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Understanding carbohydrate-carbohydrate Interactions by means of glyconanotechnology

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Carbohydrate-carbohydrate interaction is a reliable and versatile mechanism for cell adhesion and recognition. Glycosphingolipid (GSL) clusters at the cell membrane are mainly involved in this interaction. To investigate carbohydrate-carbohydrate interaction an integrated strategy (Glyconanotechnology) was developed. This strategy includes polyvalent tools (gold glyconanoparticles) mimicking GSL clustering at the cell membrane as well as analytical techniques such as AFM, TEM, and SPR to evaluate the interactions. The results obtained by means of this strategy and current status are presented.
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Abbreviations: **SSEAs: stage-specific embryonic antigens; GSLs: glycosphingolipids; carb-carb: carbohydrate-carbohydrate; Le^x: Lewis X (Galβ1-4[Fucα1-3]GlcNAc); GM3: sialylβ2-3Galβ1-4Glcβ1-Cer; Gg3: GalNAcβ1-4Galβ1-4Glcβ1-Cer; KDNGM3: KDNα2-3Galβ1-4Glcβ1-Cer; PNP: 4-nitrophenyl; *lacto*: lactose; Gal: galactose; GlcNAc: glucosamine; Fuc: fucose; SAMs: self-assembled monolayer; GNPs: glyconanoparticles; WAC: weak affinity chromatography; NMR: nuclear magnetic resonance; ITC: isothermal titration calorimetry; TEM: transmission electron microscopy; AFM: atomic force microscopy; SPR: surface plasmon resonance; *gluco*: glucose; *malto*: maltose; UV-vis: ultraviolet-visible; FT-IR: Fourier transform infrared spectroscopy; ICP: inductively coupled plasma-atomic emission spectrometry; EDTA: ethylenediaminetetraacetic acid; BSA: bovine serum albumin; HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid.**

Introduction

Carbohydrates are, together with nucleic acids and proteins, important molecules for life. Much is already known about the structure, interactions and function of nucleic acids and proteins, however, the role of carbohydrates in the cell is less clear. The surface of mammalian cells is covered by a dense coating of carbohydrates named glycocalyx [1]. In the glycocalyx, carbohydrates appear mainly conjugated to proteins and lipids (glycoproteins, glycolipids and proteoglycans) and it is as glycoconjugates that they develop their biological function. Now it is known that these complex oligosaccharides are involved in the control of many normal and pathological processes [2,3]. A variety of these processes are known to involved carbohydrate-protein interactions [4–6] and, as more recently proposed, some of them imply interactions between carbohydrate molecules [7–10]. The proposal that carbohydrate-carbohydrate interactions are involved in cell adhesion and communication is really challenging taken into account that many stage-specific embryonic antigens (SSEAs) have been identified as glycosphingolipids (GSLs). In the plasma membrane GSLs tend to form microdomains separated from glycoprotein clusters, creating in this way a multivalent array of carbohydrates [11]. GSLs probably play a significant role in defining membrane-based cell functions [12,13].

In 1989 Hakomori [14] proposed that the first phase of cell adhesion initiates through a carbohydrate-carbohydrate interaction between these multivalent GSL-domains. This initial step is followed by protein-protein interactions between adhesion receptors and protein-carbohydrate interactions between lectins and glycoconjugates. Embryogenesis, metastasis and other cellular proliferation processes are according to Hakomori, mediated by carbohydrate-carbohydrate (carb-carb) interactions [7,14–18]. Several glycosphingolipids involved in carb-carb interactions have already been identified. For example, the antigen determinant Le^X seems to mediate morula compaction in

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mouse via a carbohydrate self-interaction [14], and the interaction between the glycosphingolipids GM3 and Gg3 has been proposed to be involved in metastasis of melanoma cells in mouse [15,17]. It has been demonstrated more recently that the interaction between KDNGM3 and Gg3 is involved in the binding of sperm to egg membrane in rainbow trout fertilization [18].

Outside from the eukaryotic world other example where carb-carb interactions are involved is the specie-specific cell aggregation of marine sponges [8,19,20]. It seems that a large extra cellular acidic proteoglycan is responsible, via a Ca^{2+}-dependent, homophilic and polyvalent carb-carb interaction, for this aggregation. A sulfated disaccharide and a pyruvated trisaccharide have been identified as the repetitive epitopes of the glycan responsible for aggregation [21,22].

Characteristic features of these interactions are their specificity, their strong dependency on divalent cations, and their extreme low affinity that has to be compensated by multivalent presentation of the ligands. The study of carbohydrate-carbohydrate interactions faces the important challenge of its extreme low affinity and attempts to characterize and quantify this interaction in solution with monomeric ligands have usually failed [23–25]. Multivalent presentation has been proved to be important in the study of carbohydrate-protein interaction [26,27]. However, polyvalence seems even more mandatory in the case of carbohydrate-carbohydrate interactions.

When the original proposal by Hakomori was first presented, we were working on the complexing properties of a new type of synthetic receptors that we named glycophanes [28] because they may be considered as hybrids of two important artificial receptors: cyclodextrins and cyclophanes. These small receptors were able to bind, in water, a series of 4-nitrophenyl glycosides (PNP-glycosides) with different affinities. We observed that the presence of the pyranose ring contributed to the free energy of binding of the PNP-glycosides to the glycophanes with an additional stabilisation up to 2 kcal/mol related to the free energy of binding of 4-nitrophenol [29,30]. This stabilisation was not present in the complexes between cyclodextrins and PNP-glycosides. Thus, we concluded from these results that interactions between carbohydrate molecules in water could be responsible for the stabilizing effect. We also demonstrated that this stabilisation was due to carb-carb interactions between lipophilic patches of host and guest carbohydrate moieties [29].

These results encouraged us to go more deeply into the study of carb-carb interactions. The ideas expressed by Hakomori, that interactions between oligosaccharides constitute a mechanism for cell adhesion and recognition seemed extremely attractive despite the serious initial criticism that the proposal received from part of the scientific community. Therefore we set up to investigate, from a chemical point of view, the existence of carb-carb interactions in water between oligosaccharides proposed as the epitopes responsible for these associations *in vivo.* The logical approach to address the problem should proceed through the preparation of models with multivalent presentation of the carbohydrate ligands.

Since the seminal work of Lee on the cluster effect [4] a plethora of multivalent model systems based on peptides, proteins, liposomes, dendrimers, or polymers [26,31–33], have been prepared to study and evaluate carbohydrate-protein associations. We first approached the problem of multivalent presentation by using liposomes, but liposomes are not very stable systems and we had to face up several problems, so that we decided to investigate some other original approaches. As a result we developed a new integrated approach based on the use of nanoparticles that we have named the Glyconanotechnology strategy.

This review summarizes our efforts during the last five years, on the study of the Ca^{2+}-mediated carbohydrate-carbohydrate interactions by means of this strategy, and the results obtained in the case of the disaccharide lactose (*lacto*: Galβ1-4Glc) and the trisaccharide Lewis X (Le^X: Galβ1-4[Fucα1-3]GlcNAc) (Figure 1). Le^X antigen mediates; through a homotypic carb-carb interaction, morula compaction [14] and the aggregation of F9 teratocarcinoma cells [34] while lactosylceramide is involved in melanoma lung metastasis in mice [17]. Le^X epitopes expressed on teratocarcinoma F9 cells are carried out mainly by "embryoglycan" in addition to a relatively small amount of GSL [35]. Autoaggregation of F9 cells in the presence of Ca^{2+}, mimicking the embryonal compaction process, was shown to be due to Le^X-Le^X interaction based on Ca^{2+}-dependent autoaggregation of isolate embryoglycan per se [25]. Embryoglycan, the major carrier of Le^X epitope, has highly branched polylactosamine with many Le^X at the end. This presentation may mimic Le^X GSL cluster on the membrane. Lactose and Le^X trisaccharide are the carbohydrate components of the glycosphingolipid Le^X.

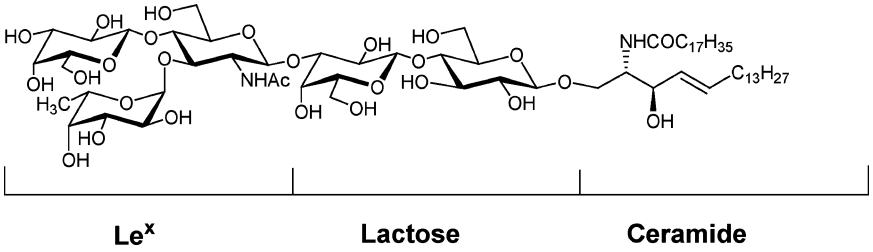

Figure 1. The structure of glycosphingolipid Le^X and its components.

The Glyconanotechnology strategy

Effectively designing model systems to study biological interactions requires a careful consideration of the special characteristics of the biological system to mimic. The low affinity of monovalent carbohydrate-carbohydrate interaction demands a careful manipulation of polyvalence to be effective. Of major importance are also the techniques used to evaluate the interaction. With this in mind, we designed an integrated approach that included both chemical models mimicking GSLs patches at the cell membrane and new surface analytical techniques to evaluate their interactions.

To create polyvalence we designed two simple systems, based on self-assembled monolayers (SAMs), by which neoglycoconjugates of the natural epitopes are attached to bi-dimensional (2D) and tri-dimensional (3D) gold surfaces creating in this way multivalent array of carbohydrates mimicking GSL patches at the cell surface (Figure 2). 2D self-assembled monolayers on gold have already been used, with success, by Whitesides and others to study different biological interactions [36,37].

The polyvalent 3D model systems are based on sugar modified gold nanoclusters [38]. The so-called glyconanoparticles (GNPs) provide a glycocalyx-like surface with globular carbohydrate display and chemically well-defined composition to study carbohydrate interactions and to interfere cell-cell adhesion processes [39]. The two models are highly complementary and the use of both of them to measure biologically relevant interactions demands the application of new surface and solution techniques. The combination of these 2D and 3D polyvalent model systems together with classical analytical techniques such as weak affinity chromatography (WAC) [40], nuclear magnetic resonance (NMR) [41], isothermal titration calorimetry (ITC) [42] and other techniques as transmission electron microscopy (TEM) [43], atomic force microscopy (AFM) [44], or surface plasmon resonance (SPR) [45] allowed us to demonstrate and to quantify Ca^{2+}-mediated carbohydrate-carbohydrate interactions (Figure 3).

The neoglycoconjugates

To construct the sugar protected 2D and 3D model systems a series of neoglycoconjugates were prepared consisting of oligosaccharides functionalized with different linkers (hydrophobic and hydrophilic in nature). The linkers were endowed with a thiol group for attaching the oligosaccharides to the gold surfaces through a Au-S covalent bonds. The neoglycoconjugates containing the disaccharide lactose and the trisaccharide determinant Le^X, the two components of the GSL-Le^X, were synthesized and isolated as disulfide derivatives (Figure 4) [38,41]. Similar neoglycoconjugates of the non-antigenic disaccharide maltose and the monosaccharide glucose were also prepared as control systems.

The spacers used were 11-thioundecanol, 11-thio-3,6,9-trioxaundecanol and (1-thioundec-11-yl)hexa(ethylene glycol). 11-Thioundecanol has been shown to be an appropriate linker to form well-organized monolayers on gold [37]. The neoglycoconjugates thus prepared were used in the preparation of both GNPs and SAMs for AFM, TEM and biosensor experiments.

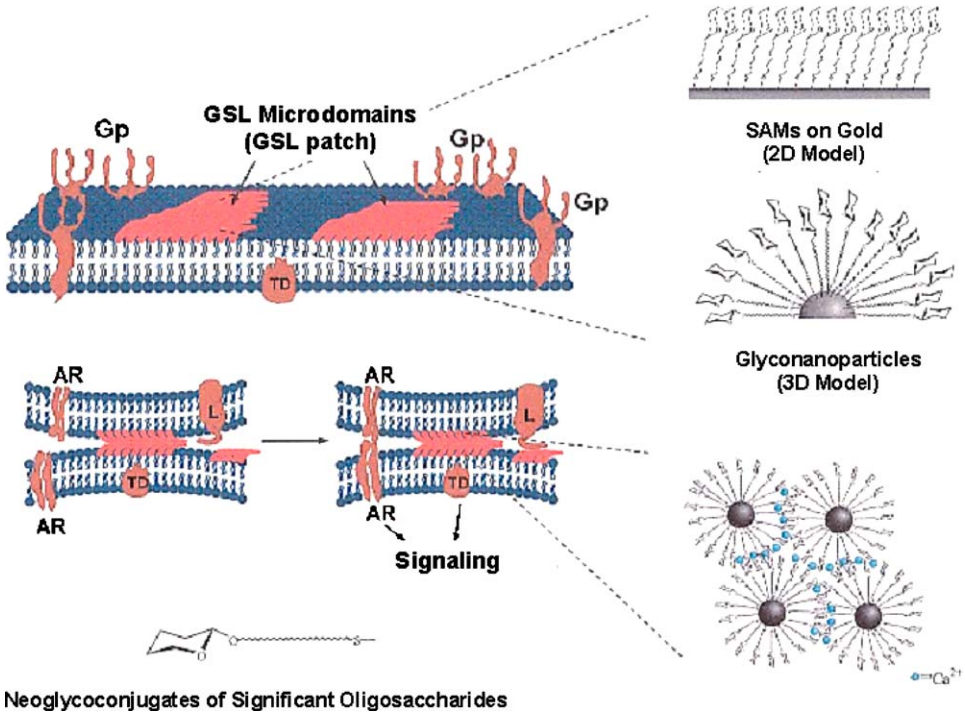

Figure 2. The 2D and 3D models that mimic GSLs presentation for studying carbohydrate-carbohydrate interactions.

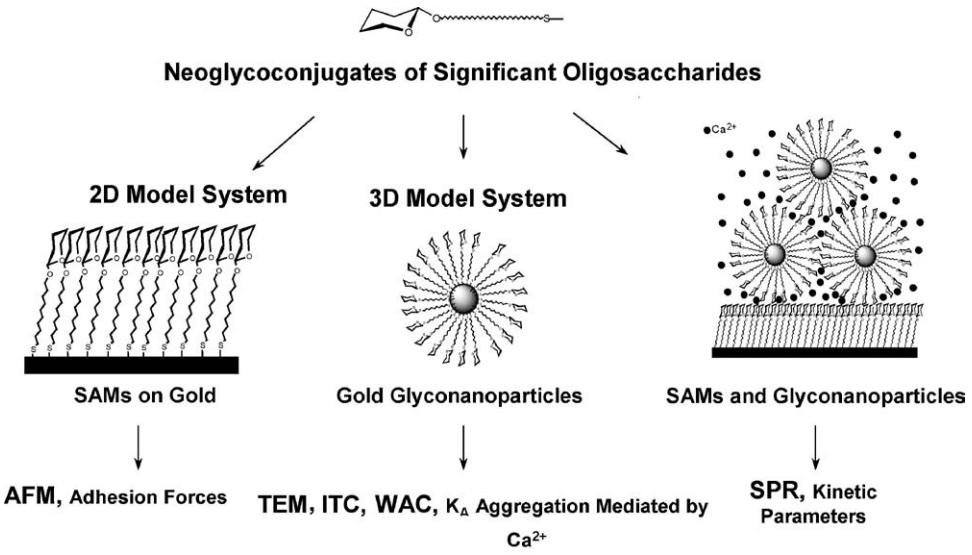

Figure 3. The glyconanotechnology strategy for studying carbohydrate-carbohydrate interactions [38].

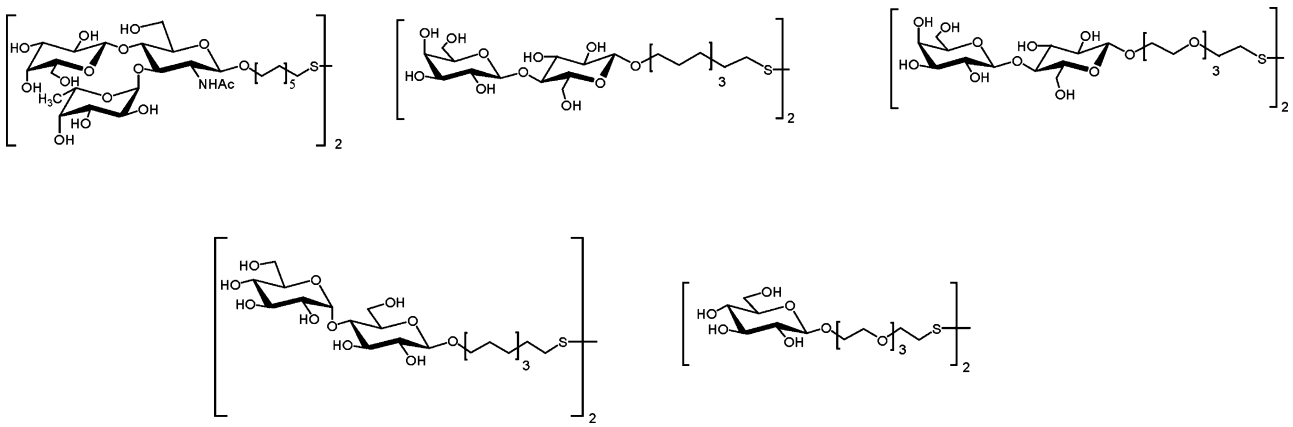

Figure 4. Neoglycoconjugates prepared to study carbohydrate-carbohydrate interactions.

Studying and evaluating the carbohydrate-carbohydrate interaction

Several studies of carbohydrate-carbohydrate interactions using model systems have already appeared in the literature [10,46–48].

Our first attempts to investigate carbohydrate-carbohydrate interactions using the above neoglycoconjugates and gold glyconanoparticles were carried out by means of Weak Affinity Chromatography (WAC) [49]. An affinity column functionalized with the Le^X neoglycoconjugate was used and Le^X glyconanoparticles were eluted through this column. An affinity constant value ($Ka = 10\ M^{-1}$) for the Ca^{2+}-mediated Le^X-Le^X interaction was obtained from the difference between the observed retention times in the presence and in the absence of calcium cations [40].

Evaluation of adhesion forces by atomic force microscopy (AFM) [44]

The adhesion forces between 2D gold surfaces functionalized with the oligosaccharides were first evaluated using AFM [44,50]. AFM is a form of scanning probe microscopy where a very sharp tip is attached to the end of a cantilever. The optical monitoring of the cantilever deflection while the tip scans the sample surface allows for producing either topographic images or force-distance curves. The microscope incorporates a sample cell to perform experiments in solution.

To evaluate the adhesion forces between carbohydrate molecules, first a thin gold layer was deposited on the tip and on a freshly cleaved mica surface. The tip and the sample were then immersed in a solution of the thiol derivatized oligosaccharides to form the self-assembled monolayers. Once the sample

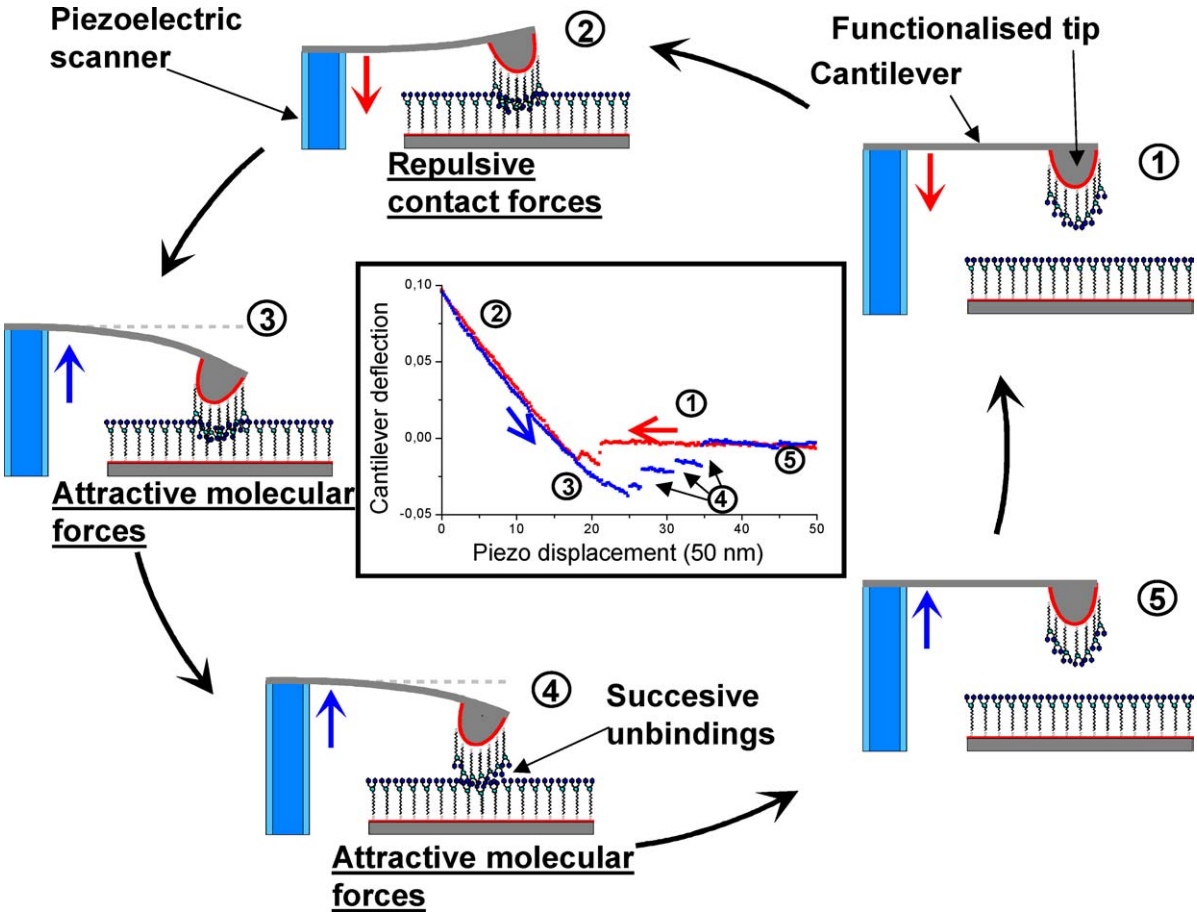

Figure 5. AFM experiment to measure carbohydrate-carbohydrate interactions.

was placed in the liquid cell, the tip was approached to the sugar functionalized sample. After the contact event the tip was withdrawn and the cantilever deflection, due to the interaction between the carbohydrate molecules, was monitored (Figure 5).

When attractive forces exist, the force-distance curves show a stepwise profile due to the successive unbinding of molecules. Thermal and mechanical vibrations make necessary the analysis of the experimental histograms to obtain an autocorrelation function. The periodicity of this function corresponds to the binding forces between two single molecules. Measurements were carried out with the tip and the sample functionalized with the Le^X neoglycoconjugate and the force-distance curves were recorded in water and 10 mM Ca^{2+} solution. In both cases an attractive interaction was observed and curves were obtained with a series of steps corresponding to successive molecules unbinding (Figure 6a and b).

A second series of control experiments were recorded on a tip and sample functionalized with monolayers formed either by the linker, the undecanol thiol chain, or by the lactose neoglycoconjugates. The curves obtained clearly indicated no adhesion forces between the tip and the sample (Figure 6c and d). For the Le^X-Le^X interaction, analysis of the histograms obtained from more than 300 experimental curves, measured in fourteen different areas of the sample, gave an autocorrelation function with a pronounced periodicity of 20 ± 4 pN in water and in Ca^+ solutions (Figure 7). The peaks of the histograms corresponded to multiples of the 20 pN: the interaction force between two single Le^X molecules.

But the most important result was the specificity observed in the Le^X self-interaction. Cross-experiments between Le^X functionalized tips and lactose functionalized samples did not show any adhesion forces between the surfaces (Figure 6d).

An adhesion force of 20 pN, although small, is significant taken into account that the adhesion forces between the proteoglycan of the *Microciona prolifera* sponge, measured also by AFM, was found to be 40 pN [51]. This value of 20 pN determined for the interaction force between two Le^X molecules indicates that only five pairs of Le^X molecules would be necessary to reach the binding strength (100 pN) between neural retina cells of embryonic chicken [52], and 16 Le^X pairs (320 pN) would be sufficient to hold T and B lymphocyte cells together in the absence of antigen stimulation [53].

These results clearly establish both the capacity and selectivity of the Le^X trisaccharide for self-recognition and confirm this antigen as an adhesion molecule. The ability of the antigen Le^X for self-recognition was also confirmed by means of TEM

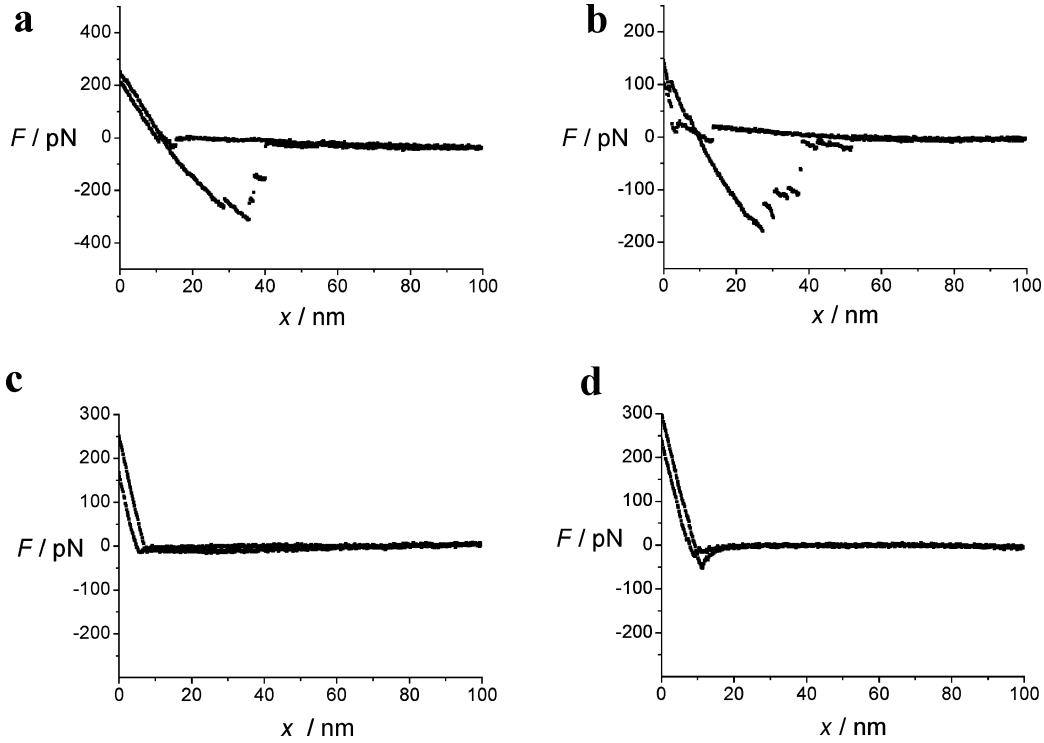

Figure 6. The adhesion curves obtained for: (a) and (b) the Le^X-Le^X; (c) lactose-lactose and (d) Le^X-lactose interactions.

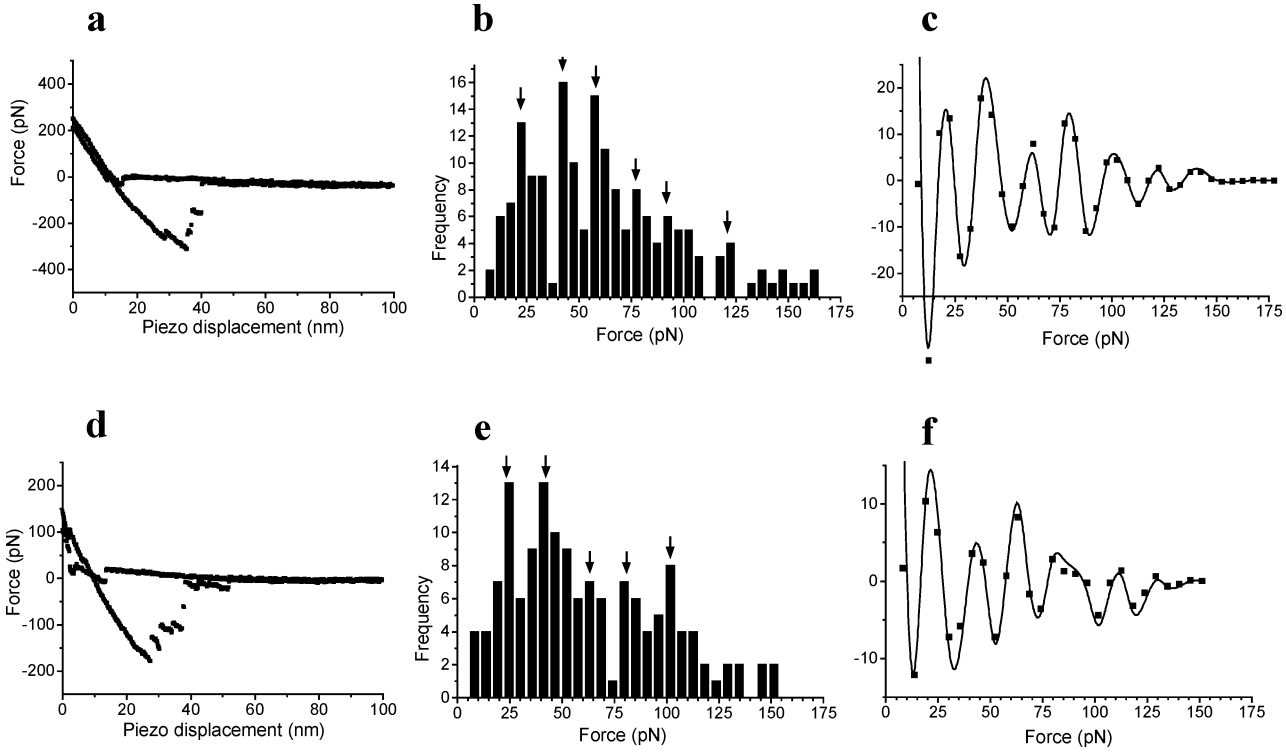

Figure 7. Quantification of the Le^X-Le^X adhesion forces by AFM: (a) and (d) Typical AFM force-distance curves obtained between an Le^X-functionalized tip and a SAM of Le^X molecules in water and in 10 mM calcium solution, respectively, (b) and (e) histogram of the unbinding force measured through 300 force-distance curves of Le^X molecules in water and in 10 mM calcium solution, respectively, and (c) and (f) corresponding auto-correlation function [44].

in a series of experiments carried out with the 3D model system: *the gold glyconanoparticles* (GNPs).

Gold glyconanoparticles

Searching for appropriate multivalent systems with well-defined chemical composition, we have developed a technology to prepare gold nanoclusters presenting carbohydrates in a globular and polyvalent configuration on their surface. The methodology is based on the Brust's procedure for the synthesis of monolayer-protected gold nanoclusters [54]. The GNPs are prepared by *in situ* reduction of a gold salt in the presence of an excess of the corresponding thiol-derivatized neoglycoconjugate [38]. The so-prepared GNPs are endowed with exceptionally small core (≈2 nm and less), are highly soluble in water, and stable for months under physiological conditions without flocculation. GNPs present some advantages over other currently available polyvalent systems incorporating carbohydrates, such as liposomes: (1) control of ligand number and nanoparticle size, (2) higher degree of multivalence and easy chemical characterization, (3) high storage stability, (4) high biological stability against enzyme degradation, and (5) they are not cytotoxic.

This methodology provides a versatile way to prepare in one step a great variety of water soluble and polyvalent carbohydrate arrays with globular shape. Hybrid glycoclusters incorporating other molecules (fluorescent probes, peptides, DNA, RNA etc.) can also be prepared by this technology [38]. Manipulation of the ratio of the ligands permits preparation of GNPs with differing carbohydrate density at the surface providing an under-control model for investigating the influence of density and presentation on molecular recognition events. We have prepared gold glyconanoparticles functionalized with the monosaccharide glucose (***gluco*-Au**), the disaccharides maltose and lactose (***malto*-Au** and ***lacto*-Au**) and the trisaccharide Le^X (**Le^X-Au**) [38] to investigate the selective self-recognition of the Le^X antigen *via* carbohydrate-carbohydrate interaction (Figure 8).

The glyconanoparticles (GNPs) were characterized using ^{1}H-NMR, UV-Vis, FT-IR, TEM, ICP and elemental analysis. Figure 9 shows the TEM images and size distribution histograms of the gold nucleus, and the corresponding ^{1}H-NMR spectra for the glyconanoparticles **Le^X-Au** and ***lacto*-Au**. The histograms indicated a narrow size distribution and an average diameter for the metallic core of 1.8 nm. This diameter corresponds with an average number of 201 gold atoms per particle [55]. Elemental analysis for **Le^X-Au** and ***lacto*-Au** confirmed a relation of 97 Le^X molecules and 75 lactose molecules per nanoparticle, corresponding to molecular weights of 109 kD and 75 kD, respectively.

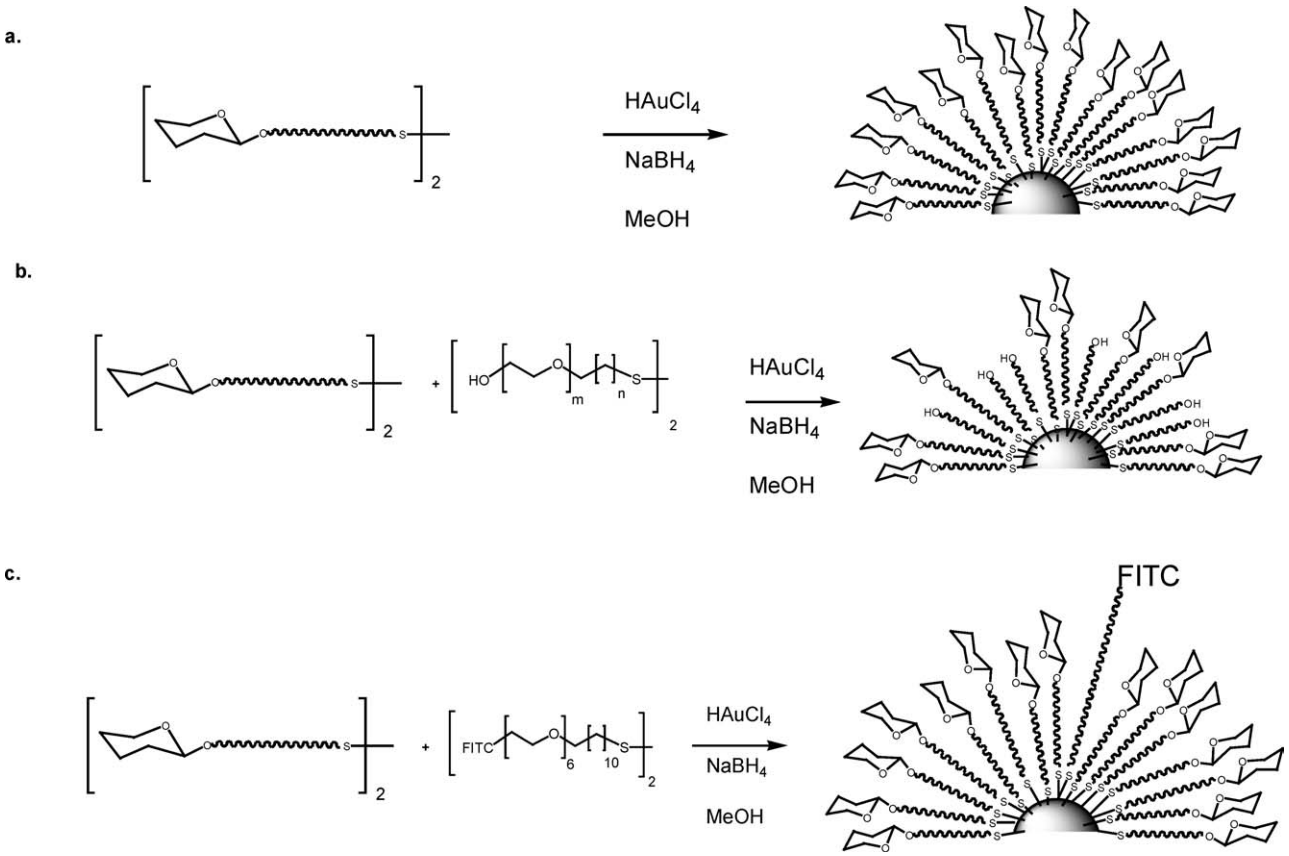

Figure 8. Some examples of the so-prepared gold glyconanoparticles.

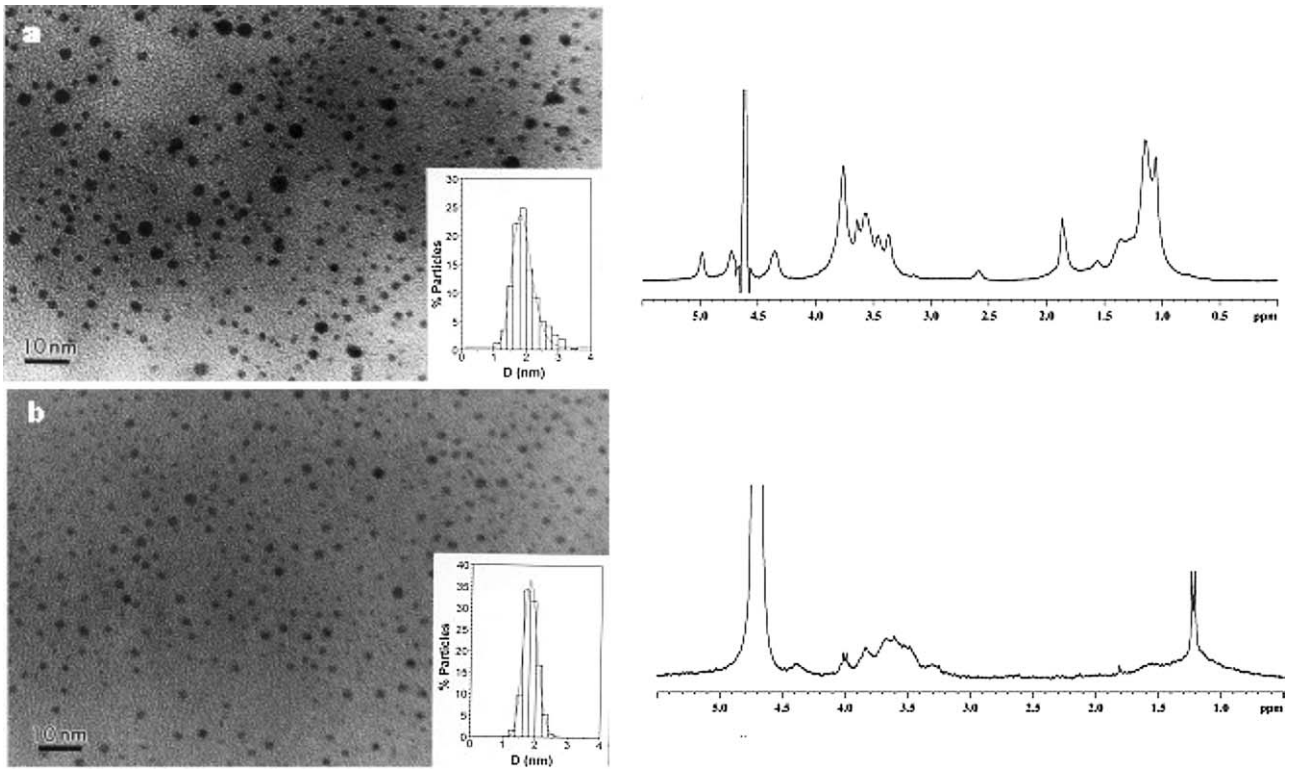

Figure 9. TEM micrographs and ^{1}H-NMR of: (a) **Le^X-Au**, and (b) ***lacto*-Au** glyconanoparticles.

The synthesized GNPs (***gluco*-Au, *malto*-Au, *lacto*-Au, Le^x-Au**) are soluble in water, but insoluble in methanol. The aqueous solutions are stable for years as it was confirmed by TEM. This stability is notable as compared with that of most gold nanoparticles described in the literature, which usually grow with the time to finally flocculate. The Le^X protected particles with their chemically well-defined matrix constitutes a polyvalent system to mimic in solution cell-cell aggregation via carbohydrate-carbohydrate interactions, while the *gluco*, *malto* and *lacto* GNPs have been used as control systems.

Self-aggregation studies of Le^{X}GNPs by TEM [43]

Transmission electron microscopy has also allowed observing directly the interaction events between glyconanoparticles in the absence and in the presence of Ca^{2+} cations. We have studied the self-aggregation of the Le^X nanoparticles (**Le^X-Au**) and compared the results to those obtained for the control system, ***lacto*-Au** [43]. The GNPs were dissolved, at different concentration, in 10 mM Ca^{2+} solution, left overnight and aliquots of the resulting solution were observed on the microscope. The TEM micrographs obtained revealed clear differences between the lactose and the Le^X samples. The Le^X particles in Ca^{2+} solution showed beautiful large three-dimensional aggregates at all concentrations, while the *lacto* particles remained dispersed as in the water solution (Figure 10).

That these aggregates are formed as a consequence of the recognition properties of Le^X and the Ca^{2+} cations and not as a result of non-specific interactions with the salt was demonstrated as follows: In contrast to the usually observed salt-induced flocculation of gold nanoclusters, neither Le^X nor lactose nanoparticles flocculate after several months either from water or from Ca^{2+} solutions. Removal of the Ca^{2+} cations from the solution by addition of EDTA results in the dispersion of the Le^X aggregates (Figure 10d). Therefore, only sequence-specific binding events between Ca^{2+} cations and the Le^X molecules dictate the self-assembly process. Isothermal titration calorimetry (ITC) has confirmed that this process in solution is a favourably enthalpy driven process [42]. Therefore, the use of TEM has also confirmed the ability of the antigen Le^X for self-recognition. Quantitative data on the kinetics of this interaction were obtained by means of SPR.

Kinetic studies of Le^X-Le^X interaction by means of biosensors with SPR detection [45]

Biosensor measurements using surface plasmon resonance (SPR) as detection provide important information about molecular interactions [56] including carbohydrate-protein interactions [57]. However, the analysis of the low affinity carbohydrate-carbohydrate interactions by means of SPR have been reported, to the best of our knowledge, only in two cases. In the first report, sensor chips functionalized with

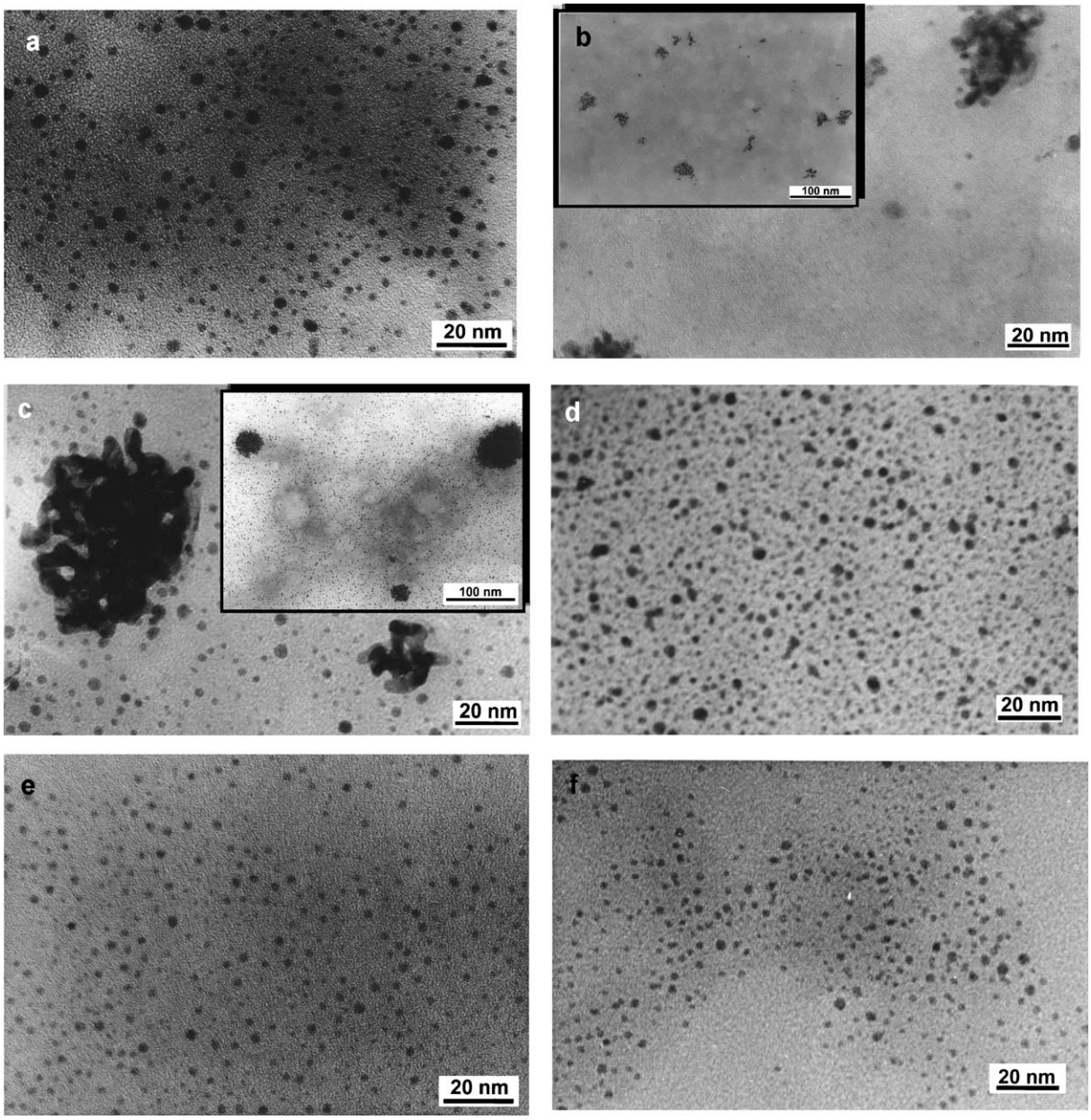

Figure 10. Transmission electron micrographs of: (a) **Le^X-Au** (0.1 mg mL^{-1} in water); (b) **Le^X-Au** (0.1 mg mL^{-1} in 10 mM $CaCl_2$ solution); (c) **Le^X-Au** (0.9 mg mL^{-1} in 10 mM $CaCl_2$ solution); (d) the same as (c) + EDTA; (e) ***lacto*-Au** (0.1 mg mL^{-1} in water); (f) ***lacto*-Au** (0.9 mg mL^{-1} in 10 mM $CaCl_2$ solution); EDTA = ethylendiaminetetraacetate [43].

glycolipid monolayers and glycoconjugate polymers were used as substrate and analyte respectively [58]. In the second, a BSA-glycoconjugate bound to dextran functionalized chips was used as substrate and the BSA-conjugate as analyte [59].

To measure the kinetics of Le^X-Le^X interaction, a simple strategy was designed by using our 2D and 3D models described above. The combination of SAMs of the neoglycoconjugates on a biosensor gold surface as substrate, gold glyconanoparticles as the analyte and SPR detection turned out to be an excellent strategy to evaluate in a well defined system the Ca^{2+}-mediated carbohydrate-carbohydrate interactions (Figure 11) [45].

Sensor chips functionalized with SAMs of Le^X and lactose neoglycoconjugates were prepared as substrates. As polyvalent analytes Le^X and *lacto* GNPs were used. The methyl glycosides of the trisaccharide Le^X and the disaccharide lactose were the monovalent analytes. Sensor chips functionalized with SAMs of the linkers, the 11-thio-tetraehylene glycol and 11-thioundecanol, were used as control surfaces (Figure 11). The SPR experiments were carried out in a Biacore instrument at 25°C. A J1 biosensor chip was functionalized with the Le^X

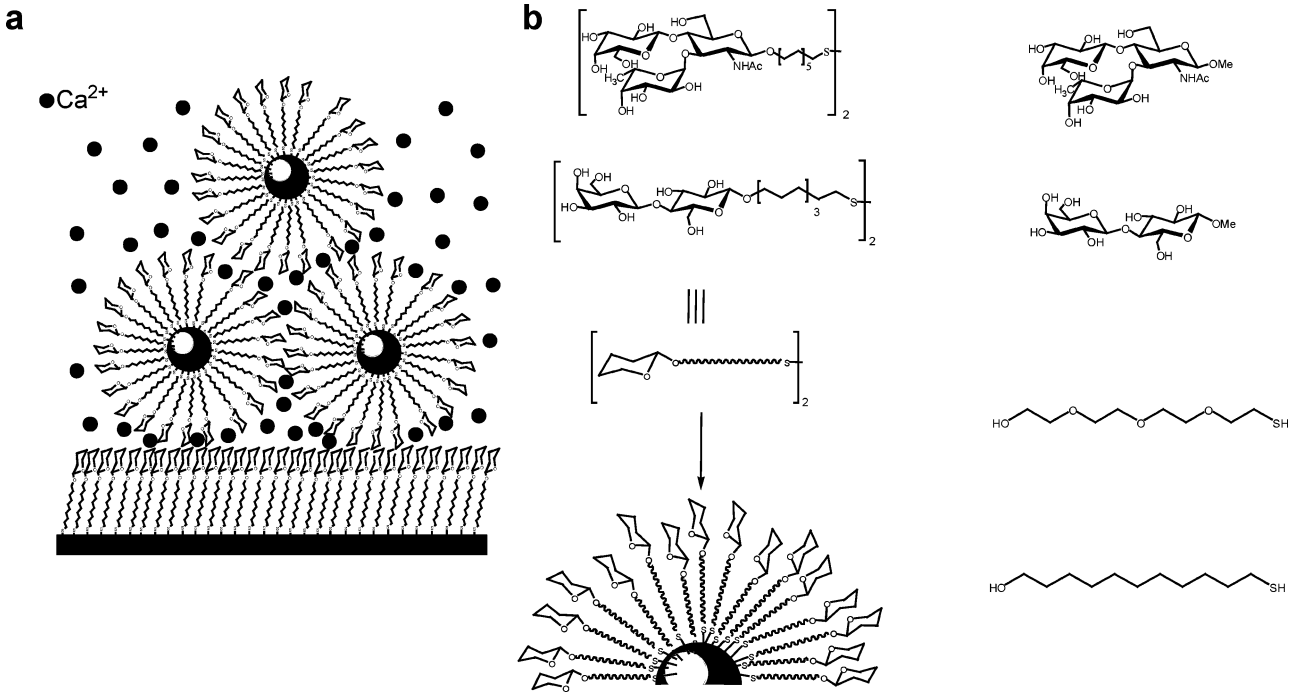

Figure 11. Strategy for measuring carbohydrate-carbohydrate interactions with biosensors: (a) Schematic representation of our binding event strategy; (b) structures of carbohydrate ligands used in this study.

and *lacto* neoglycolipids at a flow rate of 3 μL/min until a SPR response was obtained. The binding of the analytes, **Le^X-Au** and ***lacto*-Au** nanoparticles, were measured in HEPES buffer in the presence and absence of calcium cations. The sensorgrams obtained for the binding of Le^X GNPs to the Le^X -SAMs indicated a slow association phase (k_{on} 2.3 $\times$ 10^3 $M^{-1}s^{-1}$) and a gradual dissociation phase (k_{off} 1.5$\times$ $10^{-3}s^{-1}$) in the presence of calcium cations (Figure 12). The binding was of high affinity with dissociation constant in the micromolar range (*Kd*, 5.4 $\times$ 10^{-7} M). In contrast, binding of the monovalent analyte, methyl glycoside of Le^X, showed very fast association and dissociation phases, which indicated a weak interaction (*Kd*, 5.7 $\times$ 10^{-3} M). The binding of the *lacto* GNPs to the *lacto*-SAMs exhibited a rapid association phase and dissociation phases and the affinity constant (*Kd* 1.4 $\times$ 10^{-5} M) dropped two order of magnitude related to the Le^X-Le^X binding. Cross experiments between immobilized Le^X-SAMs and *lacto* nanoparticles and vice versa gave dissociation constants in the milimolar range. These results indicate that the affinity of Le^X antigen for self-recognition is in the micromolar range and 200 times higher than its affinity for *lacto*-monolayers. The Le^X-Le^X self-recognition is calcium dependent and in the absence of calcium cations Le^X GNPs bind to all functionalized surfaces as they do to the bare gold surface.

The data presented here clearly confirm that Le^X self-aggregation in water is a selective, stabilizing, multivalent, and Ca^{2+}-dependent event and support the proposal of Hakomori that carbohydrate *trans*-associations may be a mechanism for membrane to membrane interactions.

The glyconanoparticle concept

GNPs constitute a good bio-mimetic model of carbohydrate presentation at the cell surface, opening news avenues in the field of chemical Glycobiology [60] and Biomedicine. The construction of carbohydrate multivalent systems on gold nanoclusters, allows also the manipulation of both the organic shell and the metallic core to obtain nano-bioconjugates with semiconductor and magnetic properties [61]. A plethora of potential applications in Biotechnology [39] as well as in Material Science [62] can also be envisaged, using these new biomaterials (Figure 13).

The combination of a variable metallic core with a set of selected ligands may permit the preparation of anti-adhesion agents, probes for cellular labelling, polyvalent vaccines, or carriers to directly deliver drugs at their target location (Figure 13).

The development of the glyconanoparticle concept here presented is one of the main areas of research in our laboratory. The first practical development using this concept has been the application and validation of GNPs as anti-adhesion tools against metastasis progression.

Glyconanoparticles as tools in anti-adhesive therapy [39]

Metastasis is the origin of the bad prognosis of most cancers. One of the critical steps in metastasis is the adhesion of tumour cells to the vascular endothelium. After adhesion, tumour cells transmigrate and create new tumour foci (Figure 14) [63]. Interactions between tumour-associated antigens and epithelial cell selectins promote tumour cell metastasis [64]. In addition

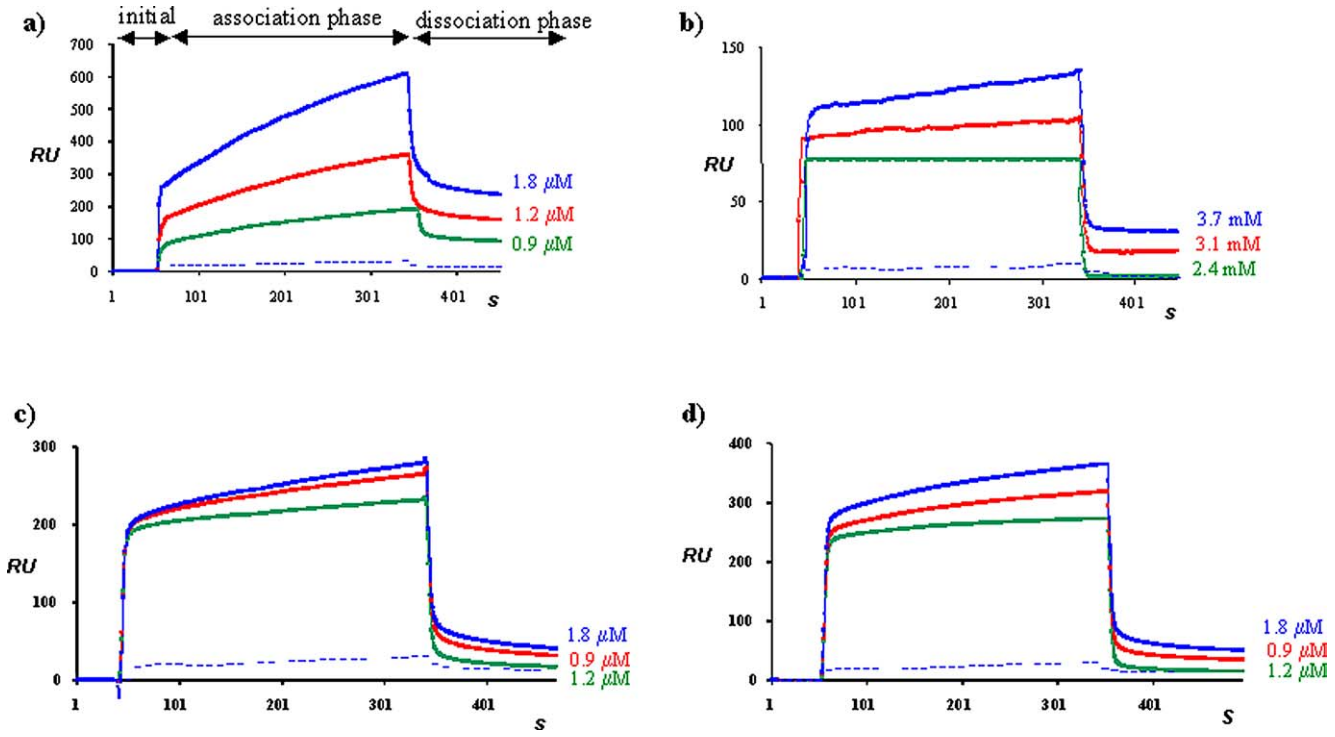

Figure 12. SPR sensorgrams for the interaction of: (7a) **Le^X-Au** to SAMs of **Le^X**; (7b) **Le^{X}OMe** to SAMs of **Le^X**; (7c) ***lacto*-Au** to SAMs of lactose; (7d) **Le^X-Au** to SAMs of lactose, without (- - - -) and with Ca^{2+} ions (—).

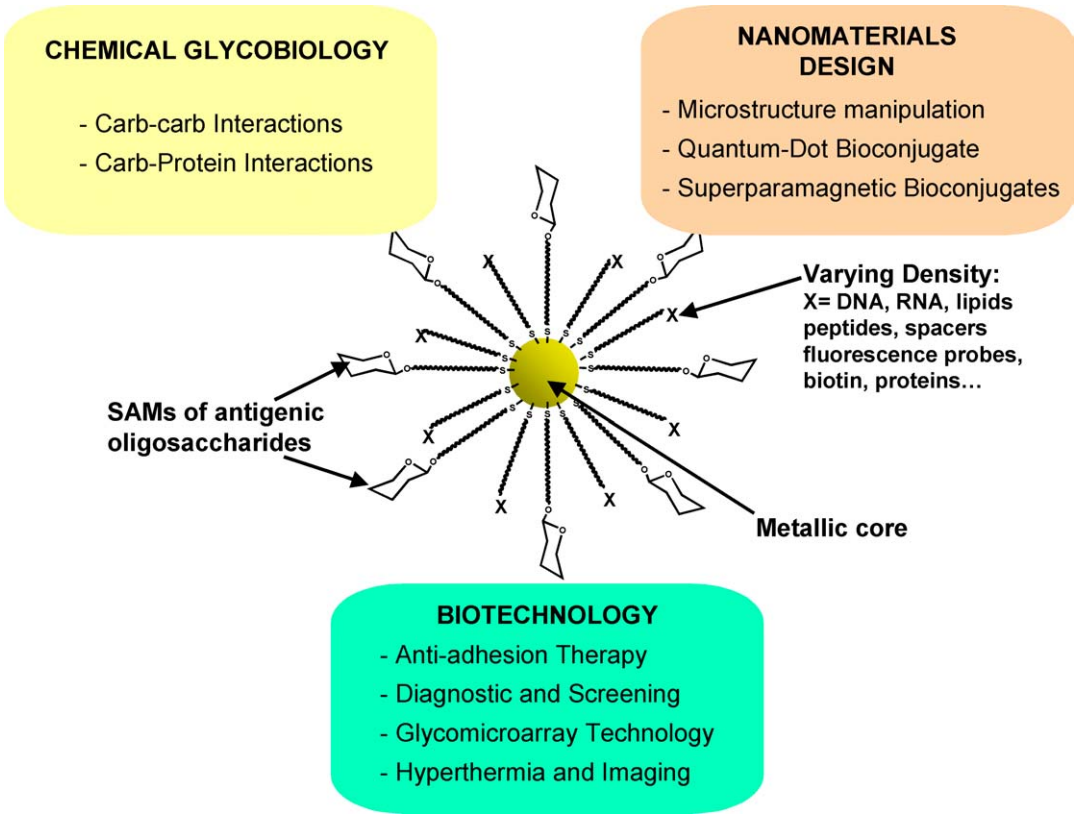

Figure 13. The glyconanoparticle concept and its potential applications.

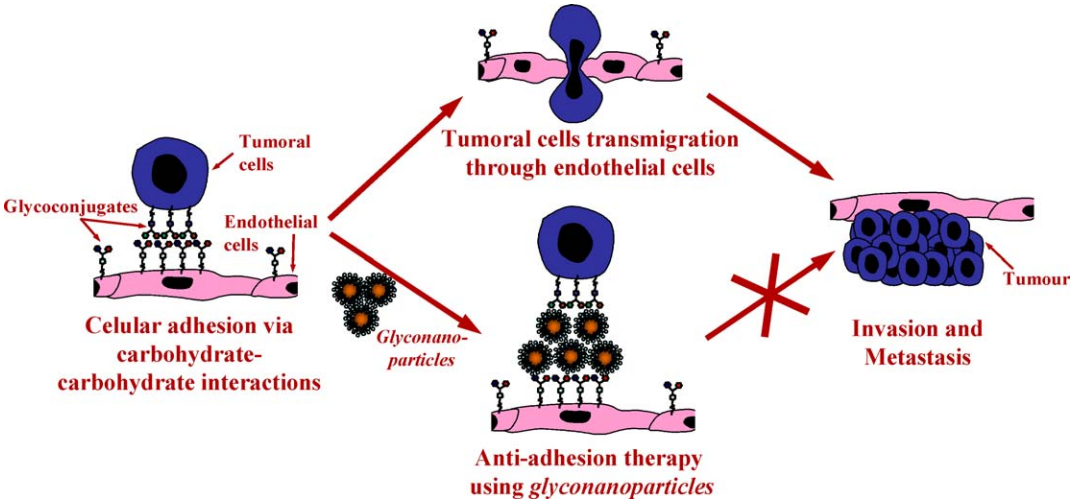

Figure 14. Possible action mechanism of ***lacto*-Au** in anti-adhesive therapy.

to this mechanism, carbohydrate-carbohydrate interactions between GSLs expressed on the tumour and endothelial cell surfaces also seem to be involved in the critical adhesion step [13]. A carbohydrate-carbohydrate interaction between GM3 expressed in a murine melanoma cell line (B16) and Gg3 or lactosylceramide of endothelium cells has been proposed to be involved in the first adhesion step of tumour cells to endothelium before transmigration [65,66].

Therefore, inhibition of this step by GNPs that present carbohydrate antigens expressed either in the tumour or the endothelium cells might provide effective anti-adhesion therapy. Based on the involvement in cell adhesion of the antigen lactosylceramide, ***lacto*-Au** GNPs were tested as a potential inhibitor of the binding of melanoma cells to endothelium. The ***malto*-Au** and the ***gluco*-Au** nanoparticles have also been tested as control systems.

Cytotoxicity studies were first carried out to see the influence of the GNPs on the morphology or growth kinetics of the melanoma cells. No significant alterations were observed when B16F10, COS-1, embryonic F9 or NIH-3T3 cells were incubated with *lacto* or *gluco* GNPs. In contrast, the *malto* GNPs induced negative effects after 6–24 h of incubation.

An *ex vivo* experiment was designed for the evaluation of the anti-metastasis potential of the ***lacto*-Au** GNPs. A control group of mice was injected with B16F10, a very aggressive melanoma cell line. Other four groups of mice were treated with single doses of melanoma cells pre-incubated with *lacto* or *gluco* GNPs respectively, the later as a positive control. The experimental metastasis process was allowed to develop during three weeks, the animals sacrificed and both lungs evaluated under the microscope for analysis of tumour foci. The groups treated with *gluco* GNPs developed a high number of new tumour foci similar the control group (only treated with melanoma cells). However, the mice treated with B16 cells pre-incubated with *lacto* GNPs at two different concentrations presented up to 70% of tumour inhibition in the case of the higher concentration, as compared with the group inoculated only with B16 cells (Figure 15).

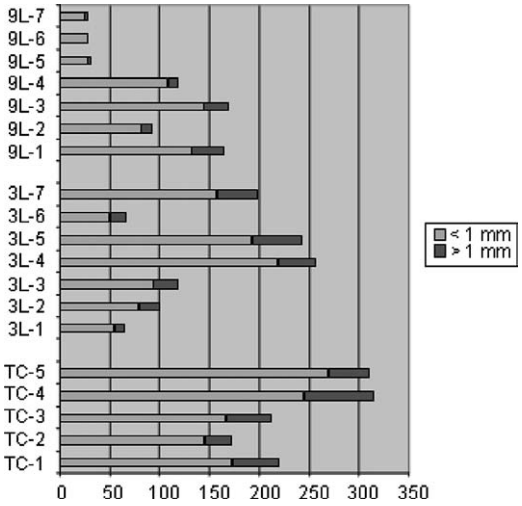

Figure 15. Quantitative analysis of the antimetastatic effect of ***lacto*-Au** on the B16F10-dependent development of lung tumoral foci. Individual scoring of tumoral foci for live animals corresponding to the TC control group [TC-1 to TC-5] and seven animals treated with 30 μM [3L-1 to 3L-7] or 90 μM [9L-1 to 9L-7] solution of ***lacto*-Au** [39].

This result is very promising and demonstrates both the specificity and the anti-adhesion properties of the ***lacto*-Au** GNPs in the selected model which can be improved in later generation of GNPs presenting GM3 or Gg3 glycosphingolipids.

Conclusions

The glycocalyx is the most outer part of the cell. Its location and complexity induces to think that carbohydrates have to be involved in cell adhesion processes. Over the last years,

the involvement of carbohydrate-protein interactions in many normal and pathological processes has been well established. Why to exclude the interaction between carbohydrates? If carbohydrate-carbohydrate interactions are involved in bacterial and virus infection, tumour cell adhesion, metastasis, etc., the understanding of the molecular basis of this association should lead to the understanding of the role of glycoconjugates in the initial step of cell recognition.

Carbohydrate-carbohydrate association is a reliable and versatile mechanism for cell adhesion and recognition. Its weak and polyvalent character makes the study of this interaction a big challenge. We accepted this challenge developing the gold glyconanoparticles (GNPs) as a tool for basic studies on specific carbohydrate-carbohydrate interactions. The globular shape and the polyvalent display, convert GNPs into powerful tools to overcome the low affinity binding of oligosaccharides. Furthermore, the glyconanoparticle concept has the potential to integrate the current knowledge and application on processes that involve a carbohydrate molecule and the recent development in biotechnology.

The "proof of principle" established here for the possible applications of GNPs allows us to anticipate important advances in a field that may be specifically named Glyconanobiotechnology and that will complement current gene-oriented Nanotechnology [67–69].

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