Physical studies of glycosaminoglycans in relation to the adhesion properties of human cancer cells

Antonio Peramo

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Physical Studies of Glycosaminoglycans in Relation to the Adhesion Properties of Human Cancer Cells

by

Antonio Peramo

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Note to Reader

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<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>iii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>iv</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract</td>
<td>vii</td>
</tr>
<tr>
<td>1. Project Scope</td>
<td>1</td>
</tr>
<tr>
<td>2. Background Elements</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Cancer, metastasis and proteoglycans</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Glycosaminoglycans</td>
<td>7</td>
</tr>
<tr>
<td>2.3 Proteoglycans</td>
<td>12</td>
</tr>
<tr>
<td>2.4 Heparanase</td>
<td>17</td>
</tr>
<tr>
<td>2.5 Ligand-receptor density</td>
<td>20</td>
</tr>
<tr>
<td>3. Experimental</td>
<td>22</td>
</tr>
<tr>
<td>3.1 Surface functionalization</td>
<td>23</td>
</tr>
<tr>
<td>3.1.1 Introduction</td>
<td>23</td>
</tr>
<tr>
<td>3.1.2 Experimental procedures for surface functionalization</td>
<td>27</td>
</tr>
<tr>
<td>3.2 Surface density quantitation of GAGs</td>
<td>35</td>
</tr>
<tr>
<td>3.2.1 Introduction</td>
<td>35</td>
</tr>
<tr>
<td>3.2.2 Experimental procedures for density quantitation</td>
<td>37</td>
</tr>
<tr>
<td>3.3 Surface characterization by ellipsometry and AFM imaging</td>
<td>40</td>
</tr>
<tr>
<td>3.3.1 Introduction</td>
<td>40</td>
</tr>
<tr>
<td>3.3.2 Experimental procedures for surface characterization by ellipsometry and AFM imaging</td>
<td>42</td>
</tr>
<tr>
<td>3.4 Static adhesion of cancer cells to the functionalized surfaces</td>
<td>44</td>
</tr>
<tr>
<td>3.4.1 Introduction</td>
<td>44</td>
</tr>
<tr>
<td>3.4.2 Experimental procedures for static adhesion of cancer cells to the</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Results and discussion
   4.1 Comments on the functionalization of the surfaces 52
   4.2 Results of the surface density quantitation of GAGs 59
   4.3 Discussion of surface analysis by AFM and ellipsometry 74
   4.4 Results of experiments of the static adhesion of cancer cells 83
      4.4.1 Immunostaining and heparanase activity and determination of cDNA transfection conditions 83
      4.4.2 Static cell adhesion to the different substrates 87
      4.4.3 Discussion of adhesion by cell line 98
      4.4.4 Comments of the adhesion of cells by the origin of the cell line 100
      4.4.5 Discussion of adhesion by type of substrate or surface 103
      4.4.6 Discussion of adhesion by the molecules involved. Heparin and heparanase effects on adhesion 106
      4.4.7 Discussion of the adhesion as a function of polysaccharide charge and chain length 108
   5. Physical model of adhesion using the radius of gyration of the biopolymers 119
   6. Future work 129
   7. Conclusions and key achievements 132
   References 137
   Bibliography 149
   Appendices 151
   Appendix A  Equipment 152
   Appendix B  Detailed steps of the chemical procedures 153
   About the Author  End Page
List of Tables

Table 1. Properties of Glycosaminoglycans 9
Table 2. Chemical structures of glycosaminoglycans 10
Table 3. Proteoglycans properties and functions 15
Table 4a. List of primary antibodies 33
Table 4b. List of secondary antibodies 33
Table 5. GAG size and length characteristic data 58
Table 6a. Surface coverage by $[^{14}C]$-Formaldehyde radiolabeling of APTES 63
Table 6b. Surface coverage by $[^{14}C]$-Formaldehyde radiolabeling of APTES (time) 63
Table 7. Surface coverage by $[^{14}C]$-Acetaldehyde radiolabeling of GAGs 69
Table 8. Surface coverage by $[^{14}C]$-Acetaldehyde of control surfaces 73
Table 9a. Variation of ellipsometric thickness of APTES with concentration and time 78
Table 9b. Variation of ellipsometric thicknesses of GAGs layers in dry state 78
Table 10. Experimental and theoretical values of surface coverage for GAGs 81
Table 11. Preferred substrates for adhesion of cancer cells 102
Table 12. Calculated values of the radius of gyration 124
List of Figures

Figure 1. Representation of perlecan, a proteoglycan on the basement membrane 14
Figure 2. Simplified scheme of the procedure of GAG surface modification 31
Figure 3. Simplified scheme of the procedure of surface modification HSP 32
Figure 4. GAGs fluorescence micrographs 55
Figure 5. AFM surface images of CSA 56
Figure 6. Simplified scheme of the procedures for quantitation of glass grafted GAGs 61
Figure 7. Scheme of the procedure for radiolabeling with $^{14}$C-Formaldehyde 62
Figure 8. Scheme for $^{14}$C-Acetaldehyde radiolabeling of polysaccharide surfaces 66
Figure 9. APTES control surfaces subjected to hydrozinolysis and deamination 72
Figure 10. Localization of heparanase in cDNA transfected cells 85
Figure 11. Heparanase activity and Western Blots. 86
Figure 12. Absolute adhesion of MCF7 cells 91
Figure 13. Adhesion of MCF7 cells relative to non-treated cells 91
Figure 14. Absolute adhesion of A431 cells 92
Figure 15. Adhesion of A431 cells relative to non treated cells 92
Figure 16. Absolute adhesion of BT20 cells 93
Figure 17. Adhesion of BT20 cells relative to non-treated cells 93
Figure 18. Adhesion of MCF7, BT20 and A431 non treated cells 94
Figure 19. Adhesion of MCF7, BT20 and A431 heparin suspended cells 94
Figure 20. Adhesion of MCF7, BT20 and A431 cDNA transfected cells 95
Figure 21. Absolute number of non-treated cells adhered to glass and APTES 95
Figure 22. Absolute number of heparin suspended cells adhered to glass and APTES 96
Figure 23. Absolute number of cDNA transfected cells adhered to glass and APTES 96
Figure 24. Adhesion vs sulfate per dimer graph for non-treated cells 113
Figure 25. Adhesion vs sulfate per dimer graph for heparin suspended cells 114
Figure 26. Adhesion vs sulfate per dimer graph for cDNA transfected cells 115
Figure 27. Adhesion vs GAG chain length 117
Figure 28. Plots of adhesion vs Rg of GAGs for non-treated cells 125
Figure 29. Plots of adhesion vs Rg of GAGs for heparin suspended cells 126
Figure 30. Plots of adhesion vs Rg of GAGs for cDNA transfected cells 127
Figure 31. Detailed reaction of silanization with APTES 153
Figure 32. Detailed reaction of reductive amination with NaBH₃CN 154
Figure 33. Detailed reaction of the procedure for radiolabeling APTES surfaces 155
Figure 34. Detailed reaction of radiolabeling via enamine 156
Figure 35. Detailed reaction of proteoglycan binding to APTES 157
Figure 36. Detailed reaction of denitrosation 158
Figure 37. Detailed reaction of deacetylation and deamination 159
List of Abbreviations

μCP: Microcontact printing
APTES: 3-aminopropyltriethoxysilane
BM: Basement Membrane
BS3: Bis[sulfosuccinimidyl] suberate
BSA: Bovine serum albumin
CSA: Chondroitin sulfate A
CSC: Chondroitin sulfate C
EDTA: Ethylenediaminetetraacetic acid
ECM: Extracellular matrix
GAG: Glycosaminoglycan
HSP: Heparan sulfate proteoglycan
HS: Heparan sulfate
KS: Keratan sulfate
M.M.: molecular mass
PDMS: Polydimethylsiloxane
PG: Proteoglycan
SAM: self-assembled monolayer
Physical Studies of Glycosaminoglycans in Relation to the Adhesion Properties of Human Cancer Cells

Antonio Peramo

ABSTRACT

The study of the processes relating glycobiology and cancer will have increased interest in coming years. To contribute to this trend the outcome of this work will be useful for investigations in glycobiology, using experimental methods exhibiting controlled carbohydrate composition, organization, and orientation, drawn from materials science and physics and that can be used in bioengineering and other technical areas in biology.

In this work, the focus has been on physical studies of some members of the family of glycosaminoglycans and their role in cancer metastasis. The project studies the static adhesion of cancer cells to substrates functionalized with cell surface glycocalyx molecules and, in particular, in the interaction of heparan sulfate, keratan sulfate and chondroitin sulfates with the cells. Surface characterization techniques are used to analyze the structure of the polymeric brushes deposited on the substrates.

The hypothesis that the adhesion of whole cancer cells to glycosaminoglycan substrates is a function of polysaccharide charge per dimer and chain length was proposed and tested. Part of the work has been dedicated to study the changes in the adhesion of tumor cells in the presence of heparanase, an enzyme expressed in the tumor cell surface.
The essential achievements of the project have been:

a) Design of a new method for the deposition and patterning of glycans to glass or silicon surfaces functionalized with a silane agent, exposing an amino terminated monolayer as functional substrate.

b) Development of a new method for the calculation of the density of the deposited molecules.

c) Physical characterization of the surfaces using a combination of surface science techniques, including ellipsometry and atomic force microscopy. These surfaces should be useful for developing additional experiments that may be helpful in understanding the adhesive properties of the cells.

d) Comparative analysis of the behavior of cancer cells to the functionalized surfaces, specifically the study of the static adhesion of the cells, in the presence or absence of the surface protein heparanase or its inhibitors.

e) Confirmation of the hypothesis that attachment of whole cancer cells, *in vitro*, depends linearly on the charge per dimer of polysaccharide.
1. Project Scope

One of the most critical factors in the malignancy of cancer is metastasis. To metastasize, cancer cells must acquire the ability to survive in the bloodstream and invade a foreign tissue. Partially, this process is prevented by the propensity of some cells to die in suspension. Cancer cells metastasize using not just blood vessels, but other vessels, as well as other mechanical processes. For example, the lymphatic system and the genitourinary track are also used in the metastatic process.

Carbohydrate to carbohydrate interaction appears to be the initial step in cell adhesion via communication between some molecules in the cell glycocalyx and the extracellular matrix. The extracellular matrix in animals is made mainly from two main classes of macromolecules: GAGs and fibrous proteins like collagen, elastin, fibronectin and laminin. These macromolecules provide structural and adhesive properties to the matrix. Specialized ECMs are basement membranes acting as a surface where cells can migrate and a barrier to cell migration.

The arterial wall contains different GAGs and PGs, like chondroitin sulfate and heparan sulfate, and heparan sulfate is found in the endothelial cell surface. Normally, methods to deposit glycocalyx molecules have consisted in growing endothelial cells on surfaces and then eliminating part of the biological material via lysis. This artificial model has permitted testing for adhesion between cancerous cells and GAGs found in the ECM. However, that method cannot study individualized interactions between cells and molecular species of choice, and developing a new technique that facilitates this analysis is within the scope of this project.
The physical characterization may be investigated by developing *in vitro* model surfaces containing molecular species of interest with the possibility of selecting the molecular composition at the interface.

Characterization of the adhesion processes in which cancer cells make use of the polysaccharide chains of proteoglycans – the glycosaminoglycans – is of interest. It has been known for some time that some proteins in cell surfaces bind glycosaminoglycans. For instance, cell adhesion proteins - i.e. integrins, laminin-, glycoproteins of the extracellular matrix and others. Where those proteins are found, GAGs are also present as side chains of PGs showing differences in their chemical composition.

Some work has been put into studying the effects of an enzyme, heparanase, on the adhesion of cancer cells to the GAGs substrates. This secreted enzyme is located on the surface of some normal and several tumor cells and can possibly function as a receptor or provide the conditions for the adhesion of the cell to the ECM. Heparanase is known to have a role in the metastatic potential of tumor cells and several tumors are known to show preferential expression of heparanase mRNA. 


\[4\] The possibility of inactive heparanase binding to heparan sulfate in the ECM was proposed by Dempsey L.A.; Plummre, T.B.; Coombes, S.L.; Platt, J.L. Heparanase expression in invasive trophoblasts an acute vascular damage. *Glycobiology*, 2000, 10, 467-475.


increase of heparanase expression in breast cancer and heparanase expressed on the surface of breast tumor cells increase metastasis\textsuperscript{10}.

Localization of the molecule on the cell membrane seems to have a major promoting effect on metastasis. Mice injected with melanoma cells incubated with pro-heparanase before injection showed a substantial increase in lung colonization\textsuperscript{11}. Parish\textsuperscript{12} and Nakajima\textsuperscript{13} have also shown that heparin, an inhibitor of heparanase, reduced the incidence of experimental metastasis in high percentages, higher than 90%.

Then, in addition to its role in the degradation of the ECM for the metastatic cellular escape from tumors\textsuperscript{14,15}, it seems there is a possibility of involvement for this enzyme in adhesion, and it is interesting to note that, although it has little activity at physiological

\begin{thebibliography}{9}
\item \textit{Cif. note 5, supra.}
\end{thebibliography}
pH, heparanase still binds to the cell surface\textsuperscript{16}. That suggests that heparanase may act in an adhesive function between tumor cells and the endothelium and this function has been, in part, studied in this project, by using cells from three different cultured cancer cell lines. The orientation was in the investigation of the binding of the tumor cells to surfaces modified with some GAGs.

It has been specifically demonstrated in the past that heparan sulfate plays an important role in the motility of some cancer cells, namely in liver metastatic cells from lung cancer\textsuperscript{17}. It is known that microvessels in the endothelium of each organ express an organ-specific membrane phenotype, providing a basis for organ specific recognition of vessels by the tumor cells\textsuperscript{18}. This recognition depends, in part, on the ability of cells to roll during the metastatic process. The rolling process involves final recognition of binding sites and there is a general consensus that this process, rolling followed by adhesion, is needed for extravasation. The arrest can also occur through adhesive bonds and several molecules are involved in this process in the endothelium, including integrins, selectins and cadherins\textsuperscript{19}.

The adhesion step during rolling is known as transient adhesion. Kojima has compared the importance of rolling or transient adhesion to static adhesion\textsuperscript{20}. In the case of integrin


\textsuperscript{20} Kojima, N.; Shiota, M.; Sadahira, Y.; Handa, K.; Hamori, S., Cell adhesion in a dynamic flow system as compared to static system. Glycospingolipid- glycosphingolipid interaction in the dynamic system predominates over lectin- or integrin-based mechanisms in adhesion of B16 melanoma cells to non-activated endothelial cells. \textit{J. Biol. Chem} 1992,
adhesion mediated mechanisms the static system seems to be predominant while for the 
selectin adhesion mechanism dynamic adhesion predomnates.

Based on the previous discussion, the specific aims of the project are:

1. Perform deposition and covalent attachment of glycosaminoglycans on silicon 
and silanized glass surfaces to simulate the structure of the extracellular 
glycocalyx. The techniques to be used are direct immersion and application of 
a pattern by means of microcontact printing (μCP).
2. Analysis of the surfaces and depositions. Immunofluorescence assays, AFM 
studies of the coated surfaces for the description of their physical 
characteristics and other surface characterization techniques will be 
performed.
3. Develop a method to evaluate surface densities of the polysaccharides on the 
surfaces. The method described has the advantage that may be used with any 
polysaccharide patterned to any surface exposing an amino terminated 
monolayer by reductive amination of their galactosamine or glucosamine 
repeating units.
4. Measure the static binding of cancer cells to substrates functionalized with 
polysaccharides. Particular attention has been put on the interaction with 
heparan sulfate, keratan sulfate, chondroitin sulfate with the surfaces.
5. Propose and verify a simple model for the adhesion of whole cells to 
polysaccharide substrates in vitro in which the adhesion has a linear 
dependence with the charge per dimer of polysaccharide and a probable 
limiting value in the length of the chain.

267, 17264-17270.
2. Background Elements

2.1 Cancer, metastasis\textsuperscript{21} and proteoglycans

The survival of tumor cells in different tissues after they move from their main tumor implications that they have the ability to recruit vasculature and stroma in those other tissues, making the local microenvironment to participate in their own proliferation, by selection and proliferation of new tumor cells\textsuperscript{22}.

Other physiological processes in the body also occur with this type of molecular cross-talk, for instance, during embryogenesis. But only malignant invasion is a persistent mechanism, with the activation of the local invasive environment. The whole process links the motility, the survival and the proliferation of tumor cells. Adhesive and de-adhesive interactions occur at the site where tumor cells cross the vessel wall and so the junctions between vascular cells are broken or retracted.

A family of proteins known as integrins exists at the junctions between cells and the ECM. The rupture of integrin mediated adhesion to the ECM, which is required for cellular motility or translocation, can trigger apoptosis if it is not followed by attachment.

\textsuperscript{21} The metastatic process includes invasion, intravasation, arrest, extravasation and neovascularization and is highly inefficient. As described by Weiss, L., (Metastatic inefficiency, \textit{Advances in Cancer Research}, 1990, \textbf{54}, 159-211), the extravasation can take place in two ways: by active migration of the cells, sometimes following the pathways of leukocytes or by intravascular proliferation of arrested cells with damage to the surrounding vessel, bursting out of the blood vessel. This process is facilitated by basement membrane-degrading enzymes released from the cancer cells themselves and by the vascular endothelium and are in these processes where proteoglycans play their role.

The transition to an invasive carcinoma is then preceded by activation of fibroblasts, immune cells and endothelial cells, and with the modification of the adjacent ECM membrane. These observations lead to the idea that tumor cells are somehow captured by vascular cells during entry and exit from the circulation and that the host interface is collaborating in the invasion.

The invasion of other tissues take place when stroma and tumor cells exchange some molecules – namely enzymes and cytokines – that modify the local extracellular matrix. The degradation of the ECM by various enzymes includes the destruction and solubilization of matrix PGs and among them HSP. Important factors for growth and angiogenesis are sequestered and stored in the microenvironment surrounding the tumors, for instance heparin-binding angiogenic proteins. The release of these proteins produces angiogenesis after the destruction of HSP by heparanase.

As mentioned in the introduction stromal therapy has emerged as a new strategy for cancer therapy. One of the categories is the use of anti-adhesive molecules where the development of PGs-based anti-cancer molecules can fit. The focus for therapies has been on blocking cell adhesion and from there the interest on the role that heparan sulfate expressed in the surface of tumor cells may have in the adhesion and extravasation of the cell, mainly via the proteoglycan syndecan\textsuperscript{23} and on the role of secreted heparanase in the adhesion of the cell by linkage to HS (or other GAGs) in the vascular endothelium.

2.2 Glycosaminoglycans

GAGs are polysaccharides that are found in animal tissues, normally in covalent association with protein, known as proteoglycans. Seven types are commonly recognized, depending upon the sugar, type of linkage between sugars and number and location of

sulfate groups. Chemically, glycosaminoglycans are polysaccharides primarily composed of the disaccharide repeats D-galactose and D-glucosamine (KS), D- or L-glucuronic acids and D-glucosamine (HS) or D-glucuronic acid and D-galactosamine (CSA and CSC). Table 1 shows their classification.

These polysaccharide types are distinguished by their monomer composition, by the position and configuration of their glycosidic linkages, and by the amount and location of their sulfate substituents. All, except one, hyaluronate, are sulfated. Hyaluronate is thought to have a role in resisting compressive forces in tissues and joints. The presence of carboxyl and sulfation groups makes GAGs highly negatively charged. Sometimes called mucopolysaccharides, GAGs are highly viscous and highly hydrophilic.

In this project, the selection of the GAGs have been based on the following criteria: in the case of HS, CSA and CSC the reason was that they are present in the arterial wall, then possibly involved in the intra or extravasation process. In the case of KS, the main reason has been that is the only GAG that does not have an acid as monosaccharide, thus greatly differentiating it from the rest. In addition, the whole group shows other interesting differences in fundamental properties that may prove useful to discern their behavior in the experiments. First, an increase in electronic charge per disaccharide in the order KS < CSC ~ CSA < HS and second, an increase in GAG chain length measured by the disaccharide number per chain, in the order KS < HS < CSA < CSC.
Table 1. Properties of glycosaminoglycans. (Adapted from Lindahl, Cif. note 1, supra.) with GAGs selected in this project in grey.

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Mol. wt (Daltons)</th>
<th>Repeating period monosaccharides</th>
<th>Sulfate per disaccharide unit</th>
<th>Other sugar components</th>
<th>Occurrence in mammalian tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronate</td>
<td>4 – 8000</td>
<td>D-glucuronic acid D-glucosamine</td>
<td>0</td>
<td>none</td>
<td>connective tissues, skin, vitreous humor, synovial fluid, cartilage</td>
</tr>
<tr>
<td>Chondroitin 4 and 6- sulfates</td>
<td>5 – 60</td>
<td>D-glucuronic acid D-galactosamine</td>
<td>0.1 – 1.3</td>
<td>D-galactose D-xylose</td>
<td>cartilage, cornea, bone, skin, arterial wall</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>15 – 40</td>
<td>D-glucuronic acid L-iduronic acid D-galactosamine</td>
<td>1.0 – 3</td>
<td>D-galactose D-xylose</td>
<td>skin, heart valve, tendon, arterial wall</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>6 – 25</td>
<td>D-glucuronic acid L-iduronic acid D-glucosamine</td>
<td>0.4 – 2</td>
<td>D-galactose D-xylose</td>
<td>lung, arterial wall, ubiquitous in several cell surfaces</td>
</tr>
<tr>
<td>Heparin</td>
<td>6 – 25</td>
<td>D-glucuronic acid L-iduronic acid D-glucosamine</td>
<td>1.6 – 3</td>
<td>D-galactose D-xylose</td>
<td>lung, liver, skin, intestinal mucosa</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>4 – 19</td>
<td>D-galactose D-glucosamine</td>
<td>0.9 – 1.8</td>
<td>D-galactosamine D-mannose L-fucose Sialic acid</td>
<td>cartilage, cornea, intervertebral disc</td>
</tr>
</tbody>
</table>
Table 2. Chemical structures of glycosaminoglycans. Abbreviations: GlcUA, glucuronic acid; IdUA, iduronic acid; GlcN, glucosamine; GalN, galactosamine; Gal, galactose. (Adapted from Lindahl, Cif. note 1, supra).

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Monosaccharide units</th>
<th>Substituents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
</tbody>
</table>

- **Hyaluronate**

- **Chondroitin 4- and 6-sulfates**

- **Dermatan sulfate**
Table 2 (cont.) Chemical structures of glycosaminoglycans. Abbreviations: GlcUA, glucuronic acid; IdUA, iduronic acid; GlcN, glucosamine; GalN, galactosamine; Gal, galactose. (Adapted from Lindahl, Cif. note 1, supra).

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>GlcUA</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>GlcUA</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>GlcN</td>
</tr>
</tbody>
</table>

[Diagram of chemical structures]
Table 2 shows the chemical structures of GAGs. GAGs are not necessarily multivalent, but that possibility exists. The binding of a second (or additional) molecule (or proteins) to a GAG has probably a small effect compared to the binding of the first molecule.

Due to this abundant variety, GAGs serve for many functions. These functions are diverse and include molecular and cell traffic regulation and help in their guidance of cells during migration. Another function is chemical signaling between cells. GAGs are essential for maintaining structural integrity of many connective tissues and the binding to macromolecules. A large number of macromolecules bind to GAGs. The majority of receptors are proteins or proteins conjugates, including the protein of interest in this study, heparanase.

The proteolysis of matrix proteins contributes to cell migration in several ways, for example: clearing a path through the matrix, expose some sites on the proteins that promote cell binding or migration, promote cell detachment or release extracellular signal proteins that stimulate cell migration. In other words, GAGs can act as selective sieves to regulate the traffic of molecules and cells according to their size, charge or both. When specific enzymes remove the GAG chains, the filtering properties of the lamina are destroyed. There are various types of binding between GAGs and other macromolecules. Cooperative electrostatic binding may involve any number of GAGs. The interaction is facilitated by increased charge density of the polysaccharides and stereochemical factors.

2.3 Proteoglycans

Proteoglycans are in a highly hydrated form, making a gel-like substance that resists compressive forces and allows diffusion of molecules between blood vessels and the surrounding tissue. In a proteoglycan, at least one of the sugar chains must be a GAG. They form a heterogeneous group because of the different number and types of attached GAG chains. Certain functions of PGs are certainly expressed by the free GAG chain itself. However, several activities of the proteoglycan depend on the core protein,
although in some cases the core protein just provides a scaffold for the appropriate immobilization of the attached GAGs chains. The protein acts as anchor, essential for the positioning of the GAG-bound ligand.

*In vivo,* instead as free polysaccharide chains, GAGs occurs as PGs, where several chains are covalently linked with its sugar terminal to a polypeptide core. The linkage region is essentially the same in PGs with CS/DS or heparin/HS chains:

\[
\text{GlcUA} \beta_1,3 \text{Gal} \beta_1,3 \text{Gal} \beta_1,4 \text{Xyl} \beta_3 \text{ L-serine bridge between the GAG chain and the polypeptide core.}
\]

Table 3 shows a list of PG with some functional and structural characteristics. The classification of PGs is complicated because of the heterogeneity of the glycan structures or the presence of different types of GAGs chains bound to the same core protein. Using the topographical distribution of the PGs respect to the cell surface, PGs are intracellular, on the cell surface and in the extracellular matrix.

Not completely within the scope of this project, some experiments have been performed with perlecan, a basement membrane PG present in vascular and epithelial basement membranes, having heparan sulfate chains and chondroitin sulfate chains with a protein core of around 400 kDa. The three GAG chains of perlecan are located at one end of the molecule, as shown in Fig 1. The N-terminal domain, domain I, contains the attachment sites for heparan sulfate chains. This region can accept either heparan or chondroitin sulfate chains, and the selection is cell specific. Perlecan expression is found mainly in mature tissues and is prominent in the endothelial cell basement membrane of all vascularized organs, specialty liver, lung, pancreas and kidney.
Fig 1. Representation of perlecan, a proteoglycan on the basement membrane. Roman numerals indicate domains. SEA, protein module; LA, LDL, receptors; Ig, immunoglobulin; LE, laminin-1; LamB globular module; LamG module in laminin-1; NtA, N terminal domain that binds laminin; FS, follistatin-like; ST, serine/threonine rich.
Table 3. Proteoglycans properties and functions. (Adapted from Kjellen et al.\textsuperscript{24}).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Core Protein size (~kDa)</th>
<th>GAG chains</th>
<th>Functions and characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracellular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage/aggrecan</td>
<td>208-221</td>
<td>&gt;100 CS, 20-30 KS</td>
<td>Mechanical support, regulate cell migration</td>
</tr>
<tr>
<td>Fibroblast/versican</td>
<td>265</td>
<td>12-15 CS</td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>&gt;200</td>
<td>DS</td>
<td>Endothelial regeneration</td>
</tr>
<tr>
<td>Family b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Connective tissue/decorin</td>
<td>36</td>
<td>1CS/DS</td>
<td>Modulate collagen fibrillogenesis; regulate cell growth</td>
</tr>
<tr>
<td>Cartilage/collagen</td>
<td>68</td>
<td>1 CS</td>
<td>Binds to collagen fibrils</td>
</tr>
<tr>
<td>Family c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblasts/perlecan</td>
<td>250-400</td>
<td>HS</td>
<td>Modulate assembly of basement membranes</td>
</tr>
<tr>
<td>Colon carcinoma cells</td>
<td>240-400</td>
<td>10-15 HS</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. (cont.) Proteoglycans properties and functions. (Adapted from Kjellen et al\textsuperscript{25}).

<table>
<thead>
<tr>
<th>Cell surface</th>
<th>Family d</th>
<th>1-2 CS, 1-2 HS</th>
<th>Link cytoskeleton to extracellular matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary epithelial cells /syndecan</td>
<td>31</td>
<td>1-2 CS, 1-2 HS</td>
<td>Link cytoskeleton to extracellular matrix</td>
</tr>
<tr>
<td>Lymphocytes/CD44</td>
<td>37</td>
<td>CS</td>
<td>Mediate cell adhesion</td>
</tr>
<tr>
<td>B-cells</td>
<td>31</td>
<td>CS</td>
<td>Antigen presentation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intracellular</th>
<th>Family e</th>
<th>CD, DS, HS,heparin</th>
<th>Store and modulate activity of granular processes. Prevent blood coagulation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage cells</td>
<td></td>
<td></td>
<td>Store and modulate activity of granular processes. Prevent blood coagulation.</td>
</tr>
</tbody>
</table>

The binding of proteins to GAGs is usually cooperative electrostatic binding although highly specific binding is also possible. In this type of binding, the polysaccharide component cannot be substituted by any other glycosaminoglycan species. One example is the binding between heparin and antithrombin. It is obvious that in any interaction of the GAGs with other macromolecules, the requirements for specificity regarding carbohydrate structure will vary. In general, the binding sites are small tracts of GlcUA and iduronic acid residues in some specific arrangements. Electrostatic is the interaction between heparanase and GAGs, as is later mentioned.

2.4 Heparanase

Few years ago, some groups\textsuperscript{26, 27, 28} reported simultaneously the cloning of the mammalian version of the enzyme. The enzyme has attracted increased interest due to its relevant role in several biological processes. The enzyme is an endo-β-glucuronidase that cleaves HS chains at well-defined sites. For HS in the ECM, that produces a degradation of the ECM that increases angiogenesis and wound healing by liberating cytokines and growth factors bound to HS.

In addition of the degradation of the HS in the ECM, heparanase also degrades syndecan-1\textsuperscript{29}, a proteoglycan found in the cell surface with chains of CS and HS. Heparanase type 1 (Hpa1) exists as a proenzyme of ~ 65 kDa that requires some proteolytic processing in

\begin{footnotes}
\end{footnotes}
order to become active, in the form of a heterodimer (protein complex with two different polypeptide chains) of two sequences of ~8 kDa and ~50 kDa. Both terminals are important for the expression of hpa1 in the cell surface. As previously indicated, the presence of hpa1 is a possible indicator of the metastatic potential of a tumor cells, but there are no specific levels of hpa1 that may give a reference to consider a cell metastatic. It has been described the change from low metastatic tumor cells to highly metastatic tumor cells when heparanase is overexpressed in the cells. Human heparanase is expressed in normal tissues mainly in placenta, lymphoid organs and during organ development because to its angiogenic potential, but it is not, or rarely expressed, in mature organs except during tissue repair and regeneration. Hpa1 is not detected in non-metastatic breast carcinoma cells, but hpa1 expression is increased in breast carcinoma cells with increased metastatic activity. These facts give an indication of the possible involvement of heparanase in cell adhesion. Additional evidence of the possible involvement of heparanase in adhesion has been given by other authors. It is know that non-anticoagulant species of heparin and polysulfated polysaccharides reduce the incidence of metastasis. However, it is not clear if the anti-metastatic activity is due to the anti-heparanase activity, in the effect on cell adhesion\textsuperscript{30} not related to heparanase or in the effect of cell adhesion related to heparanase. Miao\textsuperscript{31} reports that laminarin sulfate had no effect on melanoma in relation to endothelial cell adhesion, which is the critical step in the extravasation of metastatic cells from the capillaries.

Previously, Parish\textsuperscript{32} reported that heparanase inhibitors failed to affect adhesion of the cells to the vascular endothelium. However, as it was pointed out in the introduction, it


\textsuperscript{32} Cif. note 5, supra.
seems also that thought heparanase has little activity at physiological pH, it can work as an adhesion molecule at different pH\textsuperscript{33,34}, so the secretion of heparanase seems to be important in the adhesion of the cell. The difference between inhibition of heparanase activity and adhesion is important. It seems that heparanase inhibition is increased in the presence of species containing sulfate groups at both the N and O positions; but O sulfation is critical. That could mean that adhesion processes could be interrupted in the presence of those species, and not only by change in the pH.

In an apparent contradictory report, more recently Goldsmith\textsuperscript{35} has indicated that heparanase mediates cell adhesion independent of its enzymatic activity, if the expression of the enzyme is on the cell surface. Its results show that cell attachment of lymphoma cells with heparanase expressed on the surface was not affected after removing 85% of all ECM sulfated material from endothelial cells. The results of this work suggest that heparanase mediated cell adhesion can happen due to the effect of net cell surface charge, after interaction with HS, but not necessarily binding with HS. The work of Sandback-Pikas\textsuperscript{36} shows that in human platelets O-sulfate groups and not N-sulfated or L-iduronic residues are essential for the recognition by heparanase of the HSP. That presence is particularly required on the hexuronic acids. Specifically, substrate recognition by heparanase is performed when a 2-O-sulfated hexuronic acid is present, that may be either glucuronic or iduronic acid.

Although electrostatic binding does not use (necessarily) the same binding sites when heparanase show no enzymatic activity than when it does, it is obvious that this report is

\begin{footnotesize}
\begin{enumerate}
\item Cif. note 16, supra.
\end{enumerate}
\end{footnotesize}
significant due to the use of KS, that does not have acidic moieties. An eventual decrease of the adhesion of the cell to surfaces functionalized with KS compared to surfaces functionalized with other GAGs may indicate that the cell is effectively using heparanase as a mediator in the adhesion. However, other explanations are possible, as it will be clear in the section of the discussion of the adhesion.

2.5 Ligand-receptor density

Conditions for adhesion and migration of different types of cells have been elucidated in the past. As an example for transient adhesion, there is the so-called multistep paradigm. In this paradigm, there are mainly three steps that regulate the adhesion: first, the cell within the liquid flow approaches the vessel wall and experience rolling and labile adhesion. Eventually, it contacts chemoattractants that induce internal chemical changes in the cell and in the membrane producing adhesion molecules that can increase adhesiveness and provoke the arrest of the cell. In the case of leukocytes, for example, integrins are generated to firmly adhere to the blood vessel.

It is known that the molecular structure and substrate recognition sites of some of the molecules involved in vascular cell adhesion and metastatic processes molecules have been elucidated, as described in previous sections. Cell adhesion molecules have evolved novel binding sites that possess intrinsic association and dissociation constants that permit both high-affinity cell-cell interaction and rapid cell migration.

Because the cell adhesion is mediated by an interaction between the receptor on the cell surface and the ligand on the substrate, the surface density of both will substantially influence the adhesion process. There are several effects that have been observed that


38 Wattenbarger, M.R.; Graves D.J.; Lauffenburger, D.A., Specific adhesion of glycophorin liposomes to a lectin
depend on ligand-density. Cell attachment usually increases with ligand density until a maximum located above a threshold value and further increases in ligand density do not increase adhesion.

Among the parameters required in several adhesion models are surface density of ligands and number of receptors on the cell surface. In dynamic adhesion studies, for instance, these parameters are part of the mathematical models\textsuperscript{39,40} that provide solutions for different states of adhesion. The kinetics –rate constants of association and dissociation– of the recognition sites are then the parameters that determine cell-cell interactions, including cell adhesion. In general, those models are useful for adhesion processes that involve specific interactions between the molecules involved.

Here, heparanase density on the cell surface is unknown (as well as the origin of possible alternative adhesion processes) and the approach has been to develop a technique for the calculation of the surface density of ligands, the GAGs, a parameter useful in the calculations of adhesion numbers used in the verification of the hypothesis that cancer cells adhere to the substrates with a linear dependence of the charge density per dimer.

\textsuperscript{39} Bell, G. Models for the specific adhesion of cells to cells, \textit{Science}, 1978, \textbf{200}, 618-627.

3. Experimental

The equipment used during the experiments is shortly described in Appendix A. In Appendix B, diagrams with detailed steps of the chemical procedures used for surface functionalization and radiolabeling are included. To better address the objectives of the project, an experimental scheme in four parts was followed.

The first step was to address how to deposit the polysaccharides on the surfaces. This part is described in the section of surface functionalization. As it is mentioned latter, there is a vast experimental work that has been done with surfaces patterned with proteins, lipids and aminoacids, either using microcontact printing or by absorption. The method described in this project was needed to advance in systematic studies of the adhesion properties of cells in relation to their glycan structures.

Calculation of the number of molecules exposed on the surfaces was addressed later, and is described in the section of surface density quantitation. The surfaces posed essentially two problems: the first is that the molecules are polysaccharides, a group of molecules that lack a simple reactive group where fluorophores or other molecules can be attached for easy identification. In cases where those molecules are available, it is not always easy to perform quantitative calculations. Finally, the analysis has to be performed in 2D surfaces, not in solution. The method described performs quantitative analysis of GAGs by a radiolabeling technique.

Analysis of the surfaces was performed in the section of surface imaging by AFM and height measurement by ellipsometry. The technique deposits a silane agent in an initial layer on the surface exposing amino groups, to whom the GAGs can be later covalently linked. To have a better understanding of the processes taking place at the interface between the silanized
surface and the biological molecules, changes in the silanization conditions and analysis of heights of the layers were in place in order to achieve better reproducibility and to increase the control in which the molecules are exposed.

The behavior of cells on the surfaces and study of its adhesion was performed in the last section. The techniques for adhesion, static adhesion, have the objective of conceptualize the adhesion process of the cells to the ECM and, instead of working with a surface with all the molecules of the extracellular matrix, only the isolated GAGs are presented for attachment to the cell and its behavior evaluated by counting the number of cells attached after a specific period of time.

3.1 Surface Functionalization

3.1.1 Introduction

The initial experimental work addressed the deposition of covalently patterned glycosaminoglycans on silanized glass surfaces. Patterning of biomolecules onto solid substrates has been extensively used in previous years and is of interest for several biological applications, including controlled adhesion and growth of cells, surface functionalization, biosensors, chromatography and immunoassays41, 42, 43. The depositions were performed both by immersion and by application of a pattern by means of microcontact printing (μCP44, 45). μCP is part of a broader group of technical

methods collectively called soft lithography\textsuperscript{46}. It allows patterning proteins or other molecules on some surfaces making them available for chemical reaction with specific reactants without the need to use photolithography. There are some advantages: \(\mu\)CP has a low capital cost, can be used with several materials and surfaces chemistries and can generate patterns in non-planar surfaces.

Microcontact printing (\(\mu\)CP) has been established as a patterning technique delivering self-assembled monolayers (SAMs) onto substrates in a simple, rapid and reproducible manner. In the past, patterns of proteins\textsuperscript{47}, lipids\textsuperscript{48} and aminoacids\textsuperscript{49} have been created by this method. Along with these works, there are extensive studies on adsorption of proteins at surfaces\textsuperscript{50,51} giving clear indication that to date efforts have been focused primarily on developing patterned protein surfaces.

However, there are no known reports in the literature on reactive microcontact printing of mucopolysaccharides as reported in this project. Given the increasing importance of the study of the biological processes of polysaccharides, the introduction of this surface functionalization technique will be helpful in expanding the field of glycomics.

\textsuperscript{48} Hovis, J.S.; Boxer, S.G. Patterning barriers to lateral diffusion in supported lipid bilayer membranes by blotting and stamping, \textit{Langmuir} 2000, 16, 894-897.
\textsuperscript{50} Hlady, V.; Buijs, J. Protein adsorption on solid surfaces \textit{Curr. Opin. Biotechnol.} 1996, 7, 72-77.
It was considered necessary to ensure that GAGs were attached to the surface of patterned glass cover slips later used in the adhesion. Two possibilities were available: using microcontact printing to fabricate a master stamp and make several copies for different experiments or avoid the stamping process and simply let the reaction go by depositing the reactants on the surface. The first method has better reproducibility and control of the experimental process. The chemistry of glass is essentially the same as the silicon oxide surface, so these methods also serve to modify the surface of glass microscope cover slips, although given the different density of silicon oxide on the outer layer on the surface, surface densities will be different. In this technique, the attachment of GAGs to the glass surface is produced by reductive amination in a reaction mediated by sodium cyanoborohydride (NaBH$_3$CN) in which GAGs are bonded to the surface’s NH$_2$ groups.

The structure of the functionalized surface simulates the extracellular glycocalyx, a periodic bush-like structure located on the surface of endothelial cells that only recently has been recognized to be of great biological importance. The glycocalyx is composed of various glycosaminoglycans in the form of proteoglycans. These extracellular matrix GAGs provide structural links between fibrous and cellular elements, contribute to the viscoelasticity of the glycocalyx, and regulate the permeability of plasma elements within the matrix$^{52}$. As reported by Weinbaum$^{53}$, little was known about the glycocalyx until recently. Squire$^{54}$ and co-workers showed that the glycocalyx in essence consists of a fibrous meshwork with a 20 nm characteristic spacing with brushes of size ~10-12 nm. Models of the glycocalyx structure based solely on lengths of GAGs side chains proposed a matrix of 7 or 8 nm gap spacing, which was associated with the disaccharide repeat of the GAGs chains. In the simplified process of simulating this structure, the

Initially, focus is put on a 2D view that, observed from a zenithal position, provides a meshwork of the protein terminal chain GAGs. Within this simplification, deposition of GAGs monolayers with chains separated by 10 to 20 nm are the target of the presented experimental procedures, namely patterning of the GAGs by microcontact printing.

Although the focus will be primarily on the study of glycosaminoglycans, a parallel work has been carried out with heparan sulfate proteoglycan, known as perlecan, a protein present in the ECM. Perlecan expression is found primarily in mature tissues and is prominent in the endothelial cell basement membrane of all vascularized organs, specifically liver, lung, pancreas and kidney. Perlecan contains three GAG chains, HS and CS chains in different proportions, located at one end of the molecule, as can be seen in Figure 1. A deposition of HSP on glass will give an understanding of the orientation of the GAG chains and will provide some insights into the characteristics of the deposition of glycoproteins vs. GAGs alone. However, it is important to emphasize that HSP can expose for attachment additional amino terminal positions.

The utility of surface attachment of biomolecules for the various applications outlined above requires that the molecules retain their activity. Additionally, the attachment should be stable over long times under various environmental conditions. In this case that means that GAGs will be functional for long periods after the deposition on glass, thus a mean has been developed by which to covalently attach the polysaccharides through their reducing ends, producing an orientation that mimics that of the GAGs bound to the protein backbone of the proteoglycan.

The attachment of GAGs to the glass surface is produced by reductive amination in a reaction mediated by sodium cyanoborohydride (NaBH₃CN) in which GAGs are bonded to the amino terminated monolayer of 3-aminopropyltriethoxysilane (APTES). Two different procedures were used: 1) GAGs were transferred to surfaces by μCP and covalently bound to an amino-terminated monolayer on glass and 2) GAGs were bound to similar surfaces in continuous layers by performing all reactions by complete
immersion, without using the polymeric stamp to transfer the polysaccharides. Immunofluorescence assays were performed to verify the deposition and the quality of the patterns. In addition, AFM imaging of the coated surfaces were performed in order to observe some physical characteristics of the deposited GAGs layers. These results serve as a primary step in a possible long-term goal of characterizing the mechanical properties of GAGs in the glycocalyx and its relation with cellular migration.

Demonstration of successful attachment of GAGs and HSP was done through fluorescence microscopy. A biotinylated primary monoclonal antibody was used with streptavidin tagged with a fluorophore as the secondary marker, allowing for rapid visualization of the quality and extent of the depositions. Further characterization and analysis of these nanoscale-modified surfaces was done by means of AFM: surface images were taken and the thickness of the deposited layers was measured.

3.1.2 Experimental procedures for surface functionalization

Materials. Glass cover slips (Corning 0211) used for deposition were cleaned with a plasma cleaner (Harrick Scientific), rinsed with nanopure water, and dried under a filtered N₂ stream. Heparan Sulfate (Seikagaku America, M.M. 11 kDa), Keratan Sulfate (Seikagaku America, M.M. 13 kDa), Chondroitin Sulfate C (Seikagaku America, M.M. 60 kDa) and Chondroitin Sulfate A (Sigma Aldrich, M.M. 25 kDa) were the polysaccharides used, with Heparan Sulfate Proteoglycan (Sigma Aldrich, M.M. over 400 kDa).

Surface modification. Silanization of the glass surface with 3-aminopropyltriethoxysilane (APTES) was performed as previously described⁵⁵, with minor modifications. Briefly,

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cover slips were rinsed with ethanol and nanopure water, and placed in a plasma cleaner for 45 seconds. Immediately after, the cover slips were incubated in freshly made 0.86 mM solutions of APTES in ethanol for 15 minutes at room temperature. Afterward they were rinsed in ethanol and water (twice). Samples were used immediately. This modification produces an NH₂ terminated monolayer on the surface.

**GAG submersion deposition.** The APTES substrates were incubated for 24 hours at room temperature in solutions of 0.1 μg/ml of HS, KS, CSA and CSC in PBS with NaBH₃CN (Acros Organics) at a concentration of 3 μg/ml. After incubation, samples were rinsed copiously with water, followed by ethanol and water rinses, and dried under a nitrogen stream. In the case of HSP, additional APTES surfaces were modified by first reacting 0.1 μg/ml solutions of the proteoglycan with the cross-linker BS3 (Pierce Biotechnology) for 30 minutes followed by quenching for 15 min. with 1M Tris, following the protocol described by the manufacturer. This reaction links primary amines found on the protein core backbone of the HSP through BS3 to the primary amine bound to the substrate. At this point, all samples were ready for characterization by immunofluorescence or AFM. Figure 2 shows a schematic of the reaction.

**GAG patterning by microcontact printing.** In this experiment GAGs were transferred to the glass surface using a PDMS stamp that had been cast from a calibration grid with a ~1.5 μm spacing between ~1.5 μm wide parallel lines 500 nm in height (pitch ≈ 3 μm). Silicon gratings (TGZ03, MikroMasch) were used to cast the PDMS stamp, as described elsewhere. Briefly, PDMS (Sylgard 184, Dow Corning) was prepared with a 10:1 mass ratio of base to curing agent. The mixture was poured into the glass container in which the silicon grating was placed and allowed to degas overnight in a fume hood. Curing was performed at 65º C for at least 2 hours. The patterning of the GAGs on the glass surface takes place in the following manner. First, an APTES surface is produced as

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described in the surface modification method. Then, and separately, equal volume amounts of the GAG solutions of 0.1 μg/ml and NaBH₃CN are mixed, and 20 μl of these solutions are deposited on the patterned surface of the PDMS stamp for 15 min.

During this time, and to avoid possible evaporation, the stamp was covered with a humidified soft wiper. Later, the solution on the PDMS was removed with a stream of nitrogen and immediately put into conformal contact with the APTES functionalized glass surface, briefly exerting low pressure to help in the contact. The transfer of the patterned biomolecules from the PDMS to the glass took place overnight and afterwards experiments on the detection and analysis of the deposition were performed. When necessary, the patterned cover slips were stored before use at 4°C in 0.2μm filtered nanopure water. Samples that were used for AFM measurements were dried for measurements in air.

**Proteoglycan patterning by microcontact printing.** In the case of HSP the procedure for pattern transfer necessarily was modified. The method of transfer uses a bifunctional N-hydroxysuccinimide ester (BS3, Pierce Biotechnology, Inc.) that is primary amine reactive to perform the cross-linking of the protein. With this reactant, only the amine of the aminoacid lysine will give a stable product after reaction, although nitrogen is present on the side chains of other aminoacids. First, the crosslinker and HSP were reacted on the surface of a patterned hydrophilic PDMS for 30 min, followed by incubation with the quenching agent Tris 1M for 15 min. Excess solution was quickly blown off with N₂ stream and immediately brought into conformal contact for 5 min. with the APTES functionalized glass and then separated. The sample was ready for AFM measurements or immunofluorescence detection. This procedure works because the secondary reaction between APTES and the crosslinker can be performed at any time after reaction with the HSP.

The following procedure was also tried to deposit HSP, but different attempts failed to materialize the pattern on the glass surface. First, crosslinker was reacted on the APTES
functionalized glass for 30 min while at the same time HSP was adsorbed on the surface of PDMS for 1 min. Short times are required for the deposition of a monolayer of product. Crosslinker excess and HSP were both blown off of the surfaces by N₂ stream and immediately brought into conformal contact for 1 min. and then separated. This procedure was tried both with and without the addition of the quenching agent. Initially only one ester group react with the amine on the protein, releasing just one succinimide. There is a slight possibility that two lysine from two different proteins come close together to use both ester groups of the same BS3, but is highly unlikely. The use of PBS is to avoid excessive hydrolysis of the BS3 at basic pHs. The reaction is arrested by adding Tris, which contains more amino groups, thus using almost all BS3 remaining. Other quenching agent that may be used is lysine, which may be added in excess to the reaction.

After blowing the reactants, the HSP-linked with half of the BS3 will react with the primary amine on APTES, and there is no need for additional whole BS3, giving that the ester is already linked to the protein. In addition, there is no need for the presence of the quenching agent, given that the interest is in having all proteins containing half BS3 linked to the APTES. Giving that the BS3 is in huge molar excess, almost all proteins are linked with half BS3, thus excess proteins will produce the pattern on the surface by being able to find the APTES-NH₂.

The above explanation plus the fact that the concentration of cross-linker is kept at very low levels shows very clear why the other two reactions do not work. After blowing the APTES surface the amount of ester available for reaction is, at a maximum, the number of APTES molecules, which is much lower than the number of proteins available. Finally, while in the first reaction the ester is free to move in solution to interact with the lysine, in the second there is a clear steric restrictions for the lysine and ester to find each other. It is possible, however, that at higher concentrations of APTES it would be probable to find at least some pattern using the second reaction. As it can be seen later, this procedure does not produce these high surface densities of APTES.
Figure 2. Simplified scheme of the procedure of GAG surface modification. APTES is first deposited to glass using ethanol as solvent forming an amino terminated layer that is further modified with different GAGs for 24 hours in the presence of cyanoborohydride.
Figure 3. Simplified scheme of the procedure of surface modification for heparan sulfate proteoglycan. First, the crosslinker and HSP were reacted and immediately brought into conformal contact for 5 min. with the APTES functionalized glass and then separated. The procedure yields 2 molecules of N-hydroxysuccinimide (NHS).
Table 4a. List of primary antibodies.

<table>
<thead>
<tr>
<th>Antigen recognized</th>
<th>Label</th>
<th>Dilution</th>
<th>Host</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparan Sulfate</td>
<td>Biotin</td>
<td>1:50</td>
<td>Mouse</td>
<td>USBiological</td>
</tr>
<tr>
<td>Keratan Sulfate</td>
<td>Biotin</td>
<td>1:100</td>
<td>Mouse</td>
<td>Seikagaku America</td>
</tr>
<tr>
<td>Chondroitin Sulfate 6</td>
<td>Biotin</td>
<td>1:100</td>
<td>Mouse</td>
<td>Seikagaku America</td>
</tr>
<tr>
<td>Heparan sulfate proteoglycan</td>
<td>Biotin</td>
<td>1:50</td>
<td>Rat</td>
<td>LabVision</td>
</tr>
</tbody>
</table>

Table 4b. List of streptavidin secondary antibodies

<table>
<thead>
<tr>
<th>Antigen recognized</th>
<th>Fluorophore</th>
<th>Concentration / dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS-Biotin</td>
<td>DTAF</td>
<td>4.5 μg/ml</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>HS-Biotin</td>
<td>Qdot 525</td>
<td>1:50</td>
<td>Qdot Corp.</td>
</tr>
<tr>
<td>CSC-Biotin</td>
<td>Qdot 605</td>
<td>1:50</td>
<td>Qdot Corp.</td>
</tr>
<tr>
<td>CSA-Biotin</td>
<td>Qdot 605</td>
<td>1:50</td>
<td>Qdot Corp.</td>
</tr>
<tr>
<td>HSP-Biotin</td>
<td>Qdot 655</td>
<td>1:50</td>
<td>Qdot Corp.</td>
</tr>
</tbody>
</table>
Antibodies and fluorescence microscopy. Glass cover slips functionalized by microcontact printing with APTES and GAGs or HSP were subjected to indirect immunofluorescence detection. Tables 4a and 4b list the antibodies used. Before the addition of primary antibodies, cover slips were subjected to Streptavidin/Biotin blocking (Streptavidin/Biotin blocking kit, Vector Labs), as follows. First, cover slips were rinsed with PBS and then incubated with streptavidin solution for 15 min, rinsed with PBS and then incubated for 15 min with the biotin solution, as recommended by the manufacturer.

Biotinylated primary antibodies were applied to the surfaces as described in Table 4a for 1 hour. After incubation, samples were rinsed with cold PBS 3x5 minutes. After the deposition of primary antibodies, fluorescence labeling was performed for KS with DTAF streptavidin labeled secondary antibody (Jackson ImmunoResearch) for 30 min; for HS, Qdot 655 Streptavidin Conjugate; for CSC and CSA, Qdot 605 Streptavidin Conjugate and for HSP Qdot 525 Streptavidin Conjugate were used. Qdots were incubated for 1 hour. After rinsing with cold PBS 3x5 min, samples were taken to an inverted epifluorescence microscope (Nikon TE2000) equipped with a QImaging Retiga EX Monochrome 12-bit Digital Camera for fluorescence imaging of the reaction between GAGs and antibodies. Samples with DTAF fluorophore were mounted with Vector Labs Hard Mounting Media H1500, while samples with Qdots were mounted with a solution of 90% glycerol in PBS.

AFM surface measurements. The commercial AFM used for surface measurements and imaging was an Asylum Research MFP 3D. The silicon cantilevers used (NSC36, MikroMasch) had a nominal spring constant of 1.75 N/m and resonant frequency of 155 kHz. During each experiment, the spring constant and resonance frequency were calculated using the built-in-software, according to the thermal response method. Measurements were taken in air. Samples were dried under N₂, and images were recorded

3.2 Surface density quantitation of GAGs

3.2.1 Introduction

In the previous section, a controlled method for the deposition of glycosaminoglycans (GAGs) on glass silanized surfaces was described. In this section a method to evaluate surface densities of these polysaccharides on glass silanized surfaces in a reaction with enamine formation is presented. The method maybe extended to any polysaccharide patterned to silanized surfaces.

Surface coverage is an important parameter in the replication of surfaces with immobilized biopolymers. It is always interesting to know how surface coverage affects the biological activity of the molecules, and the best replica in these depositions would be to obtain surface coverage as close as possible to known values existing in the glycocalyx.

In general, covalent immobilization yields extended and uniform surface coverage of the deposited molecules. During this work it was necessary to find a non-optical method for the quantitation of the surface density of the linked glycosaminoglycans, an important parameter in several instances in surface science. The idea was that a procedure via enamine formation could produce a feasibly technique, that works for all type of glycosaminoglycans, regardless of disaccharide composition. Here a simple procedure for the quantitation of the surface density of polysaccharides by using a radiolabeled aldehyde that reacts with the secondary amine present in the covalent link between the surface and the GAG to form an enamine is shown.
Methods for detection and quantitation of polysaccharides not in surfaces are available. One of the most widely utilized methods for radiolabeling uses NaB\(^3\)H\(_4\) that reacts with the reducing end of the saccharide in equimolar quantities. However, once the GAG is already linked to a surface, the reducing end is not available because it has been used for the deposition during the reaction that links it to the silane, as described previously.

Tritied sodium borohydride could be used after hydroxylolation and deamination of the chains, as is described later, but there is the inconvenient that that will not work for certain type of sugars containing acids as part of their disaccharide structures, for instance heparan sulfate or heparin. Other methods are not useful in surfaces or not easily implemented to work on surfaces. For instance, HPLC by measuring their UV absorbance spectra\(^58\), chromophores linked to their reducing ends\(^59, 60\) or electrophoresis\(^61\).

The main advantage and significance of the method described here is that it only requires inexpensive chemicals and a scintillation counter, and provides an easy method to quantitate surface densities, as described in a recent report on heparinized surfaces\(^62\). In contrast, other methods for molecular surface analysis –i.e. confocal microscopy– require more complex equipment and expertise.

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60 Huang, Y.; Merchref, Y.; Novotny, M., Microscale nonreductive release of O-linked glycans for subsequent analysis through MALDI mass spectrometry and capillary electrophoresis. \textit{Anal Chem.}, 2001, 73, 6063-6069.


3.2.2 Experimental procedures for density quantitation

**Materials.** Glass cover slips (Corning 0211) used for deposition were cleaned with a plasma cleaner (Harrick Scientific), rinsed with nanopure water, and dried under a filtered N\textsubscript{2} stream. Heparan Sulfate (Seikagaku America, M.M. 11 kDa), Keratan Sulfate (Seikagaku America, M.M. 13 kDa), Chondroitin Sulfate C (Seikagaku America, M.M. 60 kDa) and Chondroitin Sulfate A (Sigma Aldrich, M.M. 25 kDa) were the mucopolysaccharides used. $[^{14}\text{C}]$-Formaldehyde with specific activity 52 mCi/mmol was purchased from PerkinElmer. $[^{14}\text{C}]$-Acetaldehyde with specific activity 52 mCi/mmol was purchased from American Radiolabeled Company. Radioactivity was measured in 5 ml scintillation fluid on a Beckman-Coulter scintillation counter.

**Surface modification.** In this technique, the glass surface is first coated with a monolayer of the silane agent APTES. The procedure for the silanization is as described previously in Section 3.1.2. “Experimental procedures for surface functionalization”. Briefly, glass cover slips were rinsed with ethanol and nanopure water, and placed in a plasma cleaner for 45 seconds. Immediately after, the cover slips were incubated in freshly made 0.86 mM solutions of APTES in ethanol for 15’ and rinsed with ethanol and water. This surface modification procedure provides a NH\textsubscript{2} terminated layer, which can be mono or multilayer depending on incubation times and concentrations.

The interest is in obtaining a good and homogeneous surface coverage, producing relatively high concentrations of amino groups on the surface. The APTES substrates were then incubated for 24 hours at room temperature in solutions of 0.1 $\mu$g/ml of HS, KS, CSA and CSC in PBS with NaBH\textsubscript{3}CN (Acros Organics) at a concentration of 3 $\mu$g/ml. After incubation, samples were rinsed with water, followed by ethanol and water, and dried under a nitrogen stream.
[\textsuperscript{14}C]-Formaldehyde radiolabeling of silanized surfaces. In separate previous experiments, the APTES substrates were labeled as previously described\textsuperscript{63}, with modifications. APTES cover slips were covered with 100 μl of a solution of 1 mM NaBH₃CN, 0.2 mM \textsuperscript{14}CH₂O in CH₃CN overnight at room temperature. After incubation, samples were thoroughly rinsed with CH₃CN first and water afterwards and dried under a nitrogen stream.

Deaminative cleavage of N-acetylated glycosaminoglycans.
After the GAGs were attached to the surface, they were N-deacetylated by treatment with hydrazine and then cleaved with HNO₂ at pH 4.0 and pH 1.5\textsuperscript{64,65}. This reaction sequence cleaved the glycosaminoglycans at their N-acetyl-D-glucosamine or N-acetyl-D-galactosamine residues. For N-deacetylation, GAG samples were treated with a solution of excess anhydrous hydrazine and hydrazine sulfate, prepared by dissolving 1 mg of NH₂-NH₂. H₂SO₄ in 1 ml anhydrous NH₂-NH₂, by depositing 200 μl of the mixture on the surfaces. Glass cover slips were placed inside small straight-sided jar glass (Fisher Scientific) and heated at 90ºC in a sand bath for 10 hours.

Samples were cooled, rinsed with water to eliminate residual hydrazine and quickly dried under a nitrogen stream. Deamination was performed with the addition of HNO₂ freshly prepared and kept at 0º. Nitrous acid at pH 1.5 was prepared by extracting the supernatant of the centrifuged mixture of equal amounts of separately kept and ice cooled solutions of H₂SO₄ 0.5M and Ba(NO₂)₂ 0.5M. Nitrous acid at pH 4.0 was prepared by mixing 5 ml of 5.5M NaNO₂ and 2ml of 0.5M H₂SO₄. In a first step, samples for all GAGs were


deaminated by with 200 μl of nitrous acid at pH 4.0. In the case of HS samples, an additional deamination with nitrous acid at pH 1.5 is necessary. Samples were deaminated for 1 hour\textsuperscript{66}. Samples were abundantly rinsed with water to wash off the surface the saccharide chains cleaved. Again, samples were dried under a nitrogen stream.

Denitrosation of polysaccharide surfaces. Nitrous acid also attacks the secondary amine linking the silane and the GAG, forming an N-nitrosamine. Quantitative denitrosation is then performed by depositing on the surface, at room temperature and overnight, a solution containing an excess mixture of NaN\textsubscript{3}, SC(NH\textsubscript{2})\textsubscript{2} and H\textsubscript{2}SO\textsubscript{4} prepared by dissolving 7.15 mg NaN\textsubscript{3}, 85.3 mg SC(NH\textsubscript{2})\textsubscript{2} and 100 μl H\textsubscript{2}SO\textsubscript{4} in 10 ml acetic acid\textsuperscript{67}.

\([^{14}\text{C}]\)-Acetaldehyde radiolabeling of polysaccharide surfaces in the presence of cyanoborohydride. The remaining mono or disaccharide substrates were radiolabeled as follows. Previously denitrosated cover slips were covered with 200 μl of a solution containing 1mM NaBH\textsubscript{3}CN and 0.2 mM \(^{14}\text{CHO}^{14}\text{CH}_3\) in CH\textsubscript{3}CN overnight at room temperature. In those conditions the concentrations of acetaldehyde and GAG were in molar ratios of 100:1. After incubation, samples were thoroughly rinsed with CH\textsubscript{3}CN first and water afterwards and dried under a nitrogen stream. During reaction, samples were kept in a dissecator containing P\textsubscript{2}O\textsubscript{5} to avoid moisture and hydrolysis of the formed enamine.

\([^{14}\text{C}]\)-Acetaldehyde radiolabeling of control APTES surfaces. Experiments were repeated using control APTES surfaces only, with no glycosaminoglycans. One set of samples were subjected to hydrazinolysis, deamination, denitrosation and radiolabeling, while

\textsuperscript{66} Shively, J.E.; Conrad, H.E. Stoichiometry of the nitrous acid deaminative cleavage of model amino sugar glycosides and glycosaminoglycuronans *Biochemistry* 1970, 9, 33-43.

other set was only subjected to radiolabeling. As additional background control, clean glass surfaces were also reacted with $[^{14}\text{C}]-\text{Acetaldehyde}$.

3.3 Surface characterization by ellipsometry and AFM imaging

3.3.1 Introduction

In previous sections the techniques used for the deposition of GAGs on glass silanized surfaces have been described and a method for quantitation of the density of the deposited molecules. In this section the modified surfaces are studied by performing null ellipsometry measurements and AFM imaging. All measurements were performed in air.

Patterning of biomolecules onto solid substrates is of interest for several biological applications. Given the possibility of commercial applications in which mucopolysaccharides can be used as detection agents i.e. in kits for immunoassays and to establish a standard procedure for the deposition of GAGs to simplify further analysis, it was considered necessary to carry out an additional study to determine the effects on the coatings of concentration and incubation time of the silane coupling agent used (APTES) that could be helpful for the ulterior characterization of the end-grafted polymer GAGs chains using different polymer models and comparison with our experimental results.

Ellipsometry and AFM were used for this characterization. Ellipsometry measurements were used to confirm values obtained by AFM for the incompressible layer thickness of some of the coatings. Studies of the coated surfaces provided information of the thickness of the deposited GAGs layers. Changes and improvements in the deposition technique are described, as well as studies analyzing the effect of variation in the concentration of APTES, the silane agent used for the functionalization.

The purpose behind the analysis is as follows. Surfaces prepared by covalent immobilization have an architecture in which functional interfaces of interest in biology
are easily replicated and maintained. However, it is necessary to have good knowledge of the processes taking place at the interface between the silanized surface and the biological molecules in order to achieve better reproducibility and to increase the control in which the final molecule is presented and exposed. Unlike proteins, polysaccharides have the advantage that they do not denaturate, thus keeping their biological activity, and the covalent attachment impedes molecules washing off the surface as it happens when they are not irreversibly adsorbed. This type of immobilization usually yields extended and uniform surface coverage of the molecules and our objective is to study the reliability and repeatability of the silanization, and the best conditions for the attachment of GAGs.

Following the description of Squire\textsuperscript{68}, the glycocalyx is composed of brushes of 10-12 nm width, which correspond to the diameter of the fiber of the core proteins. These brushes are spaced at 20 nm from each other. GAG chains seem to be strongly stretched due to possible electrostatic interactions with solvent molecules and repulsive interactions between dimers in adjacent chains. This picture agrees with studies of nanomechanics of human cartilage\textsuperscript{69} indicating dominance of electrostatic repulsive interactions in GAG-GAG chains.

The structure of this section is as follows: initially, best conditions for surface modification are shown in order to obtain reproducible coatings of a monolayer of APTES and this is checked by ellipsometry measurements to get information on the height of the brushes of GAGs in dry state and with AFM measurements for one of the glycosaminoglycans (CSA).

\textsuperscript{68} Cif. note 52 supra.

\textsuperscript{69} Dean, D.; Seog, J.; Ortiz, C; Grodzinsky, A.J.; Molecular-level theoretical model for electrostatic interactions within polyelectrolyte brushes: applications to charged glycosaminoglycans, \textit{Langmuir} 2003, 19, 5526-5539.
3.3.2 Experimental procedures for surface characterization by ellipsometry and AFM imaging

**Materials.** Glass cover slips (Corning 0211) used for deposition were cleaned with a plasma cleaner (Harrick Scientific), rinsed with nanopure water, and dried under a filtered N\textsubscript{2} stream. Heparan Sulfate (Seikagaku America, M.M. 11 kDa), Keratan Sulfate (Seikagaku America, M.M. 13 kDa), Chondroitin Sulfate C (Seikagaku America, M.M. 60 kDa) and Chondroitin Sulfate A (Sigma Aldrich, M.M. 25 kDa) were the mucopolysaccharides used, along with the proteoglycan Heparan Sulfate Proteoglycan (Sigma Aldrich, M.M. over 400 kDa). Silicon wafers used in ellipsometry measurements were also cleaned in hot piranha solutions.

**Procedure for surface modification.** Silanization with APTES of the glass surface was performed as previously described in section 3.1, with some modifications. Briefly, cover slips were rinsed with ethanol and nanopure water, and placed in a plasma cleaner for 45 seconds. Immediately after, the cover slips were incubated in freshly made 0.43 mM, 0.86 mM, 2.10 mM, 4.20 mM and 21.0 mM solutions of APTES in ethanol for different incubation times at room temperature. Afterward they were rinsed in ethanol and water. Samples were used immediately or stored in pure ethanol no longer than two hours before ellipsometric measurements. This surface modification procedure provides a NH\textsubscript{2} terminated layer, which can be mono or multilayer depending on incubation times and concentrations. Silanization of silicon wafers was performed in the same manner, but they were cleaned by immersion in hot piranha solution for 30 minutes, followed by water rinses before being placed in the plasma cleaner.

**GAGs direct deposition.** The APTES substrates were incubated for 24 hours at room temperature in solutions of 0.1 μg/ml of HS, KS, CSA and CSC in PBS with NaBH\textsubscript{3}CN (Acros Organics) at a concentration of 3 μg/ml. After incubation, samples were rinsed copiously with water, followed by ethanol and water rinses, and dried under a nitrogen
stream. At this point, all samples were ready for characterization by ellipsometry or AFM. When necessary, cover slips were stored before use at 4°C. Samples taken to the AFM for measurements were dried for measurements in air.

**Ellipsometry measurements.** For ellipsometric measurements, APTES and GAGs were deposited, using the same procedure, on silicon wafers. The thickness of the layers was calculated with a null spectroscopic ellipsometer, Rudolph Research III EL, equipped with a He-Ne laser of $\lambda = 632.8$ nm. The angle of incidence was set at 70° and the polarizer angle was 45°. Measurements were taken for samples in air in five spots for six separate samples. A thermal oxide layer of ~26 Å covered the native silicon surface.

For the ellipsometric measurements of GAGs layers, the substrate (SiO$_2$/Si) was assumed to be a single layer having a set of optical constants that is a combination of the contributions from both silicon and the surface oxide$^{70}$. Indexes of refraction used were taken from the literature or from the manufacturer of the products. Whenever possible, those values were checked with a refractometer (Abbe-3L Refractometer, Milton Roy, Rochester). Possible decreases in the value of the refractive index were also considered, that could happen as a result of disordered layers of product. The accuracy of the measurements was +/- 0.1 nm.

**AFM imaging.** The commercial AFM used for surface measurements and imaging was Asylum Research MFP 3D. The silicon cantilevers used (NSC12/50, MikroMasch) had a nominal spring constant of 0.20 N/m and resonant frequency of 20 kHz. During each experiment, the real spring constant and resonant frequency were calculated using the built-in-software, according to the thermal response method. For measurements in air, samples were dried under N$_2$ and images recorded. All experiments were performed with

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bare cantilever tips at room temperature. Experiments were performed in triplicate and results are given with the corresponding standard errors of means.

3.4 Static adhesion of cancer cells to the functionalized surfaces

3.4.1 Introduction

The final part of the experiments consisted of the study of the static adhesion of three cancer cell lines to surfaces functionalized with GAGs. As a secondary study, the possible involvement of heparanase in this process was analyzed. Four different glycosaminoglycans (heparan sulfate, chondroitin sulfate A, chondroitin sulfate C and keratan sulfate) were deposited on glass surfaces that had been previously coated with 3-aminopropyltriethoxy-silane (APTES), and those substrates were used in static adhesion experiments. BT20, a moderately metastatic breast cancer cell line, MCF7 a non-metastatic breast cancer cell line, and A431, a highly metastatic epidermoid skin carcinoma cell line, were selected because of their different metastatic activity.

Static cell adhesion is generally associated with integrins on the cell surface and the process may include several other type of molecules, for instance hyaluronan, a member of the glycosaminoglycan family. For cells in the vasculature, for example, the adhesion is considered to be a multi-step process, that includes a first rapid contact—of low affinity binding that does not involve integrins—followed by a persistent binding—of high affinity binding—that is mediated by integrins and that initiates the signaling cascade. During this work the static adhesion of selected cancer cells with glycosaminoglycans was investigated with attention to the role of heparanase in this interaction.

The experimental approach has been as follows. GAGs were covalently bonded to

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previously silanized glass cover slips, using the technique described in previous sections. The molecular structure of the functionalized surface can be understood as a reduced section of the outer areas of the extracellular glycocalyx or the ECM containing proteoglycans.

Adhesion experiments were performed in three different conditions: untreated cells; cells suspended in media with heparin and cells transfected with heparanase cDNA to induce overexpression of endogenous levels of the enzyme. The first was devised to analyze how untreated cells react to the surfaces, mainly by comparing their response with respect to each. The last two conditions were used to determine the effect of heparin on the adhesion and the possible involvement of heparanase. Previous works involving heparanase have been performed usually with transfected cells, generally with overexpression of the enzyme. Recently, a model for secreted heparanase uptake on the cell surface by binding with heparan sulfate chains was proposed\textsuperscript{72}. A strategy was devised to demonstrate if cells showing different levels of heparanase affected the number of cells adhering to glycosaminoglycan substrates.

Use of heparin in the experiments is based on the following reasons. Heparin is a well-known inhibitor of the enzymatic activity of heparanase and its inclusion was justified as a control to analyze if inactivation or reduction of the enzymatic activity did not substantially affect adhesion, in case heparanase played a role in the binding process. It is known that non-anticoagulant species of heparin and polysulfated polysaccharides reduce the incidence of metastasis, up to 90\% in some cases\textsuperscript{73}. However, it is not clear if the anti-metastatic activity is due to the anti-heparanase enzymatic activity or to another effect on cell adhesion\textsuperscript{74}. Miao\textsuperscript{75} reports that laminarin sulfate had no effect on melanoma


\textsuperscript{73} \textit{Cif.} note 13 \textit{supra}.

\textsuperscript{74} \textit{Cif.} note 30 \textit{supra}.
in relation to endothelial cell adhesion, which is the critical step in the extravasation of metastatic cells from the capillaries.

In another report, Parish\textsuperscript{76} shows that heparanase inhibitors failed to affect adhesion of the cells to the vascular endothelium. It seems that heparanase inhibition is increased in the presence of species containing sulfate groups at both the N and O positions and that O sulfation is critical for this process. For this reason, experiments were conducted at physiological pH, were the enzymatic activity of the molecule is suspended, but adhesive functions are present and, as indicated by Ihrcke\textsuperscript{77}, in physiological conditions heparanase inactivation does not affect its binding to heparin.

Although other inhibitors of heparanase have been used, for instance heparan sulfate solutions, the attention was on heparin because it has been introduced as a therapeutic agent and much interest has been generated with the use of heparin as possible therapeutic antimetastatic agent. Heparin is proposed to interfere with the selectin-mediated interaction of the cancer cells and their stroma\textsuperscript{78}. This role has been put to work reaching even clinical trials with the use of low-weight heparins\textsuperscript{79}. Then, the experiments with heparin here will have a dual purpose: observation of a change in the adhesion may be due to blocking of cell surface receptors (including heparanase) and/or binding to GAGs on the substrates.

\textbf{3.4.2 Experimental procedures for static adhesion of cancer cells to the functionalized surfaces}

\textsuperscript{75} Cif. note 32 supra.

\textsuperscript{76} Cif. note 5 supra.

\textsuperscript{77} Cif. note 35 supra.


**Tumor cell lines.** Human BT20 breast tumor cells and A431 human epidermoid carcinoma cells were purchased from ATCC, while MCF7 breast tumor cells were kindly provided by Dr. William Dalton, Moffit Cancer Center. All cell lines are adherent and were maintained in tissue culture using the specifications recommended by ATCC. MEM medium supplemented with 10% FBS was used for BT20 cells; DMEM supplemented with 10% FBS for A431 cells and RPMI 1640 supplemented with 5% FBS for MCF7 cells. Cells were cultured at 37°C in 5% CO₂ atmosphere and harvested using standard trypsinization procedures and passaged near confluency.

**Reagents and materials.** Glass cover slips (Corning 0211) used for deposition were cleaned with a plasma cleaner (Harrick Scientific) or with piranha solution (H₂SO₄:H₂O₂, 70:30 v/v), rinsed with nanopure water, and dried under a filtered N₂ stream. Heparan sulfate, chondroitin sulfate C and keratan sulfate were from Seikagaiku America. Chondroitin sulfate A and Heparin from Sigma Aldrich. Common reagents were from Fisher Scientific.

**Glass surface modification.** The procedure for the silanization was described previously in Section 3.1.2. “Experimental procedures for surface functionalization”. Briefly, cover slips were rinsed with ethanol and nanopure water, and placed in a plasma cleaner for 45 seconds or immersed in hot piranha solution for 20 minutes. After rinsing with water, the cover slips were incubated in freshly made 0.86 mM solutions of APTES in ethanol for 15 minutes at room temperature. Afterward they were rinsed in ethanol and water. This modification produces an NH₂ terminated submonolayer or monolayer on the glass surface. Samples were used immediately for adhesion experiments with cells or for the deposition of GAGs, as described below.

**GAG submersion deposition.** This method has been described in Section 3.1.2. “Experimental procedures for surface functionalization”. The APTES substrates were incubated for 24 hours at room temperature in solutions of 0.1 μg/ml of HS, KS, CSA.
and CSC in PBS with NaBH₃CN (Acros Organics) at a concentration of 3 μg/ml. After incubation, samples were rinsed with water and dried under a nitrogen stream. At this point, all samples were ready for adhesion experiments.

**Heparanase staining.** Cell surface expression of heparanase in transfected cells was determined by indirect immunocytochemistry as described⁸⁰ with modifications. Cells were first fixed with 3.7% paraformaldehyde for 30’ and later incubated with BSA 2% as blocking agent for another 30’. Cells were then incubated overnight at 4ºC with goat, anti-human Heparanase 1 HPA1 (C20), polyclonal antibody (Santa Cruz Biotechnology, California) diluted 1:50 in PBS and then rinsed with PBS, followed by incubation (1:500 dilution) for 45 minutes at room temperature with horseradish peroxidase-conjugated secondary antigoat antibody  (Jackson Immunoresearch). Color was developed using EAC substrate (Lab Vision Corp. California), followed by counter-staining with Mayer’s hematoxylin. Cells were visualized using bright field light microscopy with a Leica DMLB microscope and photographed with a Diagnostic Instruments RT color camera. Controls for negative expression were taken without addition of primary antibody.

**Heparanase activity.** Activity of heparanase was quantified using a commercial presentation of the assay that monitors the degradation of heparan sulfate (Heparan Sulfate Enzyme Assay Kit, Takara Mirus Bio, Winsconsin). The method is based on the fact that heparan sulfate losses its binding activity to basic fibroblast growth factor after degradation by heparanase. The procedure followed the protocol described by the company.

cDNA Transfection. The cDNA expression construct⁸¹ used in these experiments was kindly provided by Professors Robert Parish and Mark D. Hulett, Australian National University, Canberra. Human heparanase was subcloned into the EcoRI site of the vector

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⁸⁰ *Cif.* note 7 supra.

⁸¹ *Cif.* Note 27 supra.
pCDNA3. After plasmid recovery\textsuperscript{82}, competent bacteria cells were transformed and DNA obtained for transient transfection of the cancer cell lines. Optimization of cDNA transfection conditions was performed by incubating all cell lines with 1.5 μg of DNA per million cells in 10 cm dishes for 24, 36 and 48 hours to produce Western Blots. Maximum efficiency of transfection was assigned to cells transfected for 24 hours for A431 and BT20 cells, and for 36 hours for MCF7 cells. In all cases, appropriate amounts of FuGene transfection reagent (Roche Diagnostics, Indiana) with ratio 1 μg DNA:4 μl reagent were used. After selection of appropriate time, and for adhesion experiments, the corresponding amounts of DNA were used for transfection of culture flasks where cells were incubated for 24 or 36 hours at 37 °C and transfected with the appropriate amount of FuGene reagent in serum free media, as per manufacturer instructions. All adhesion experiments of cDNA heparanase transfected cells were performed using transiently transfected cells with the times previously indicated.

**BFA incubation.** Lysates for Western Blots were obtained for all cell lines with cells treated with 10 μg/ml of Brefeldin A (Fluka) for 3 hours in 10 cm dishes with near confluent cells.

**Western Blots.** Heparanase presence in tumor cells was also detected using Western Blots. For immunoblotting, cells were incubated in the following conditions: control (no transfection); control cells (no transfection) incubated for 3 hours previous lysis with Brefeldin A; and cDNA transfected cells incubated for the specified times. After incubation the medium was collected and cells were washed with PBS twice. Lysates were prepared using RIPA buffer (solution containing 50mM Tris pH 8, 200 mM Nacl, 5mM EDTA, 1% Igepal, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitors (Roche Diagnostics). Lysates were then subjected to SDS-PAGE (4-12% Tris Acetate Gel membranes NuPAGE, Invitrogen) and transferred to nitrocellulose membranes. Proteins were probed against goat, anti-human Heparanase 1 HPA1 (C20),

\textsuperscript{82} Rosman, G.J.; Miller, A.D. Improved method for plasmid shipment. *Biotechniques*. 1990, 8, 509.
polyclonal antibody (Santa Cruz Biotechnology, California) at 1:100 dilution for 1 hour at room temperature, followed by incubation at 1:10000 dilution for 45 minutes at room temperature with horseradish peroxidase-conjugated secondary antigoat antibody (Jackson Immunoresearch).

**Static cell adhesion assays.** Adhesion assays were performed using conflually grown tumor cells, in three different groups. In one set, heparanase cDNA transfected cells were used. In another set, cells non-transfected were used. In a third set, non-transfected cells were resuspended in heparin containing media, as described below.

Cells were detached from culture flasks with trypsin and resuspended in the corresponding complete media (for cDNA transfected or non transfected) or in a solution of complete media containing 10 μg/ml of heparin for expensive assays with heparin. Trypsin inactivates the enzimatic activity of heparanase, \( t_{1/2} = 5h \) but that was not relevant in our assays. The solutions were always kept warm at 37\(^{\circ}\)C. Cells resuspended in heparin containing solution were incubated for 30’ in an incubator previous to the assay. Cells then were transferred to either clean cover slips, APTES only coated cover slips or GAGs coated cover slips placed on the bottom of Petri dishes. The cover slips were previously coated with APTES or GAGs as described and were kept warm at 37\(^{\circ}\)C prior use. Deposited cells were allowed to incubate at 37\(^{\circ}\)C for 2 hours for A431 and BT20 cells and 8 hours for MCF7 cells. Seeding time was determined by comparing the number of cells adhered at 30’, 1h, 2h and 4h (for A431 and BT20) and between 4, 6, 8 and 10 hours (for MCF7), and selecting between the times that showed stable trend of adhesion, determined by 15% or less difference in the number of cells adhered. For BT20 and A431, seeding time was decided to be 2 hours, while for MCF7 was 8 hours. These are the times used in the adhesion experiments.

The number of cells originally deposited on each cover slip was 2.5x10\(^5\). Cells were carefully washed with PBS kept at room temperature to remove non-adherent cells. The remaining cells were detached using 250 μl of a solution of PBS-EDTA and then
counted. For each experimental group, the results are expressed as the mean percentage (+/- SD) of bound tumor cells in 9 cover slips. Given that the whole surface of the cover slips had been previously coated with APTES the values shown were compared relative to the values found for APTES surfaces. An additional control was performed using clean glass cover slips.
4. Results and discussion

In this section a detailed discussion of the results obtained in the experiments is presented.

4.1 Comments on the functionalization of the surfaces

Surface modification and GAGs deposition. Silanization in ethanol was chosen because the same procedure in aqueous media yields a low surface concentration of amines. This deposition of APTES produces a monolayer or submonolayer of the product on the surface. The thickness of the layers of silane depositions and the number of reactive -NH$_2$ groups present on the surface have been quantified previously. To avoid APTES polymerization, the concentration of APTES was kept at the low level used. As can be seen in the AFM images in Figure 5, APTES aggregates are not observed over the glass surface. APTES multilayer formation could result in undesirable aggregates, giving unreliable results in force measurements. Work with increasing concentrations of APTES will show that it is not convenient to work with APTES multilayers to perform GAG depositions.

µCP. The use of 1.5 µm gratings is justified because resolving features at this length scale is more than sufficient for most applications in cell biology and biosensing. PDMS stamps were plasma cleaned before deposition of biomolecule solutions to make them hydrophilic and to increase wettability. This is an important step in cases where aqueous solutions of biomolecules are used and that provides a more efficient transfer of the

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83 Cif. note 63 supra.
84 Cif. note 55 supra.
GAGs to the functionalized glass surface. The polymeric stamp makes good conformal contact with the APTES-glass substrate, resulting in the relief pattern of its surface being translated to the glass as a pattern of GAG molecules. In all cases, GAGs have been covalently bonded providing long-term fixation to the surface. Evidence of covalent binding is indirect, deriving from control experiments in which the stamping process was carried out in the absence of either the APTES monolayer or the catalyst cyanoborohydride.

The covalent attachment compares favorably with the process of physical adsorption – physisorption- in that many biomolecules, such as peptides or oligonucleotides, do not retain activity after the surface adsorption process. However, it must be noted that the potential effects of adsorption of GAGs to PDMS have not been tested. An additional advantage of the covalent bonding is that in most instances the performance of patterning in fixation of the molecules is higher than with coating and similar or better than immersion. It is important to note that the pattern generated remains even after 1) sonication in saturated salt solution for 24 hours and 2) after contact mode and lateral force microscopy is performed over several hours of work, abrading the patterned surface with the cantilever tip.

**Immunofluorescence.** Figure 4 shows fluorescence images that reveal the pattern of the GAGs by DTAF (for KS) or Quantum Dots (in all other cases). In all cases, the pattern formed shows high contrast and resolution. The ~1.5 μm wide lines were transferred over distances of many tens of hundreds of microns. No diffusion of the molecules into the open spaces of the pattern is observed, and the edges of the pattern are very well defined at this scale. The use of biotinylated mAb and the use of DTAF labeled streptavidin provided an efficient method for immunofluorescence detection of the pattern, based on the strong affinity of the biotin-streptavidin binding. Streptavidin conjugated Quantum Dots provided also a variation of the method that allowed photographic imaging of the detection at different wavelengths. In one of the cases (CSA, Fig 4.E) it is possible to observe a small increase in the thickness of the patterned lines.
This is due to the process of exerting low pressure between the glass and the patterned PDMS stamp when in conformal contact. Further experimentation using defined forces will give a standard procedure to overcome this difficulty. Both the PDMS stamps and silicon gratings can be reused. To avoid dropouts in the pattern, PDMS stamps were cleaned in a sonicator for 30 min in ethanol, while the silicon gratings were also sonicated in pure acetone for 30 min. after their use. However, defects can be found in the pattern, possibly indicating incomplete cleaning of the stamp prior to use.

As mentioned earlier, control experiments were carried out to eliminate the possibility of GAGs being adsorbed on the glass surface, instead of covalently linked. Immunofluorescence control experiments were performed with the following sample types: clean glass with no deposition of GAGs; glass functionalized with APTES with no deposition of GAGs and APTES functionalized glass with a deposition of GAGs without the reactive NaBH₃CN. All cases tested negative to the presence of patterned GAGs by immunofluorescence detection and by surface analysis by AFM.
Figure 4. GAGs fluorescence micrographs. In all cases, products were deposited on a monolayer of APTES functionalized glass cover slip and reacted with specific biotinylated monoclonal primary antibodies (See text). A) 40X epifluorescence microscopy image of KS with DTAF labeled streptavidin. DTAF is a fluorescein derivative with the same emission and excitation wavelengths. B) 40X epifluorescence microscopy image of HS with Quantum Dot 655 labeled streptavidin. C) 40X epifluorescence microscopy image of CSC with Quantum Dot 605 labeled streptavidin. D) 63X epifluorescence microscopy image of HSP with Quantum Dot 525 labeled streptavidin. E) 63X epifluorescence microscopy image of CSA with Quantum Dot 605 labeled streptavidin. The pitch of the lines on all patterns is \( \approx 3 \mu m \).
Figure 5. AFM surface images of CSA. Selected tapping-mode topographic AFM images in air of deposited layers of CSA on APTES functionalized surfaces, with cantilevers described in the text. A) 20 x 20 μm scan and 6 nm z-scale, scan rate 1 kHz and B) 10 x 10 μm scan. The free amplitude of the cantilever was chosen to be about 100 nm. Two samples were analyzed and for each sample, measurements were taken at two different positions.
**AFM imaging.** AFM images of cover slip surfaces of one the GAGs, CSA, were collected, and the patterned structures are shown in Figure 5. Table 5 shows a theoretical calculation of the contour lengths of the GAGs, where estimated values of the molecular mass of the products indicated by the manufacturers were used. For the number of sulfates per disaccharide unit, Lindahl\(^85\) was used as a primary source to calculate molecular mass of dimers. The estimation assumes a mean disaccharide monomer length ranging from 1 nm, using values from Squire\(^86\), to 1.28 nm\(^87\), reported in the CS-GAGs present in aggrecan in the cartilage, although calculations made using data reported by Arnott and Scott\(^88\) and from Rees\(^89\) give values between 0.92 and 1.16 nm, more in line with the 1 nm estimate.

Analysis of cross-sections for CSA, as shown in Fig.4, gave a mean deposited height of 2.1 +/- 0.6nm. For measurements in air, this height can be considered the thickness in the dry state for CSA. Given our contour lengths and GAGs chain molecular weights, our results are within the range of values reported by Seog and co-workers who used CS chains with contour lengths of 35 nm and indicated an estimated value of 1.5 nm for the incompressible layer thickness of the GAG in air using AFM isoforce imaging, while reporting a value of 3.18 nm by ellipsometry.

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85 Cif. note 1, supra
86 Cif. note 52, supra
Table 5. GAG size and length characteristic data. \( L_c \) is the total (contour) length in nanometers.

<table>
<thead>
<tr>
<th></th>
<th>M.M. (kDa)</th>
<th>Estimated dimer M.M. (Da)</th>
<th># Disaccharides per chain</th>
<th>( L_c ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS</td>
<td>13</td>
<td>403</td>
<td>32</td>
<td>32 to 42</td>
</tr>
<tr>
<td>CSC</td>
<td>60</td>
<td>456</td>
<td>131</td>
<td>131 to 171</td>
</tr>
<tr>
<td>CSA</td>
<td>25</td>
<td>456</td>
<td>54</td>
<td>54 to 70</td>
</tr>
<tr>
<td>HS</td>
<td>11</td>
<td>496</td>
<td>22</td>
<td>22 to 29</td>
</tr>
</tbody>
</table>
4.2 Results of the surface density quantitation of GAGs

The general schematic of the method developed to evaluate the surface density of GAGs is shown below in Fig. 6 containing a total of six steps that are summarized as follows:

1. After cleaning, the surface is silanized using APTES at concentrations of 0.86 mM for 15’.
2. Immediately after, GAGs are deposited by reductive amination with cyanoborohydride for 24 h.
3. GAGs chains contain acetamido groups that are eliminated by hydrazinolysis at 90ºC for 10 h.
4. Deaminative cleavage of the GAG chains is performed with nitrous acid for 1 h. For HS, an additional step is necessary at lower pH for full chain cleavage.
5. At this point there is only one remaining disaccharide whose bond with the silane has to be denitrosated overnight.
6. Radiolabeling is performed using an aldehyde overnight in a reaction with enamine formation.

First part: [14C]-Formaldehyde radiolabeling of silanized surfaces.
This procedure is well known and based on the reaction of primary amines with ketones or aldehydes to produce imines or secondary amines to produce enamines. In the first case, the Schiff base (imine) is then converted to a secondary amine with cyanoborohydride, a very mild reductor that readily reduces the Schiff base but not the aldehyde or ketones. The reaction takes place at neutral pH and is shown in Figure 7. The use of sodium borohydrade has essentially two disadvantages: the reaction depends on pH and its strong reductive action reduces formaldehyde to methanol.

Under ideal conditions, and assuming that all sites available on the glass surface react with the silicon to form a perfect monolayer, with a distance for the Si-O-Si bond of 5.0 Å with a linear chain of propilamine, the maximum density of available amine terminals
for reaction with GAGs will be around 2 sites/nm², which in this ideal situation seems high compared with the density in the glycocalyx. As it is shown later in the section of ellipsometry thickness, the experimental limitations permit a surface density of ~ 0.08 to 0.16 sites/nm² for APTES. With the technical method described, this would be a good approximation to produce an APTES substrate as base for the replication of an actual glycocalyx meshwork. This good replica could then be used for studies of the mechanical properties of the molecules in simulated physiological conditions.

0.86 mM solutions of APTES had been used during the work with GAGs to prepare the surfaces. Here, radiolabeling was performed on surfaces at four different concentrations, as shown in Table 6a. The experimental methodology followed the description of Xiao, but keeping the ratio of sodium cyanoborohydride / formaldehyde as low as possible for maximum efficiency, as described by Jentoff. The objective is to have 1 molecule incorporated for every 12 added.

For calculations, the initial assumption was made that the surface was covered with 2 sites/nm² in a cover slip with an area of 5.06 x 10⁻⁴ m², that is equivalent to 1.677 nanomol of APTES, and that reaction GAG:APTES was 1:1. The reaction mediated by cyanoborohydride is slow and has to be done overnight allowing for slow evaporation of the solvent from the surface at room temperature. The ratio of cyanoborohydride / formaldehyde was 5 to 1.
1) Surface Cleaning (piranha or plasma)
2) Silanization (15’ with AP1ES)
3) Glycosaminoglycan deposition (with NaBH3CN for 24h)
4) Hydrazinolysis at 90°C for 10 h
5) Deamination with nitrous acid
6) Denitrosation
7) Radiolabeling

Figure 6. Simplified scheme of the procedures for quantitation of glass grafted glycosaminoglycans.
Figure 7. Scheme of the procedure for radiolabeling the silane surface with $[^{14}\text{C}]$-Formaldehyde. APTES surfaces prepared with different concentrations or times (as shown in Table 6a and 6b) were used in the experiment.
Table 6a. Surface coverage by $^{14}$C-Formaldehyde radiolabeling of APTES (concentration). Time for deposition was 15' in all cases.

<table>
<thead>
<tr>
<th>APTES concentration</th>
<th>0.43mM</th>
<th>0.86 mM</th>
<th>2.10 mM</th>
<th>4.20 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface density (sites/ nm$^2$)</td>
<td>0.10 +/- 0.03</td>
<td>0.14 +/- 0.01</td>
<td>0.16 +/- 0.02</td>
<td>0.14 +/- 0.02</td>
</tr>
</tbody>
</table>

Table 6b. Surface coverage by $^{14}$C-Formaldehyde radiolabeling of APTES (time). APTES concentration for deposition was 0.43 mM in all cases. Mean value of two samples.

<table>
<thead>
<tr>
<th>Time</th>
<th>15'</th>
<th>1 hour</th>
<th>4 hours</th>
<th>12 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface density (sites/ nm$^2$)</td>
<td>0.09</td>
<td>0.10</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>
The thickness of the layers of silanes depositions and the number of reactive NH$_2$ groups present on the surface have been quantified previously by Xiao, with a value of 0.5 nm and a surface coverage of 0.22 nMol NH$_2$ groups per cm$^2$ for a submonolayer of product (approximately 1.3 amino terminals per nm$^2$) using a concentration of 2.15 mMol of APTES and incubation time of 3 hours in dry toluene with reflux. Then the method presented here yields surface densities ten times lower, essentially because refluxing dry toluene was not used.

An additional analysis was performed varying the time of APTES deposition before radiolabeling. It is known that increasing deposition times increases the amount of APTES on the surface, generally by forming successive layers over time. This fact is demonstrated in Table 6b where increased times, up to 12 hours, of incubation do not produce significant variation on surface densities. That means that the structure of the amino layer presented by the surface does not have a significant change, at least respect to the ability of small molecules like formaldehyde to find reactive sites. However, experiments with extended incubation times with higher concentrations were not performed, so it is not possible to assess the stability and homogeneity of the surface and their exposed amino groups in those cases.

Second part: $[^{14}\text{C}]$-Acetaldehyde radiolabeling of polysaccharide surfaces.
As can be observed in Figure 7, in the first part of the surface modification GAGs are deposited by the well-known method of reductive methylation$^{90}$, using cyanoborohydride, of the Schiff base produced in the reaction between the primary amine and the aldehyde. The secondary amines present in the GAGs may also react to form an enamine, thereby masking the true surface density. The process of deamination$^{91}$ of N-deacetylated GAGs is necessary given that several acetamido groups are present in the GAG chains.


$^{91}$ Cif. note 66 supra.
As mentioned, N-deaminative cleavage of deacetylated polysaccharides cleaves the glycosaminoglycans at their N-acetyl-D-glucosamine or N-acetyl-D-galactosamine residues leaving only a disaccharide or a monosaccharide attached to the surface\textsuperscript{92}. Exploiting the fact that the secondary amine can react with a ketone or aldehyde, a final reaction produces a \textsuperscript{14}C labeled enamine, using the same method described in the radiolabeling of APTES. The reaction conditions used for the deacetylation and deamination are the ones standard in the literature, with the only change made here of extending reaction times for the deamination up to 1 hour to ensure total reaction. It is a little more difficult to eliminate residual hydrazine when the reaction is performed on surfaces, and after several rinses with water deamination was performed for 1 hour, instead of 15’.

The deamination process is also beneficial to help in the enamine formation, for two reasons. First, given the structural limitations of the GAG-APTES chain, formation of the enamine tautomeric group will be favoured (\textit{versus} formation of the imine group) by using small aldehydes or ketones and minimizing steric hindrances\textsuperscript{93}.

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Figure 8. Simplified scheme of the procedures for $^{14}$C-Acetaldehyde radiolabeling of polysaccharide surfaces. In A) APTES is first fixed to glass in ethanol forming an amino terminated layer that is further modified with different GAGs for 24 hours in the presence of cyanoborohydride (B). In C.1) deacetylation of GAG chains is performed by hydrozinoysis and in C.2) the deamination reaction is shown. Deamination at pH 1.5 is performed only for HS GAGs. The nitrosamine formed is then reverted to the secondary amine by denitrosation in a reaction overnight (D). Finally, in E), radiolabeling can be performed by enamine formation using $^{14}$C-Acetaldehyde.
It has been shown that, in general, reaction yields are higher by using aldehydes and that in some reactions enamine does not form by using ketones. The rate of enamine formation essentially depends on two factors: the basicity of the amine and steric hydrances. These considerations favoured the use of the aldehydes. Secondary reactions like aminal formation were not within the scope of this study and were not analyzed, however aminals do not form in reactions involving non-cyclic amines.

A secondary reaction involves the formation of a N-Nitrosamine in the secondary amine in Figure. 8C that is quantitatively reverted to the secondary amine by denitrosation of the N-nitrosamine with removal with a trap (sodium azide) of the nitrous acid produced in the denitrosation in the presence of a good nucleophile (thiourea) in highly acidic conditions94.

Concentration of the reactants was in molar excess in several cases. Conrad and coworkers used the following values for their reactions: 300 μg GAG: 20 μl NH2-NH2 : 0.2 mg NH2-NH2. H2SO4 which represents between 5 and 30 molar excess of GAG over hydrazine sulfate when performing the reactions in closed reacti-vials in suspension. However, 20 μl of pure anhydrous hydrazine does not yield the volume necessary to cover a glass slip. Thus, the reactions were designed in order to deposit 200 μl of total volume on the surfaces and the solution used contained the same ratios of NH2-NH2 and NH2-NH2. H2SO4 employed by Conrad.

The benefit of using small molecules like acetaldehyde is to have easier access to reactive sites, an important factor given that the reaction is performed on a surface. In this case, azeotropic elimination of water is not possible but, given the small volumes and molar quantities of the species over the surface, it suffices to eliminate water using a dissecator containing P2O5 or any other strong dissecant. After reaction, samples are rinsed with

acetonitrile and blown with a nitrogen stream. To avoid moisture and possible reversibility of the reaction, samples were immediately immersed in scintillation liquid and its radioactivity measured.

Surface coverage obtained for polysaccharide surfaces is shown in Table 7. Essentially the results indicate that the ratio of deposited GAG per amino terminal present on the surface is low and that there are small differences between GAGs, with larger molecules like CSC having slightly higher surface densities.
Table 7. Surface coverage by $[^14\text{C}]-\text{Acetaldehyde radiolabeling of GAG.}$ 15' deposited APTES surfaces were used in all cases.

<table>
<thead>
<tr>
<th>GAG</th>
<th>HS</th>
<th>KS</th>
<th>CSA</th>
<th>CSC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>478.5 +/- 59.5</td>
<td>766.4 +/- 142.5</td>
<td>690.4 +/- 136.8</td>
<td>1060.2 +/- 290.2</td>
</tr>
</tbody>
</table>
Given that denitrosation is a fully quantitative process, improvements in the values can be made in the reaction yields of enamine formation by a strong elimination of water during enamine formation.

A secondary aspect of the reaction, shown in Figure 9A, is the possibility of a competing process occurring by the reaction between excess primary amines on the APTES surface, previously unreacted with GAGs, and the acetaldehyde. This possibility is eliminated because the deamination also cleaves primary amines on the APTES molecules. The reason is that the primary step in the deamination reaction is the nitrosation of the enamine\textsuperscript{95},

\[
\text{RNH}_2 + \text{HNO}_2 \rightarrow \text{RNH}_2\text{NO}^+ + \text{OH}^- 
\]

and continues in subsequent steps with final elimination of N\textsubscript{2} and conversion of the alkane to an aldehyde eliminated in subsequent washes. As mentioned, the conditions for the production of other species are complex and include formation of NO and NO\textsubscript{2}. As a result, the deamination process cleaves all NH\textsubscript{2} groups that may remain on the surface and on the GAG chains or convert them into nitrosamines that are later eliminated with the denitrosation procedure. To test this assessment, APTES control surfaces were subjected to direct hydrozinolysis, deamination, denitrosation and radiolabeling and the levels of radioactivity were similar to control glass cover slips that did not have amines on their surfaces, in contrast to control APTES samples not subjected to hydrozinolysis and deamination that showed lowered levels of radioactivity after formation of the Schiff’s base, as shown in Fig. 9B.

Levels of radioactivity shown in Table 8 indicate two things: first that hydrozinolysis treatment of an APTES surface effectively eliminates the amino groups from the surface

\textsuperscript{95} Horton, D.; Philips, K.D. The nitrous acid deamination of glycosides and acetates of 2-amino-2-deoxy-D-glucose

(with levels on the order of clean glass samples) and thus no contribution from amino groups to the surface density of GAG can be expected. The second is that when APTES surfaces are not treated with hydrazine, the reaction produces higher levels of radioactivity than in the case of no hydrazynolisis treatment but lower than those found in the experiment with formaldehyde. This means that, in the conditions of the reaction, formaldehyde is more effective in producing the Schiff base than acetaldehyde, as it could be expected.
Figure 9. APTES control surfaces subjected to hydrozinolysis, deamination, denitrosation and radiolabeling (A) did not produce any labelled material due to the non reactivity of the silanol with the acetaldehyde. In contrast, direct reaction between the primary amine and the acetaldehyde could be done via reductive amination (B).
Table 8. Surface coverage by [¹⁴C]-Acetaldehyde radiolabeling of control APTES and glass surfaces. 15’
deposited APTES surfaces of 0.86mM concentration were used in all cases.

<table>
<thead>
<tr>
<th>Surface</th>
<th>APTES (treatment)</th>
<th>APTES (no treatment)</th>
<th>Glass (no treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface density (sites/μm²)</td>
<td>127.2 +/- 58.4</td>
<td>12477 +/- 549.7</td>
<td>36.9 +/- 7.3</td>
</tr>
</tbody>
</table>
4.3 Discussion of surface analysis by AFM and ellipsometry

Surface modification and GAGs deposition. Silanization in ethanol was chosen because the same procedure in aqueous media yields low surface concentration of amines. The initial low concentration of APTES, 0.43 mM, during an incubation of 15 minutes produces a submonolayer of product, which is not very convenient. To assess what is the maximum concentration of APTES that can be used in the deposition process that provides consistent results, silanization was done with APTES concentrations of 0.43 mM, 0.86 mM, 2.10 mM, 4.20 mM and 21.0 mM. Simultaneously, the effect of time on the deposition was analyzed by observing height changes of the deposited layer. Times varied from 15 minutes to 15 hours.

To avoid high rates of APTES polymerization, the concentration of APTES needs to be kept at low levels. The reason is that APTES hydrolyzes in aqueous solution forming aggregates that are even visible at high concentrations. APTES aggregates are not observed over the glass or silicon surfaces at very low concentrations. APTES multilayer formation could result in undesirable aggregates and structures giving unreliable results in imaging or force measurements. The result would be that immobilization of biomolecules using silanes will see loss of activity because of the hydrolysis of the siloxane layers. This problem –the multilayer formation- disappears after APTES is reacted with the glycosaminoglycans, assuming that a homogenous deposition of the GAGs is achieved. Methods to avoid polymerization of silanes include curing96 to produce the cross-linking of non reacted silanol groups, but here no attempt was made for curing given the homogeneous thickness obtained and the shorter time span of incubation.

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Ellipsometry measurements and silanization conditions. Single wavelength null ellipsometry was used, as is common practice in investigation of biological materials. Ellipsometry measurements have been successful using deposited monolayers of silanes on silica surfaces, with the assumption that the monolayers are uniform and homogeneous\textsuperscript{97, 98}. In general, data obtained by ellipsometry of the deposited layers should be consider relative thickness due to the difficulty in establishing the real refractive index of the films and under the assumption that $k_f$ is zero and that there is no light adsorption. This estimation of film thickness is then highly dependent on an appropriate choice of the refractive index of the material, especially in cases where $n_f$ and layer thickness are correlated. Even if the deposition is a monolayer of product, the fact is that ellipsometric measurements are averaged over the area of the laser beam $\approx 0.6 \text{ mm}^2$. The thickness of the silane layer was calculated assuming a refractive index of 1.422, verified with an Abbe refractometer.

Under equal conditions, a disordered structure gives lower refractive indexes, so to verify that changes in the index of refraction had little effect on the thickness, some measurements were performed at 1.440, 1.422 and 1.380, which are in the range of values for the refractive indexes of alkilamine silanes (around 1.46)\textsuperscript{99}. For instance, the height for 0.86 mM depositions was 1.07 nm assuming a refractive index of 1.380, 1.04 nm with 1.422 and 0.97 nm with 1.440, which means that even if the depositions are disordered, the measurements in the heights wouldn’t change appreciably. With respect to the GAGs, the index of refraction was measured with an Abbe refractometer at concentrations of 10mg/ml. Values obtained were 1.344 for HS and KS and 1.346 for CSA and CSC. At very diluted concentrations of 0.1 $\mu$g/ml the refractive index changed slightly to 1.335 in all cases, which is in practice the value for PBS.

\textsuperscript{97} Wasserman, S.R.; Tao, Y.T.; Whitesides, G.M. Structure and reactivity of alkylsiloxane monolayers formed by reaction of alkyltrichlorosilanes on silicon substrates \textit{Langmuir} 1989, \textit{5}, 1074-1087
Table 9a shows APTES thicknesses obtained with variation of concentration and time. The thicknesses found are lower than the length of the fully extended molecule (~ 1.3 nm) for the case of a monolayer, as expected, and in agreement with previous results for low concentrations and incubation times. Concentration and reaction times affected differently the thickness of the layers. On one side, the deposition of 15’ at 0.43 mM gave a submonolayer of product. On the other side, high concentrations of APTES (21.0 mM) were used for comparison purposes only, given the high degree of polymerization that produces stacks of visible APTES flocks. The conclusion is that the parameter to control is the time of incubation, unless high concentrations are used. The best conditions to obtain a uniform deposition of APTES were obtained with incubation times and concentrations of 15’ and 0.86mM or 0.43 mM and 1 hour, respectively.

An additional possibility that was tested in order to obtain homogeneous depositions was to interrupt the incubation time to wash the surface with water, and then continue the incubation again without drying the sample. Two tests were done with 0.86 mM solutions with 15’ or 1 hour initial incubation times. After water rinses samples were incubated during another 15 hours. That resulted in a very similar height, 9.61 nm for the 15’ sample and 9.58 nm for the 1h sample.

This similarity is in contrast with the case were no water rinses are done, where the differences in height are near 50% after the 15’ and 1 hour incubation times. This of course conforms to the known fact that excess water increases polymerization of silanes. This procedure can be used as an alternative method to produce consistent and uniform stacks of APTES layers. If the interest is in obtaining high surface coverage, this method has the drawback of producing lower concentrations of amino groups on the surface.

Thickness and surface coverage. It has been already commented that the thickness of the layers of silanes depositions and the number of reactive NH₂ groups present on the
surface have been quantified in previous experiments using different experimental conditions, with values of 0.5 nm for height and surface coverage of 1.3 NH₂ groups per nm² using a concentration of 2.15 mMol of APTES and incubation time of 3 hours in dry toluene. As indicated previously, an experimental surface coverage for APTES of 0.14 amino terminals per nm² was obtained and multilayer formation with increased concentration only slightly changes surface coverage.
Table 9a. Variation of ellipsometric thickness of APTES layers with concentration and time.

<table>
<thead>
<tr>
<th>Thickness of layers (nm)</th>
<th>Times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15'</td>
</tr>
<tr>
<td>APTES concentration</td>
<td></td>
</tr>
<tr>
<td>0.43 mM</td>
<td>0.85 +/- 0.13</td>
</tr>
<tr>
<td>0.86 mM</td>
<td>1.04 +/- 0.22</td>
</tr>
<tr>
<td>2.10 mM</td>
<td>1.47 +/- 0.22</td>
</tr>
<tr>
<td>4.20 mM</td>
<td>1.87 +/- 0.27</td>
</tr>
<tr>
<td>21.0 mM</td>
<td>2.33 +/- 0.34</td>
</tr>
</tbody>
</table>

Table 9b. Variation of ellipsometric thicknesses of GAGs layers in dry state. GAGs were deposited on APTES layers of 0.86mM concentration and 15’ incubation time.

<table>
<thead>
<tr>
<th>GAGs</th>
<th>HS</th>
<th>KS</th>
<th>CSA</th>
<th>CSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness (nm)</td>
<td>1.54 +/- 0.21</td>
<td>2.18 +/- 0.44</td>
<td>2.20 +/- 0.46</td>
<td>2.41 +/- 0.25</td>
</tr>
</tbody>
</table>
Some theoretical considerations follow. The thickness of the deposition is highly
dependent on the concentration, time and solvent used. A theoretical calculation using
simple bond lengths shows that a monolayer of product will be around 0.9 nm height
assuming all atoms oriented approximately normal to the surface. If there is any
difference between the calculated and experimental values, it may be due to the fact that
some APTES chains are not in a completely all-trans configuration. In some experiments
it has been found that the alkyl chains are oriented approximately normal to the surface,
giving a high density packed APTES\textsuperscript{100}. However, some groups\textsuperscript{101} have reported that
approximately 25% of chains may be oriented toward the surface. In this description,
GAGs chains can be considered either horizontal or vertical (it is assumed that no
molecule can attach at 45\degree and that no molecule will have kinks allowing being vertical
and horizontal).

Maximum distances of the link O-Si-O in the oxide layer are in the order of 0.4–0.5 Å,
assuming a non-linear configuration. Ellipsometry measurements show that when having
a monolayer of APTES the limiting factor in the increase of surface coverage of GAG is
not the APTES surface coverage. Thus, it is not possible to assume, in first
approximation, that in the reductive methylation via NaBH\textsubscript{3}CN all available APTES sites
react with GAGs. Then it is possible to calculate the maximum GAGs chains that can be
accommodated in this surface. Although our GAGs have not been crystallized and
information of the crystal cell dimensions is not available, a rough estimate for the
maximum and minimum dimensions can be made by using some available cell
dimensions of similar sulfated monosaccharides. With the standard description of the cell
parameters used in the literature, and assuming that around 75\% of APTES chains do not
bend towards the substrate, as indicated previously, cell dimensions for β-D-mannan are

\textsuperscript{100} Henke, L.; Piunno, P.A.E.; McClure, A.C.; Krull, U.J. Covalent immobilization of single-stranded DNA onto
\textsuperscript{101} Bergkvist, M., Carlsson, J. Karlsson, T. TM-AFM Threshold Analysis of Macromolecular Orientation: A Study of
the Orientation of IgG and IgE on Mica Surfaces \textit{J. Colloid Interface Sci.} 1998, \textbf{206}, 475-481. See also Xiao, Cif. note 63 \textit{supra}.

79
in the order of 8 Å\textsuperscript{102}, which mean that each GAG chain will occupy the linear space of at least 2 silane groups. Cells with sulfate groups have bigger cell dimensions and, using data from X-ray structure analysis of an N-sulfated monosaccharide sugar\textsuperscript{103}, it is estimated that each GAG chain could occupy the linear space of at least 4 silane groups. In other words, the upper limit for surface coverage for highly sulfated GAGs (HS or heparin) when covalently deposited on APTES may be around 1/4 of the experimentally found APTES surface density, while for less sulfated GAGs (chondroitins, KS) or non-sulfated (hyaluronian) the upper limit may be one half. Steric effects between GAGs during incubation could produce additional reductions in these ratios but these have not been quantified.

The results can be compared with some values calculated for the glycocalyx and for adsorbed molecules. With the distance between chains of 2–4 nm in the glycocalyx, the graft density for some GAGs chains in the glycocalyx can be at a maximum 0.25 chains/nm\textsuperscript{2}, which can be considered very high. In comparison, most common experimental depositions of adsorbed molecules are in the range of 0.001 to 0.05 chains/nm\textsuperscript{2}. In this case, an experimental APTES surface coverage of 0.14 chains/nm\textsuperscript{2} has been obtained. Assuming a reaction yield between GAGs and APTES of only 25%, then a reasonable expected surface coverage for GAGs will be for KS, CSA and CSC of ~ 0.017 chains/nm\textsuperscript{2} and of ~ 0.008 chains/nm\textsuperscript{2} for HS.


Table 10. Comparison between experimental and theoretical values of surface coverage for GAGs. Theoretical values are based in the experimental results of 0.14 sites/nm² for APTES.

<table>
<thead>
<tr>
<th>GAG (sites/μm²)</th>
<th>HS</th>
<th>KS</th>
<th>CSA</th>
<th>CSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>478.5 ± 59.5</td>
<td>766.4 ± 142.5</td>
<td>690.4 ± 136.8</td>
<td>1060.2 ± 290.2</td>
</tr>
<tr>
<td>Theoretical</td>
<td>8,800</td>
<td>17,500</td>
<td>17,500</td>
<td>17,500</td>
</tr>
</tbody>
</table>
A comparison of results using the experimental values and the theoretical calculations using as a base the experimental results of APTES (0.14 sites/nm²) are shown in Table 10. They are in a ratio varying from 1:25 to 1:15. It must be noted that the expected results have been calculated with figures for the size of the elementary monosaccharide as if all contiguous chains were packed as in a crystal giving a distorted, to the low end, view of the experimental results of the measured density of GAGs.

As a conclusion, although the GAG surface density can be considered low, it has to be noted that full or highly packed surface coverage is not the situation in which cell surface recognition occurs and that our intention is replication of the glycocalyx density, not the maximization of surface coverage.

**AFM and ellipsometry thickness comparison.** AFM images of chondroitin sulfate A patterned structures are shown in Figure 5. AFM images were obtained only for CSA, then the comparison will be given only for this GAG. Contour length, estimated molecular mass and number of charges per dimer in CSA are as follows: CSA (Lc =54 nm, charges= 2, MM dimer = 456). Values of the molecular mass of the products were taken from the manufacturers. Classical estimates of level of sulfation given by Lindahl\textsuperscript{104}, were used to calculate molecular mass of dimer.

Using our results for 0.86mM APTES concentration and 15’ deposition time, table 9b shows the ellipsometric thicknesses obtained for the deposition of GAGs. This height is in dry state and gives an idea of how the total length of the extended molecule affects the process of laying down on the APTES surface. As it could have been expected intuitively, the longer the contour length, the higher is the average stack formed by the molecules. These dry brushes, of course, represent the incompressible layer of the polymer chains. Given that the thickness determination by ellipsometry is calculated over the area of the laser spot, which is around 0.6mm², it is convenient to compare it with the

\textsuperscript{104} Cif. note 1 supra.
thickness calculated from AFM using the analysis of cross-sections for CSA. As shown in Fig. 5, heights for CSA gave a mean value of 2.05 +/- 0.58 nm, very close to the ellipsometer value of 2.20 +/- 0.46 nm.

4.4 Results of experiments of the static adhesion of cancer cells

Three cell lines, three different conditions (non treated, cDNA transfected and heparin resuspended cells) and 6 different surfaces were used in the experiments. At least 9 results were acquired for each combination of cell line/condition/surface for a total of more than 500 samples. The results of the experiments are essentially divided in three parts. In the first part, expression of heparanase in the cells is demonstrated using different methods. In the second part, Western Blots permit the identification of best transfection times. Results and discussion of the adhesion experiments are described later in a third part.

4.4.1 Immunostaining and heparanase activity and determination of cDNA transfection conditions

Presence of heparanase on the BT20, A431 and MCF7 cells was detected. Fig. 10 demonstrates the presence of heparanase in all cells. Generally, endogenous levels of heparanase are low for immunostaining with non transfected cells, and the immunostaining experiments were performed with cDNA transfected cells. Low natural levels of heparanase and instability of the molecule do not simplify identification and work.

It is of interest to compare the levels of heparanase of the three cell lines and then if a correlation between amounts of heparanase expressed by the cells and number of adherent cells could be established. A commercial presentation of the assay that monitors the degradation of heparan sulfate (Takara Miro Bio) was used. A standard curve was prepared for the assay fitted to a sigmoid with 4-parameters, as recommended by the
manufacturer.

The assay monitors the relative amount of enzymatically active heparanase, and as mentioned earlier, the adhesion assays were performed at physiological pH in order to analyze the involvement of inactive heparanase on the adhesion. As mentioned by Ihrcke\textsuperscript{105}, inactivation of heparanase is due to a change in conformational state. It is possible that such conformational state changes during the lysis of the cells during the assay. It is assumed that the activity, as measured with this method, and the amount of heparanase have linear proportionality. Thus, levels of heparanase activity could provide only a partial indication of the involvement of heparanase in the adhesion when assessing whether higher amounts of heparanase in one cell line are correlated with increased adhesion levels. The reason is that part of the secreted heparanase binds to the cell surface, and part is released to the media. However, the exact mechanism of surface expression is not known. Recently, a model for heparanase biosynthesis was proposed\textsuperscript{106} where endogenous heparanase is secreted and interacts with cell surface heparan sulfate proteoglycans (syndecan). A possible explanation of the activation of the hpa1 latent in cells was given by Fairbanks\textsuperscript{107}.

\textsuperscript{105} Cif. note 35 supra.

\textsuperscript{106} Cif. Note 75 supra.

Fig 10. Immunohistochemical localization of heparanase in A431 (A), BT20 (B) and MCD7 (C) cDNA transiently transfected cells. Identification was performed as described in the Experimental Procedures. Cell surface expression of heparanase in transfected cells was determined by indirect immunocytochemistry as described\textsuperscript{108} with modifications. Cells were first fixed with 3.7% paraformaldehyde for 30’ and later incubated with BSA 2% as blocking agent for another 30’. Cells were then incubated overnight at 4°C with goat, anti-human Heparanase 1 HPA1 (C20), polyclonal antibody (Santa Cruz Biotechnology, California) diluted 1:50 in PBS and then rinsed with PBS, followed by incubation (1:500 dilution) for 45 minutes at room temperature with horseradish peroxidase-conjugated secondary antigoat antibody (Jackson Immunoresearch). Color was developed using EAC substrate (Lab Vision Corp. California), followed by counter-staining with Mayer’s hematoxylin. Cells were visualized (micrographs amplifications are 40X in all cases) using bright field light microscopy with a Leica DMLB microscope and photographed with a Diagnostic Instruments RT color camera. Controls for negative expression were taken without addition of primary antibody.

\textsuperscript{108} Cif. note 5, supra.
**Fig.11 Heparanase activity and Western Blots.** Levels of heparanase activity (in arbitrary units per ml) of BT20, A431 and MCF7 non treated cells (top) and blots identifying best cDNA transfection times (bottom). Selected transfection times were 36h for MCF7 cells and 24h for BT20 and A431 cells, as described in Experimental Procedures section.
4.4.2 Static cell adhesion to the different substrates

To address the question of how the adhesion of cells differs for different GAGs, adhesion experiments were performed in which cells were seeded on glass cover slips functionalized with GAGs and the number of cells attached were counted after a specified period of time. Glass cover slips were coated with a monolayer of APTES over which covalently attached monolayers of GAGs were deposited with the technique described in previous sections.

Results are first presented independently for each cell line and later a comparison is made for all cell lines. For each cell line, results are presented for each different condition tested during the adhesion experiments. The following considerations should be noted:

a) Adhesion information is broken-down into two parts, control surfaces and GAG substrates. The reason is that GAG substrates figures must be normalized to equivalent surface densities, to make the analysis meaningful. All calculations were adjusted to the CSC surface density. (See Table 7).

b) Given that there are three different experimental conditions, further analysis will be performed by presenting GAG adhesion information relative to non-treated cells.

c) In all cases, the number of cells seeded was 2.5 x10^5 on the cover slips.

Results for MCF7 cells.

Figure 12 represents absolute number of cells attached (in cells per ml) to the four different GAG substrates, adjusted for surface densities of GAGs. The main observations are:

1) Non-treated MCF7 cells attach in higher numbers to HS and CSA but not to CSC and KS substrates. Globally, HS appears to be a preferred substrate for adhesion.

2) Taking each substrate individually, and for all substrates, the number of adherent cells follows the decreasing sequence: non-treated – cDNA – heparin suspended.
3) Heparin suspended cells decreased adhesion with respect to untreated cells and cDNA transfected cells up to 250%.

Figure 13 shows the relative percentage of cells cDNA transfected or heparin suspended with respect to non-treated cells, for the four different GAG substrates, adjusted for surface densities.

The main observations are:

1) Compared to non-treated cells, MCF7 cDNA transfected cells show a 50% increase in adhesion to CSC and KS, and a reduction in adhesion around 50% for CSA and HS.

2) Compared to non-treated cells, MCF7 heparin suspended cells show about 60-70% decrease in adhesion, except for CSC, only 25%.

Results for A431 cells.

Figure 14 represents the absolute number of cells attached (in cells per ml) to the four different GAG substrates, adjusted for surface densities. The main observations are:

1) Non-treated A431 cells attach in higher numbers to HS and CSA but not CSC and KS substrates. Globally, HS appears to be a preferred substrate for adhesion, as in the case of MCF7 cells.

2) Taking each substrate individually, and for all substrates, the number of adherent cells follows the decreasing sequence: non-treated – cDNA – heparin suspended, except for KS.

3) Heparin suspended cells decrease adhesion with respect to untreated cells and cDNA transfected cells except for KS. This reduction is less dramatic than in MCF7 cells.

Figure 15 shows the relative percentage of cells cDNA transfected or heparin suspended with respect to non-treated cells, for the four different GAG substrates, adjusted for surface densities.

The main observations are:
1) Compared to non-treated cells, A431 cDNA transfected cells show small increases in adhesion to HS and CSA and no changes in adhesion for CSC and KS.
2) Compared to non-treated cells, A431 heparin suspended cells show about 20-50% reduction in adhesion, except for KS, that shows 25% increase.

Results for BT20 cells.
Figure 16 represents the absolute number of cells attached (in cells per ml) to the four different GAG substrates, adjusted for surface densities. The main observations are:

1) BT20 cells show strong preference for HS substrates and low preference for KS, as with the other two cell lines, for all conditions.
2) Heparin suspended cells reduction in adhesion respect to untreated cells is small in all cases.
3) Cells do not change adhesion to CSA or CSC substrates.

Figure 17 shows the relative percentage of cells cDNA transfected or heparin suspended with respect to non-treated cells, for the four different GAG substrates, adjusted for surface densities.

The main observations are:
3) Compared to non-treated cells, BT20 cDNA transfected cells show almost 50% increase in adhesion to CSA and KS, and a reduction in adhesion around 50% for HS.
4) Compared to non treated cells, BT20 heparin suspended cells show about 30-40% reduction in adhesion, except for CSA, with an increase of 25%.

Comparison of all cells.
Figure 18, 19 and 20 show a comparison of absolute number of cells attached (in cells per ml) to the four different GAG substrates, adjusted for surface densities, for the three cell lines, for non treated, heparin resuspended and cDNA transfected cells, respectively.
a) For untreated cells (Figure 18):
a.1) HS is the preferred substrates for all cells.

a.2) All cells attach similarly to KS and CSC.

a.3) KS is not a preferred substrate for attachment for any cell line.

b) For heparin suspended cells (Figure 19):

b.1) MCF7 cells are substantially more affected by suspension in heparin than the other two cell lines and show decreased levels of adhesion to all substrates.

b.2) Heparin increases substantially the adhesion of A431 cells to KS and BT20 to HS, compared to the other two cell lines.

b.3) BT20 cells show preferred adhesion to HS even after suspension in heparin.

c) For cDNA transfected cells (Figure 20):

a.1) As with heparin, MCF7 cells attach in lower or equal number to all substrates, except KS.

a.2) All cells attach similarly to CSC.

a.3) All cells continue showing preferred adhesion to HS.

Comparison of all cells with glass and APTES substrates.

Figures 21, 22 and 23 show a comparison of absolute number of cells attached (in cells per ml) to glass and APTES, for the three cell lines, for non treated, heparin resuspended and cDNA transfected cells, respectively.

a) For untreated cells, glass is a similar substrate, but APTES shows substantial differences between cell lines, with A431 strongly adhering and BT20 not.

b) For heparin suspended and cDNA transfected cells lower adhesion is observed for all cell lines compared to glass and a similar pattern of increased adhesion going from MCF7 to A431 cells.
Fig. 12. **Absolute adhesion of MCF7 cells.** Absolute number of MCF7 cells adhered to the specified substrates after 8 hours of incubation time. The three different conditions tested –non treated, heparin resuspended and cDNA transfected cells- are shown.

Fig. 13. **Adhesion of MCF7 cells relative to non-treated cells.** Cells adhered to the specified substrates after 8 hours of incubation time, compared to untreated cells. Heparin resuspended and cDNA transfected cells percentages are shown.
**Fig. 14 Absolute adhesion of A431 cells.** Absolute number of A431 cells adhered to the specified substrates after 2 hours of incubation time. The three different conditions tested – non-treated, heparin resuspended and cDNA transfected cells – are shown.

**Fig. 15. Adhesion of A431 cells relative to non-treated cells.** A431 cells adhered to the specified substrates after 2 hours of incubation time, compared to untreated cells. Heparin resuspended and cDNA transfected cells percentages are shown.
Fig. 16 Absolute adhesion of BT20 cells. Absolute number of BT20 cells adhered to the specified substrates after 2 hours of incubation time. The three different conditions tested—non-treated, heparin resuspended and cDNA transfected cells—are shown.

Fig. 17. Adhesion of BT20 cells relative to non-treated cells. BT20 cells adhered to the specified substrates after 2 hours of incubation time, compared to untreated cells. Heparin resuspended and cDNA transfected cells percentages are shown.
Fig. 18. Comparison of adhesion of MCF7, BT20 and A431 non-treated cells.

Fig. 19. Comparison of adhesion of MCF7, BT20 and A431 heparin resuspended cells.
Fig. 20. Comparison of adhesion of MCF7, BT20 and A431 cDNA transfected cells, normalized to equal surface densities.

Fig. 21. Absolute number of MCF7, BT20 and A431 non-treated cells adhered to glass and APTES.
Fig. 22. Absolute number of MCF7, BT20 and A431 heparin suspended cells adhered to glass and APTES.

Fig. 23. Absolute number of MCF7, BT20 and A431 cDNA transfected cells adhered to glass and APTES.
Discussion. Cell adhesion requires ligand-receptor interaction. A full description of the adhesion at the molecular level requires information of the substrates (that are known in this study) and possible adhesion receptors on the cell surface. Several molecules bind to heparan sulfate (and possibly to other glycosaminoglycans) in a list that include chemokines, FGF, integrins and the selectin family, but in this work the focus is not in the elucidation of the precise mechanism used by each cell line, although some insights are given. To simplify the discussion, the following assumptions and clarification are made. Even after, some complexity in the analysis will remain given that the work has been performed with three cell lines with three conditions and six surfaces:

1) It is clear that the type of adhesion performed here corresponds to firm, permanent, static adhesion. Then, the first assumption is to discard the possibility that the adhesion is mainly due to the involvement of the abundant set of molecules participating in transient adhesion, that are well known in transient leukocyte and cancer cell adhesion to the endothelium: that includes sLe\(^\alpha\) and sLe\(^\beta\) binding to E- and P-selectins and L-selectin binding to sialylated or sulfated glycans.

2) It is known that glycosaminoglycans may bind, electrostatically, to other polysaccharides. However, a second assumption will be that cell surface glycosaminoglycans will not be involved in the adhesion to GAGs substrates. That is, it is assumed that receptors and substrates have different chemical and functional compositions.

3) Introducing heparanase-inhibiting molecules in cell cultures to analyze any reduction in the binding processes was performed by resuspension of the cells in heparin for 30’. In addition, in some instances where cells have been transfected with heparanase\(^{109}\), heparin enhances accumulation of heparanase in cell culture medium, but there is no information at what rate. Values provided by these authors are for 24 hours, not thirty minutes nor during the time the cells are adhering (two hours or eight

\(^{109}\) Cif. note 75 supra.
hours). Thus, it is assumed that resuspension in heparin will have little impact on the accumulation of heparanase on the media or on its distribution on the cell surface, and that its regular trafficking is not substantially affected during this time.

4) Also, it is emphasized that incubation temperature was 37°C and pH in physiological conditions. This situation ensured that heparanase was not enzymatically active but could bind or mediate binding to the ligands on the substrates and also to heparin present in the medium during resuspension.

4.4.3 Discussion of adhesion by cell line

*MCF7.* MCF7 cells attach in substantially higher numbers when cells are non-treated to HS and CSA. In general the effect of heparin is more intense than with the other cell lines. Because transfection of the cells and resuspension in heparin was not performed simultaneously, it is not possible to claim that transfection had the net effect of overcoming, albeit partially, the reduction in the adhesion in the presence of heparin. However, for two substrates the adhesion with cDNA transfected cells is higher than with non treated cells. This result, along with the result that HS is a strongly preferred substrate for adhesion in all cases, provides strong indication that MCF7 cells use heparanase –preferentially or as a mediator or secondary adhesive mechanism- in their binding to glycosaminoglycans. The increased adhesion to CSC after transfection may be due to the ability of the cell to find appropriate attachment sites with extended, longer molecules.

In all cases, MCF7 cells resuspended in heparin containing media show decreased adhesion with respect to untreated cells. Except for the case of CSC, this decrease is around 50%. There are two reasons that may explain this behaviour. If the adhesion is mediated by heparanase, then the reduction could be explained mainly by heparin blocking heparanase. If the adhesion is due to other molecules, then those molecules are effectively affected by heparin in the media, with the net effect of reducing the adhesion. This effect is clearly shown in the cases of CSC and KS. However, the results for HS and
CSA remain to be explained, because after cDNA transfection an increase should be expected.

Of special interest is the additional observation that the number of adherent cells in heparin containing media does not vary substantially between different substrates. This indicates that cells are probably using more than one mechanism for adhesion, and that a secondary mechanism blocked by heparin is the same for all surfaces. The mechanism could be heparin blocking integrin chains\textsuperscript{110,111}. Heparin has been shown to block P-selectin mediated adhesion of tumor cells to endothelial cells. But, as mentioned before, mechanisms involving P-selectins, receptors that are known to bind glycosaminoglycans and that are found in MCF7 cells, are discarded here because they are associated to transient adhesion.

\textit{A431}. Apart of the high natural expression of heparanase in these cells, other receptors that are known to bind glycosaminoglycans are found in A431 cells\textsuperscript{112}. There is moderate expression of sLe\textsuperscript{x} and sLe\textsuperscript{a}. On the other side A431 cells strongly express \(\beta1\), \(\alpha3\), \(\alpha6\), and \(\alpha\nu\) and moderately express \(\alpha2\) and \(\alpha5\) integrin chains with no significant expression of \(\alpha4\) and \(\alpha1\) integrins.

Generally, A431 show similarities with MCF7 cells for cells suspended in heparin containing media, showing decreased adhesion respect to untreated cells. Also, the substrate with the highest binding of cells is HS, again in support of the heparanase mediated adhesion. In contrast to MCF7 cells, where almost all substrates showed deep

\begin{flushright}
\begin{tabular}{l}
\textsuperscript{111} Brockbank, E.C.; Bridges, J.; Marshall, C.J.; Sahai, E. Integrin beta1 is required for the invasive behaviour but not proliferation of squamous cell carcinoma cells in vivo. \textit{British Journal of Cancer.} 2005, 92, 102-112. \\
\end{tabular}
\end{flushright}
reduction in adhesion for heparin suspended and cDNA transfected cells, A431 cells show small increases, no change or small reduction in almost all substrates respect untreated cells. This smaller reduction in the adhesion when cells are resuspended in heparin medium may be due to the different levels of heparanase activity in the cells - much higher in A431 and then the same concentration of heparin leaves unblocked more heparanase that can be used for adhesion to the substrates.

**BT20.** Two main aspects can be mentioned about the adhesion of this cells. First, again indicating heparanase involvement in the adhesion, cells strongly attach to HS substrates with numbers higher than in the other cell lines. The adhesion to KS and CSC follow the same patterns of the other two cell lines, with low adhesion levels. It is interesting to note that in this cell line, except in HS, cDNA transfected cells show the highest attachment.

### 4.4.4 Comments of the adhesion of cells by the origin of the cell line

It is of interest now to try to relate the observed preferences for adhesion of the cell lines with the substrates and some basic aspects of tumor origin and tissue distribution of the glycosaminoglycans, with the reminder that experiments here were performed *in vitro*. It is of course highly unlikely that the adhesion results obtained here can be of any direct application to the complex analysis of *in vivo* metastases. But at least it would be interesting to answer the question if it is possible to obtain a basic link between preferential adhesion of the cells (*in vitro*) to GAG substrates and the experimental metastases observed for these cell lines. In addition, could these results be indicative of preferential locations for metastasis after a hypothetical treatment with heparin or when cells overexpress heparanase?.

After adjusting for equal surface density of GAGs and, excluding the values for control substrates (glass and APTES), non treated cells showed preferential adhesion to heparan sulfate substrates in all cell lines. All cells show CSA as secondary preference (with two exceptions). HS is present in several tissues and organs, mainly in lung, liver, arterial
wall and in several cell surfaces. As expected, the affinity of the cells for HS chains shown here implies that this type of adhesion could prove helpful for the cells during metastasis.

Heparin suspended cells show preferential adhesion to HS for MCF7 and BT20 and to CSA for A431 cells. CSA is mainly present in cartilage, bone and skin. This results may indicate that it may prove more difficult to reduce metastases of MCF7 and BT20 cells using heparin than to do it with A431 because they seem to have higher affinity for HS substrates after heparin resuspension –assuming that the cells use binding to HS chains at some point when their tumors metastasize-. Also, given that A431 shows preferential adhesion for CSA after resuspension it would be possible to observe a tendency for A431 cells to metastasize to tissues with abundant CSA content. cDNA transfected cells showed preferential adhesion to HS for BT20 and A431 cells and KS for MCF7 cells. This may indicate preferential locations for metastasis for these cell lines.
Table 11. **Preferred substrates for adhesion of cancer cells.** The preferred and secondary GAG substrate for *in vitro* adhesion of MCF7, A431 and BT20 cells under three different conditions. Preferences are equal for all cell lines (preferred:secondary $\rightarrow$ HS:CSA) except for the two exceptions noted.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MCF7 Breast carcinoma</th>
<th>BT20 Breast carcinoma</th>
<th>A431 Epidermoid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non treated cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preferred adhesion</td>
<td>HS</td>
<td>HS</td>
<td>HS</td>
</tr>
<tr>
<td>Secondary adhesion</td>
<td>CSA</td>
<td>CSC-CSA*</td>
<td>CSA</td>
</tr>
<tr>
<td><strong>Heparin suspended cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preferred adhesion</td>
<td>HS</td>
<td>HS</td>
<td>CSA</td>
</tr>
<tr>
<td>Secondary adhesion</td>
<td>CSC-CSA*</td>
<td>CSA</td>
<td>KS</td>
</tr>
<tr>
<td><strong>cDNA transfected cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preferred adhesion</td>
<td>HS</td>
<td>HS</td>
<td>HS</td>
</tr>
<tr>
<td>Secondary adhesion</td>
<td>CSC</td>
<td>CSA</td>
<td>CSA</td>
</tr>
</tbody>
</table>

* close results
4.4.5 Discussion of adhesion by type of substrate or surface

*KS surfaces*: Low adhesion to KS in all conditions and for all cell lines is a very broad result. In all cases, -non transfected cells, with heparin or for cDNA transfection- cells show little affinity for attachment to KS, with the exception of A431 cells in heparin and the more moderate attachment of cDNA transfected cells. This anti-adhesive property of KS has been observed and established previously\textsuperscript{113,114}. The confirmation of this observed property in the experiments conducted here provides good support for the quality of the techniques used in this project. Levels of adhesion to KS substrates are very similar in all cases, except for A431 heparin suspended cells; a result not too surprising given that A431 heparin suspended cells show similar levels of adhesion to all substrates, in contrast with the other two cell lines.

A possible explanation of the lack of adhesion between the cell lines tested and the surfaces with KS would be to assume that the adhesion of the tumor cells is mediated by selectin receptors. In this case, E-selectin would try to find syalyl Lewis x antigens whose main structural difference with KS is that they are fucosylated on the glucosamine near the nonreducing terminus of the molecule. These types of glycosaminoglycan forms are present on endothelial cells and serve as selectin receptors.

However, as was mentioned in the introduction of this section, selectin mediated adhesion is due to transient adhesion and it was assumed not to be the type of adhesion under consideration. As it will shown later, the reasons for the low level of adhesion are related to dimer charge, sulfation levels and chain length.

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\textsuperscript{114} Burg M.A.; Cole G.J. Clastrin, an anti-adhesive neural keratan sulfate proteoglycan, is structurally related to
**HS surfaces:** Apart of the enzymatic activity of heparanase on heparan sulfate chains (cleavage in the ECM or cell surface), Goldshmidt\(^{115}\) has proposed that heparanase-mediated cell adhesion most likely could involve heparan sulfate chains. However, in one experiment, removing 85% of all ECM sulfated material from endothelial cells did not affect adhesion. The results of that work suggest that heparanase mediated cell adhesion could happen due to the effect of net cell surface charge after interaction with HS, but not necessarily by binding with HS.

As has been mentioned before\(^{116}\), the current understanding of heparanase trafficking includes a step in which secreted heparanase is sequestered by cell surface proteoglycans by binding to their heparan sulfate chains. The presence of heparin in the medium increases accumulation of heparanase in the culture medium after several hours, and the process competes with heparanase binding to HS chains. Here, experiments were performed with heparin resuspension time of 30’ and adhesion times of two hours and eight hours. Then, it is unclear how much competition heparin could have and levels of heparanase on the cell surface should not be affected.

The previous discussion indicates that secreted heparanase is able to bind HS chains on the cell surface, and then it is highly presumable that it also could do so to HS substrates. This is very clear, and a general result of the experiments. The precise interaction of heparanase binding to heparin is unknown, but O-sulfation of heparin chains is not necessary, while N-sulfation is.

It is now possible to analyze cell attachment to HS substrates in light of the previous discussion. As it can be observed in Figure 18, HS can be considered a good substrate for the adhesion of non-treated cells, in all cell lines. This result is likely to be related to the

\(^{115}\) Cif. note 36 supra.

\(^{116}\) Cif. note 75 supra.
total electronic charge present in the molecule, an aspect that will be discussed later. Figure 19 shows a reduction in adhesion for MCF7 and A431 cells with heparin, but the decrease is similar for all substrates (except for BT20 cells). That shows that heparin is binding to heparanase on the cell surface. The differences between cell lines and substrates in the level of reduction is due to the different levels of heparanase expressed and how other mechanisms participating in the adhesion are affected.

Chondroitin surfaces: Chondroitins used in the experiments differ essentially in two aspects: chain length and structural sulfation. They are similar in number of charges and sulfation groups per dimer. CSA contains approximately 90% of its approximately 54 dimers with 4-sulfated groups, while CSC has its approximately 131 dimers with 90% 6-sulfated groups. The possible relation with chain length and sulfation level and structure is analysed in a later section. As it can be seen in the results, CSA and CSC are the surfaces least affected by the presence of heparin in the media or by increased heparanase on the cell surface. There is another trend in this adhesion. MCF7 cells attach in lower numbers than BT20, and BT20 cells less than A431, or at the same level. This tendency is observed for all conditions in both substrates, with the exception of non treated MCF7 cells. Chondroitins are not know for their anti-adhesive properties, like KS. But their small changes in adhesion with these cells in the experimental conditions used are in line with the lack of information regarding heparanase binding to chondroitins. In particular, CSC, a long molecule with mainly 6-sulfate groups seems particularly unaffected by the conditions.

Glass. Clean glass surfaces offer a monolayer of hydroxyl groups surrounded by water molecules on top of the silicon substrate that provides a neutral or slightly negatively charged surface. The main difference between glass and APTES surfaces and the rest of the substrates is that in glass and APTES adhesion is non specific and in absolute terms adhesion can be higher. This is shown in Figure 21 for non transfected cells, where MCF7 and BT20 cells show the highest levels of adhesion and high, but not the highest for A431 cells. It is interesting to note that heparin reduces adhesion essentially to
APTES surfaces (see next paragraph) but not (except for MCF7) to glass. This is clearly
due to the opposite charge on the surfaces.

*APTES*. APTES surfaces provide a highly positively charged surface. Although it is
known that cells can attach to positively charged surfaces, the type of attachment here is
by non-specific adsorption. Given that cells are cultured in regular media and deposited
resuspended on it, calcium and magnesium are present and electrostatic repulsions
between surface and charges in the medium present a barrier for adhesion.

For non-treated cells, adhesion to APTES is low for MCF7 cells, the lowest for BT20
cells and the highest for A431 cells as shown in Figure 21. Assuming that the adhesion is
electrostatically affected or mediated, Figure 22 shows that the the net effect of heparin is
to block domains or regions having negatively charged groups, with significant reduction
levels in MCF7 and A431, and a small increase in BT20.

Increased presence of heparanase also continues with this trend. As it has been
mentioned, heparanase binding to proteoglycans on the cell surface may block exposure
of other negatively charged domains and the reduction is more pronounced in MCF7 and
A431 cells, as shown in Figure 22.

4.4.6 Discussion of adhesion by the molecules involved. Heparin and heparanase
effects on adhesion

1. Heparin had the effect of reducing the adhesion of the cells to all substrates and all
cell lines respect to the levels of non-treated cells, with the exception of KS for A431
cells and CSA and APTES for BT20 cells.

In the case of the MCF7 cell line, the reduction is similar to all substrates, indicating that
the methods used by the molecule to block adhesion are virtually substrate independent,
but this is not the case of the two other cell lines. The reduction in adhesion when heparin
is used is increasingly less intense in BT20 cells and A431 cells, in that order. Given that heparin binds to heparanase and that the level of heparanase activity in regular cells (Figure 11) in these three cell lines are inversely proportional to the reduction caused by heparin, the results provide indication that heparanase is participating in the adhesion.

But because heparin also binds several other molecules in the cell surface, including integrins, the only conclusive assertion that can be made is that these experiments provide additional evidence that heparin blocks the observed adhesion and that the reduction is cell dependent.

As has been mentioned before, in the theoretical introduction of this document, heparin has reached the phase of use in clinical trials because it has been shown to reduce the incidence of metastasis. The anti-metastatic activity of heparin has two sides: by reduction of P-selectin adhesion\textsuperscript{117,118} and by inactivation of heparanase activity, that has the effect of preventing degradation of the ECM by heparanase secreted by the cancer cells.

However, the effect of heparin on the adhesive properties of heparanase is unclear. The results of the adhesion experiments shown here, albeit non conclusive respect to this role, are in the trend of previous works\textsuperscript{119,120} indicating that heparanase activity inhibitors failed to affect adhesion of cells to the vascular endothelium. The main reason for which it is unclear is because the expression of heparanase on the cell surface is not completely understood, seems to be cell line dependent with only 25\% of the total heparanase secreted in the cell bound to the surface. Heparanase in the media is uptaken by heparin, but it is not clear if heparin will bind heparanase already bound to HS chains in the cell


\textsuperscript{118} Cif. note 79, \textit{supra}.

\textsuperscript{119} Cif. note 5, \textit{supra}.

\textsuperscript{120} Cif note 32 \textit{supra}.
surface. The exact chain location of the binding between heparanase and glycosaminoglycans is not known and it is highly unlikely that heparanase can work as a multivalent molecule.

2. cDNA transfected cells showed mixed results in adhesion reduction or adhesion increase with respect to non-treated cells.

The cell line with the lowest level of heparanase activity in regular cells –MCF7- shows substantial reduction in the level of adhesion to all substrates, except to CSC and KS, and the reduction in the adhesion is close, but lower, than when heparin was used. On the other side, the cell line expressing the highest level of heparanase activity, A431, shows similar or increased levels of adhesion to the substrates respect non-treated cells and the same is found with the other cell line BT20. As mentioned before, the expression of heparanase on the cell surface seems to be cell dependent and it is not known in the case of these cell lines. Moreover, the differential expression of the enzyme on the cell surface after cDNA transfection is not known. These mixed results show that heparanase overexpression only partially enhances cell adhesion respect to non-treated cells, and that, depending on the cell line and substrate, heparanase overexpression actually reduces cell adhesion.

4.4.7 Discussion of the adhesion as a function of polysaccharide charge and chain length

Three important aspects were mentioned in the introductory part of this document that prompt for cell adhesion analysis under a perspective that differs from what has been written in this document so far. It is of high interest to determine if adhesion levels of the cells have some type of underlying functional dependence on some of the physical and chemical properties characterizing glycosaminoglycans. Essentially, the analysis that follows will focus on the electronic charge per dimer of glycosaminoglycan and its chain
length, measured using the number of dimers that constitute the polysaccharide backbone.

It has been known for some time that charge interaction between glycosaminoglycans and proteins may require conformational changes in proteins, and that these interactions increase with the length and charge density of the molecules\textsuperscript{121,122}. It is also known that if L-iduronic acid is present, the binding has higher affinity than when it is not present. Thus, the results presented here give indication that cancer cells may have a functional dependence of their adhesion with the number of charges per dimer of the polyelectrolyte to which they are attaching and that this dependence takes the form of a linear function that increases with the number of charges of the dimer.

Specific parameters of this linear function appear to be cell line dependent and are probably modulated by factors related to cell surface density of ligands. In addition, as a secondary hypothesis, a more subtle relationship between cell adhesion and length of the polysaccharide chain may exist, but this relationship is not as clear as the previous one. A third aspect included in this analysis is how the presence or absence of glucuronic acid and the sulfation of the glucosamine residue affect adhesion levels and is implicitly included in central hypothesis presented here.

The hypothesis then can be rewritten in the following form: for a fixed density of cell surface receptors that bind to glycosaminoglycans and a fixed density of GAG ligands, cell adhesion to the polysaccharides is linearly dependent on the number of charges per disacharide in the chain and also dependent on the length of the chain.

For the analysis, the number of disaccharides (dimers) and charges per dimer of GAGs


\textsuperscript{122} Gelman, R. A.; Blackwell, J. Interactions between mucopolysaccharides and cationic polypeptides in aqueous
are as follows: HS (dimers =22, charges=2.5, sulfate groups= 1.5) ; KS (dimers =32, charges=0.5, sulfate groups=0.5); CSA (dimers =54, charges= 2.0, sulfate groups=1); CSC (dimers =131, charges=1.75, sulfate groups=0.75). For this calculation, as it was done for the average molecular masses of the dimers, classical estimates of levels of sulfation given by Lindahl123 have been used.

The total number of charges directly depend on sulfation levels, given that except for the charge contribution of the COO\(^{-}\) groups of the glucuronic or iduronic acids in heparan and chondroitins, the rest of the contribution to the charge is due to the presence of the SO\(_3\)\(^{-}\) groups. The existence of other charged groups present on the polysaccharides, which is possible, is uncommon. The calculations have been made for the products used during the experiments and represent a fair, good average estimate for the polysaccharide length and charge. As will be shown in the discussion of the results by analysis of the graphs below, small deviations (~ 0.25 charges/dimer) of these values will not change the general trend that supports the hypotesis of linear dependence of adhesion with charge per dimer.

In order to show the proposed dependence of the adhesion with charge per dimer, an individualized plot of adhesion versus sulfate and chain length is presented in Figures 24 to 29 for each cell line. Charge per dimer, as shown , increases in the sequence KS<CSC<CSA<HS and chain length in the sequence HS<KS<CSA<CSC.

It must be clarified that no intention has been made of obtaining the precise mathematical form of the dependence, for any case. Rather, a simple linear best fit has been plotted to support the central hypothesis in the case of the adhesion versus charge diagrams, and a “trend” line in the case of the chain lengths. The inclusion in these results of adhesion data for two more polysaccharide substrates will give enough information for the fitting solution: Hyaluronic acid, heparitin sulfate, and keratan sulfate. Biopolymers, 1974, 13, 139-156.

123 Cif note 1 supra.
values and extraction of the precise form of the mathematical relation. However, as shown, it has been possible to give, qualitatively, the approximate form of the relation and then provide a rationale for it.

Figure 24 shows a plot of the adhesion vs. sulfate per dimer for MCF7 (bottom), BT20 (middle) and A431 (top) of non treated cells. The fact that CSC and CSA have similar charge values per dimer but differ in adhesion values is what provided a lead to study the effects of chain length on the adhesion. In the case of the MCF7 cell line, the plot shows a line that has been plotted assuming that the value for CSC may be higher. With that exception, linearity is fairly clear.

Figure 25 shows a plot of the adhesion vs. sulfate per dimer for MCF7 (bottom), BT20 (middle) and A431 (top) of heparin suspended cells. Again, the similarity of the lines between cell lines and, more importantly, between different conditions –non treated and heparin resuspend- substantiates the assessment made before about the existence of this relationship. In the case of the A431 cell line, the plot shows a line with negative slope. It is possible that the value for KS may be distorting the plot.

That leads to the discussion of the possibility of finding cell lines or conditions where the adhesion does not increase or decreases with polysaccharide charge. That situation indicates that a modification of conditions, or cDNA transfection changing the molecular expression of heparanase, may disrupt the tendency of regular cells to attach to polysaccharide chains depending on the charge density of the chain.

Figure 26 shows a plot of the adhesion vs. sulfate per dimer for MCF7 (bottom), BT20 (middle) and A431 (top) of heparanase cDNA transfected cells. All plots look fairly similar, with similar slopes.

The y intercepts in these plots may have the following significance. If the y-intercept is not at zero level for zero charge (assuming that the extrapolation to zero can be
performed), that would mean that adhesion levels do not drop to zero if the molecule is neutral. This residual level then can be understood as the level of adhesion of the cells not associated to the specific binding that provides the bulk of the adhesion numbers. If a mathematical relation can be deduced, this could predict the levels of adhesion of the homologous desulfated polysaccharide. If the y-intercept is at negative levels, that could mean that the cells require substrates with substantial charge density in order to be able to attach.

This discussion is clearly understood when looking at the plots, because they have been constructed using adhesion levels versus sulfate groups. If the graph is plotted using total charge per dimer, instead of sulfate groups only, the y-intercept may cross over the zero level (but not necessarily in all cases). In that case, the y-intercept will show the adhesion levels when the total number of sulfate groups is zero. As indicated, this second type of plot would be more useful in two cases: when plotting adhesion of naturally desulfated polysaccharides (i.e. hyaluronan) or when plotting adhesion levels of artificially desulfated GAGs.
Fig. 24. Adhesion vs. sulfate per dimer graph for A431, BT20, and MCF7 non-treated cells. Individualized plot of the adhesion vs. charge (sulfates) per dimer for A431 (top), BT20 (center), and MCF7 (bottom) non-treated cells. Number of samples per GAG/substrate/cell line was nine.
Fig. 25. Adhesion vs sulfate per dimer graph for A431, BT20 and MCF7 heparin suspended cells. Individualized plot of the adhesion vs. charge (sulfates) per dimer for A431 (top), BT20 (center) and MCF7 (bottom) heparin suspended cells. Number of samples per GAG/substrate/cell line was nine.
Fig. 26. Adhesion vs sulfate per dimer graph for A431, BT20 and MCF7 cDNA transfected cells. Individualized plot of the adhesion vs. charge per dimer for MCF7 (top), BT20 and A431 cDNA transfected cells. Number of samples per GAG/substrate/cell line was nine.
Figure 27 shows individualized plots of adhesion vs. GAG length for BT20 (top) and a proposed trend in a series with different degrees of polymerization (bottom). Obviously, four results do not provide enough indication to construct a curve to fit the values, mainly because the results adjust to more than one type of curve, and what has been represented are two possible trends.

One trend, represented in with a continous blue line and the other with a continous red line. In both cases cells seems to experience a limiting value for adhesion levels –that is, increased polysaccharide chain length will not result in an increase in the number of adherent cells. This limiting value is around 50-70 dimers, which in this case, assuming 1 nm length for the dimer, gives 50-70 nm for the extended length. Also, this same curve type clearly indicates that a minimum length of about 8-10 dimers may be necessary to observe adhesion to polysaccharides.

Clearly, the modulation of the adhesion levels by chain length is of lesser importance compare to the charge density per dimer and this dominant effect is observed in the other trend. The best way to prove the existence of this limiting effect would be to perform adhesion experiments with short chondroitins (of equal charge density) to truly observe adhesion behaviour independent of the charge per dimer. This proposed trend has been plotted in the same figure, representing what could be the adhesion number of an arbitrary cell line versus the degree of polimerization of an arbitrary GAG.

In conclusion, a subtle relationship between cell adhesion and length of the polysaccharide chain may exist, but this relationship is certainly not as clear as the linear relationship that exists with the charge per dimer.
Fig. 27. Adhesion vs GAG chain length. Individualized plot of the adhesion vs. GAG length for BT20 (top) and proposed possible trend in a series with different degrees of polymerization.
To summarize, the existence of a possible limiting value restricting the number of adherent cells to substrates containing polysaccharides by the length of the chain may prove interesting in several instances. Indeed, the use of very large or very short molecules for adhesion is not observed in nature, and quite the opposite, some very large molecules are used to initiate a signaling cascade to avoid adhesive contacts in cancer cells, for instance MUC1. It is interesting to note how, at constant charge density per dimer, like in the case of chondroitins, almost in all cases the longer molecule shows the higher level of adhesion.

In previous pages it has been shown that cells may have no tendency (a possibility mentioned for A431 cells and/or for cDNA transfected cells) to increase adhesion when the charge density increases, but that the most likely linear relationship is to increase the adhesion. Polysaccharides may have a very high-density charge, but rarely surpassing four charges per dimer (i.e. heparin). Then, the range of the charge per dimer studied here includes the major part of the spectrum of biological polyelectrolytes. A third aspect included in this analysis is how the presence or absence of glucuronic acid and the levels of sulfation affect adhesion levels and is implicitly included in the central hypothesis presented here. First, the absence of a hexuronic acid in KS is directly responsible for the reduction in adhesion levels, because of the reduction in charge density per dimer. This relation is direct and clear and, along with having a medium to short chain, produces a GAG that has some anti-adhesive properties. That is the reason why, proteins bind proteoglycans in the cell surface (syndecans) using HS residues but do not do it with the KS chains. Second, high levels of sulfation provide the necessary charges to increase adhesion. It must be noted that no experiments have been performed here to compare adhesion levels between molecules showing different sulfation location, for instance between 2 and 6 sulfated heparan sulfates. Although CSA is 4 sulfated and CSC is 6 sulfated, and both have similar charge density per dimer, the difference in chain length impedes any conclusion about the effect of 4 and 6 sulfation on adhesion levels.
5. Physical model of adhesion using the radius of gyration of the biopolymers

The previous discussion has shown that there seems to be a dependence of the adhesion of the cells respect to the length of the biopolymer on the substrate and also respect to the total charge (or sulfatation level). A possible model to accommodate both parameters is at hand using the concept of the radius of gyration of a polymer. The intention is to calculate the radius of gyration of the GAGs using polymer models and then analyze how the adhesion of the tumor cells changes with the radius of gyration.

The idea here is to apply a well known polymeric model that describes the values of the radius of gyration $R_g$ as a function of the two parameters studied in the previous section: chain length $L_c$ and electronic charge, that here it will identified by $f$ and corresponding to the charge per disaccharide. Two main options are available: the freely jointed chain (FJC) and the worm-like chain model (WLC). The WLC\textsuperscript{124} model describes the polymer as a curved, continuous string of irregular shape but that remains linear in the range of a length known as persistence length $L_p$. Within this context, the longer the persistence length, the stiffer or more rod-like the polymer chain will be or resemble. In the case of the FJC model, the polymer is treated consisting of a specified number of segments joined by flexible joints with a characteristic length known as Kuhn length, which is a measure of the stiffness of the molecule.

It is possible to use modified WLC or FJC models that account for the elasticity of the polymer, but the variations introduced on them will not provide significant changes in the results presented here for several reasons. On is the comparative high change in the number of adherent cells to the substrates respect to the small change of the radius of

gyration. Tipically, the FJC model has been used to describe molecules that show great flexibility, for example oligonucleotides$^{125}$, while the WLC model has been applied, for instance, to DNA$^{126}$, that has a substantially stiffer chain. However, polysaccharides have been described by both models but here the WLC model will be used. The GAGs used here are highly charged polysaccharides with large variations in their lengths and there are recently proposed models for the electrostatic persistence length that can be applicable to the them using the WLC model. In addition, in adhesion experiments there are not big extensions of the molecules (they are not subjected to big stretching forces) and then the differences between the FJC and WLC model that may appear because of this reason will not be of application.

It can be shown (Doi, 1996; Flory, 1953) that the radius of gyration of a polymer molecule is given by

$$R_g = \left( \frac{ \langle r^2 \rangle }{6} \right)^{1/2}$$

(1)

Where $\langle r^2 \rangle$ is the mean square end-to-end distance of the molecule. The radius of gyration is defined as the root-mean-square distance of an end of the chain from the center of gravity of the chain. Equation (1) is valid for all type of chains, ideal or real, and what changes between different chain statistical models is the value of $\langle r^2 \rangle$. For the WLC model, the mean square end-to-end distance is given by

$$\langle r^2 \rangle_{WLC} = 2L_pL_c \left( 1 - \frac{L_p}{L_c} + \frac{L_p}{L_c} e^{-L_c/L_p} \right)$$

(2)

equation where the only fit parameter is the persitence length, because the contour length is fixed and assumed to be well defined and given by

$^{125}$ Lee, G.U.; Chrisey, L.A.; Colton, R.J. Direct measurement of the forces between complementary strands of DNA. *Science* 1994, **266**, 771-773.

\( L_c = n l \)  

where \( l \) is the disaccharide length (that corresponds to the theoretical segment of the chain). Equation (3) was already used in previous sections for the calculation of GAGs contour length. Application of the model requires the calculation of the persistence length, a parameter that cannot be measured experimentally. As mentioned, in the case of the persistence length the chain direction is preserved on its length scale and below that value the polymer is considered linear.

Originally, the models describing persistence lengths did not include specific terms to account for the effect of the ionic atmosphere surrounding the polymer backbone like in the case of polyelectrolytes. However, Odijk\textsuperscript{127} and Skolnick and Fixman\textsuperscript{128} introduced the concept of electrostatic persistence length. Essentially, the conformational properties of a polymer chain that contains ionizable groups may be described using the Debye-Huckel potential, where electrostatic interactions in the media are screened –exponentially- with a length scale of the order of the Debye screening length \( \kappa^{-1} \). Apparently, the increased stiffness of the chains highly charged, respect to non charged chains, may be due to an extension of the range of interaction of different segments. The model for the persistence length proposed by Odijk, Skolnik and Fixman introduced a quadratic dependence of the electrostatic persistence length with the Debye screening length and this dependence has been recently modified\textsuperscript{129} to describe a semiflexible polyelectrolyte under the conditions of the WLC model, with a total persistence length given as

\begin{align*}
\text{References:} \\
128\text{ Skolnick, J.; Fixman, M. Electrostatic Persistence Length of a Wormlike Polyelectrolyte } Macromolecules, 1977, 10, 944-948. \\
\end{align*}
\[ L_p = L^0_p + L^{\text{elect}}_p = L^0_p + 0.32 \left( \frac{l_B}{l} \right) f^2 \kappa^{-1} \]  

(4)

where \( L^0_p \) is the bare persistence length (of a similar polyelectrolyte without charged groups), \( L^{\text{elect}}_p \) is the electrostatic persistence length, \( f \) is disaccharide charge, \( \kappa^{-1} \) is Debye length, (which represents the thickness of the ionic atmosphere or double layer surrounding the polymer), \( l_B \) is the Bjerrum length, (distance at which the Coulomb interaction between two elementary charges in a dielectric medium of dielectric constant \( \varepsilon \) is equal to the thermal energy \( K_B T \) and \( l \) is length of the disaccharide.

In the situation of low forces applied to the polymers, which is of application here, the persistence length and the Kuhn length are related by

\[ L_K = 2L_p \]  

(5)

where it should be noted that there is no simple observable correlation between Kuhn or persistence lengths and real measurable quantities like the bond length or the disaccharide length. In order to apply the model given by equation (4) to GAGs, some assumptions are necessary. Values of parameters (some have been taken from the literature) and the assumptions made follow:

- Contour lengths \( L_c \) and charges per disaccharide will be as previously assigned, HS (dimers =22, charges=2.5, sulfate groups= 1.5) ; KS (dimers =32, charges=0.5, sulfate groups=0.5); CSA (dimers =54, charges= 2.0, sulfate groups=1); CSC (dimers =131, charges=1.75, sulfate groups=0.75).
- The bare persistence length used will be 0.22 nm, taken dividing by 2 the persistence length of dextran (0.44 nm), a polysaccharide containing all glucose residues with the sequence \( \alpha(1,3)\text{-Glu-}\alpha(1,3)\text{-Glu} \).
- Debye length, using estimated values given in Israelachvili, 1992. In general \( \kappa^{-1} \) lies between the following values, 100Å (10^{-3} Molar solutions) < \( \kappa^{-1} \) < 3 Å
(1Molar solutions). Assuming physiological conditions of around 0.150 Molar in solution, the estimated value is $\kappa^{-1} \sim 0.8$ nm. To observe the possible effect on the linearity of the adhesion of the Debye length, two other values have been used for calculations: 0.5 and 1.5 nm.

- **Bjerrum length 0.7 nm** (value taken is for water)
- **HS** is assumed to have a rod like conformation due to its high charge per dimer and it is assumed to be a stiff chain applying the limit of the WLC for stiff chains given by $<r^2> = L^2$ which is also of application for short chains.
- **CSA, CSC and KS** are assumed semiflexible charged chains, applying the WLC full equation (2).

Using the mentioned parameters, calculated values for the persistence length, the mean square end-to-end distance and the radius of gyration are shown in Table 12. The value of the Debye length that will later be used in the plots of the adhesion vs Rg is 0.8 nm. The other two are shown for comparison purposes. Some aspects can be commented from these results:

- The radius of gyration increases with the square of the charge per dimer, making this parameter more important than the chain length. For instance, albeit the length of CSA is 1.5 times the length of KS, the radius is 2.5 bigger.
- The Debye length of 0.8 nm was calculated assuming physiological conditions. When a Debye length smaller –0.5nm- is used, there are small changes in radius of gyration, but the final results keep the proportionality observed with the calculations with 0.8 nm. This indicates that albeit the effect of increased salt presence certainly has an impact on the radius of gyration, the proportionality observed between the radius of gyration of different GAGs with different Debye lengths doesn’t change and that will keep the linearity observed in the plots of the of the adhesion vs Rg.

123
Table 12. Calculated values of the radius of gyration. Results shown have been estimated using the assumptions made in the text. Values are shown in three groups, with variations in the Debye length and calculations of the persistence length and radius of gyration.

<table>
<thead>
<tr>
<th>Model Parameters (nm)</th>
<th>Debye length $\kappa^{-1} = 0.5$</th>
<th>Debye length $\kappa^{-1} = 0.8$</th>
<th>Debye length $\kappa^{-1} = 1.5$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lp</td>
<td>$&lt;r^2&gt;$</td>
<td>Rg</td>
</tr>
<tr>
<td>GAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KS</td>
<td>0.248</td>
<td>15.87</td>
<td>1.62</td>
</tr>
<tr>
<td>CSA</td>
<td>0.668</td>
<td>72.14</td>
<td>3.46</td>
</tr>
<tr>
<td>CSC</td>
<td>0.563</td>
<td>147.5</td>
<td>4.95</td>
</tr>
<tr>
<td>HS</td>
<td>0.920</td>
<td>302.5</td>
<td>7.10</td>
</tr>
</tbody>
</table>
Fig. 28. Plots of adhesion vs Rg of GAGs for non treated cells. Individualized plot of the adhesion vs. radius of gyration of the corresponding GAG as calculated in Table 12 for A431 (top), BT20 (center) and MCF7 (bottom). Number of samples per GAG/substrate/cell line was nine.
Fig. 29. Plots of adhesion vs $R_g$ of GAGs for heparin suspended cells. Individualized plot of the adhesion vs. radius of gyration of the corresponding GAG as calculated in Table 12 for A431 (top), BT20 (center) and MCF7 (bottom). Number of samples per GAG/substrate/cell line was nine.
Fig. 30. Plots of adhesion vs $R_g$ of GAGs for cDNA transfected cells. Individualized plot of the adhesion vs. radius of gyration of the corresponding GAG as calculated in Table 12 for A431 (top), BT20 (center) and MCF7 (bottom). Number of samples per GAG/substrate/cell line was nine.
Figures 28, 29 and 30 shows plot of the adhesion vs. radius of gyration for MCF7 (bottom), BT20 (middle) and A431 (top) of non treated cells, heparin suspended cells and cDNA transfected cells. The values of the radius of gyration used are the ones with a Debye length of 0.8 nm, as mentioned previously. As is very clear, all figures are very similar to the figures plotted using only the charge per disaccharide. In all cases it seems to be a linear relationship between the adhesion of the whole cells and the calculated radius of gyration of the glycosaminoglycan on the substrate.

The results can be analyzed in the following manner. Adhesion levels are generally higher for HS. Using the WLC model in the limit of stiff chains the radius of gyration is also high enough to make this relatively short GAG stiff and rod-like enough to allow the receptors in cell to have access to the disaccharide sequences to permit an increased adhesion. On the other side, KS, with a low value of the radius of gyration and more amenable to be treated as a linear semiflexible chain has a much more limited ability to work as a good substrate for the adhesion. In the intermediate area, CSA and CSC increase the adhesion levels, but in the case of CSC chain length increase does not correspond to similar increase in the adhesion levels.

One of the difficulties to assess the correctness of this analysis is that the experiments are performed with whole cells and the relative variation in adhesion numbers by cell is comparatively much higher than the relative variations introduced by different polymer models, changes in the Debye length or in the total charge per disaccharide. This become especially more difficult with only four polysaccharides studied.
6. Future work

This project has established basic experimental methods that will permit additional work in other aspects of the adhesion of these cancer cells. Here some of these additional aspects are mentioned.

The most apparent aspect to be studied is the dependence of the static adhesion with increasing or decreasing GAG surface concentration. The reason behind this consideration is the still unclear density of GAGs present in the glycocalyx. Given our results, where GAG concentration depends on the concentration of APTES, increasing GAG concentration would be possible by using a different technique for APTES deposition, for instance by reflux with dry toluene.

The most interesting study would be to include additional polysaccharides to precisely obtain the linear relationship of the adhesion vs. charge per dimer. The use of hyaluronan, that contains an acidic moiety but no sulfate group, can provide an idea of the contribution of the carboxylic group by itself and observe the effect on the adhesion when no sulfate group is present. To assess the effect of higher charge density in the sequence, heparin can be used. In addition, to completely verify the hypothesis that adhesion has a limiting value in the chain length, the same GAG in a series with different degrees of polymerization can be tested.

An additional study may be conducted to analyze the effect on the adhesion when the medium contains additional factors, known to be present in the microenvironment of the glycocalyx of endothelial cells of the blood vessel of the primary tumor, and whose presence at different concentration may disrupt the adhesion process. Specifically, the effect of some proteolytic factors derived from the solubilization of the ECM and present
in the blood stream as well as changes in pH and ionic strength on the attachment to different surfaces may be analyzed\(^{130}\).

There is abundant information to justify the study of the adhesion of cancer cells to the glycocalyx in the presence of these factors. Essentially, metastatic cells produce or wait for a change in pH, from physiological to slightly acidic, for the cleavage of HSP and other PGs and the solubilization of the ECM to help them intravasate. This is clear since the ECM retains heparanase in an inactive form at physiological pH but after a decrease in pH—or maybe the presence of other factors- the enzyme regains the catalytic activity.

The proteolytic factors that may be studied are mainly disaccharide or oligosaccharide sequences derived from GAGs, galactose, glucosamine, glucuronic acid, galatosamine and xylose. There is no information available in the literature about the concentrations of proteolytic fragments of glycosaminoglycans in microvessels or in the areas surrounding primary tumors but any polymer present in the media and highly sulfated could affect adhesion.

The elevation of ionic strength can block a possible electrostatic binding between glycosaminoglycan/receptor, so it may also be of interest to conduct cell adhesion studies with changing physiological salt conditions to characterize if that could affected electrostatic interactions responsible for the adhesion. The reason for this test is based on reported results\(^{131}\) showing that cell attachment of lymphoma cells with heparanase expressed on the surface was not affected after removing 85% of all ECM sulfated material from endothelial cells. The results of that work suggest that heparanase mediated cell adhesion could happen due to the effect of net cell surface charge after interaction with HS, but not necessary by binding with HS.

Another obvious aspect of interest is the study of transient adhesion. It is common

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\(^{130}\) Cif. note 3, supra.

\(^{131}\) Cif. note 35 supra.
knowledge that, previous to the attachment, viruses and cells use rolling as means to identify specific binding sites. Rolling of tumor cells in the presence of shear flow in the blood vessels may play a role in the adhesion of tumor cells. Cell rolling under shear flow could also be an important factor in the ability of the cancer cells to extravasate from the vessel into the epithelium. This is because the effectiveness of the adhesion is dependent on the shear force created by the fluid in the circulatory system, as has already been demonstrated in the ability of leukocytes to roll over surfaces\textsuperscript{132}. Additional experimental studies for cell rolling can be performed based on the measured properties of the adhesion at the single molecule level. Results of this project indicate that statically heparanase seems to preferentially bind heparan sulfate surfaces. However, since the dynamic cell adhesion role of the heparan sulfate/heparanase binding has not been investigated previously, these force experiments are of particular interest and constitute a natural extension of the work performed in this project. The relationship between the molecular mechanical properties, the energy landscape of the bond, and the ability to support rolling interactions could be investigated.

Because the static adhesion of the cells is probably mediated by integrin receptors, additional use of function-blocking integrin antibodies to prevent adhesion could provide more information regarding the specificity of the bond between these receptors and GAGs.

\textsuperscript{132} Chen, S.Q. An automatic braking system that stabilizes leukocyte rolling by an increase in selectin bond number with shear. \textit{Journal of Cell Biology}, 1999, \textbf{144}, 185-200.
7. Conclusions and key achievements

The study of the processes relating glycobiology and cancer will have increased interest in coming years. To contribute to this trend the outcome of this study will be useful for investigations in cancer glycobiology using experimental methods exhibiting controlled carbohydrate composition, organization, and orientation, drawn from materials science and physics. The following paragraphs summarize the research performed in this project.

Contribution to the field of glycomics

As it was mentioned in the introduction, typical methods to deposit molecules have generally consisted in growing endothelial cells on surfaces and then eliminating part of the biological material via lysis. This artificial model has permitted testing for adhesion between cancerous cells and GAGs found in the ECM. However, that method cannot study individualized interactions between cells and molecular species of choice, and developing a new technique that facilitates this analysis has been one of the main contributions in this project.

There are no known reports in the literature on reactive microcontact printing of mucopolysaccharides as reported in this project. Given the increasing importance of the study of the biological processes of polysaccharides, the introduction of this surface functionalization technique will be helpful in expanding the field of glycomics.

In essence, there are several advantages in using this type of surface: individualized study of molecular interactions at the molecular level –carbohydrate-carbohydrate, carbohydrate-protein or even carbohydrate-nucleic acid; manipulation of the density of the carbohydrate on the surface; manipulation of the structural composition of the
Development of novel method for preparation and characterization of biopolymeric surfaces.

During the course of this study a new method was devised for the covalent attachment and patterning of glycosaminoglycans to surfaces previously coated with amino terminated monolayers via reductive amination. Four glycosaminoglycans – keratan sulfate, heparan sulfate, chondroitin sulfate A and chondroitin sulfate B, and one proteoglycan, perlecain – were successfully deposited and characterized. Confirmation of attachment and patterning of the molecules to the substrates was demonstrated with fluorescence imaging, while variations in silane agent concentrations and adjustment in deposition times provided information on the best concentration and incubation times. Characterization of the surfaces was performed by ellipsometric measurements and a representative patterned surface was characterized and imaged by AFM.

These functionalized surfaces may prove useful in the study of the adhesive properties of cells, and provide the basis for the development of a model system to study cancer cell adhesion to glycans and a broader list of polysaccharides, beyond glycosaminoglycans.

A novel experimental technique for the determination of glycosaminoglycan surface densities was developed.

In the absence of instrumentation for spectroscopy measurements, this surface density radiolabeling technique can be used with any N-acetylated sugar surface exposing an amino terminated monolayer. The method uses known chemical procedures for glycosaminoglycan chain cleavage. Surface coverage is an important parameter in the replication of surfaces with immobilized biopolymers. Changes in surface densities of the deposited biopolymers affects the biological activity of the molecules and thus the
adhesion of the cancer cells.

Results indicate that APTES surfaces had a density of amino groups of 0.14 groups/nm² and that this surface density did not change with increased incubation times –from 15’ to 12 hours- indicating that silanization was performed with uniform layers. GAGs surface densities were in the range of 500 to 1000 sites per μm² depending on GAG. Glycocalyx meshwork descriptions assume a 20 nm spacing between chains, thus the density obtained was one order of magnitude lower than the known density existing in the glycocalyx.

In vitro experimental measurement of adhesion levels of cancer cells to the glycosaminoglycan functionalized surfaces was performed.

Specifically, the comparative analysis of the static adhesion of the cells in regular conditions, in medium containing heparin and with heparanase cDNA transfected cells was performed. A combination of six substrates and three different conditions provided information on cancer cell adhesion for eighteen different cell environments. Results indicate that heparanase participates in the cancer cell adhesion to the glycosaminoglycans but that this is not the only mechanism for the adhesion.

Two are the factors that indicate heparanase increases cell adhesion. First when natural levels of heparanase in the cell are high, like in A431, then the adhesion of cDNA transfected cells surpasses or equals in all cases the adhesion of non transfected cells. Second, when natural low expression is found, like in MCF7 cells, the overexpression does not produce enough heparanase to make the level of adhesion higher than non transfected cells, but always better than heparin suspended cells.

Non treated, heparin suspended and heparanase cDNA transfected cells show reduced levels of attachment to keratan sulfate substrates.
Results show that keratan sulfate substrates have anti-adhesive properties and that this properties are common for all cell lines and conditions.

**Preferential adhesion of cells is to heparan sulfate substrates**

After adjusting for equal surface density of GAGs and, excluding the values for control substrates (glass and APTES), non treated cells showed preferential adhesion to heparan sulfate substrates in all cell lines. Heparin suspended cells show preferential adhesion to HS for MCF7 and BT20 and to CSA for A431 cells. cDNA transfected cells showed preferential adhesion to HS for BT20 and A431 cells and KS for MCF7 cells. Taken together, all cells showed preferential adhesion to heparan sulfate substrates. Physiologically, the reasons behind these preferences may lie in the type and location of the tumor and in the distribution of the glycosaminoglycans in tissues.

**Heparin effectively reduces adhesion while cDNA transfection shows mixed results**

Heparin had the effect of reducing the adhesion of the cells to all substrates in all cell lines with respect to the levels of non-treated cells, with the exception of KS for A431 cells and CSA and APTES for BT20 cells. Results also show that heparanase overexpression only partially enhances cell adhesion respect to non-treated cells. However, cDNA transfection always increased adhesion levels with respect to heparin suspended cells.

It is hypothesized that the adhesion of cells to polysaccharide chains has a linear dependence with the number of charges per disaccharide.

Results indicate that the relationship may be cell dependent and that usually takes the form of linear increase when the number of charges (sulfate groups) per disaccharide increases. These results are valid within the range of 0.5 to 2 sulfates (0.5 to 3 charges) per dimer. Extrapolation of values to zero charge per dimer –neutral polysaccharides-
indicates that adhesion will not necessarily drop to zero levels when the molecule is neutral. These residual levels of adhesion are also cell line dependent.

It is hypothesized that the adhesion of cells to polysaccharide chains have a maximum and a minimum limiting values in the length of the chain.

Also depending on the specific cell line, for very short oligosaccharides, below 8-10 disaccharides, adhesion levels may drop to zero. Cell adhesion will increase up to a limiting value of 50-70 disaccharides and beyond that mark the adhesion levels of the cells will remain constant, independent of the chain length, at least up to 130 disaccharides.

All together, adhesion of whole cells shows linear dependence with the radius of gyration of glycosaminoglycans on the substrates.

Using the WLC model for polymer chains to calculate the radius of gyration of the GAGs on the substrates it has been possible to confirm that the adhesion of the cells has a linear response with the radius of gyration and is essentially controlled by the charge per dimer –with a secondary contributing effect due to the chain length- and that this dominating mechanism is not eliminated when the cells are subjected to heparanase cDNA transfection or resuspension in media with heparin.

The purpose here has been to show that there is a physical underlying mechanism dominating the adhesion of the cells, mainly the charge per dimer, that works independently of the conditions the cells are subjected to, and that this mechanism can be studied and observed macroscopically working with whole cells, not necessarily having to use isolated molecules as receptors. This in fact indicates that the cellular environmental conditions may play a small role in the adhesion process and are unable –at least to the extent studied here- to disrupt cellular adhesion.
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Appendices
Appendix A: Equipment

Only major equipment is listed in this page.

**Atomic Force Microscope.** Asylum Research MFP 3D AFM model with liquid tapping mode imaging, Q-control for cantilever resonance enhancement, reduced coherence length light source for reduced noise in force measurements, real time hand operated control over the tip placement, ability to sit atop a fluorescence microscope.

**Inverted Optical Fluorescence Microscope.** Epifluorescence microscope (Nikon TE2000) equipped with a QImaging Retiga EX Monochrome 12-bit Digital Camera for fluorescence imaging

**Automatic Ellipsometer.** AutoEL III Automatic Ellipsometer. Automated nulling ellipsometer (Rudolph Research).
Appendix B: Detailed Steps of the Chemical Procedures

Diagrams with detailed steps of the chemical procedures used for surface functionalization and radiolabeling are included here.

Figure 31. Detailed reaction of silanization with APTES.
Appendix B: (cont.)

Figure 32. Detailed reaction of reductive amination with NaBH₃CN.
Figure 33. Detailed reaction of the procedure for radiolabeling Aptes surfaces. A) with $[^{14}\text{C]}$-Formaldehyde and B) with $[^{14}\text{C]}$-Acetaldehyde.
Figure 34. Detailed reaction of radiolabeling via enamine.
Figure 35. Detailed reaction of proteoglycan binding to APTES. In A) the crosslinker and HSP are reacted on the surface of a patterned hydrophilic PDMS stamp with release of a N-hydroxysuccinimide. In B) the crosslinker and HSP are transferred to the APTES surface with release of an N-hydroxysuccinimide.
Figure 36. Detailed reaction of denitrosation.
Figure 37. Detailed deacetylation and deamination. The example corresponds to a chondroitin sulfate C sample.
About the Author

ANTONIO PERAMO received a Bachelor’s Degree in Chemistry and a M.S. in Electrochemistry from the University of Barcelona. Later he studied European Union Law and received a Master in European Union Policies after focusing in the Scientific Policy the of Union. Before entering the Ph.D. program in Applied Physics at the University of South Florida in 2002 he received a Bachelor in Physics by the UNED in Madrid.