Adhesion Forces between Lewis^{X} Determinant Antigens as Measured by Atomic Force Microscopy**

Christophe Tromas, Javier Rojo, Jesús M. de la Fuente,
Africa G. Barrientos, Ricardo García, and
Soledad Penadés*

A carbohydrate coat named glycocalix covers the outer membrane of most types of cells. These carbohydrates at the cell surface are responsible for communication with other cells and with the extracellular matrix through interaction with proteins.[1] Recently, carbohydrate to carbohydrate interactions have been proposed as the initial step of cell adhesion.[2, 3] Embryogenesis, metastasis, and other proliferation processes are, according to Hakomori,[2] mediated by carbohydrate–carbohydrate interactions. Some of the structures involved in this novel mechanism are the trisaccharide Lewis^{X} determinant (Galβ1 →4[Fucα1 →3]GlcNAcβ1) and the glycosphingolipids GM3 (NeuAcα2 →3Galβ1 →4Glcβ1Cer) and Gg3 (GalNAcβ1 →4Galβ1 →4Glcβ1Cer). The Lewis^{X} antigen (Le^{X})—previously defined as the stage-specific embryonic antigen (SSEA-1)—is found in a wide variety of systems[4] such as human cancers, pre-implantation mouse embryos, embryonic carcinoma cells, and human erythrocytes. This antigen mediates, by a homotypic interaction, morula compaction and teratocarcinoma metastasis in mouse cells.[2, 5] A heterotypic interaction between GM3 and Gg3 also seems to be involved in metastasis of melanoma cells in mice.[6, 7] Characteristic features of these interactions are a strong dependency on divalent cations (Ca^{2+}), a high specificity and a low affinity. Nature solves these problems by a multivalent presentation of the carbohydrate antigen at the cell surface. These characteristics make it difficult to study and to quantify this process.[8, 9] Nevertheless, in previous studies using synthetic receptors we have shown that the interaction between lipophilic patches of simple carbohydrate units in water contributes to the free energy of binding with a stabilization of up to 7.1 kJ mol^{-1}.[10, 11]

Recently, atomic force microscopy (AFM)[12] has been successfully applied to measure adhesion forces between complementary DNA strands,[13] avidin–biotin derivatives,[14–16] antigen–antibody systems,[17] adhesion proteoglycans of marine sponges,[18] and cadherins[19] under physiological conditions. The study on the adhesion proteoglycans is, to our knowledge, the only example in which a carbohydrate–carbohydrate interaction has been determined by AFM.[18]

Herein, we present a quantitative AFM study of the specific interaction forces between individual Le^{X}-antigen molecules in aqueous solution using chemically well defined, two dimensional (2D), self-assembled monolayer (SAM) surfaces. This study demonstrates the self-recognition ability and selectivity of the Le^{X} antigen. To measure these adhesion forces, flat gold surfaces were functionalized with the Le^{X} determinant containing neoglycoconjugate **2** to form self-assembled monolayers. For comparison purposes a parallel study was carried out by similarly functionalizing gold surfaces with lactose (Galβ1-4Glc) containing neoglycoconjugate **1** (Figure 1). Neoglycoconjugates **1** and **2** were synthesized (manuscript in preparation) by glycosidation of conveniently protected lactose and Le^{X} derivatives, respectively, with 11-mercapto undecanol using the trichloroacetimidate method.[20] The thiol group was incorporated to attach these molecules to the gold surfaces. A micro-fabricated AFM cantilever[21] and a gold-surface sample were functionalized with neoglycoconjugates **1** and **2** by immersion for at least 2 h,

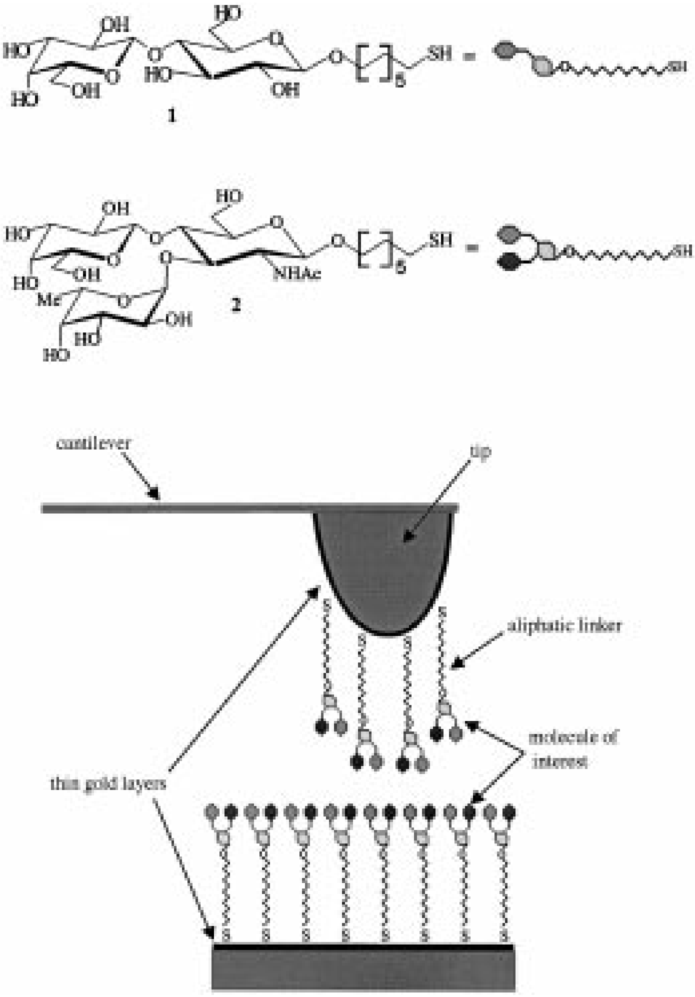

Figure 1. Neoglycoconjugates **1** and **2**, and schematic representation of the sample and the AFM tip which is functionalized with self-assembled monolayers of the neoglycoconjugates.

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[*] Dr. S. Penadés, Dr. C. Tromas, Dr. J. Rojo, J. M. de la Fuente, A. G. Barrientos
Grupo de Carbohidratos, Instituto de Investigaciones Químicas, CSIC, Isla de La Cartuja
Américo Vespucio s/n, 41092 Sevilla (Spain)
Fax: (+34) 95-448-95-63
E-mail: penades@cica.es

Dr. C. Tromas, Dr. R. García
Instituto de Microelectrónica de Madrid, CSIC
Parque Tecnológico, 28760 Tres Cantos, Madrid (Spain)

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in 1 mM solutions of **1** and **2** in methanol and water, respectively, then rinsed several times with the same solvent and dried under a stream of nitrogen.

The neoglycoconjugates organize themselves as monolayers both on the tip and on the sample.[22] Although this model may be considered faraway from the natural glicosphingolipids presentation, it provides a polyvalent functionalized surface with chemically well-defined structure to evaluate carbohydrate–carbohydrate interactions. In a typical procedure, a series of approach and retract cycles with the Le^X functionalized tip and sample were carried out in water and in 10 mM aqueous calcium solution in a liquid cell. Unloading rates of about 100 $nm\,s^{-1}$ were used. For each force scan, the functionalized tip was maintained in contact with the molecular surface at a constant force (150 pN) for two seconds before unloading to ensure intermolecular contact and chemical equilibrium. The unloading curve, representing the adhesion force as a function of the distance, shows a steplike behavior (Figure 2). Each step corresponds to an out of equilibrium cantilever movement because of the unbinding events among the molecules of the tip and the molecules of the sample. Measurement of the step height leads to the determination of the intermolecular unbinding force. For each sample, more than 300 curves over 15 different areas of the sample surface were recorded.

Typical force–distance curves obtained with a tip and a sample functionalized with Le^X determinant are presented in Figure 2A and 2D. The stepwise profile of the curves is characteristic of molecular interaction, and was observed in water as well as in calcium solution (Figure 2A and 2D, respectively). A statistical analysis of the step height[14] reveals that the measured unbinding forces are entire multiples of 20 ± 4 pN (Figure 2B and 2E). The strong periodicity of the auto-correlation function (Figure 2C and 2F) is an important evidence that the interaction observed in these experiments results exclusively from specific adhesion forces. The 20 ± 4 pN force has been assigned to the binding force between two individual Le^X-determinant molecules. The value of the force was independent of the presence of calcium ions in the solution. This result was not expected based on the described characteristics of carbohydrate–carbohydrate interactions. However, in experiments using many different tips and different samples the same 20 ± 4 pN interaction force was obtained.

Carbohydrate to carbohydrate interaction is based mainly on van der Waals contacts between the corresponding polyamphiphilic surfaces involved in the process.[2, 23] The complementary nature of these surfaces makes the interaction highly specific and calcium ions seem to be crucial for this process to occur in biological and other model systems.[2] The role of calcium in carbohydrate–carbohydrate interactions is not well understood. Calcium ions could be responsible for the approach and organization of the sugar moieties which provide the adequate surfaces for interaction, or may enhance the adhesion force between complementary surfaces, acting as a bridge between specific hydroxy groups.

In the AFM experiments presented here, the formation of a well-packed self-assembled monolayer provides the organization of carbohydrates on the surfaces. Furthermore, interaction between the molecules is established by applying an initial contact force in the cantilever which makes the Le^X-functionalized surfaces approach. Therefore, if the role of calcium ions is to favor the approach and organization of the molecules at the surface, it could not be detected in the AFM experiment. However, if the role of calcium is to reinforce the contact between the interacting molecules, an increase in the

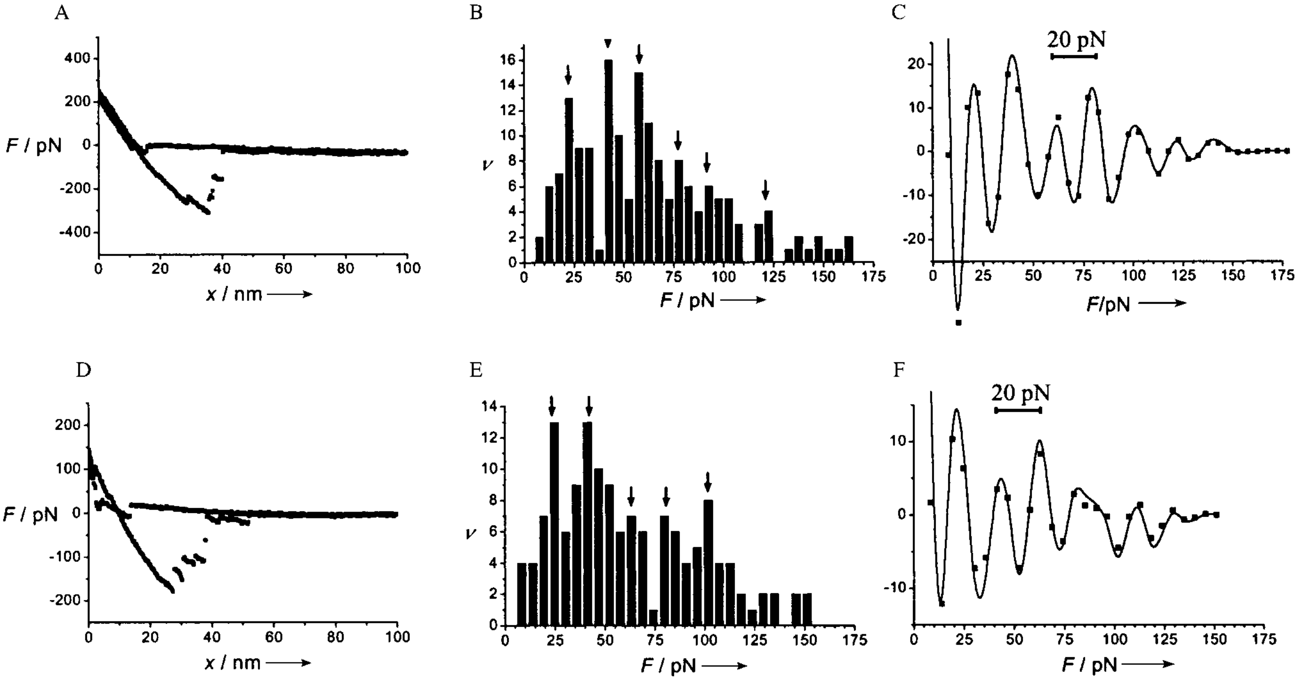

Figure 2. A) and D) Typical AFM force–distance curves obtained between an Le^X-functionalized tip and a SAM of Le^X molecules in water and in 10 mM calcium solution, respectively, B) and E) histogram of the unbinding force measured through 300 force–distance curves of Le^X molecules in water and in 10 mM calcium solution, respectively, and C) and F) corresponding auto-correlation function.

adhesion-force values in calcium solution should be observed.[18] In these experiments, the presence of calcium ions does not contribute significantly to the adhesion force. This result may indicate that calcium is only responsible for the approach and organization of the carbohydrates in the cell membrane. This proposal should be considered as a model that can be refuted with new experimental evidence. It is interesting to note that in experiments with a three dimensional (3D) polyvalent Le^X model (glyconanoparticles) in aqueous solution, where the molecules move freely, we have observed that self-aggregation of the Le^x molecules only occurs in the presence of calcium cations.[24]

Control experiments confirmed that the adhesion force of 20 pN corresponds to the Le^X–Le^X interaction. Force distance curves were measured between an Le^X-functionalized tip and a 11-mercapto undecanol functionalized sample. 500 force scans were performed over 12 different areas of the sample surface; in none of them was interaction observed among functionalized surfaces, thus indicating that the observed adhesion force is really because of the Le^X–Le^X specific interactions and not because of unspecific ones in which the linker could be involved. On the other hand, in control experiments, no interaction was observed between the lactose-functionalized tip and the sample, either in water or in aqueous calcium solution (Figure 3 A), showing the adhesive properties of the Le^X determinant in comparison with the lactose derivative. Additionally, AFM crossed experiments between lactose-functionalized tips and Le^X-functionalized samples or vice-versa gave the same force–distance curves as in the lactose–lactose experiments (Figure 3 B). No interactions were observed either in water or in aqueous calcium solution. In other words, the interaction between the Le^X determinant molecules appears to be highly selective, supporting the proposal that carbohydrate–carbohydrate interactions could be an additional mechanism in specific recognition processes in nature.

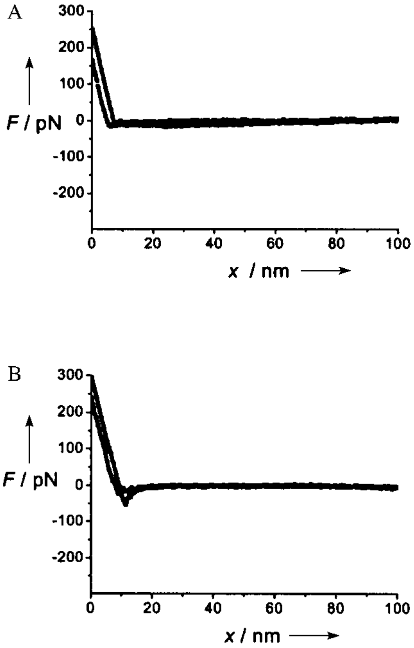

Figure 3. Typical AFM force–distance curve obtained with a lactose-functionalized tip and a SAM of A) lactose molecules. B) Le^X molecules or vice-versa.

The existence of an interaction force of 20 ± 4 pN between individual molecules of Le^X determinant has been clearly demonstrated in water, as well as in 10 mM aqueous calcium solution. This value is the smallest adhesion force reported to date. However, it is in the same order of magnitude as the forces of a single binding event measured in the proteoglycan of marine sponges[18] and other systems such as cadherine–cadherine,[19] adenine–thymine,[25] glycosphingolipid GM1–cholera toxin B (CTB),[26] and the ferritin–antiferritin antigen–antibody system.[17] A further step to obtaining thermodynamic information would be to correlate the measured forces with the energy of binding. Moy et al.,[15] and Chilkoti et al.[27] found a linear correlation between experimental rupture forces and enthalpy in different avidin and biotin derivatives. Preliminary studies based on weak affinity chromatography have allowed us to measure a Le^X–Le^X binding constant around 10 M^{-1} at room temperature (unpublished results). This value corresponds to an energy of 5.7 kJ mol^{-1} and according to the equation $E = Fd$, the interaction distance could be determined taking into account that the maximum force (F) found by AFM is 20 pN.[28] The distance (d) obtained, using this approach,[28] is 0.5 nm which approximately corresponds to the size of a pyranose ring of the Le^X trisaccharide.

In summary, we have been able to quantify the adhesion forces between polyvalent surfaces of Le^X trisaccharide by using atomic force microscopy. The adhesion force measured between two Le^X molecules (20 ± 4 pN) represents a new limit in the sensitivity of force microscopy. The results obtained establish the capacity and selectivity of Le^X antigen for self-recognition supporting the concept of carbohydrate–carbohydrate interactions as a mechanism for cell adhesion and recognition. Furthermore, these results confirm AFM as a powerful tool to measure weak adhesion forces. The measurements of adhesion forces using other carbohydrate antigens are in progress.

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[21] The experiments were performed with a PicoSPM atomic force microscope, from Molecular Imaging, in a Teflon liquid cell. Commercial silicon nitride cantilevers (Olympus) were used for this study. They were rectangular shaped cantilever, 200 μm long, with a 0.05 $N m^{-1}$ nominal spring constant. This real spring constant was calibrated according to the dynamic behavior of the cantilever and to the equipartition theorem.[29, 30] The tips were coated with a five nanometer thick layer of chromium and then a 25 nm thick layer of gold.

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The CH_3N Diradical: Experimental and Theoretical Determinations of the Ionization Energies**

Wang Jing, Sun Zheng, Zhu Xinjiang, Yang Xiaojun, Ge Maofa, and Wang Dianxun*

Methylnitrene, the CH_3N diradical, is one of the most interesting of all organic free radicals, and it has drawn a lot of attention in both experiments[1–5] and theory.[6–11] As the simplest alkyl nitrene, the general class of molecules with the form RN, CH_3N is a dielectronic radical similar to carbene CH_2:, and is thought to be an important intermediate in many organic and inorganic reactions.[12–14] Berry suggests[1] that the alkyl nitrene should have electron states similar to NH which has been well characterized by spectroscopists. However, much less is known about alkyl nitrenes such as CH_3N, $CH_3CH_2{-}N$, $(CH_3)_2CH{-}N$, or $(CH_3)_3C{-}N$. There are no reports on the ionization energy of the nitrenes until now, but these ionization energies, especially the first ionization energy, are important physical and chemical data.

When combined with ab initio molecular orbital calculations, HeI photoelectron spectroscopy (PES) gives a lot of information on the electronic and vibrational structures, the configuration, the bonding characteristics, as well as the relative photoionization cross sections of different molecular orbitals. Of course, the most important information obtained from the PES study is the ionization energy of the different molecular orbitals. Pople[15]and Ng[16] and their co-workers pointed out that the ab initio Gaussian 2 (G2) calculation can correctly predict the first ionization energy.

We have shown how to generate a continuous flowing beam of short-lived species by the microwave discharge or pyrolysis of the parent species, allowing us to perform PES studies on reactive open-shell species such as NO_3,[17] $(CH_3)_2N$,[18] CH_3O,[19] CH_3S,[19] CH_3SS,[20] CH_3CH_2S,[21] $(CH_3)_3CO$[22] and further demonstrated that the G2 calculation can also predict the ionization energies of different ionic states. Herein, we present the experimental and theoretical determinations of the ionization energies of different ionic states for the CH_3N diradical; this is also the first report on ionization energy for the nitrenes.

Calculations on the CH_3N_3 molecule indicate that the bond between the CH_3N and N_2 units is the weakest present and therefore pyrolysis of the CH_3N_3 offers a simple route to the CH_3N diradical through loss of the N_2 unit.[23–26] However, in the pyrolysis study of CH_3N_3 carried out by Bock and co-workers,[24–26] no PES signals of the CH_3N diradical could be detected and PES signals attributed to CH_2NH appeared when CH_3N_3 was heated to 770 K.

Through careful analysis of the experimental process used by Bock et al.[24–26] it is clear that: 1) the distance from the point of pyrolyzation of the parent compound to the photoionized point for the new species generated is too far (maybe more than half meter); 2) the pyrolysis temperature (770 K) for maintaining the new species is also too high. Obviously, it is difficulty to record the PES signals of transient species which have a short life-time, such as the CH_3N diradical. In fact, mixed PE spectra showing CH_3N_3 and CH_2NH (these spectra are the same as Figure 1 reported by Bock and Ralph in ref. [26]) were always obtained from the pyrolysis of the CH_3N_3 at $650(\pm 0.5)\,^{\circ}C$ with the heater installed outside our PE spectrometer.[27] When the PES of the species generated by pyrolysis of CH_3N_3 are recorded in situ (see Experimental Section) a lot of the short-lived active species is detected at a lower temperature in our PE spectrometer.

Figure 1 gives the PE spectrum of the product obtained from the pyrolysis of CH_3N_3 at $145\ (\pm 0.5)\,^{\circ}C$, the spectrum is recorded in situ under protection of the NO species. Noteworthy is that the mixed PE spectra showing both CH_3N_3 and CH_2NH are also obtained at $215\ (\pm 0.5)\,^{\circ}C$ if NO is not

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[*] Dr. W. Dianxun, W. Jing, S. Zheng, Z. Xinjiang, Y. Xiaojun, G. Maofa
The Center for Molecular Sciences
Institute of Chemistry
The Chinese Academy of Sciences, Beijing 100080 (PR China)
Fax:(+86)10-62559373
E-mail: wangdx@xx1.icas.ac.cn

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