Abstract: Thermodynamic evidence for the selective Ca$^{2+}$-mediated self-aggregation via carbohydrate–carbohydrate interactions of gold glyconanoparticles: a model for cell adhesion via carbohydrate–carbohydrate interaction

Introduction

Cells use multiple mechanisms to bind to extracellular matrices and to other cells. Adhesion and recognition are important events in migration, differentiation, self-assembly, and communication of cells. Protein–protein interactions constitute an important mechanism for cell adhesion that controls cellular behavior. Because most cell surface proteins are highly glycosylated (glycoproteins), carbohydrate–protein interactions are also involved in cell adhesion and recognition. Glycoproteins and glycolipids are ligands for toxins, bacteria, viruses, and other cells through carbohydrate–protein interactions. In addition, interactions between carbohydrates are emerging as a versatile mechanism for cell adhesion and recognition. Carbohydrate–carbohydrate interactions between cell surface glycolipids mediate cell adhesion during embryogenesis, metastasis, and signal transduction. Most of the structures involved in this novel mechanism are glycosphingolipids (GSL) organized in the plasma membrane as microdomains separated from the glycoprotein clusters.

Characteristic features of carbohydrate–carbohydrate interactions are a strong dependency on divalent cations (Ca$^{2+}$), a variable specificity, and an extremely low affinity that is compensated in nature by a multivalent presentation of the carbohydrate ligands. As in the study of other biological interactions, the use of model systems has been a productive approach to understanding carbohydrate interactions. Qualitative studies on carbohydrate–carbohydrate interactions with model systems, although limited, span from simple monovalent to polyvalent systems and even whole cells. The first thermodynamic data on carbohydrate–carbohydrate interactions in water were obtained with a model system that consisted of a new type of synthetic receptor named glycophanes and a series of 4-nitro-
Eggens, I.; Fenderson, B. A.; Toyokuni, T.; Dean, B.; Stout, M. R.; teratocarcinoma cells in the presence of Ca$^{2+}$. These model systems are based on self-valent model systems as well as analytical techniques to evaluate this interaction.\textsuperscript{10} These model systems include polyvalent carbohydrate molecules.\textsuperscript{10a} The 3D polyvalent system is based on assembled monolayers (SAMs) of gold surfaces creating a polyvalent presentation of carbohydrate molecules.\textsuperscript{10b} The adhesion forces between carbohydrate molecules and surface plasmon resonance (SPR),\textsuperscript{10b} the adhesion forces and the kinetics of the self-recognition of the antigen Lewis X (Le$^X$, Gal$^-$(1-4)Fuc$^-$(1-3)GalNAc$^-$(1)). Auto-aggregation of F9 teratocarcinoma cells in the presence of Ca$^{2+}$, mimicking the embryonal compaction process, was shown to be due to homotopic Le$^X$-Le$^X$ interactions.\textsuperscript{11} Water-soluble GNPs functionalized with the antigen Lewis X represent a good model to mimic the auto-aggregation of F9 carcinoma cells and morula compaction via carbohydrate-carbohydrate interaction.

The first data on self-aggregation of Le$^X$ functionalized GNPs were obtained by mean of transmission electron microscopy (TEM).\textsuperscript{10b} TEM micrographs of these GNPs dissolved in 10 mM calcium chloride solution showed 3D aggregates, the sizes of which depended on the concentration of GNPs. In contrast, control GNPs presenting the disaccharide lactose did not aggregate in the presence of calcium ions. The self-aggregation of the Le$^X$ GNPs was calcium dependent, and addition of EDTA resulted in dispersion of the aggregates. It was argued that the aggregation events as observed on a carbon grid might be rather different from those of biological systems in physiological conditions.

To further demonstrate the selective Ca$^{2+}$-mediated self-aggregation in solution of GNPs presenting the Le$^X$ antigen, we now report the first thermodynamic data of this aggregation obtained by isothermal titration calorimetry (ITC) in aqueous solution and visualize this process by means of AFM. As control systems, we have also studied the aggregation of GNPs presenting the disaccharides lactose (lacto, Gal(1-4)Glc) or maltose (malto, Glc(1-4)Glc). The results now presented: (a) corroborate the aggregation of the Le$^X$ glyconanoparticles in aqueous solution; (b) provide, for the first time, a thermodynamic estimation for the Ca$^{2+}$-mediated self-aggregation of the Le$^X$ antigen; and (c) confirm the Ca$^{2+}$ selectivity of this association.

Experimental Section

Materials. Neoglycoconjugates 1, 2, and 3 were prepared as previously reported.\textsuperscript{10c,12} The neoglycoconjugates were isolated as disulfide derivatives and in this form used to prepare the corresponding glyconanoparticles, Le$^X$-Au, lacto-Au, and malto-Au.\textsuperscript{10c}

ITC Experiments. The calorimetric measurements were performed with a Microcal MCS-ITC at 25 °C. In a typical run, aliquots of an aqueous solution of the glyconanoparticles Le$^X$-Au, lacto-Au, and malto-Au were added into the calorimetric cell (operating volume 1.35 mL) with a 10 mM CaCl$_2$, MgCl$_2$, or NaCl solution in MilliQ water. The aqueous solution of GNPs was loaded into the injection syringe at 25–50 μM. All of the experiments were performed at 25 °C with data points taken every 2 s. For Le$^X$-Au experiments, typically three injections of 15 μL were made at 4000 s intervals after a first 1 μL injection. In lacto-Au or malto-Au experiments, 15 μL injections of the GNP solution were added into the calorimetric cell every 380 s after a first injection of 1 μL. The total heat evolved for each injection was corrected for the heat of dilution of the GNPs in water. The apparent enthalpy of association was estimated as the heat evolved per mole of GNP injected using the ITC-Origin software provided by Microcal Inc.:

$$\Delta H_{\text{app}} = -Q/([M] \cdot v)$$

where $Q$ (μcal) is the experimental heat of injection after subtracting the heat of dilution, M is the glyconanoparticle concentration in the syringe (25 or 50 μM), and $v$ is the injected volume (15 μL). The maximum number of injections per run for Le$^X$-Au was three due to the slowness of Le$^X$-Au association and the limits imposed by the size of the experimental-data collecting files.

AFM Experiments. AFM measurements were made with a Topometrix explorer AFM (Veeco Instruments, Sunnyvale, USA) in noncontact mode in air. The noncontact mode\textsuperscript{13} was used to avoid any compression of the sample that may have occurred with contact mode AFM. Rectangular silicon cantilevers with integrated tips of spring constant 4.5 N/m (MikroMasch, Tallinn, Estonia) were used, oscillating at just below their natural resonant frequency (nominally 150 kHz, but measured for each cantilever) throughout the work. The radius of curvature of the tips was specified as less than 10 nm, but given the very small radius of the glyconanoparticles, significant image dilation is likely to have occurred.\textsuperscript{14} Therefore, particle heights rather than lateral dimensions were used to characterize particle diameter. This has been shown to be the best method of measuring small particle diameters.\textsuperscript{15,16}

In the case of pure gold nanoparticles, it has been found that AFM height measurements and TEM measured diameters match closely.\textsuperscript{16} Untreated gold glyconanoparticles were prepared for imaging by dissolving in pure water (18 ΩΩ, Sigma-Aldrich, UK), followed by centrifugation at 10 000g for 60 s to remove undissolved impurities. GNPs were deposited onto freshly cleaned mica from dilute (0.2 μM) solution, and left to air-dry. Ca$^{2+}$, Mg$^{2+}$, and Na$^+$ treatment was carried out by incubating the GNPs in 10 mM CaCl$_2$, MgCl$_2$, or NaCl solutions for 24 h. The solution was filtered by centrifugation through a
membrane filter (MICROCON 30 000 Mw cutoff, 10 000g). This was followed by washing with water and by centrifugal filtering (six times) to remove the excess of nonbound salts, which would otherwise interfere with the AFM imaging. It is worth noting that the same centrifugal filtering process was used as part of the synthesis of the nanoparticles to purify the product, and therefore no size selection occurred via loss of small nanoparticles during preparation of the samples for AFM analysis.10b This was confirmed by TEM analysis of the filtrate.

Results and Discussion

Neoglycoconjugates 1, 2, and 3 of the trisaccharide Lewis X (LeX) and the disaccharides lactose (lacto) and maltose (malto), and the corresponding polyvalent gold glyconanoparticles LeX–Au, lacto-Au, and malto-Au (Figure 1), were synthesized and characterized as previously described.10a,12 LeX–Au GNPs, containing an average number of 97 LeX molecules per nanoparticle, were our model system to mimic cell–cell adhesion via carbohydrate–carbohydrate interactions. The lacto-Au and malto-Au glyconanoparticles, with an average number of 70 and 55 lactose and maltose molecules, respectively, were the controls to confirm the selectivity of the Ca2+‐mediated LeX–Au self-aggregation in solution.

lacto-Au GNPs were tested as control to evaluate the contribution of the lactose disaccharide in carbohydrate–carbohydrate association. Lactose is the common disaccharide of all GSLs, and its contribution to biological interaction cannot be excluded. malto-Au GNPs should be considered as the negative control.

Calorimetric titrations were carried out for the system formed by the GNPs and 10 mM solutions of different cations (Ca2+, Mg2+, or Na+). The microcalorimetry curves are represented as a function of time in Figure 2. A slow aggregation process with a favorable enthalpy term of around −160 ± 30 kcal mol−1 per mole of GNP injected (average of seven injections; n = 7) was observed when LeX–Au glyconanoparticles were added to a 10 mM calcium chloride solution (Figure 2Ai). The heat emission observed when LeX–Au GNPs were added to a magnesium chloride solution was more than 5 times lower (∆Happ = −30 ± 20 kcal mol−1; n = 4) than in the case of calcium chloride (Figure 2B). A similar result was obtained (∆Happ = −50 ± 30 kcal mol−1; n = 2) when LeX–Au was added to a sodium chloride solution (Figure 2C), indicating the Ca2+‐mediated selectivity of the LeX aggregation. The aggregation process needed more than 1 h after each injection to reach the equilibrium. As indicated in the Experimental Section, the number of injections per run was limited by the size of the calorimeter acquisition data files due to the slowness of LeX–Au dilution/association underlying events.

The heat evolved upon addition of lacto-Au or malto-Au GNPs to a calcium solution (Figure 2D) was rather low, and thermal equilibrium was quickly achieved. It is worth noting that the thermal signal in the case of lacto-Au increases after the 4–7 first injections, suggesting that some interparticle interactions could take place as the GNPs concentration increases by successive injections of lacto-Au in the Ca2+ solution. This behavior was not observed in the case of the malto-Au GNPs. The results obtained clearly reveal the different thermodynamic behavior of the LeX, lacto, and malto GNPs and demonstrate the selectivity of the Ca2+‐mediated self-aggregation of the LeX–Au GNPs in aqueous solution.

The selectivity of the aggregation process of LeX–Au GNPs was also demonstrated by AFM. At least six images were recorded for each type of GNP under each condition. Representative images are shown in Figure 3 for each type of nanoparticle before (left) and after (center) salt treatment with CaCl2. Before salt treatment, the images are very similar (Figure 3, left). The estimated average particle heights for the LeX–Au and lacto-Au GNPs appear very similar (2.7 ± 0.7 nm), the resolution of the instrument is estimated as 1–2 Å, and both are larger than malto-Au (2.0 ± 0.9) GNPs. These values match the trend in the results obtained by TEM,10b although the heights are rather larger than the particle diameters observed by TEM (1.8 and 1.6, respectively). This is presumably because TEM only images the gold core. However, AFM sizes (2.7 and 2.0 nm) do not match the measured gold core sizes plus the expected length of the neoglycoconjugates (3 nm). This suggests that the GNPs did not have all attached molecules in an extended configuration in air. This may have been due to the affinity of the carbohydrate molecules for the mica surface leading to a more flattened topography of the GNPs.

The auto-aggregation of the LeX–Au, lacto-Au, and malto-Au was studied by incubating the GNPs in 10 mM calcium, magnesium, or sodium chloride solutions for 24 h, followed by washing and drying. After incubation with Ca2+ cations, LeX–Au GNPs developed very large aggregates (Figure 3A, center column). The size of the aggregate shown is approximately 700 nm of diameter, but a broad range of aggregate diameters was observed. Although this aggregate is much larger that those obtained previously by TEM at similar concentration, the results obtained with both techniques agree qualitatively. In the case of the lacto-Au GNPs, some aggregation occurred, limited...
apparently to a few nanoparticles (Figure 3B). The largest aggregates observed for lacto-Au GNPs measure approximately 100 nm in diameter, and no larger aggregates such as that for LeX nanoparticles could be observed. This observation agrees with the result observed in calorimetry, suggesting that some interparticle interaction takes place in the lacto GNPs in the presence of Ca2+. Lactose and the trisaccharide LeX built up the carbohydrate moiety of the LeX GSL, and its contribution to the biological interaction cannot be excluded. The images obtained for the malto-Au GNPs did not show aggregation at all after incubation with Ca2+ (Figure 3C, center column).

In the presence of Mg2+, LeX GNPs showed some limited aggregation. However, no large aggregates such as those observed with Ca2+ could be detected (Figure 3A, right column). There was no definitive evidence of aggregation with either lactose or maltose GNPs in the presence of Mg2+ (Figure 3B and C, right column). Treatment of LeX, lacto, or malto GNPs with 10 mM NaCl solution did not result in any aggregation, and the GNPs appeared dispersed over all of the observed surface (data not shown). These results clearly show that Ca2+-mediated self-aggregation of LeX–Au nanoparticles can be observed independently of the measurement technique or the deposition substrate (here, mica versus amorphous carbon in TEM10b), further reinforcing that the aggregates also form in solution. Both calorimetry and AFM results indicate that specific self-recognition events between LeX molecules in the presence of Ca2+ ions dictate the aggregation process.

The calorimetric results obtained for the Ca2+-mediated aggregation of LeX GNPs clearly indicate that this association is an enthalpically favorable process ($\Delta H \approx -160 \pm 30$ kcal per mole of GNP), even though calorimetric evidence suggests that carbohydrate association in water is an unfavorable process (the excess enthalpies of mono- and oligosaccharide aqueous solutions are positive).17 In our previous studies on carbohydrate–carbohydrate interactions using different model systems and techniques, a free energy change ($\Delta G$) of binding ranging from $-3.0$ kcal mol$^{-1}$ in the monovalent glycophane system8b to $-8.5$ kcal mol$^{-1}$ in the polyvalent system10d was measured. However, in all of these systems, the association of saccharide molecules was assisted either by the presence of additional aromatic–aromatic interactions8b or by the immobilization on a surface of one (SPR)10d or two of the carbohydrate partners (AFM).10c The Ca2+-mediated LeX–Au self-aggregation at 25 °C proceeds with a favorable decrease of the enthalpy ($\Delta H \approx -160$ kcal mol$^{-1}$) and probably with an unfavorable decrease of the entropy, as it may be inferred from the free energy of binding ($\Delta G = -8.5$ kcal mol$^{-1}$) obtained by SPR measurements for the interaction of LeX GNPs with LeX SAMs.10d

In the Ca2+-mediated aggregation of LeX–Au, a cooperative behavior may promote the association. That is, the aggregation of the first two GNPS leads to a structure in which the next added GNP can be further stabilized by multiple interactions with the neighboring GNPs. This cooperativity may explain the average high favorable enthalpy term observed for the aggregation of LeX GNPs in water. The molecular basis of LeX–LeX

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**Figure 2.** ITC curves for LeX–Au added to: (A) (i) water and (ii) 10 mM CaCl$_2$; (B) 10 mM MgCl$_2$; (C) 10 mM NaCl; (D) ITC curves for malto-Au (i) and lacto-Au (ii) added to 10 mM CaCl$_2$. 15 μL injections of 50 μM GNPs were added after a first injection of 1 μL.
interactions is not well established. However, it has been proposed that interactions between two lipophilic complementary surfaces and subsequent cross linking of two LeX molecules via coordination of a calcium cation by four oxygen could be a mechanism that stabilizes the interaction.\(^\text{18}\) Alternatively, the coordination of calcium cations may be the necessary first step to bring together complementary surfaces of the oligosaccharide in a suitable conformation to establish the interaction. Polyvalent presentation of the oligosaccharide seems, in any case, mandatory to stabilize and to observe the self-recognition process in water. Previous attempts to demonstrate the self-association of monomeric LeX antigen molecules have failed.\(^\text{18,19}\)

The present study provides the first thermodynamic evidence and visualization for the selective Ca\(^{2+}\)-mediated self-aggregation in solution of LeX oligosaccharides in a trans-configuration, as was previously observed by TEM on a surface.\(^\text{10}\) Interaction between complementary surfaces and coordination of oxygen atoms by calcium cations probably dictate the binding events of this enthalpy-driven process in aqueous solution. The results herein described confirm the trisaccharide LeX as a homophilic adhesion molecule, which may be able to bring together cells in a specific configuration. Previously, we determined by AFM that the adhesion force between two LeX antigen molecules was around 20 pN.\(^\text{10}\) This magnitude, although small, is significant taking into account that the adhesion force between the proteoglycan involved in the species-specific cell aggregation of the Microciona prolifica sponge, measured also by AFM, was found to be 40 pN.\(^\text{9}\) This further means that only five pairs of LeX molecules would be enough to provide the binding strength (100 pN) between neural retina cells of embryonic chicken,\(^\text{20}\) or that the adhesion force between 16 pairs of LeX (320 pN) would be sufficient to hold T and B lymphocyte cells together in the absence of antigen stimulation.\(^\text{21}\)

The extremely slow aggregation process (see Figure 2; more than 1 h to reach equilibrium) observed for the LeX nanoparticles in the presence of calcium cations may be compared either to


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**Figure 3.** Noncontact AFM images of (A) LeX–Au, (B) lacto–Au, and (C) malto–Au GNPs before (left column) and after incubation with 10 mM CaCl\(_2\) solution (center column), and 10 mM MgCl\(_2\) solution (right column). The bars show the height scale of the images.
the Mg$^{2+}$-induced isodesmic self-association process of the FtsZ bacterial protein$^{22}$ or to a nucleation-elongation process, which are known to be the mechanism of, for example, actin$^{23}$ and flagellin$^{24}$ polymerization. These processes are commonly used in nature as a means to assemble in a dynamic fashion. This mechanism process requires the existence of multiple non-covalent interactions and the presence of divalent cations as is the case of Le$^X$–Au self-aggregation. Therefore, the aggregation of gold glyconanoparticles presenting Le$^X$ epitopes may represent a synthetic model for mimicking dynamic association processes via carbohydrate–carbohydrate interactions. The particular features of this interaction (e.g., low affinity, reversibility, existence of repulsive and attractive interactions) provide a reliable mechanism for a dynamic self-assembly process. Indeed, in nature the possibility of creating multiple low-energy interactions would facilitate the necessary association/dissociation events for cell adhesion and function before the onset of more stable cell–cell interactions and transduction signaling.

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