consecutive HA−surface links. The biological effect can be molecular-weight- or sequence-length-dependent,[55−63] meaning that, in principle, different sequence lengths of surface-linked HA can induce different effects; according to the Scott model, the biological informa-

tion content of HA is carried by structuring−destructuring of HA chains.[79] According to the Day and Sheehan model, it is the specific HA conformation engendered by interaction with a given hyaladherin that provides function.[89] Of course, both models require chain mobility and conformational freedom. In both models the presence of the field of force stemming from a solid surface and/or the constrained mobility due to surface-linking can provide significant perturbations.

Such considerations will be discussed again later on, after literature approaches to HA immobilization are reviewed.

Adsorption. Given the high solubility of the HA molecule in aqueous solutions, its very hygroscopic nature, and its negative charge, it can come as a surprise to the surface chemist that the most commonly adopted technique for surface immobilization of HA, widely used in a significant part of the work on the discovery of HA properties just described, is the simple passive adsorption of HA to plastic labware. This approach was probably serendipitously introduced, mimicking typical protocols of immunoassay, where a given protein or antigen is passively adsorbed to the polystyrene surface of microtiter wells. However, contrary to proteins, whose hydrophobic domains in general supply the required driving force for passive adsorption through hydrophobic interactions, hydrophilic polysaccharides in general, and HA in particular, would not be considered the ideal candidates for stable adsorption to plastic surfaces. Yet, a number of data suggest that this approach works, at least for the short lifetime of the tests involved, even if, as often happens in multidisciplinary fields, the relevant papers tackle just the biological side of the problem and definitely lack the required surface characterization work, in a surface chemistry sense. Delpech and co-workers, in 1985, described an enzyme immunoassay based on the immobilized HA−hyaluronectin interaction.[93] The paper reports that polysytyrene (PS) microwells were coated with a 50 mg/L (0.005%) solution of HA in 0.1 M bicarbonate. Incubation of the immuno reagents on the HA-coated microtest plate lasted 4 h. Interestingly, maximum sensitivity was achieved when incubation was carried out at 4 °C; the low temperature could possibly decrease the rate of dissolution of adsorbed HA. The assay was applied to determination of HA in sera, and it demonstrated specificity, showing for the sake of this review that HA was indeed on the surface. Goetinck and co-workers in 1987 evaluated link protein binding to HA, using immobilized HA obtained by applying in each well 60 μL of a solution containing 50 mg/mL HA.[43] Results show that the link protein−HA interaction is a decreasing function of ionic strength, as discussed in the previous section.

Passive adsorption of HA has been widely used since these pioneering works, without, as already reported, in-depth surface characterization (or, more precisely, without any surface characterization at all). Some indication that the properties of the underlying substrate play a role in the adsorption process can be found in an interesting paper by Catterall and co-workers:[94] While binding of ovarian cancer cells to immobilized HA was being investigated, a study related to the previously referred to role of HA in tumor progression, several types of microtiter wells were screened

to check which one could maximize immobilized-HA-mediated cell adhesion (in 5 min experiments). HA immobilized on tissue culture microwells was much more effective than HA immobilized on Maxisorp (which is basically γ-ray-treated PS used for immunoassays, very close, in terms of surface hydrophobicity, to untreated PS; as evaluated by X-ray photoelectron spectroscopy (XPS), the surface of Maxisorp microwells shows 2−3 atom % oxygen, while tissue culture plastic surfaces usually contain more than 10 atom % oxygen). These findings suggest that HA adsorption is favored on "polar" as compared to "nonpolar" surfaces, a result recently substantiated by the first thorough study on HA immobilization by chemisorption, by Suh, Kademhosseini, and co-workers.[95,96] In a series of interesting studies, aimed at the exploitation of the cell-resistant properties of HA for micropatterning and cell culture, these authors have shown that HA is indeed chemisorbed to solid surfaces, where it is stabilized by hydrogen bonding between carboxylate or hydroxyls from HA and electron donor−electron acceptor groups on the substrate. In these studies, HA is applied by spin coating from 5 mg/mL (0.5%) unbuffered aqueous solutions and then dried, a practice that is clearly more favorable for the establishment of interfacial interactions than direct adsorption from aqueous solutions. It is shown that, despite the water solubility, a 3 nm thick chemisorbed HA layer remains on the surface even after prolonged washing, and it is stable on highly hydrogen bonding surfaces such as glass or silicon oxide for at least 7 days in phosphate-buffered saline (PBS). In a very interesting comparison, it was shown that HA is adsorbed on these surfaces in much higher quantities than other polysaccharides such as dextran sulfate, heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and alginic acid, because of the molecular entanglement and intrinsic stiffness of HA. Two consideration here are mandatory: From one side, these interesting studies finally put the passive adsorption of HA on a sound ground, showing once again the role of short-range forces (interfacial hydrogen bonding) in interfacial interactions in aqueous solutions. On the other hand, it is made clear that even in this case the stability of immobilized HA is limited to a few days at maximum in PBS, while in biological media exchange reactions between solution and surface-adsorbed biomolecules could further reduce the lifetime of the adsorbed layer. This occurrence sets an upper time limit to the experiments in which it can be used.

Passive adsorption of HA to microtiter well surfaces has been used to probe, as already discussed, link protein−HA interactions, receptor−HA interactions,[44−46] and cell−HA interactions.[65,94] This is a clear indication that HA immobilized in this way can still communicate with biological counterparts. Unfortunately, the lack of detailed surface characterization does not allow investigators to extract significant information on, for instance, the surface density of adsorbed HA, and its fine structure in terms of relevant parameters such as conformational mobility and length of segments available for interactions.

Ionic Coupling. If not for the compelling evidence recently presented in the papers by Suh, Khadamhusseini, and co-workers,[95,96] no surface chemist would even think

about immobilizing HA without some form of surface pretreatment that could increase the binding strength of HA to the surface and prevent its rapid dissolution in aqueous solutions. Given the polyanionic nature of HA, the most obvious approach is to provide a cationic surface and stabilize HA by the formation of a surface-linked polyelectrolyte layer. Immobilization of anionic polysaccharides to positively charged surfaces is probably the oldest approach to carbohydrate immobilization.[97] Immobilization of HA via formation of an ionic complex with polycationic surfaces has been widely studied, especially in the formation of alternate polycationic−polyanionic layers, an approach that is enjoying much popularity today. Sequential adsorption of oppositely charged colloids was reported in 1966,[98] and sequential adsorption of polycationic poly(ethylenimine) (PEI) and polyanionic dextran sulfate and heparin has been widely used for many years in the biomedical industry, for instance, in the Carmeda process for heparin coating.[99] Polyelectrolyte multilayers involving HA have been widely investigated. Picart and co-workers have presented detailed characterization of chitosan (CH)−HA and poly(L-lysine) (PLL)−HA multilayers,[100,101] while Burke and Barrett have presented a nice description of the pH-responsive properties of PLL−HA multilayers.[102] In particular, it has been shown that CH−HA multilayers grow by an exponential increase in mass and thickness with the number of layers, an occurrence that is basically related to the ability of the polycation to diffuse in and out of the whole film at each deposition step. Evaluation by AFM shows a nice evolution of the film surface toward a smooth morphology. Each polyion deposition induces a reversal of surface charge, providing the driving force for the adsorption of the next layer. In the study by Picart and co-workers,[100,101] the ζ potential switches from about +50 to about −25 mV after deposition of each CH or HA layer, respectively, in 0.15 M NaCl. As for biological properties, homogeneous HA films obtained by CH−HA ionic buildup show very low adhesion of primary chondrocytes and of *Escherichia coli* bacterial cells. Resistance to nonspecific biological adhesion is a typical property that HA shares with other highly hydrated or hydrogel-like surfaces. In another study, that will be discussed again later in the section on immobilization by cross-linking, PLL−HA ionic multilayers show resistance to adhesion of chondrosarcoma cells. Interestingly, these same surfaces are degraded by hyaluronidase. This shows that HA ionically surface immobilized according to this process can still be recognized by hyaluronidase, and that the minimum sequence length required for specific interaction and/or the required chain mobility, as discussed in the previous section, is still available despite restriction of conformational freedom. Immobilized HA, thus, still has regions that look like an information-rich biomolecule, at least for this kind of interaction, when surface-immobilized in this way.

Tabrizian and co-workers have presented another interesting example of a polyionic multilayer based on HA.[71] To improve the biocompatibility of nitinol stents used in percutaneous transluminal coronary angioplasty (PTCA), possibly taking advantage of the previously referred to bioactive properties of HA toward smooth muscle cells,

multilayer buildup was used to prepare CH−HA-coated stents. Interestingly, to optimize adhesion to the metal substrate, the first polycationic layer is PEI rather than CH. Multilayer buildup follows a linear growth, a few μg/cm^2 being deposited at each step. Surface characterizations by fime-of-flight secondary ion mass spectroscopy (TOF-SIMS), AFM, and contact angle measurement are reported to show the uniformity of the surface layer.[71] An in vitro test shows that immobilized HA decreases platelet adhesion as compared to the control, due to its antifouling and protein-resistant properties. However, an ex vivo test shows that the HA overlayer does not prevent neutrophil adhesion, a result that, as correctly underlined by the authors, could be due either to nonspecific interactions or to specific adhesion involving the CD44 receptor expressed on leukocytes. As for coating stability, exposure of the coating to PBS for several days does not cause coating detachment or delamination. Interestingly, when a coated sample was kept in contact with a porcine coronary wall for 3 days in PBS, the HA layer was translocated to the arterial wall, providing a way to achieve a high local concentration of HA that could trigger all the complicated mechanisms related to HA bioactivity in restenosis.

Cross-Linking. In general, long-term stability of implant coatings requires some form of covalent bonding to stabilize the biomolecular overlayer, not only against dissolution, but also against exchange phenomena that occur between (bio)macromolecules adsorbed and those in the aqueous environment (the Vroman effect is probably the most famous example in the biomaterials field, yet even synthetic polymers and polysaccharides can undergo exchange phenomena in solution[103]). The literature shows that surface immobilization of HA by some form of covalent bonding can be roughly itemized into three different subheadings: surface cross-linking, photochemical immobilization, and covalent bonding. While it is not always easy to drive a clear-cut line between surface cross-linking and covalent bonding, for the sake of the present paper covalent bonding methods are those approaches aimed at building up a monomolecular overlayer of HA on a properly functionalized substrate, while surface cross-linking involves the establishment of covalent bonding between thicker layers.

From a general point of view, surface cross-linking involves the establishment of multiple covalent bondings between a water-resistant surface layer and HA. Halpern and Beaver pioneered this approach by patenting the so-called "bilaminar graft" configuration:[104−107] In short, an adhesive polymer coating, bearing, for example, diisocyanate groups, is first applied to the surface from an organic solution. Aqueous HA is then applied and cross-linked with the previously applied layer by moderate heating. Also, the acidic form of HA can be cross-linked to polymeric layers bearing epoxides, aziridine, or alcohol groups. In principle, the frequency of cross-linking can be tuned to tie HA at multiple points along the chain and to provide different lengths of free segments. An interesting paper by Lowry and Beavers shows that coatings obtained in this way can resist hyaluronidase attack for experimental times as long as 28 months in PBS at 37 °C.[108] In these experiments, hyaluronidase

concentrations ranged from that normally found in human serum up to 60 times that level. The most obvious explanation of the hyaluronidase-resistant behavior is that the periodic urethane links between HA and a substrate copolymer hinder HA chain mobility or reduce the available free-segment lengths to the point that the enzyme is unable to position its active site with the immobilized HA molecule. While, in light of the previous discussion, this result could be interpreted as evidence that HA immobilized in this way is no longer able to "communicate" with the biological counterpart (while keeping, of course, most of its interesting physical properties such as wettability and lubricity), a more recent development of the same technology must be credited as a very notable example of exploitation of specific interactions involving surface-immobilized HA. In particular, HA coatings through isocyanate cross-linking were applied to assay slides to evaluate sperm−HA binding.[109] It is known that HA binds to two specific surface receptors on mature sperm, PH20 and RHAMM. HA−sperm binding increases swimming vigor by increasing the cross beat frequency of tail motion, and is critical for fertilization.[110] In the tests performed,[109] mature sperm that encounter the HA layer bind to it and can easily be distinguished from immature, non HA binding sperm through observation by fluorescence microscopy. This is very clear evidence that, by proper tuning of immobilization conditions, surface-linked HA can still offer the information required to establish specific interactions with biological counterparts.

Picart and co-workers give another example of how properties of immobilized HA can be affected by cross-linking and relevant modification of the HA structure.[111] The previously described cell-resistant and hyaluronidase-degradable HA−PLL ionic multilayer[100,101,111] can be turned into a cell-adhesive, hyaluronidase-resistant coating by carbodiimide−succinimide cross-linking. Carbodiimide chemistry has been widely used in the surface immobilization of HA and will be discussed later in the section on covalent immobilization. When used in combination with hydroxy-, or sulfohydroxysuccinimide, it promotes the condensation reaction between amino groups and carboxylate groups. In the Picart et al. paper,[111] carbodiimide-promoted cross-linking increases the rigidity of the surface polyelectrolyte films as compared to those obtained by purely ionic interactions, as evaluated by quartz crystal microbalance dissipation (QCM-D). While it has been argued that rigidity alone (which is a purely physical attribute) can control cell movements,[112] it is clear that any change of rigidity is necessarily a consequence of changes of the film chemistry and the molecular structure. (In the cited paper,[112] rigidity was changed by adjusting the amount of bisacrylamide cross-linker, hence modifying the polymer structure and conformational freedom of the polyacrylamide molecules in the gel.) Given the importance of conformational details engendered by the HA molecular structure on HA properties, as evidenced by the molecular dynamics simulation previously described,[86−89] surface rigidity alone may not be enough to interpret the quoted data. Rather, the combined evidence of resistance to hyaluronidase and loss of the resistance to nonspecific bioadhesion, which is generally shown by immobilized HA,

suggests that cross-linking in the experimental conditions adopted changes the molecular nature of immobilized HA to the point that it no longer shows the properties of less constrained systems, i.e., those closer to native HA from a molecular point of view.

Photochemical Immobilization. Attachment of photoreactive groups to the HA chain, which, upon being illuminated, react with a substrate surface and/or yield insoluble gel layers by cross-linking, has been reported. Kito and Matsuda synthesized photocurable HA by partial derivatization with UV-light-sensitive moieties, such as thymine, cinnamate, and coumarin groups, to coat the luminal surface of small-caliber vein grafts.[113] In vitro studies showed significant reduction of platelet adhesion, while minimal cell adhesion was observed during acute-phase implantation into dogs for up to 1 week. Barbucci and co-workers have used photosensitive HA obtained by 4-azidoaniline coupling to prepare HA-photopatterned surfaces.[114] HA-coated areas resisted adhesion of melanocyte cells, which were able to spread on the silanized glass substrate. In another experiment, taking advantage of the resistance to nonspecific binding to immobilized HA, lymphatic endothelial cells were aligned on microstructures of alternating HA and aminosilanized glass stripes.[115] Cells consistently spread and proliferated only on aminosilanized glass, thereby orienting parallel to the longitudinal axis of the stripe, forming a pattern of alternating stripes of aminosilanized glass uniformly covered by elongated cells and of cell-free HA. Thus, HA-micropatterned surfaces can potentially orient the growth of cells and allow the regeneration of lymphatic endothelium in the desired direction.

Anderson and co-workers have compared several different photolinked coatings on silicone rubber, among them an HA-based one.[116] Coating reagents were synthesized with 4-benzoylbenzoic acid (BBA) as the photoreactive moiety. The tested coating significantly inhibited fibrinogen and immunoglobulin G adsorption and fibroblast adhesion in vitro. However, neither the HA-based coating nor the other hydrophilic coatings tested are able to resist leukocyte adhesion. It would be tempting to account for the extensive leukocyte colonization of the HA-coated surface with the specific interaction that plays such an important role in wound healing, as previously described.[6] However, significant leukocyte adhesion was also found on the other tested surfaces,[116] so it is not possible to conclude whether the phenomenon is driven by specific or nonspecific interactions, as remarked also in the paper by Tabrizian and co-workers.[71] As a further finding, none of the tested coatings, HA included, reduced fibrous capsule formation when implanted in rats. Surface characterization was limited to contact angle measurements that, while confirming that HA coatings can impart surface hydrophilicity, do not allow any significant insights into the photoimmobilized HA fine structure and conformation, which previous sections have shown to play a critical role in the outcome of the investigated phenomena.

Covalent Linking. Among the methods of surface modification, covalent linking of HA chains to a properly functionalized surface is, in principle, the most suitable for implant devices. In light of the previous descriptions, it is

clear that, ideally, coupling schemes should not interfere with the structure and dynamic properties of HA chains. The molecular structure of HA offers reactive carboxyl and hydroxyl groups that can be exploited for surface coupling reactions. Early reports on covalent immobilization of HA were aimed at the preparation of affinity chromatography gels (carbodiimide chemistry)[117] and at the comparison between heparin- and HA-coated surfaces obtained by reductive amination.[118,119] An interesting paper in 1986 suggests that covalently linked HA exerts the expected bioactive effects on selected cell lines: According to Kujawa and Kaplan, HA covalently bonded to cell-culture surfaces stimulates chondrogenesis in stage 24 limb mesenchyme cell cultures, and digestion of the substrate with hyaluronidase abolishes the stimulation.[120] More recently, Guo and Hildreth reported enhanced and reproducible binding of CD44-rich tumor cells to microwell plates bearing covalently linked HA, HA immobilization being obtained by carbodiimide-mediated condensation to amino groups on the well surfaces.[121] While the papers do not present thorough surface characterization, it is important at this stage to come back to the already discussed carbodiimide coupling reaction. Dimethylamino-propylethylcarbodiimide (EDC), a water-soluble carbodiimide, is most frequently used. While it has been widely used in attempts to link HA to aminated surfaces, it has been underlined and demonstrated that the coupling reaction by EDC alone does not lead to formation of amide linkages between HA carboxyls and amino groups.[111,122,123] Actually, EDC reacts with carboxyl groups to form active *O*-acyli-sourea intermediates. In the absence of nucleophiles, the reactive *O*-acylisourea can convert to the stable *N*-acylurea, by an irreversible reaction that hinders formation of amide linkage. The EDC-mediated condensation can be directed toward the desired carboxyl−amino coupling simply by adding to the reaction solution a nucleophile, most commonly *N*-hydroxysuccinimide (NHS), or its sulfo derivative.[111,123] The latter is often suggested because of its greater solubility, even if the solubility of straight NHS is more than enough for the typical experimental conditions required. A direct comparison of HA immobilization to aminated surfaces by EDC chemistry in unbuffered water, with or without the addition of NHS, shows that only the reaction involving NHS is successful.[123]

From a general point of view, covalent linking of biomolecules requires two ingredients: suitable chemical groups on the substrate surface and a coupling reaction. Since most substrates of interest in the biomedical field do not bear the required kind and density of reactive groups, they must be introduced in a proper surface functionalization step. In the case of HA immobilization, the most commonly used functionalization steps involve surface amination, and the processes most frequently used are deposition from plasma of aminated molecules, silane chemistry, and PEI adsorption. Other methods have been used as well: Grigoreas and co-workers have recently presented a method for HA bonding to mictrotiter plates involving first a glutaraldehyde coating followed, via a Schiff's base bond, by a spermine layer to introduce amino groups.[124] HA was added to the activated microwells in the presence of carbodiimide. The paper

contains interesting results and reflections on the need to preserve enough carboxyl groups on the surface-linked HA molecule to obtain a high yield in the biospecific HA reactions.

Concerning coupling reactions, the most frequently reported are the already cited EDC condensation and the reaction of hydrazide derivatives.

Plasma deposition using allylamine, heptylamine, or other aminated precursors can introduce amino groups on the substrate surface by a dry step.[125] The surface density of amino groups can be controlled by deposition conditions. HA has been immobilized on metallic stents by EDC chemistry to an aminated, plasma-deposited layer by Verheye and co-workers, yielding a hyaluronidase-resistant coating.[126,127] A primate thrombosis model shows reduced thrombus formation as compared to control stainless steel devices. Platelet adhesion to coated devices was significantly reduced as compared to that of the control in a baboon AV shunt model.

Sweeney and co-workers have tested, among other biological coatings, HA immobilized to polycarbonate membranes surface-functionalized by deposition of heptylamine plasma.[128] Samples were used in a feline model to investigate the effect on corneal epithelialization of a synthetic polymer surface in vivo. In this study, HA coating did not perform well as compared to other biological coatings, such as collagen I, collagen IV, and laminin. HA-coated implants (together with chondroitin sulfate and collagen III coatings) showed persistent postsurgical irritation and inconsistent epithelialization of the implant surface. The authors suggest that the persistent ocular irritation may have compromised the biological functions of the surface coatings and therefore affected the overall performance.

Thierry and co-workers have investigated the immobilization of diethylenetriamine pentaacetic acid (DTPA)-conjugated HA (HA−DTPA) complexed with a γ or β radionuclide to nitinol stents and polymeric surfaces, aimed at combining the hemocompatibility of HA-coated surfaces and the antiproliferative effects of an appropriate radiotherapy.[129] The approach involves first surface functionalization by plasma polymerization of acetaldehyde, followed by reaction of the surface aldehyde groups with polyallylamine hydrochloride using sodium cyanoborhydride as a reducing agent for the intermediate Schiff bases. HA and HA derivatives were then coupled to the aminated surface via EDC−NHS chemistry (some of the presented surface composition data are shown in Table 2).

Mason and co-workers have adopted a different strategy for the surface immobilization of HA to different polymer surfaces.[130] On the basis of the chemistry developed by Prestwich and co-workers,[131] HA was first modified to obtain the reactive adipic dihydrazide HA (HA-ADH). Pendant reactive idrazide groups were then reacted with Ar and NH_3 plasma treated polymer surfaces (polypropylene, PS, and poly(tetrafluoroethylene)). The results show that ammonia plasma treated polymers were more reactive toward HA attachment, especially when the reactive groups were extended away from the surface with succinic anhydride, followed by reacting the newly formed carboxylic acid group

Table 2. Surface Composition (atom %) As Detected by X-ray Photoelectron Spectroscopy Analysis of Functionalized Substrates before and after HA Coupling

sample	C	O	Si	N	S	remarks on the substrate	ref
functionalized substrate	75.3	13.4		7.2		poly(allylamine) reacted with plasma-deposited acetaldehyde film on NiTi alloy	129
after HA coupling	65.5	27.1		7.4			129
functionalized substrate	28.8	43.3	15.5	2.3		aminosilane-treated glass	135
after HA coupling	38.2	35.8	11.7	6.7	1.2		135
functionalized substrate	75.0	15.3		9.7		poly(ethylenimine)-adsorbed polystyrene	136
after HA coupling	63.7	28.9		7.4			136

with HA-ADH. The authors claim that, since the latter scheme proved to be more effective, steric effects were involved with the reactivity of the HA with surface groups. However, it must be noted that no thorough surface characterization of the HA-coupled surfaces was presented (except for attenuated total reflectance infrared spectroscopy, ATR-IR, which does not have the required surface sensitivity, its sampling depth being a few hundred nanometers).

Cen and co-workers have discussed the covalent coupling of HA to electrically conductive polypyrrole (PPY) films.[132,133] The coupling reaction involves, first, plasma treatment of PPY, followed by UV-promoted 2-hydroxyethylacrylate (HEA) grafting to the PPY surface. ATS ((3-aminopropyl)-triethoxysilane) is then chemisorbed to the HEA-grafted surface, through formation of Si−O bonds. Finally, EDC-activated HA is coupled to the surface amino groups. Interestingly, Cen and co-workers provide results on the assessment of the immobilized-HA bioactivity by biotinylated HA binding protein (bHABP) assay. The amount of bHABP specifically bound increases with the amount of immobilized HA, but it gradually approaches an asymptotic level. According to the authors, this effect may be due to the conformational changes of HA after immobilization that affect its effectiveness in protein binding.[132]

HA immobilization to metallic surfaces through silane chemistry has been presented by Pitt and co-workers.[134] In particular, an epoxysilane was applied to a clean stainless steel surface, followed by conversion of the terminal epoxy group to aldehyde by periodate oxidation and linking of HA-ADH. The rationale for HA coating, as suggested by the authors, is the exploitation of the biopassive, or bioinert nature of HA, which can turn into a bioactive surface by attachment of bioactive peptides. Also in this case no data on the actual surface composition were presented, yet ATR-IR, fluorescence labeling, and in vitro platelet adhesion data confirm the successful coupling of HA and its resistance to platelet adhesion.

An extensive characterization by XPS of the surface chemistry of HA coupled by EDC−NHS to aminosilane-treated glass surfaces has been presented by Stile and co-workers.[135] In particular, HA was coupled to glass substrates chemisorbed with (*N*-(2-aminoethyl)-3-aminopropyl)tri-methoxysilane (EDS). Investigating different experimental conditions and using different controls, the authors provide a clear picture of the surface stoichiometry of immobilized HA. HA immobilization is clearly detected by the increase of the 286.5 C−O component in the C1s peak. Overall stoichiometry does not reach the value expected from the molecular formula of HA, because the thickness of the HA layer is thinner than the XPS sampling depth (a few nanometers; see also Table 2 and the discussion below). This is in agreement with the nature of the reaction, which is limited to the boundary between the aminated layer and HA and leads to the formation of a "monomolecular" layer of HA over the aminated glass.

Incidentally, perusal of Table 3 of this interesting paper[135] shows that XPS analysis does not detect any significant amount of HA after the aminated surface is soaked in the same HA-containing solution for the same experimental time but without the required chemicals (pH 7, sample coded, adsorbed HA control in Table 3 of the cited paper). If 16 h of adsorption to an aminated glass surface does not lead to immobilization of any detectable amount of HA (by XPS), the surface chemist keeps wondering how much HA, and how reproducibly, would be adsorbed to the anionic PS surfaces of microtiter plates used to unravel the specificity of HA-based interactions, as described in the beginning of this section.

Previous results on aminosilane-coupled HA are in agreement with XPS analysis of HA linked by EDC−NHS chemistry to PEI adsorbed to air plasma treated polymeric surfaces.[136] Also in this case it is shown that the overall stoichiometry is in agreement with a multilayer model, that is, the few nanometers probed by XPS contain signal from the underlying substrate, the aminated layer, and the surface-linked HA. Table 2 shows the surface composition, as detected by XPS, taken from some of the cited papers on covalent linking of HA, showing the relationship between the surface compositions of the functionalized surface and of the HA-coupled one. HA coupling usually leads to an increase of the oxygen-to-carbon ratio, except for data taken from ref 137, where the substrate is glass that has an O/C ratio higher than that of organic substrates.

Coming back to HA linked to PEI, interestingly, while XPS data show that the thickness of the surface HA layer is on the order of nanometers in the ultra-high-vacuum environment of the analysis chamber, force−distance curves obtained in saline by AFM show a "diffuse" aqueous interface spanning a few tens of nanometers[137,138] (note that this is not an absolute distance, rather a distance measured from an arbitrary point that corresponds to zero compressibility of the surface layer by the AFM tip[139]). This is direct evidence that the HA layer immobilized in this way is swollen by water and that, despite constraints imposed by surface immobilization, it still keeps a certain degree of conformational freedom and chain mobility. Comparison of force−distance curves between HA ionically and covalently coupled to adsorbed PEI shows, in the former case, a more
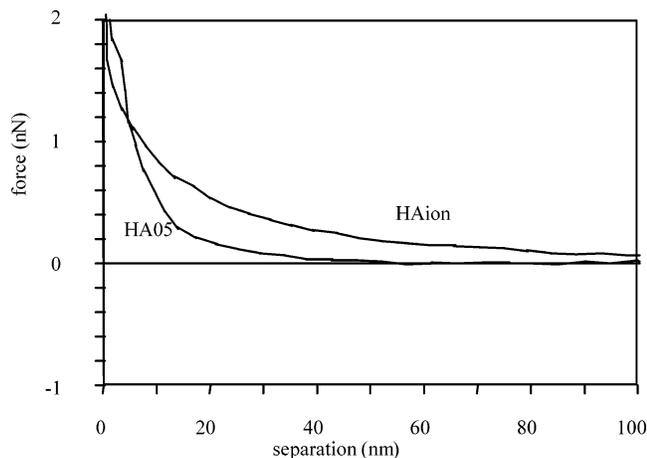
Figure 3. Force−separation approaching curves measured in 0.001 M NaCl between the AFM tip and HA covalently (HA05) or ionically (HAion) linked to a PEI-adsorbed surface.
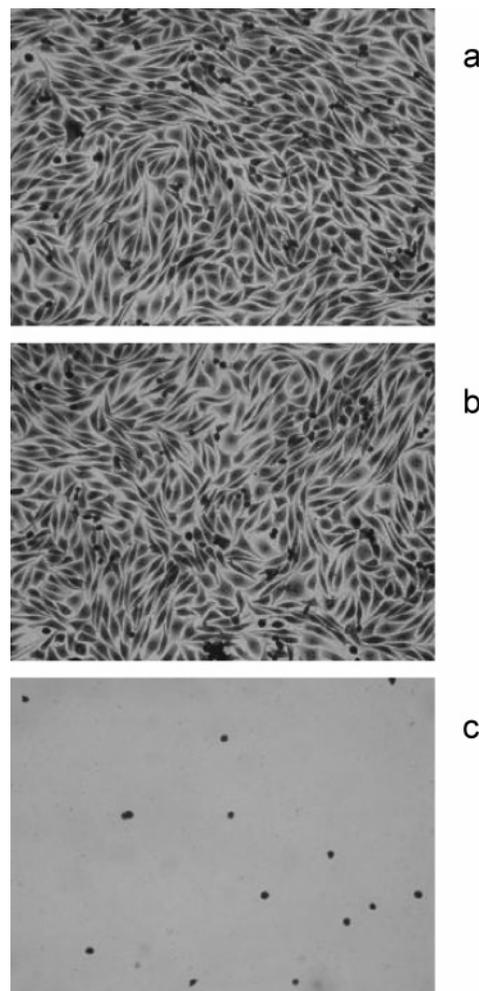

Figure 4. Inverted microscope images showing the results of in vitro L929 fibroblast adhesion experiments, after 72 h of contact between the samples and the cell suspension: (a) PEI-coated glass; (b) ionically coupled HA; (c) covalently coupled HA (original magnification 200×; reproduced at 86% of original size).

diffuse and/or softer aqueous interface[138] (Figure 3), in agreement with the results from Picart and co-workers that indicate increased rigidity of PLL−HA multilayers after EDC−NHS cross-linking.[111] Covalently linked HA, according to this process, shows significant resistance to nonspecific adhesion of cells and bacteria. Ionically coupled surfaces, while showing a certain degree of resistance to cell adhesion in the short time frame, lose their properties with increased time, and after 72 h they are completely covered by cells and indistinguishable from control tissue culture PS (Figure 4). These results confirm the need for stable linking of HA monolayers for medium- to long-term exposure to biological, protein-containing media.[138]

AFM force measurements provide further information on the molecular structure of the aqueous interface of HA covalently linked to adsorbed PEI.[138] The discussion in the previous sections indicates that the length of free segments and of chains may play a pivotal role in aqueous HA behavior and properties. AFM force−distance curves obtained on tip retracting show the typical "sawtooth" appearance attributed to the stretching of polymeric segments,[139] or, in general, to the tip winning its freedom from adhesive interactions with interacting species of different lengths. This again indicates that HA is not "monolithically" immobilized to the aminated surface, and that detachment events occur for distances as long as about 40 nm. This could indicate that the coupling conditions adopted yield a length of free sequence of at least 40 disaccharide units (MW ≈ 16000). Does this mean that, from a biological point of view, HA immobilized in this way, independently from its actual molecular weight (∼300000 in this case), corresponds to "free" HA no heavier than 16000 Da? There is presently no clear answer to this question; this topic will be discussed again in the last section.

Poly(ethylene terephthalate) (PET) and expanded-poly-(tetrafluoroethylene) (ePTFE) surgical meshes, HA-surface-modified according to the just presented approach, have been tested in an in vivo rabbit model of postsurgery adhesion.[140] HA is widely used (mostly as a gel) in the prevention of postsurgery adhesion, which is a major cause of morbidity and expense in clinical practice.[2−4] Its incidence is very high in abdominal surgery, when biomaterials are used to replace vessels or to repair abdominal wall defects in the absence

of a peritoneal layer or other viable tissue. In such cases, intestinal loops adhere to the foreign body, yielding permanent adhesion. The in vivo test involved four groups of 18 rabbits each, each with a 4 × 3 cm full thickness parietal defect produced by abdominal surgery.[140] Two months after surgery the extent of adhesions was blindly assessed and rated by a site-specific scoring system. The results are reported in Table 3, while Figure 5 shows typical results. Clearly, the nanometer thick HA overlayer was able to reduce significantly postsurgery adhesion. According to Larsen, the scientific rationale for the use of HA in the prevention of adhesion formation is based on several biological effects:[141] modulation of fibrous tissue formation, barrier effect to fibrinogen and ensuing lack of scaffolding for fibrous tissue formation, nonprocoagulant activity, and inhibition of superoxide release from granulocytes. It is not possible to state if and how much these biological effects contributed to the results, yet it seems reasonable to conclude that, despite surface immobilization, HA was able to exert an antiadhesive effect in vivo as a result of some of the known biological properties of free HA.

Another example of performance improvement by HA immobilization in vivo has recently been presented by Ohri

Table 3. Mean and Standard Deviation of the Total Site Adhesion Score in a Rabbit Model of Adhesion Prevention of Abdominal Surgery[a]

sample	mean ± STD of adhesion score
ePTFE mesh, HA-coated	0.33 ± 0.33
ePTFE mesh	3.00 ± 1.31
polyester mesh, HA-coated	2.50 ± 1.08
polyester mesh	6.25 ± 1.39

Adhesion Score System		
	description	score
extent	none	0
	25%	1
	50%	2
	75%	3
	100%	4
type	none	0
	filmy, transparent, avascular	1
	opaque, tanslucent, avascular	2
	opaque, capillaries present	3
	opaque, large vessels present	4
tenacity	none	0
	falls apart	1
	lysed with traction	2
	requires sharp dissection	3
total		11

[a] The scoring system was adapted from ref 140.

and co-workers.[142] On the basis of evidence that HA and other GAGs exert a calcification-mitigating and antidegeneration role in the extracellular matrix of bioprosthetic valve material,[143,144] the study evaluated the calcification-resistance potential of glutaraldehyde-fixed bovine pericardium tissue (GFBP) after HA grafting. To this end, the already discussed HA-ADH was linked to free aldehyde goups of GFBP, and control materials and HA-modified ones were implanted subcutaneously in osteopontin-null mice, an established model for acceleration of eptopic calcification. A dramatic 84.5% reduction in calcification was found between GFBP-implanted and HA-modified-GFBP-implanted animals, after 2 weeks. Concerning the mechanism of the reduction of calcification, and hence of the HA role in this experiment, the authors suggest that it is not clear whether it involves some intrinsic property of HA, or simply the capping of free aldehyde groups, or a combination of the two. While free aldehyde groups obtained upon glutaraldehyde fixation are known to contribute to calcification, it is speculated that two known properties of HA could have also contributed to the results: Ca^{2+} chelation and HA-induced inhibition of an inflammatory/immunogenic response. The authors suggest that the latter contribution is unlikely to account for the observed effects of HA, because the subcutaneous implantation model used did not involve any circulatory stress. It must also be noted that ADH reacts with HA carboxylates to form HA-ADH, and according to the paper, the yield of ADH substitution of HA carboxyl groups was about 80%. This means that the surface-immobilized HA described in this paper contains a very low number of carboxylate groups, which are the obvious sites for the Ca^{2+}-related role of GAGs in mineralization,[145] and it is not clear if it is reasonable to account for the immobilized HA-ADH−Ca^{2+} interaction on the basis of the properties of "native" HA.
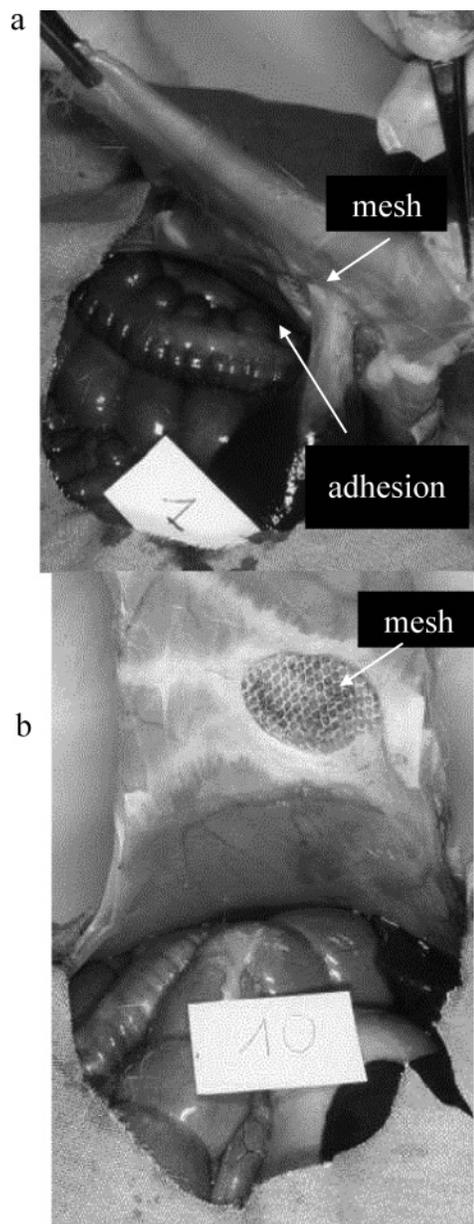

Figure 5. Results of in vivo experiments on postsurgery adhesion formation on polyester surgical meshes in a rabbit model: (a) control uncoated polyester mesh; (b) HA-coated polyester mesh. (See Table 3 for the obtained adhesion score and scoring system.)

Biospecific Immobilization. Specific biorecognition interactions have been used to link HA to solid surfaces. A very interesting approach describes HA binding to a surface coated by the natural ligand p32.[146,147] The latter, a hyaluronan binding protein including three HA binding sites,[148] was synthesized by genetic engineering both to anchor HA and to interact with the substrate, glass cover slides coated by lipids through the conventional Langmuir−Schaefer technique. The upper lipid layer contained a Ni chelating headgroup that can interact with the engineered p32 through its histidine tag. The study investigates the viscoelastic properties of the HA film in terms of surface viscosity and surface elasticity as a function of film thickness.

Some very interesting findings on the biological immobilization of HA have been recently presented by the Israelachvili group.[149] Briefly, while devising a method to link HA to the mica substrate commonly used in Israelach-

vili's surface force apparatus (SFA), the authors tried a first approach based on the interaction between a supported lipid bilayer covered with streptavidin and biotinylated HA (b-HA). Results suggest that, while streptavidin was firmly attached to the lipid bilayer, its interaction with b-HA was not strong enough to provide stable linking. The comparatively large size of HA used (700 kDa) is suggested among the possible reasons for this behavior. A successful approach was then devised, by first binding bHABP[148] to the streptavidin-coated bilayer, followed by HA binding to the surface-linked HABP. Results show that this approach provides a strength of interaction high enough to resist pushing out of the HA layer as the SFA surfaces are moved together.[149]

Reflections and Conclusions

Previous sections have addressed some issues related to modification of (bio)material surfaces by HA. The initial *detour* presenting a short description of HA-based interactions with biological moieties is, in my view, a necessary introduction to capture all the complexity and broadness of the field, and the need for a truly multidisciplinary approach to surface modification by HA, not bounded by traditional approaches of parent disciplines. It is clear that three fields contribute significantly to modern HA surface science: (i) the strict biomedical science, which addresses the effects and the mechanisms of interactions of HA molecules with tissue components; (ii) the study and molecular modeling of HA in aqueous solutions, based on molecular-resolution analytical techniques and molecular-dynamics simulations; (iii) biomaterials surface science, which should grow to the point to be able to target the processes discovered in (i) on the basis of inputs obtained from (ii), and on the basis of its own background and development. It is clear that the exploitation of bioactive HA properties on biomaterial surfaces requires a more comprehensive approach as compared to "classical" surface modification and analysis, and that findings from (i) and (ii) above suggest that successful HA immobilization is not just a matter of finding the right stoichiometry on an HA-modified surface, even if surface spectroscopies are and still will be an integral part of every surface modification approach. Incidentally, it is a matter of reflection that, for all the complexity and peculiarities of interactions involving HA, as described above, proper surface chemistry characterization can be found only in a small number of the cited papers, and that direct or indirect information, or even just speculation, on the structure and conformation of surface-linked HA at the aqueous interface is even more rare. The initial sections of this paper open many questions that do not have any answer in present-day biomaterial surface science. For instance, given the different HA sequence lengths required by different hyaladherins, are surface modification methods that, e.g., yield three free consecutive disaccharide units intrinsically abolishing interactions with hyaldherins that require longer chains for HA recognition? Given the molecular-weight-dependent effects of HA, will those surface modification processes that yield a high frequency of chain–surface bonds intrinsically select properties typical of low-molecular-weight HA? Will devices surface-modified by these processes be suitable to accelerate

wound-healing through the angiogenic properties of HA oligosaccharides, and will other applications require a lower frequency of HA–surface bonds to achieve properties of high-molecular-weight HA? If the Day and Sheehan model is correct, are HA–surface binding sites or HA–surface interactions already bringing some order to the polysaccharide chaos, restricting the number of highly dynamic, chaotically interchanging, semiordered states? If yes, will this decrease the driving force for selective protein assembly along the HA chain, and how will this affect the suggested quaternary structure obtained on hyaladherin binding and the associated biological information content? If the Scott model is correct, is surface linking of HA intrinsically inducing irreversible denaturation of its tertiary structure, and what are the consequences for the biological properties of immobilized HA? In one model or the other, since bioactive HA properties are directly emerging from the interplay of its molecular structure with the water structure and forces, is surface immobilization intrinsically denaturing HA? There are presently no clear answers to these and many other issues, which make, however, the very basis of the emerging field of biomolecular surfaces and implant biomaterials. The present lack of definite answers to the previous questions should be seen not just as a shortcoming, but also as a clear indication of the opportunities that lie ahead once the biological language of HA, and its relationship with surface immobilization, is more completely understood.

References and Notes

(1) *The Biology of Hyaluronan*; Evered, D., Whelan, J., Eds.; Wiley: Chichester, U.K., 1989.

(2) *The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives*; Laurent, T. C., Ed.; Portland Press Ltd.: London, 1998.

(3) *Redefining Hyaluronan*; Abatangelo, G., Weigel, P. H., Eds.; Elsevier: Amsterdam, 2000.

(4) *Hyaluronan*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Hascall, V., Eds.; Woodhead Publishing Ltd.: Cambridge, U.K., 2002.

(5) Chen, W. Y. J. Functions of hyaluronan in wound repair. In *Hyaluronan*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Hascall, V., Eds.; Woodhead Publishing Ltd.: Cambridge, U.K., 2002; pp 147−156.

(6) Chen, W. Y. J.; Abatangelo, G. Function of hyaluronan in wound repair. *Wound. Rep. Reg.* **1999**, 79−89.

(7) Ratner, B. D. The blood compatibility catastrophe. *J. Biomed. Mater. Res.* **1993** 27, 283−287.

(8) Ratner, B. D. New ideas in biomaterials science, a path to engineered biomaterials. *J. Biomed. Mater. Res.* **1993**, 27, 837−850.

(9) Plant, A. L.; Chen, C. S.; Groves, J. T.; Parikh, A. N. The biomolecular interface. *Langmuir* **2003**, *19*, 1449−1450.

(10) Vogler, E. A. On the biomedical relevance of surface spectroscopies. *J. Electron Spectrosc. Relat. Phenom.* **1996**, *81*, 237−242.

(11) *Water in Biomaterials Surface Science*; Morra, M., Ed.; Wiley: Chichester, U.K., 2001.

(12) Lee, J. Y.; Spicer, A. P. Hyaluronan: a multifunctional, megaDalton, stealth molecule. *Curr. Opin. Cell Biol.* **2000**, *12*, 581−586.

(13) Meyer, K.; Palmer, J. The polysaccharide of the vitreous humor. *J. Biol. Chem.* **1934**, *107*, 629−634.

(14) Rowen, J. W.; Brunish, R.; Bishop, F. W. Form and dimensions of isolated hyaluronic acid. *Biochim. Biophys. Acta* **1956**, *19*, 480−489.

(15) Laurent, T. C. A comparative study of physico-chemical properties of hyaluronic acid prepared according to different methods and from different tissues. *Ark. Kemi* **1957**, *11*, 487−496.

(16) Preston, B.; Davies, M.; Ogston, A. G. The composition and physicochemical properties of hyaluronic acids prepared from ox synovial fluid and from a case of mesothelioma. *Biochem. J.* **1965**, *96*, 449−471.

(17) Ogston, A. G.; Preston, B. N. The exclusion of protein by hyaluronic acid. Measurement by light scattering. *J. Biol. Chem.* **1966**, *10*, 17−19.

(18) *Chemistry and Molecular Biology of the Intercellular Matrix*; Balazs, E. A., Ed.; Academic Press: London, 1970; Vols. 2 and 3.

(19) Comper, W. D.; Laurent, T. C. Physiological function of connective tissue polysaccharides. *Physiol. Rev.* **1978**, *58*, 255−315.

(20) Nichol, L. W.; Ogston, A. G.; Preston, B. N. The equilibrium sedimentation of hyaluronic acid and of two synthetic polymers. *Biochem. J.* **1967**, *102,* 407−416.

(21) Toole, B. P.; Gross, J. The extracellular matrix of the regenerating newt limb: synthesis and removal of hyaluronate prior to differentiation. *Dev. Biol.* **1971**, *25*, 57−77.

(22) Toole, B. P.; Jackson, G.; Gross, J. Hyaluronate in morphogenesis: inhibition of chondrogenesis in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1384−1386.

(23) Scott, J. E. Chemical morphology of hyaluronan. In *The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives*; Laurent, T. C., Ed.; Portland Press Ltd.: London, 1998; pp 7−15.

(24) Hardingham, T. E.; Muir, H. The specific interaction of hyaluronic acid with cartilage proteoglycans. *Biochim. Biophys. Acta* **1972**, *279*, 401−405.

(25) Hardingham, T. E.; Muir, H. Binding of oligosaccharides of hyaluronic acid to proteoglycans. *Biochem. J.* **1973**, *135*, 905−908.

(26) Hascall, V. C.; Heinegard, D. Aggregation of cartilage proteoglycans. I. The role of hyaluronic acid. *J. Biol. Chem.* **1974**, *249*, 4232−4241.

(27) Hascall, V. C.; Heinegard, D. Aggregation of cartilage proteoglycans. II. Oligosaccharide competitors of the proteoglycan-hyaluronic acid interaction. *J. Biol. Chem.* **1974**, *249*, 4242−4249.

(28) Hascall, V. C.; Heinegard, D. Aggregation of cartilage proteoglycans. III. Characteristics of the proteins isolated from trypsin digests of aggregates. *J. Biol. Chem.* **1974**, *249*, 4250−4256.

(29) Pessac, B.; Defendi, V. Cell aggregation: role of acid mucopolysaccharides. *Science* **1972**, *175*, 898−900.

(30) Toole, B. P. Hyaluronan is not just a goo! *J. Clin. Invest.* **2000**, *106*, 335−336.

(31) Turley, E. A. Hyaluronic acid stimulates protein kinase activity in intact cells and in an isolated protein complex. *J. Biol. Chem.* **1989**, *264*, 8951−8955.

(32) Turley, E. A. Purification of a hyaluronate-binding protein fraction that modifies cell social behaviour. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 1016−1024.

(33) Underhill, C.; Dorfman, A. The role of hyaluronic acid in intercellular adhesion of cultured mouse cells. *Exp. Cell Res.* **1978**, *117*, 155−164.

(34) Underhill, C. B.; Toole, B. P. Binding of hyaluronate to the surface of cultured cells. *J. Cell Biol.* **1979**, *82*, 475−484.

(35) Underhill, C. B.; Toole, B. P. Physical characteristics of hyaluronate binding to the surface of simian virus 40-transformed 3T3 cells. *Biol. Chem.* **1980**, *255*, 4544−4549.

(36) Day, A. J. The structure and regulation of hyaluronan-binding proteins. *Biochem. Soc. Trans.* **1999**, *27*, 115−121.

(37) Kohda, D.; Morton, C. J.; Parkar, A. A.; Hatanaka, H.; Inagaki, F. M.; Campbell, I. D.; Day, A. J. Solution structure of the link module: a hyaluronan-binding domain involved in extracellular matrix stability and cell migration. *Cell* **1996**, *86*, 767−775.

(38) Peach, R. J.; Hollenbaugh, D.; Stamenkovic, I.; Aruffo, A. Identification of hyaluronic acid binding sites in the extracellular domain of CD44. *J. Cell Biol.* **1993**, *122*, 257−264.

(39) Banerji, S.; Day, A. J.; Kahmann, J. D.; Jackson, D. G. Characterization of a functional hyaluronan-binding domain from the human CD44 molecule expressed in *Escherichia coli*. *Protein Expression Purif.* **1998**, *14*, 371−381.

(40) Grover, J.; Roughley, P. J. The expression of functional link protein in a baculovirus system: analysis of mutants lacking the A, B and B′ domains. *Biochem. J.* **1994**, *300*, 317−324.

(41) Watanabe, H.; Cheung, S. C.; Itano, N.; Kimata, K.; Yamada, Y. Identification of hyaluronan-binding domains of aggrecan. *J. Biol. Chem.* **1997**, *272*, 28057−28065.

(42) Bajorath, J.; Greenfield, B.; Munro, S. B.; Day, A. J.; Aruffo, A. Identification of CD44 residues important for hyaluronan binding and delineation of the binding site. *J. Biol. Chem.* **1998,** *273,* 338−343.

(43) Goetinck, P. F.; Stirpe, N. S.; Tsonis, P. A.; Carlone, D. The tandemly repeated sequences of cartilage link protein contain the sites for interaction with hyaluronic acid. *J. Cell Biol.* **1987**, *105*, 2403−2408.

(44) Underhill, C. CD44: The hyaluronan receptor. *J. Cell Sci.* **1992**, *103*, 293−298.

(45) Lesley, J.; English, N.; Hascall, V. C.; Tammi, M.; Hyman, R. Hyaluronan binding by cell surface CD44. In *Hyaluronan*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Hascall, V., Eds.; Woodhead Publishing Ltd.: Cambridge, U.K., 2002; Vol. 1, pp 341−348.

(46) Lesley, J.; English, N. M.; Gal, I.; Mikecz, K.; Day, A. J.; Hyman, R. Hyaluronan binding properties of a CD44 chimera containing the link module of TSG-6. *J. Biol. Chem.* **2002**, *277*, 26600−26608.

(47) Lesley, J.; Gal, I.; Mahoney, D. J.; Cordell, M. R.; Rugg, M. S.; Hyman, R.; Day, A. J.; Mikecz, K. TSG-6 modulates the interaction between hyaluronan and cell surface CD44. *J. Biol. Chem.* **2004**, *279*, 25745−25754.

(48) Entwistle, J.; Hall, C. L.; Turley, E. A. HA receptors: Regulators of signaling to the cytoskeleton. *J. Cell Biochem.* **1996**, *61*, 569−577.

(49) Zhang, S.; Chang, M. C.; Zylka, D.; Turley, S.; Harrison, R.; Turley, E. A. The hyaluronan receptor RHAMM regulates extracellular-regulated kinase. *J. Biol. Chem.* **1998**, *273*, 11342−11348.

(50) Jackson, D. G.; Prevo, R.; Ni, J.; Banerji, S. Novel endothelial hyaluronan receptors. In *Hyaluronan*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Hascall, V., Eds.; Woodhead Publishing Ltd.: Cambridge, U.K., 2002; Vol. 1, pp 335−364.

(51) Mohamadzadeh, M.; DeGrendele, H.; Arizpe, H.; Estess, P.; Siegelman, M. Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *J. Clin. Invest.* **1998**, *101*, 97−108.

(52) Weigel, P. H.; Frost, S. J.; McGary, C. T.; Le Boeuf, R. D. The role of hyaluronic acid in inflammation and wound healing. *Int. J. Tissue React.* **1998**, *10*, 355−365.

(53) Presti, D.; Scott J. E. Hyaluronan- mediated protective effect against cell damage caused by enzymatically produced hydroxyl (OH·) radicals is dependent on hyaluronan molecular mass. *Cell Biochem. Funct.* **1994**, *12*, 281−288.

(54) Wisniewski, H. G.; Hua, J. C.; Popper, D. M.; Naime, D.; Vicek, J.; Cronstein, B. N. TNF/IL-1 induced protein TSG-6 potentiates plasmin inhibition by inter-α-inhibitor and exerts a strong anti-inflammatory effect *in vivo*. *J. Immunol.* **1996**, *156*, 1609−1615.

(55) West, D. C.; Hampson, I. N.; Arnold, F.; Kumar, S. Angiogenesis induced by degradation products of hyaluronic acid. *Science* **1985**, *228*, 1324−1326.

(56) West, D. C.; Kumar, S. The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. *Exp. Cell Res.* **1989**, *183*, 179−196.

(57) Sattar, A.; Rooney, P.; Kumar, S.; Pye, D.; West, D. C.; Scott, I.; Ledger, P. Application of angiogenic oligosaccharides of hyaluronan increases blood vessel numbers in rat skin. *J. Invest. Dermatol.* **1994**, *103*, 576−579.

(58) Lees, V. C.; Fan, T. P.; West, D. C. Angiogenesis in a delayed revascularization model is accelerated by angiogenic oligosaccharides of hyaluronan. *Lab. Invest.* **1995**, *73*, 259−266.

(59) Montesano, R.; Kumar, S.; Orci, L.; Pepper, M. S. Synergistic effect of hyaluronan oligosaccharides and vascular endothelial growth factor on angiogenesis *in vitro*. *Lab. Invest.* **1996**, *75*, 249−262.

(60) Slevin, M.; Krupinski, J.; Kumar, S.; Gaffney, J. Angiogenic oligosaccharides of hyaluronan induce protein tyrosine kinase activity in endothelial cells and activate a cytoplasmic signal transduction pathway resulting in proliferation. *Lab. Invest.* **1998**, *78*, 987−1003.

(61) Slevin, M.; Kumar, S.; Gaffney, J. Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. *J. Biol. Chem.* **2002**, *277*, 41046−41059.

(62) Liu, D.; Pearlman, E.; Guo, E. D. K.; Mori, H.; Haqqi, S.; Markowitz, S.; Willson, J.; Sy, M. S. Expression of hyaluronidase by tumor cells induces angiogenesis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7832−7837.

(63) West, D. C.; Chen, H. Is hyaluronan degradation an angiogenic/metastatic switch? In *Hyaluronan*; Kennedy J. F., Phillips G. O., Williams P. A., Hascall V., Eds.; Woodhead Publishing Ltd.: Cambridge, U.K., 2002; Vol. 2, pp 165−172.

(64) Savani, R. C.; Caos, G.; Pooler, P. M.; Zaman, A.; Zhou, Z.; DeLisser, H. M. Differential Involvement of the Hyaluronan (HA) Receptors CD44 and Receptor for HA-mediated Motility in Endothelial Cell Function and Angiogenesis. *J. Biol. Chem.* **2001**, *276*, 36770−36778.

(65) Lokeshwar, V. B.; Selzer, M. G. Differences in Hyaluronic Acid-mediated Functions and Signaling in Arterial, Microvessel, and Vein-derived Human Endothelial Cells. *J. Biol. Chem.* **2000**, *275*, 27641−27649.

(66) Wight, T. N.; Evanko, S. P. Hyaluronan is a critical component in atherosclerosis and restenosis and in determining arterial smooth muscle cell phenotype. In *Hyaluronan*; Kennedy J. F., Phillips G.

O., Williams P. A., Hascall V., Eds.; Woodhead Publishing Ltd.: Cambridge, U.K., 2002; Vol. 2, pp 173−176.

(67) Evanko, S. P.; Angello, J. C.; Wight, T. N. Formation of Hyaluronan- and Versican-Rich Pericellular Matrix Is Required for Proliferation and Migration of Vascular Smooth Muscle Cells. *Arterioscler., Thromb., Vasc. Biol.* **1999**, *19*, 1004−1013.

(68) Savani, R. C.; Turley, E. A. The role of hyaluronan and its receptors in restenosis after balloon angioplasty: development of a potential therapy. *Int. J. Tissue React.* **1995**, *17*, 141−151.

(69) Chajara, A.; Levesque, H. Role of hyaluronic acid in the arterial response to angioplasty. *Pathol. Biol. (Paris)* **1998**, *46*, 561−570.

(70) Heublein, B.; Evagorou, E. G.; Rohde, R.; Ohse, S.; Meliss, R. R.; Barlach, S. Haverich A. Polymerized degradable hyaluronan—a platform for stent coating with inherent inhibitory effects on neointimal formation in a porcine coronary model. *Int. J. Artif. Organs* **2002**, 25, 1166−1173.

(71) Thierry, B.; Winnik, F. M.; Merhi, Y.; Silver, J.; Tabrizian, M. Bioactive coatings of endovascular stents based on polyelectrolyte multilayers. *Biomacromolecules* **2003**, *4*, 1564−1571.

(72) Xu, H.; Ito, T.; Tawada, A.; Maeda, H.; Yamanokuchi, H.; Isahara, K.; Yoshida, K.; Uchiyama, Y.; Asari, A. Effect of hyaluronan oligosaccharides on the expression of heat shock protein 72. *J. Biol. Chem.* **2002**, *277*, 17308−17314.

(73) Termeer, C.; Benedix, F.; Sleeman, J.; Fieber, C.; Voith, U.; Ahrens, T.; Miyake, K.; Freudenberg, M.; Galanos, C.; Simon, J. C. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J. Exp. Med.* **2002**, *195*, 99−111.

(74) Huang, L.; Cheng, Y. Y.; Koo, P. L.; Lee, K. M.; Qin, L.; Cheng, J. C. Y.; Kumta, S. M. The effect of hyaluronan on osteoblast proliferation and differentiation in rat calvarial-derived cell cultures. *J. Biomed. Mater. Res.* **2003**, *66A*, 880−884.

(75) Taylor, K. R.; Trowbridge, J. M.; Rudisill, J. A.; Termeer, C. C.; Simon, J. C.; Gallo, R. L. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J. Biol. Chem.* **2004,** *279,* 17079−17084.

(76) Fieber, C.; Baumann, P.; Vallon, R.; Termeer, C.; Simon, J. C.; Hofmann, M.; Angel, P.; Herrlich, P.; Sleeman, J. P. Hyaluronan-oligosaccharide-induced transcription of metalloproteases. *J. Cell Sci.* **2004**, *117* (Part 2), 359−367.

(77) Nasreen, N.; Mohammed, K. A.; Hardwick, J.; Van Horn, R. D.; Sanders, K.; Kathuria, H.; Loghmani, F.; Antony, V. B. Low molecular weight hyaluronan induces malignant mesothelioma cell (MMC) proliferation and haptotaxis: role of CD44 receptor in MMC proliferation and haptotaxis. *Oncol. Res.* **2002**, *13*, 71−78.

(78) Ghatak, S.; Misra, S.; Toole, B. P. Hyaluronan Oligosaccharides Inhibit Anchorage-independent Growth of Tumor Cells by Suppressing the Phosphoinositide 3-Kinase/Akt Cell Survival Pathway. *J. Biol. Chem.* **2002**, *277*, 38013−38020.

(79) Scott, J. E.; Heatley, F. Biological properties of hyaluronan in aqueous solution are controlled and sequestered by reversible tertiary structures, defined by NMR spectroscopy. *Biomacromolecules* **2002**, *3,* 547−553.

(80) Scott, J. E.; Heatley, F. Hyaluronan forms specific stable tertiary structures in aqueous solution: A 13C NMR study. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4850−4855.

(81) Jacoboni, I.; Valdre, U.; Mori, G.; Quaglino, D., Jr. Pasquali-Ronchetti, I. Hyaluronic acid by atomic force microscopy. *J. Struct. Biol.* **1999**, *126*, 52−58.

(82) Morra, M. On the molecular basis of fouling resistance. *J. Biomater. Sci., Polym. Ed.* **2000**, *11*, 547−570.

(83) Gribbon, P.; Heng, B. C.; Hardingham, T. E, The analysis of intermolecular interactions in concentrated hyaluronan solutions suggest no evidence for chain-chain association. *Biochem. J.* **2000**, *350*, 329−335.

(84) Gribbon, P.; Heng, B. C.; Hardingham, T. E. The Molecular Basis of the Solution Properties of Hyaluronan Investigated by Confocal Fluorescence Recovery After Photobleaching, *Biophys. J.* **1999**, *77*, 2210−2216.

(85) Laurent, T. C. The interaction between polysaccharides and other macromolecules. 9. The exclusion of molecules from hyaluronic acid gels and solutions. *Biochem. J.* **1964**, *93*, 106−112.

(86) Almond, A.; Sheehan, J. K.; Brass, A. Molecular dynamics simulations of the two disaccharides of hyaluronan in aqueous solution. *Glycobiology* **1997**, *7*, 597−604.

(87) Almond, A.; Brass, A.; Sheehan, J. K. Dynamic exchange between stabilised conformations predicted for hyaluronan tetrasaccharides: comparison of molecular dynamic simulations with available NMR data. *Glycobiology* **1998**, *8*, 973−980.

(88) Almond, A.; Brass, A.; Sheehan, J. K. Oligosaccharides as model systems for understanding water-biopolymer interaction: hydrated dynamics of a hyaluronan decamer. *J. Phys. Chem. B* **2000**, *104*, 5634−5640.

(89) Day, A. J.; Sheehan, J. K. Hyaluronan: polysaccharide chaos to protein organisation. *Curr. Opin. Struct. Biol.* **2001**, *11*, 617−622.

(90) Ponnuraj, K.; Jedrejas, M. J. Mechanism of hyaluronan binding and degradation: structure of *Streptococcus pneumoniae* hyaluronate-lyase in complex with hyaluronic acid disaccharide at 1.7 Å resolution. *J. Mol. Biol.* **2000**, *299*, 885−895.

(91) Markovic-Housley, Z.; Miglierini, G.; Soldatova, L.; Rizkallah, P. J.; Muller, U.; Schirmer, T. Crystal structure of hyaluronidase, a major allergen of bee venom. *Structure* **2000**, *8*, 1025−1035.

(92) Huang, W.; Boju, L.; Tkalec, L.; Su, H.; Yang, H. O.; Gunay, N. S.; Linhardt, R. J.; Kim, Y. S.; Matte, A.; Cygler, M. Active site of chondoitin AC lyase revealed by the structure of enzyme-olygosac-charide complexes and mutagenesis. *Biochemistry* **2001**, *40*, 2359−2372.

(93) Delpech, B.; Bertrand, P.; Maingonnat, C. Immunoenzymoassay of the hyaluronic acid-hyaluronectin interaction: application to the detection of hyaluronic acid in serum of normal subjects and cancer patients. *Anal. Biochem.* **1985**, 149, 555−565.

(94) Catterall, J. B.; Gardner, M. J.; Jones, L. M. H.; Turner, G. A. Binding of ovarian cancer cells to immobilized hyaluronic acid. *Glycoconjugate J.* **1997**, *14*, 867−869.

(95) Suh, K. P.; Yang, J. M.; Khademhosseini, A.; Berry, D.; Tran, T. T.; Park, H.; Langer, R. Characterization of chemisorbed hyaluronic acid directly immobilized on solid substrates. *J. Biomed. Mater. Res., B: Appl. Biomater.*, in press.

(96) Suh, K. Y.; Khademhosseini, A.; Yang, J. M.; Eng, G.; Langer, R. Soft Litographic patterning of hyaluronic acid on hydrophilic substrates using molding and printing. *Adv. Mater.* **2004**, *16*, 584−588.

(97) Hersh, L. S.; Gott, V. L.; Najjar, F. Thermal and ionic methods of heparinizing small-diameter dacron grafts. *J. Biomed. Mater. Res.* **1972**, *6*, 85−96.

(98) Iler, R. K. Multilayers of colloidal particles. *J. Colloid Interface Sci.* **1966**, *21*, 569−594.

(99) West, R. H.; Paul, A. J.; Hibbert, S.; Cahalan, P.; Cahalan, L.; Verhoeven, M.; Hendriks, M.; Fouache, B. Correlation of surface chemistries of polymer bioactive coatings with their biological performances. *J. Mater. Sci., Mater. Med.* **1995**, *6*, 63−67.

(100) Picart, C.; Mutterer, J.; Richert, L.; Luo, Y.; Prestwich, G. D.; Schaaf, P.; Voegel, J. C.; Lavalle, P. Molecular basis for the explanation of the exponential growth of polyelectrolyte multilayers. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12531−12540.

(101) Picart, C.; Lavalle, P.; Hubert, P.; Cuisinier, F. J. G.; Decher, D.; Schaaf, P.; Voegel, J.-C. Buildup mechanism for poly(L-lysine)/hyaluronic acid films onto a solid surface. *Langmuir* **2001**, *17*, 7414−7421.

(102) Burke, S. E.; Barrett, C. J. pH-responsive properties of multilayered poly(L-lysine)/hyaluronic acid surfaces. *Biomacromolecules* **2003**, *4*, 1773−1783.

(103) Ball, V.; Schaaf, P.; Voegel, J. C. Mechanism of interfacial exchange phenomena for proteins adsorbed at solid-liquid interfaces. In *Biopolymers at Interfaces*; Malmsten, M., Ed.; Surfactant Science Series Vol. 110; Dekker: New York, 2003; pp 295−320.

(104) Beavers, E. M. Lens with Hydrophilic Coating. U.S. Patent 4,663,233, May 5, 1987.

(105) Halpern, G.; Campbell, C.; Beavers, E. M. Method of Hydrophilic Coating of Plastics. U.S. Patent 4,801,475, Jan 31, 1989.

(106) Halpern, G.; Campbell, C.; Beavers, E. M. Method of Interlaminar Grafting of Coatings. U.S. Patent 5,037,677, Aug 6, 1991.

(107) Hoekstra, D. Hyaluronan-Modified Surfaces for Medical Devices. *Med. Device Diagn. Ind.* **1999**, Feb, 23−26.

(108) Lowry, K. M.; Beavers, E. M. Resistance of hyaluronate coatings to hyaluronidase. *J. Biomed. Mater. Res.* **1994**, *28*, 861−864.

(109) Johnston, J. B.; Huszar, G. Male infertility. Paper available at http://www.biocoat.com/spermattech.asp.

(110) Ranganathan, S.; Bharadwaj, A.; Datta, K. Hyaluronan mediates sperm motility by enhancing phosphorylation of proteins including hyaluronan binding protein. *Cell. Mol. Biol. Res.* **1995**, *41*, 467−476.

(111) Richert, L.; Boulmedais, F.; Lavalle, P.; Mutterer, J.; Ferreux, E.; Decher, G.; Schaaf, P.; Voegel, J. C.; Picart, C. Improvement of stability and cell adhesion properties of polyelectrolyte multilayer films by chemical cross-linking. *Biomacromolecules* **2004**, *5*, 284−294.

(112) Lo, C. M.; Wang, H. B.; Dembo, M.; Wang, Y. L. Cell Movement Is Guided by the Rigidity of the Substrate. *Biophys. J.* **2000**, *79*, 144−152.

(113) Kito, H.; Matsuda, T. Biocompatible coatings for luminal and outer surfaces of small-caliber artificial grafts. *J. Biomed. Mater. Res.* **1996**, *30*, 321−330.

(114) Barbucci, R.; Magnani, A.; Lamponi, S.; Pasqui, D.; Bryan, S. The use of hyaluronan and its sulphated derivative patterned with micrometric scale on glass substrate in melanocyte cell behaviour. *Biomaterials* **2003**, *24*, 915−926.

(115) Weber, E.; Rossi, A.; Gerli, R.; Lamponi, S.; Magnani, A.; Pasqui, D.; Barbucci, R. Micropatterned hyaluronan surfaces promote lymphatic endothelial cell alignment and orient their growth. *Lymphology* **2004**, *37*, 15−21.

(116) DeFife, K. M.; Shive, M. S.; Hagen, K. M.; Clapper, D. L.; Anderson, J. M. Effects of photochemically. immobilized polymer coatings on protein adsorption, cell adhesion, and the foreign body reaction to silicone rubber. *J. Biomed. Mater. Res.* **1999**, *44*, 298−307.

(117) Tengblad, A. Affinity chromatography on immobilized hyaluronate and its application to the isolation of hyaluronate binding properties from cartilage. *Biochim. Biophys. Acta* **1979**, *578*, 281−289.

(118) Larsson, R. Biocompatible surfaces prepared by immobilized heparin or hyaluronate. *Acta Otolaryngol. Suppl.* **1987**, *442*, 44−49.

(119) Fagerholm, P.; Koul, S.; Trocme, S. Corneal endothelial protection by heparin and sodium hyaluronate surface coating of PMMA intraocular lenses. *Acta Ophthalmol. Suppl.* **1987**, *182*, 110−114.

(120) Kujawa, M. J.; Caplan, A. I. Hyaluronic acid bonded to cell-culture surfaces stimulates chondrogenesis in stage 24 limb mesenchyme cell cultures. *Dev. Biol.* **1986**, *114*, 504−518.

(121) Guo, M. M.; Hildreth, J. E. Assessment of cell binding to hyaluronic acid in a solid-phase assay. *Anal. Biochem.* **1996**, *233*, 216−220.

(122) Pouyani, T.; Kuo, J. W.; Harbison, G. S.; Prestwich, G. D. Solid-state NMR of *N*-acylureas derived from the reaction of hyaluronic acid with isotopically-labeled carbodiimides. *J. Am. Chem. Soc.* **1992**, *114*, 5972−5976.

(123) Morra, M.; Cassinelli, C.; Benedetti, L.; Callegaro, L. Process for the coating of objects with hyaluronic acid, derivatives thereof, and semisynthetic polymers. U.S. Patent 6,129,956, Oct 10, 2000.

(124) Grigoreas, G. H.; Anagnostides, S. T.; Vynios, D. H. A solid-phase assay for the quantitative analysis of hyaluronic acid at the nanogram level. *Anal. Biochem.* **2003**, *320*, 179−184.

(125) *Plasma Deposition, Treatment and Etching of Polymers*; d'Agostino, R., Ed.; Academic Press: New York, 1995.

(126) Verheye, S.; Markou, C. P.; Salame, M. Y.; Wan, B.; King, III, S. P.; Robinson, K. A.; Chronos, N. A. F.; Hanson, S. R. Reduced thrombus formation by hyaluronic acid coating of endovascular devices. *Arterioscler., Thromb., Vasc. Biol.* **2000**, *20*, 1168−1172.

(127) Wan, B.; McGregor, H.; Clavin, M.; Chang, G.; Shiedlin, A.; Nickerson, C. M. B.; Skinner, K.; Chronos, N. A. F.; Verheye, S.; Robinson, K. A.; Hanson, S. R. Evaluation of immobilized hyaluronic acid: stability and platelet deposition studies. *Soc. Biomater. Trans.* **1999**, *22*, 228.

(128) Sweeney, D. F.; Xie, R. Z.; Evans, M. D. M.; Vannas, A.; Tout, S. D.; Griesser, H. J.; Johnson, G.;. Steele, J. C. A Comparison of Biological Coatings for the Promotion of Corneal Epithelialization of Synthetic Surface In Vivo. *Invest. Ophthalmol. Visual Sci.* **2003**, *44*, 3301−3309.

(129) Thierry, B.; Winnik, F. M.; Merhi, Y.; Silver, J.; Tabrizian, M. Radionuclides-hyaluronan-conjugate thromboresistant coatings to prevent in-stent restenosis. *Biomaterials* **2004**, *25*, 3895−3905.

(130) Mason, M.; Vercruysse, K. P.; Kirker, K. R.; Frisch, R.; Marecak, D. M.; Prestwich, G. D.; Pitt, W. G. Attachment of hyaluronic acid to polypropylene, polystyrene, and polytetrafluoroethylene. *Biomaterials* **2000**, *21*, 31−36.

(131) Pouyani, T.; Harbison, G. S.; Prestwich, G. D. Novel hydrogels of hyaluronic acid: Synthesis, surface morphology, and solid-state NMR. *J. Am. Chem. Soc.* **1994**, *116*, 7515−7522.

(132) Cen, L.; Neoh, K. G.; Li, Y.; Kang, E. T. Surface functionalization of electrically conductive polypyrrole film with hyaluronic acid. *Langmuir* **2002**, *18*, 8633−8640.

(133) Cen, L.; Neoh, K. G.; Li, Y.; Kang, E. T. Assessment of in vitro bioactivity of hyaluronic acid and sulfated hyaluronic acid functionalized electroactive polymer. *Biomacromolecules* **2004**, *5*, 2238−2246.

(134) Pitt, W. G.; Morris, R. N.; Mason, M. L.; Hall, M. W.; Luo, Y.; Prestwich, G. D. Attachment of hyaluronan to metallic surfaces. *J. Biomed. Mater. Res.* **2004**, *68A*, 95−106.

(135) Stile, R. A.; Barber, T. A.; Castner, D. G.; Healy, K. E. Sequential robust design methodology and X-ray photoelectron spectroscopy to analyze the grafting of hyaluronic acid to glass substrates. *J. Biomed. Mater. Res.* **2002**, *61*, 391−398.

(136) Morra, M.; Cassinelli, C. Simple model for the XPS analysis of polysaccharide coated surfaces. *Surf. Interface Anal.* **1998**, *26*, 742−746.

(137) Cassinelli, C.; Morra, M.; Pavesio, A.; Renier, D. Evaluation of the interfacial properties of hyaluronan coated poly(methylmethacrylate) intraocular lenses. *J. Biomater. Sci., Polym. Ed.* **2000**, *11*, 961−977.

(138) Morra, M.; Cassinelli, C.; Pavesio, A.; Renier, D. Atomic force microscopy evaluation of aqueous interfaces of immobilized hyaluronan. *J. Colloid Interface Sci.* **2003**, *259*, 236−243.

(139) Butt, H. J.; Kappl, M.; Mueller, H.; Reiteri, R.; Meyer, W.; Ruhe, J. Steric forces measured with the atomic force microscope at various temperature. *Langmuir* **1999**, *15*, 2559−2568.

(140) Lise, M.; Belluco, C.; Pucciarelli, S.; Meggiolaro, F.; Codello, L.; Pressato, D.; Dona′, M.; Bigon, E. Prevention of adhesions in abdominal surgery. In *Redefining Hyaluronan*; Abatangelo, G., Weigel, P. H., Eds.; Elsevier: Amsterdam, 2000; pp 339−344.

(141) Larsen, N. E. management of adhesion formation and soft tissue augmentation with viscoelastics: hyaluronan derivatives. In *The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives*; Laurent, T. C., Ed.; Portland Press Ltd.: London, 1998; pp 267−282.

(142) Ohri, R.; Hahn, S. K.; Hoffman, A. S.; Stayton, P. S.; Giachelli, C. M. Hyaluronic acid grafting mitigates calcification of glutaraldehyde-fixed bovine pericardium. *J. Biomed. Mater. Res.* **2004**, *70A*, 328−334.

(143) Vyavahare, N.; Ogle, M.; Schoen, F. J.; Zand, R.; Gloeckner, D. C.; Sacks, M.; Levy, R. J. Mechanisms of bioprosthetic heart valve failure: Fatigue causes collagen denaturation and glycosaminoglycan loss. *J. Biomed. Mater. Res.* **1999**, *46*, 44−50.

(144) Lovekamp, J.; Vyavahare, N. Periodate-mediated glycosaminoglycan stabilization in bioprosthetic heart valves. *J. Biomed. Mater. Res.* **2001**, *56*, 478−486.

(145) Boskey, A. L.; Maresca, M.; Wikstrom, B.; Hjerpe, A. Hydroxyapatite formation in the presence of proteoglycans of reduced sulphate content: studies in the brachymorphic mouse. *Calcif. Tissue Int.* **1991**, *49*, 389−393.

(146) Sengupta, K.; Schilling, J.; Marx, S.; Markus, F.; Sackmann, E. Supported membrane coupled ultra-thin layer of hyaluronic acid: viscoelastic properties of a tissue-surface mimetic system. *Biophys. J.* **2003**, *84*, 381a−381a.

(147) Sengupta, K.; Schilling, J.; Marx, S.; Fischer, M.; Bacher, A.; Sackman, E. Mimicking tissue surfaces by supported membrane coupled ultrathin layer of hyaluronic acid. *Langmuir* **2003**, *19*, 1775−1781.

(148) Jha, B. K.; Salunke, D. M.; Datta, K. Structural Flexibility of Multifunctional HABP1 May Be Important for Regulating Its Binding to Different Ligands. *J. Biol. Chem.* **2003**, *278*, 27464−27472.

(149) Benz, M.; Chen, N.; Israelachvili, J. Lubrication and wear properties of grafted polyelectrolytes, hyaluronan and hylan, measured in the surface force apparatus. *J. Biomed. Mater. Res.* **2004**, *71A*, 6−15.