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Modulating glycosidase degradation and lectin recognition of gold glyconanoparticles

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ABSTRACT

Glyconanoparticles (GNPs) are water-soluble carbohydrate-functionalized gold nanoclusters with a promising potential to serve as versatile tools in studies ranging from basic chemical glycobiology to clinical applications. In this paper we evaluate the influence of ligand density and presentation on the recognition by protein receptors by examining the interaction of lactose-functionalized GNPs with two different galactose-specific carbohydrate-binding proteins: an enzyme, *Escherichia coli* β-galactosidase, and a lectin, *Viscum album* agglutinin. The results suggest that the proper selection of ligand densities and spacers in GNP functionalization is an important requisite to match the topological requirements of the target receptor while escaping glycosidase degradation.

1. Introduction

Carbohydrate-mediated interactions are central to many important biological phenomena, such as pathogenic infections, inflammation processes, metastasis and (glyco)protein regulation, and trafficking.[1] Characteristic features of most interactions involving carbohydrates (either protein–carbohydrate or carbohydrate–carbohydrate interactions) are high specificity and low affinity. Nature overcomes this low affinity by clustering ligands and receptors at the cell surface.[2] Therefore, the concept of multivalence becomes an important issue in glycobiology. To understand the mechanism of these interactions, a variety of model systems comprising spherical or linear arrays of carbohydrates have been developed.[3] These include glycodendrimers,[4] glycopolymers,[5] glycopeptides, and glycoproteins,[6] as well as self-assembled systems, for example, liposomes,[7] micelles,[8] rotaxanes,[9] and nanoparticles.[10] Some of these multivalent systems have been shown to be excellent ligands for binding endogenous lectins[11] and bacterial toxins, such as Shiga-like toxin[12] or cholera toxin,[13] suggesting potential clinical applications. The presentation and density of the ligands in these recognition processes have been shown to critically influence the binding.[14] Such an influence is intimately related to the particular arrangement and architecture of carbohydrate-binding sites in the receptor.

In the search for new multivalent systems with well-defined chemical composition, we developed an integrated strategy (glyconanotechnology)[15] for the construction of bi- (2D) and three-dimensional (3D) multivalent tools, based on self-assembled carbohydrate monolayers on gold. The 3D polyvalent systems, named glyconanoparticles (GNPs),[10a,10d] are water-soluble gold nanoclusters functionalized with carbohydrate antigens, the preparation of which is based on the work of Brust and colleagues on monolayer-protected gold nanoclusters.[16] With a well-defined chemical composition and globular shape, GNPs display a multivalent presentation of carbohydrates that mimic glycosphingolipid (GSL) clusters at cell surfaces. Due to their exceptionally small gold core (⩽2 nm) and hydrophilic functionalization, GNPs are highly soluble in water and exhibit a long-term stability in solution, thus emerging as new and versatile polyvalent tools for basic studies in chemical glycobiology. Furthermore, since they are non-cytotoxic, GNPs may be regarded as promising biological vectors for intervening in carbohydrate-mediated processes.[17] Thus, as a first example of application of GNPs as anti-adhesive

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agents, we reported some time ago that lactose-functionalized GNPs are able to inhibit up to 70% metastasis of melanoma cells in mice.[17a]

When designing GNPs to study in vivo biological processes, specificity, affinity and stability against glycolytic enzymes (glycosidases) are important factors to be considered. Besides obvious structural considerations and the above-mentioned impact of ligand clustering, the orientation of the oligosaccharide on the metallic surface may also decisively affect recognition by carbohydrate-binding proteins.[18,19] By manipulating the ratio and characteristics of the neoglycoconjugates used in the GNPs construction process, these GNPs can be prepared with different carbohydrate density and presentation at the surface, providing an under-control model for investigating the influence of these factors on the recognition by lectins and enzymes. On the other hand, the stability of GNPs to enzyme hydrolysis is crucial to circumvent lysosomal degradation when they are internalized in the cell by endocytosis.

In this paper we evaluate these questions by examining the interaction of lactose-functionalized GNPs with two different galactose-specific carbohydrate-binding proteins: an enzyme, β-galactosidase from *Escherichia coli*,[20] and a lectin, the agglutinin from *Viscum album*.[21] The carbohydrate-binding site architecture and mode of recognition of these two representative proteins are different. Thus, the binding sites of lectins typically are shallow grooves on the protein surface, while enzymes usually present more deep or narrow binding pockets. Furthermore, glycolytic enzymes display a higher activity toward monovalent substrates, while lectins are often involved in multivalent interactions. The results here presented illustrate the potential of modulating the behavior of GNPs toward these two types of receptors, opening a multitude of perspectives for the application of GNPs in carbohydrate-mediated biological processes.

2. Results and discussion

2.1. Synthesis

We have prepared and characterized a series of water-soluble gold GNPs from lactose neoglycoconjugates containing spacers of diverse length and nature (Fig. 1). Three different linkers, an aliphatic chain, 11-mercaptoundecanol ($HOC_{11}SH$), a hydrophilic chain, 11-mercapto-3,6,9,-trioxaundecanol ($HOEG_4SH$), and a mixed linker, containing 11-mercaptoundecanol and hexa-ethylene glycol ($HOEG_6C_{11}SH$), were used for the preparation of neoglycoconjugates **1, 2**, and **3**. For the preparation of GNPs of differing densities, a mixed aliphatic linker formed by 11-mercaptoundecanol and tri-ethylene glycol ($HOEG_3C_{11}SH$) was used. The neoglycoconjugates were isolated as disulfide derivatives, and therefore they can be considered as divalent ligands (Fig. 1). By varying the proportion and/or nature of the neoglycoconjugates used for the construction of the GNPs, a diversity of GNPs was prepared. These include GNPs **1-Au, 2-Au, 3-Au** with 100% density of neoglycoconjugates **1**, **2**, and **3**, respectively, which mimic GSL clustering at the cell membrane. By varying the ratio of neoglycoconjugate **3** and the mixed linker $HOEG_3C_{11}SH$, GNPs with 15% (**3-Au**-15) and 30% (**3-Au**-30) density of lactose (Fig. 1) have also been prepared. The GNPs thus prepared are water soluble, stable and can be manipulated as a water-soluble biological macromolecule. Lactose GNPs have previously been shown to be non-cytotoxic against glioma C6, melanoma B16F10, COS-1, embryonic F9, or NIH-3T3 cells.[17a]

GNPs (**4-Au**) presenting the disaccharide maltose were also prepared from neoglycoconjugate **4** as a negative control. The neoglycoconjugates and GNPs used in this study are represented in Figure 1.

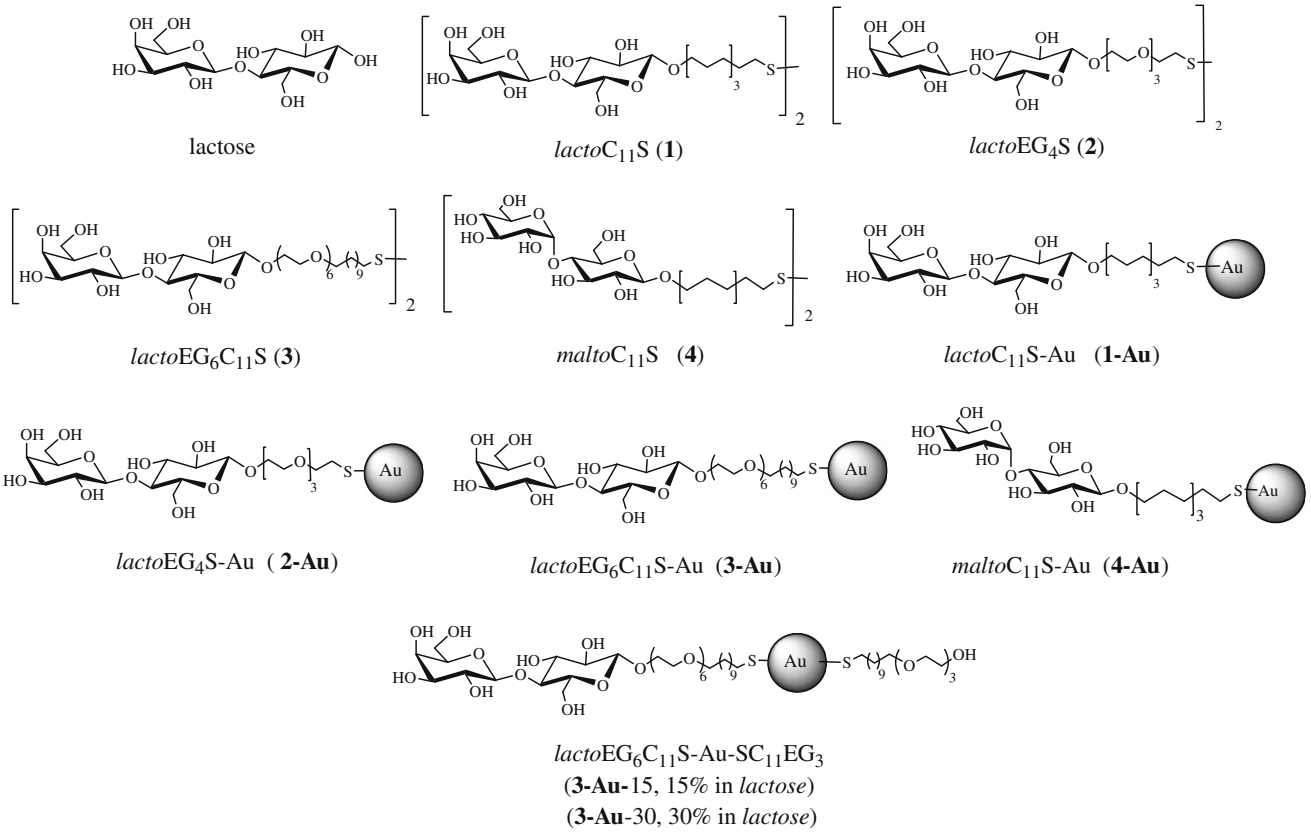

Figure 1. Neoglycoconjugates and glyconanoparticles used in this study.

2.2. Enzyme recognition

In contrast to the considerable number of studies on interactions of multivalent carbohydrate systems involving lectins,[22] studies with enzymes are rather scarce.[5d,14c,23] A few reports have appeared on the recognition of GNPs by proteins.[10b–j] However, as far as we know, the susceptibility of GNPs to enzymatic hydrolysis has never been evaluated. In this study we have assessed the hydrolysis of lactose-presenting gold GNPs by β-galactosidase from E. coli (*E.* coli), in comparison with the hydrolysis of the corresponding neolactoconjugates and lactose itself. This galactosidase recognizes and hydrolyses β-galactosyl moieties at the non-reducing end of oligosaccharide structures.[20] The activity of the enzyme can be easily evaluated using a coupled galactose-dehydrogenase reaction,[24] in which the formation of NADH, proportional to the amount of galactose released, is followed spectrophotometrically. Neoglycoconjugates **1**, **2**, and **3** and GNPs **1-Au**, **2-Au**, **3-Au,** and **3-Au-**30 (30% of lactose) (Fig. 1) were tested as substrates. Lactose, a natural substrate of β-galactosidase, was used as positive control. The kinetics of galactose release and relative velocities of β-galactosidase hydrolysis of these substrates are compiled in Table 1 and Figure 2, respectively.

Neoglycoconjugates **1** and **2** were processed by the enzyme at levels comparable to lactose, while *lacto*-conjugate **3** was not degraded under the same conditions, showing a great influence of the spacer on the enzyme activity. Only upon increasing 10-fold the enzyme concentration, some hydrolysis of **3** could be observed after incubation for 1 h (Table 1). On the other hand, GNPs *lacto*C_{11}S-Au (**1-Au**) and *lacto*EG_4S-Au (**2-Au**), with 100% density of lactose, were barely processed. The same behavior was observed for GNP **3-Au** with 100% density of lactose (Table 1). One possible reason for the poor hydrolysis of **3** may be due to its aggregation into micelles or other supramolecular structures. In fact, translational diffusion studies of the disulfide neoglycoconjugates and the corresponding GNPs indicate that compound **3** is in aggregated form at the concentration used in the enzymatic assays.[25] As it would happen with the GNPs, the attachment of the first enzyme molecule to the lactose residue in the aggregate can reduce the accessibility of the other lactose residues to new enzyme molecules.

Interestingly, when the density of lactose in the GNP was reduced (**3-Au-**30, with 30% lactose density), the activity was comparable to that exhibited by the corresponding neoglycoconjugate **3** (Fig. 2, Table 1). However, the observed specific activity was still very low, indicating a clear increased resistance of the lactose moiety to hydrolysis. This low activity hampered the determination of kinetic parameters for the hydrolysis of GNPs. Very large incubation times and increasing amounts of enzyme were necessary to observe some hydrolysis. Because of that the V_{max} was determined only at one concentration (Table 1). The different behavior of the high- and low-density GNPs point to steric crowding of carbohydrate molecules at the GNP surface contributing to the impairment in the galactosidase activity. The results indicate that lactose-GNPs resist *E. coli* β-galactosidase hydrolysis even under drastic conditions. It seems plausible that the GNPs could exhibit a similar increased resistance to other glycosidases although these results cannot be directly extrapolated to glycosidases with different topologies. In particular, resistance to circulating and lysosomal glycosidases would represent an advantage for the in vivo application of these tools to intervene in carbohydrate-mediated processes. Thus, a future extensive investigation of the resistance of GNPs to other glycosidases is definitely warranted.

Table 1
Specific activity of *E. coli* β-galactosidase in the enzymatic hydrolysis of lactose, neoglycoconjugates, and GNPs

Substrate	Lactose equivalents (mM)	βgalactosidase (μg/mL)	Vabs[a] (galactose μM /min)	Specific activity (μmol/min mg)
Lactose	2	2	10.90	5.45
LactoC_{11}S (1)	2	2	5.75	2.88
LactoEG_4S (2)	2	2	6.49	3.25
LactoEG_6C_{11}S (3)	2	20	3.20	0.161
1-Au	2	20	0.83	0.042
2-Au	2	20	0.56	0.028
3-Au	2	20	0.41	0.020
LactoEG_6C_{11}S (3)	1	36	2.96	0.082
3-Au-30	1	36	1.75	0.048
3-Au	1	36	0.15	0.004

[a] All the measurements are corrected for unspecific reaction in the secondary enzymatic reaction.

2.3. Lectin recognition

The possibility of modulating the recognition of GNPs by lectins was evaluated using the galactose-specific agglutinin from *V. album* (VAA). VAA belongs to an interesting group of cytotoxic lectins, which share a similar protein sequence and structure. They are composed of two different subunits; the A subunit exhibits ribosome-inactivating activity, while the B subunit contains two galactose-binding sites located at the N-terminal and C-terminal domains, respectively, of the subunit.[26] In addition, VAA forms a homodimer through contacts between the N-terminal domains of two B chains.[26a,27] The overall arrangement of sugar-binding sites in the dimer makes VAA a suitable, frequently used system to investigate the performance of neoglycoconjugate ligands.[11]

Neolactoconjugates and GNPs presenting lactose were tested as inhibitors of the binding of ^{125}I-VAA to asialofetuin (ASF) coated onto microtiter plate wells. This asialoglycoprotein presents a heterogeneous glycan population, containing several N-linked

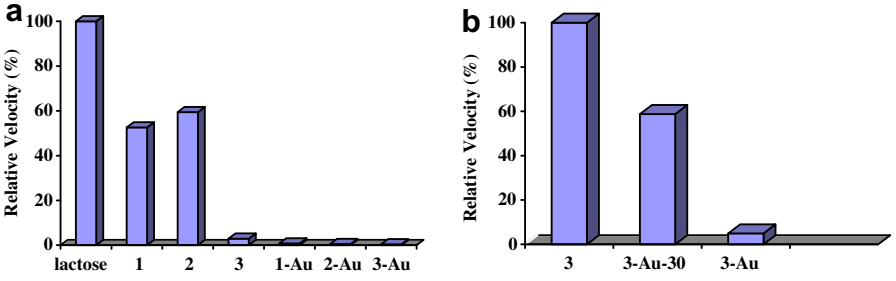

Figure 2. Relative velocities of enzymatic hydrolysis of: (a) lactose, neoglycoconjugates **1**, **2**, and **3**, and glyconanoparticles **1-Au**, **2-Au**, and **3-Au** (2 mM in lactose equiv) with 100% density in lactose; and (b) neoglycoconjugate **3** and glyconanoparticles with 100% (**3-Au)** and 30% (**3-Au-**30) lactose density (1 mM in lactose equivalents).

and O-linked galactose-terminated oligosaccharides, although only the N-glycan chains have been shown to be involved in the cross-linking of ASF by lectins.[28] Inhibition curves obtained for lactose, as monovalent ligand, neoglycoconjugates **2** and **3** as divalent ligands, and GNPs **1-Au**, **2-Au**, and **3-Au** as multivalent ligands are shown in Figure 3. All *lacto*-nanoparticles were competent to inhibit the binding of VAA to ASF, whereas neoglycoconjugate of maltose **4** and *malto*-nanoparticle **4-Au** used as controls did not produce inhibition (curves not shown), evidencing the ability of the *lacto*-GNPs to behave as specific ligands for the lectin. Furthermore, the inhibition curves reveal different binding avidities for the GNPs. The inhibitory potency of **1-Au**, which contains an aliphatic chain and 70 half-molecules of neoglycoconjugate **1**,[10d] was somewhat smaller than that for free lactose, as clearly observed when data were normalized with respect to the disaccharide content (Fig. 3a). This indicates that clustering of lactose molecules on the nanoparticle surface is not by itself sufficient to enhance lectin binding. On the other hand, the inhibitory activity of the GNPs with the same lactose density (100%) but with more flexible spacers (**2-Au** and **3-Au**) was noticeably higher, independently on the spacer length, clearly indicating that the flexibility of the spacer facilitates matching the appropriate topological requirements of the receptor. Moreover, the inhibitory potency of the GNPs with 15% (**3-Au**-15) and 30% (**3-Au**-30) in lactose surpassed that of **3-Au**, particularly at high GNP concentrations (Fig. 3b). Thus, avoiding an excessive crowding of lactose molecules on the nanoparticle surface further facilitates the GNP–lectin interactions.

In order to further evaluate the impact of immobilization and presentation of the neoglycoconjugates on the nanoparticle surface on recognition by the lectin, the inhibitory potency of the disulfide neoglycoconjugates **2** and **3**, containing two lactose units per molecule, was determined (Fig. 3c). The activity of neoglycoconjugate **2** was similar to that exhibited by the corresponding nanoparticle **2-Au**, which contains 63 lactose molecules. Thus, the higher valence of the glyconanoparticle does not correlate with a higher inhibitory potency. On the contrary, immobilization and clustering of neoglycoconjugate **2** on the GNP seemingly reduce lectin binding to some extent. Similarly, the behavior of neoglycoconjugate **3**, which is also a more potent inhibitor than lactose, is comparable to that of **3-Au**. However, the activity of the GNPs **3-Au**-15 and **3-Au**-30, with smaller density of lactose molecules, was higher, supporting the notion that, provided the appropriate distances among lactose molecules is ensured, immobilization and multiple presentation of lactose molecules on the nanoparticle may improve the interaction with the lectin.

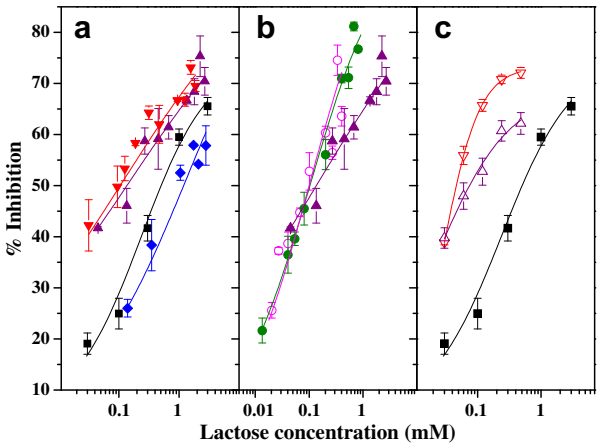

Figure 3. Inhibition of the binding of VAA to ASF by lactose and lactose-containing GNPs and neoglycoconjugates. The binding of ^{125}I-VAA to ASF-coated microtiter plate wells was measured in the absence and presence of increasing concentrations of (a) lactose (■), and GNPs with 100% (**1-Au**, ♦) (**2-Au**, ▼), (**3-Au**, ▲); GNPs with 30% (**3-Au**-30, ●) and 15% (**3-Au**-15, ○) density of lactose compared to those with 100% density **3-Au** (▲); and (c) neoglycoconjugates **2** (▼) and **3** (△) compared with lactose (■). The percentage of inhibition was calculated taking as 100% the complete inhibition of VAA binding in the absence of inhibitor.

3. Conclusions

The selective recognition of different *lacto*-nanoparticles by VAA points out the importance of tailoring GNPs to the precise topological requirements of the target receptor. The results clearly show that ligand clustering is not necessarily correlated with improved binding affinity and a higher valence of the GNP does not imply a superior inhibitory potential. In fact, a weaker binding of VAA to the nanoparticles with 100% lactose density compared to nanoparticles with lower densities is observed. Similar observations had been made previously for the binding to VAA and galectin-1, a mammalian β-galactoside-specific lectin, of wedgelike glycodendrimers containing an increasing number of lactose moieties.[11] On the other hand, the carbohydrate ligand presentation on the nanoparticle surface appears as a key factor in the design of superior inhibitors. In this regard, the use of flexible spacers may facilitate matching the geometrical requirements of the receptor. Bearing in mind the preceding observations, GNPs could be considered a convenient lectin-targeting system. In this regard, a detailed comparative analysis of the recognition of GNPs by lectins with different topological requirements is still needed. The *lacto*-nanoparticles, in addition, have proved to be a poor substrate for *E. coli* β-galactosidase. The observed stability toward degradation, together with the lack of toxicity against several cell lines, makes the GNP technology a promising strategy for the development of potential drugs to intervene in carbohydrate-mediated processes in vivo.[17]

4. Experimental

4.1. General reagents

β-Galactosidase (EC 3.2.1.23) from *E. Coli*., β-NAD, galacto-dehydrogenase (EC 3.2.1.23) from *Pseudomonas fluorescens*, BSA and ASF were acquired from Sigma–Aldrich.

4.2. Synthesis

The synthesis of neoglycoconjugates and GNPs was carried out by using the methodology previously developed in our laboratory.[10d] The purified GNPs were characterized by NMR, IR, and UV spectroscopy, and transmission electron microscopy (TEM) and their composition were confirmed by elemental analysis and ICP. A mean diameter of 1.8 nm was found for the gold core, which corresponds to an average number of 201 gold atoms. Elemental analysis confirmed an average number of 70, 63, and 90 lactose molecules for **1-Au**, **2-Au**, and **3-Au** nanoparticles, respectively.

4.3. Enzyme assay

The amount of galactose released by β-galactosidase hydrolysis was evaluated using a coupled galactose-dehydrogenase reaction.[24] *E. coli* β-galactosidase (0.01–0.1 mg/mL) in sodium phosphate buffer (50 mM, pH 7.2, 1 mM $MgCl_2$) was added to a GNP or neoglycoconjugate solution (1–2 mM in lactose equivalents) in the same buffer, in a final volume of 50 μL. Final galactosidase concentrations are indicated in Table 1. After incubation at 37 °C for 30 or 60 min, the reaction was stopped by diluting eight times with cold Tris–HCl buffer (0.1 M, pH 8.8) containing NAD 10 mM, and the enzyme was removed by ultrafiltration through MICROCON

with a molecular weight cut-off of 30,000. The filtrate was transferred to a spectrophotometer quartz cuvette and 7 μL of galactose dehydrogenase (1.1 mg/mL) was added. The mixture was incubated at 30 °C for 45 min. Absorption at 340 nm due to the generation of NADH was recorded and converted into galactose concentration by interpolation in a calibration curve constructed with galactose.

4.4. Lectin assay

V. album agglutinin was purified from mistletoe extracts by affinity chromatography on α-lactose immobilized on 6% beaded agarose (Sigma), as previously described.[29] The lectin was labeled with ^{125}I in presence of 0.1 M lactose using IODO-GEN (Pierce), according to the manufacturer's recommendations. The radiolabeled protein was separated from free iodine by gel-filtration chromatography on a Sephadex G-25 column, PD10 (Pharmacia), equilibrated with 5 mM sodium phosphate, pH 7.2, 0.2 M NaCl (PBS). The ^{125}I-lectin was undistinguishable from the unlabeled protein by SDS–PAGE and autoradiography and was stored at −20 °C until use.

The avidity of VAA for the GNPs was estimated by determining the amount of ^{125}I-VAA bound to immobilized ASF in the presence of different concentrations of the sugar derivatives.[30] Microtiter plate wells were coated overnight at 4 °C with 50 μL of ASF (0.1 mg/mL) in PBS. The plates were then washed five times with PBS and blocked with 200 μL of BSA (3% BSA/PBS w/v) for 1 h at 37 °C. After that, BSA was removed, and the wells were incubated at 20 °C for 2 h with 50 μL of ^{125}I-VAA (20,000 cpm) in the absence or presence of different concentrations of the GNPs (0.5–40 μM in GNP) or neoglycoconjugates (15–240 μM). Plates were washed five times, and bound radioactivity was measured in an LKB mini-γ counter. Nonspecific binding in the presence of 0.1 M lactose was always below 2%.

Acknowledgments

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