ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 8620–8622

www.rsc.org/chemcomm

COMMUNICATION

Multivalent glyconanoparticles with enhanced affinity to the anti-viral lectin Cyanovirin-N†

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Received 21st May 2011, Accepted 19th June 2011
DOI: 10.1039/c1cc12981c

Low-mannose (LM) structures were coupled to gold nanoparticles (Au NPs) to amplify the affinity of LMs with Cyanovirin-N (CV-N) lectins and to study the structures of CV-N variants CVN^{Q50C} and CVN^{MutDB}.

Lectins, carbohydrate-binding proteins, play critical roles in a plethora of biological processes.[1] An in-depth understanding of carbohydrate–lectin interactions is not only fundamentally important for elucidating their biological functions, but also of outstanding practical value in the design and development of therapeutics and diagnostic tools. Cyanovirin-N is an 11 kDa cyanobacterial lectin that exhibits inhibitory activity against a number of viruses, including HIV, at concentrations as low as nanomolar. Its anti-HIV activity is mediated by binding to the high-mannose (HM) structures on the envelope glycoprotein gp120.[2–6] Previous studies established that the binding epitope(s) on *N*-linked high oligomannosides for CV-N comprised α-D-Man*p*-(1 → 2)-α-D-Man*p* moieties on the glycan's D1 and D3 arms.[7–10]

Multivalency, resulting in cooperative interactions of multiple ligands with multiple receptors, is a general phenomenon that occurs in many biological processes involving molecular recognition. Multivalent interactions are often significantly stronger than the corresponding monovalent interactions, and, as such, the design and creation of multivalent reagents is an important strategy for generating diagnostic and therapeutic tools.[11] In glycobiology, these kinds of approaches are especially relevant given the commonly observed weak affinities between glycans and lectins.[12,13] On the other hand, high glycan structures exhibit drastically enhanced apparent affinities, compared to the monovalent ligands. However, the synthesis of HM glycans is tremendously demanding, involving multiple protection/deprotection steps and complex stereochemistry control. As such, their availability is limited. An alternative approach for creating multivalency is to use a scaffold, such as polymers, lipids or nanomaterials, on which multiple copies of a ligand can be presented, thereby generating a multivalent ligand.[14–18] For example, Melander and coworkers prepared small molecule-coated Au NPs as effective inhibitors for HIV fusion,[19] and Gervay-Hague's group reported that galactosyl- and glucosyl-functionalized Au NPs exhibited 300 times better binding to gp120.[20] In previous studies from our group, we showed that carbohydrate ligands conjugated to Au NPs exhibited affinities up to five orders of magnitude higher than those of the corresponding monomeric ligands with lectins.[21]

Here, we conjugated two low-mannoses, Man2 and Man3, to the Au NP scaffold, and investigated the binding affinity of the resulting **GNP-M2** and **GNP-M3** with CV-N lectins (Fig. 1). In order to derive quantitative numbers for the affinity enhancement caused by Au NPs, we developed a fluorescence competition assay and determined the apparent dissociation constant of GNP binding to CV-N (K_D). The results from this assay were compared with the K_d values of monomeric glycan binding to CV-N using isothermal titration calorimetry (ITC).

GNP-M2 and **GNP-M3** were prepared following a previously established procedure,[22] outlined in Fig. 1. Uniform, ~22 nm Au NPs (Fig. S1, ESI†) were synthesized by the Turkevich method[23] and were subsequently functionalized

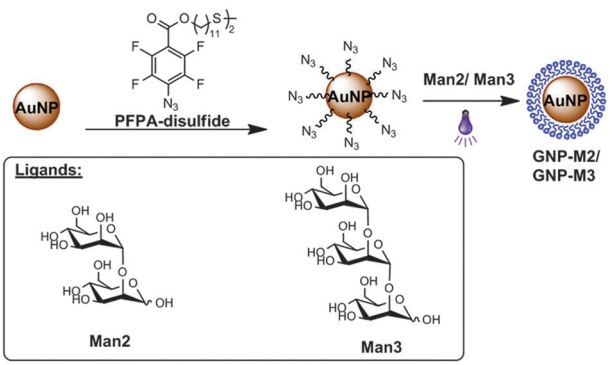

Fig. 1 Synthesis of Man2- and Man3-conjugated Au NPs **GNP-M2** and **GNP-M3**.

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† Electronic supplementary information (ESI) available: Experimental details, nanoparticle characterization, ITC results. See DOI: 10.1039/c1cc12981c

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with PFPA-disulfide (Fig. 1). Man2 and Man3 were then conjugated to the PFPA-functionalized Au NPs using a photocoupling method[22] (see experimental details in ESI†) by way of a CH insertion reaction of the photogenerated perfluorophenylnitrene.[24,25] The ligand density was determined using a colorimetric assay with anthrone/sulfuric acid.[22] Values of 1516 ± 232 Man2 and 1037 ± 148 Man3 per particle were obtained for **GNP-M2** and **GNP-M3**, respectively.

Binding affinities of **GNP-M3** to CV-N were evaluated using two CV-N variants: CVN^{MutDB} and CVN^{Q50C}. CVN^{Q50C} is essentially a wild-type variant, comprising two separate glycan binding sites, one on Domain A and one on Domain B.[8,10] Domain A exhibits a slight preference for the Man3 units and domain B for the Man2 units.[10,26] The Cys substitution at position 50 was introduced to allow for specific fluorescence labeling of CV-N without interfering with glycan binding. In the CVN^{MutDB} variant on the other hand, the glycan binding site on domain B is completely eliminated, while the site on domain A still can bind glycan ligands. Since this variant no longer can cross-link glycans on gp120, it has lost its anti-HIV activity.[27] Therefore, in interactions with GNPs, we would expect that CVN^{Q50C} can act as a crosslinker and form a complex with **GNP-M3**, while no such crosslinking should be possible between **GNP-M3** and CVN^{MutDB}. Indeed, **GNP-M3** treatment with CVN^{Q50C} caused a red shift from 529 nm to 542 nm in the surface plasmon resonance (SPR) band of Au NPs (Fig. 2a), indicative of particle size growth.[28] Such an increase in particle size was further confirmed by TEM (Fig. 2c) which revealed the presence of clusters of aggregated particles. When **GNP-M3** was treated with CVN^{MutDB}, however, no SPR shift was observed (Fig. 2b). TEM images were devoid of aggregates and only isolated single particles were observed in this case (Fig. 2d). Dynamic light scattering (DLS) measurements of CV-N treated **GNP-M3** particles yielded average particle sizes of 25.9 ± 3.5 nm and 38.3 ± 4.6 nm for CVN^{MutDB} and CVN^{Q50C}, respectively (Fig. S2, ESI†). These results are all consistent with our previous structural studies on CVN^{MutDB} that revealed a single glycan binding site.[27]

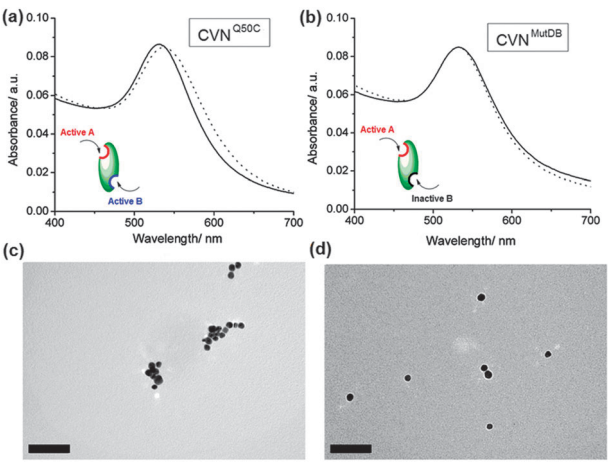

Fig. 2 UV-vis spectra of **GNP-M3** before (solid line) and after (dotted line) treatment with (a) CVN^{Q50C} and (b) CVN^{MutDB}. TEM micrographs of **GNP-M3** treated with (c) CVN^{Q50C} and (d) CVN^{MutDB}. Scale bars: 100 nm.

The binding affinities of the GNPs to the CV-N variants were evaluated using a recently developed fluorescence-based competition assay.[21] In the experiment, a free ligand competitor (Man2 for **GNP-M2**, Man3 for **GNP-M3**) together with varying concentrations of **GNP-M2** or **GNP-M3** was incubated at a fixed concentration of Cy5-CVN^{Q50C}, Cy5-labeled CVN^{Q50C} (Fig. 3a, see ESI† for experimental details). The solution was centrifuged to remove all GNPs and the fluorescence intensity of the supernatant was measured. The difference in fluorescence intensity of Cy5-CVN^{Q50C} before and after incubation with GNPs corresponds to the amount of the bound CVN^{Q50C}. Concentration response curves for **GNP-M2** or **GNP-M3** permit the determination of IC_{50} values (Fig. 3c).

In order to extract the binding constants of GNPs with CVN^{Q50C}, it is necessary to know the K_d values of the monomeric ligands, Man2 and Man3, with CVN^{Q50C} (Fig. 3b). These values were determined by ITC and the dissociation constants, K_{d1} (glycan-binding site on Domain A) and K_{d2} (glycan-binding site on Domain B), were calculated based on a two-site binding model (see Experimental section and Fig. S3, ESI† for details). Values for K_{d1} and K_{d2} of 700 μM and 64 μM for Man2, and 3.4 μM and 43 μM for Man3, respectively, were calculated (Table 1). These values agree well with our previous observation that slightly stronger binding of Man2 to the site on Domain B than to that on Domain A occurs, while the opposite is true for Man3.[8,10,27] These data, together with the IC_{50} values determined from the data shown in Fig. 3c, were then used to calculate the apparent dissociation constants for

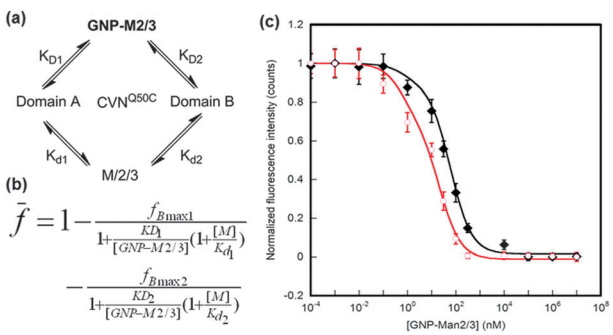

Fig. 3 Fluorescence competition assay. (a) Schematic representation of a binding scenario. (b) Modified Cheng–Prusoff equation based on a competitive two-site binding model, where [M] is the concentration of the free ligand, and K_{d1} and K_{d2} are the dissociation constants of the free ligand for the glycan-binding sites on Domains A and B of CVN^{Q50C}, respectively. The data were fitted using the maximum bound fractions, f_{Bmax1} and f_{Bmax2}, corresponding to the two binding sites, and dissociation constants K_{D1} and K_{D2} as adjustable parameters. (c) Concentration response curves of **GNP-M2** (black) and **GNP-M3** (red).

Table 1 Affinities for Man2/3 (K_d) and **GNP-M2/3** (K_D) binding to CVN^{Q50C}. Numbers in parentheses correspond to EF ($= K_{d1}/(K_{D1} \times$ number of ligands on GNP))

Ligand	K_{d1} or K_{D1} (Domain A)	K_{d2} or K_{D2} (Domain B)
Man2	700 ± 50 μM	64 ± 4 μM
GNP-M2	56.4 ± 7 nM (8.2)	0.24 ± 0.1 nM (176)
Man3	3.4 ± 0.2 μM	43 ± 2 μM
GNP-M3	0.011 ± 0.007 nM (309)	11.8 ± 2.3 nM (3.6)

the site on Domain B, K_{D1} and K_{D2}, based on a two binding site model (Fig. 3b, see Experimental section in ESI† for details). The data summarized in Table 1 demonstrate that both GNPs, **GNP-M2** and **GNP-M3**, exhibit an affinity enhancement by several orders of magnitude compared to the affinities measured for the isolated, monomeric sugars interacting with CVN^{Q50C}. Taking into account the number of ligands on the particles, *i.e.* considering the affinity/ligand, an increase up to several hundred times is still present for the Au NP-bound glycan (Table 1). In addition, **GNP-M3** exhibited a higher affinity than **GNP-M2** for both domains. These results correlate well with the general affinity ranking of the free ligands Man2 and Man3, and are consistent with observations in our previous study with a different GNP-lectin system.[21] Interestingly, for both **GNP-M2** and **GNP-M3**, the affinity enhancement is more pronounced for the better binding domain. For example, Man2 exhibits a higher affinity for the binding site on Domain B, and with **GNP-M2**, the affinity enhancement factor (EF) is 178 for the Domain B site *vs.* 8.3 for the Domain A site (Table 1). For **GNP-M3**, on the other hand, the opposite was observed that the EF is higher for the Domain A site (340) than for the Domain B site (3.8).

In conclusion, we have successfully grafted LM ligands onto Au NPs *via* an efficient photocoupling reaction. The resulting GNPs interacted with the CV-N variants CVN^{MutDB} and CVN^{Q50C} in a manner that is consistent with the expected behavior of one- and two-site binders. Crosslinked complexes and aggregates were observed when **GNP-M3** was treated with the two-site CVN^{Q50C} while only single particles were seen after treatment with the single-site variant CVN^{MutDB}. Furthermore, these GNPs exhibited significantly higher affinity towards the CV-N lectins, compared to the free glycan ligands, demonstrating that Au NPs serve as an efficient multivalent scaffold that significantly enhances the apparent affinity. This affinity enhancement compares well with that of other synthetic multivalent ligands. Therefore, a general strategy can be envisioned which uses simple glycans, rather than large and complex sugars, for grafting onto a multivalent scaffold for affinity amplification. These types of approaches will aid in development of effective new glyconanomaterials for diagnostic and therapeutic applications.

This work was supported by National Institutes of Health Grants R01GM080295 and 2R15GM066279 (to M.Y.) and R01GM080642 (to A.M.G.). L.D. thanks the China Scholarship Council for a special scholarship award.

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