

Probing Multivalent Lectin-Carbohydrate Binding via Multifunctional Glycan-Gold Nanoparticles: Implications for Blocking Virus Infection

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ABSTRACT: Multivalent lectin-glycan interactions are widespread in biology and are often exploited by pathogens to bind and infect host cells. Glycoconjugates can block such interactions and thereby prevent infection. The inhibition potency strongly depends on matching the spatial arrangement between the multivalent binding partners. However, the structural details of some key lectins remain unknown and different lectins may exhibit overlapping glycan specificity. This makes it difficult to design a glycoconjugate that can potently and specifically target a particular multimeric lectin for therapeutic interventions, especially under the challenging *in vivo* conditions. Conventional techniques such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) can provide quantitative binding thermodynamics and kinetics. However, they cannot reveal key structural information, *e.g.* lectin's binding site orientation, binding mode, and inter-binding site spacing, which are critical to design specific multivalent inhibitors. Herein we report that gold nanoparticles (GNPs) displaying a dense layer of simple glycans are powerful mechanistic probes for multivalent lectin-glycan interactions. They can not only quantify the GNP-glycan-lectin binding affinities *via* a new fluorescence quenching method, but also reveal drastically different affinity enhancing mechanisms between two closely-related tetrameric lectins, DC-SIGN (simultaneous binding to one GNP) and DC-SIGNR (inter-crosslinking with multiple GNPs), *via* a combined hydrodynamic size and electron microscopy analysis. Moreover, a new term, potential of assembly formation (PAF) has been proposed to successfully predict the assembly outcomes based on the binding mode between GNP-glycans and lectins. Finally, the GNP-glycans can potently and completely inhibit DC-SIGN-mediated augmentation of Ebola virus glycoprotein-driven cell entry (with IC₅₀ values down to 95 pM), but only partially block DC-SIGNR-mediated virus infection. Our results suggest that the ability of a glycoconjugate to simultaneously block all binding sites of a target lectin is key to robust inhibition of viral infection.

Introduction

Multivalent lectin-glycan interactions are widespread and mediate many important biological functions which include cell-cell communication, pathogen-host cell recognition, attachment and infection, and modulation of immune responses.¹⁻⁹ As most monovalent lectin-glycan binding events are often too weak to be bio-functional, many lectins form multimeric structures to cluster their carbohydrate-binding-domains (CRDs) for efficient binding with spatially matched multivalent glycans to enhance binding affinity and specificity.¹⁰⁻¹⁴ The overall multivalent affinity is not only directly linked to the monovalent affinity, but also the glycan valency and the mode of binding. In general, a pair of spatially matched multivalent binding partners can bind simultaneously with each other and form a single entity. This gives the highest affinity enhancement and selectivity

due to the most favorable enthalpy and entropy terms.¹³ While those without such spatial and orientation match may inter-crosslink with each other to maximize binding enthalpy and form large scale assemblies, but this typically gives lower affinity enhancement and binding specificity due to an unfavorable entropy penalty.¹⁵ Therefore, understanding the structural mechanism underlying the affinity enhancement in multivalent protein-ligand binding is key to the design of potent, specific multivalent inhibitors against a target multivalent receptor. Notably, the development of potent glycoconjugates to block virus binding and infection of host cells can prevent virus mutation and developing resistance, a unique advantage over other anti-viral strategies.⁹

A number of different scaffolds, including DNAs, proteins, polymers, dendrimers, C₆₀ derivatives, vesicles, and inorganic

nanoparticles, have been employed to construct glycoconjugates^{14, 16-22} to study multivalent lectin-glycan recognition and develop effective interventions against certain diseases. Some of these have exhibited excellent potency in inhibiting pathogen infection.^{9, 11, 13-14, 16-20, 23-24} Most of the bindings have been evaluated by conventional biophysical techniques such as surface plasmon resonance (SPR),^{14, 22} and/or isothermal titration calorimetry (ITC).^{14, 25-27} Despite their wide use and good capacity in providing quantitative binding affinity, thermodynamic and kinetic data, these techniques cannot reveal key structural information of the target lectin, *e.g.* binding site orientation, inter-binding site distance, and binding mode which are critical to design potent, specific multivalent glycan inhibitors. Meanwhile, the unique size-dependent physical properties of nanomaterials have been harnessed to study lectin-glycan interactions. A good example here are gold nanoparticles (GNPs)²⁸ whose strong, size- and aggregation-state dependent absorption,²⁹⁻³⁰ powerful signal amplification in SPR,³¹ and surface enhanced Raman scattering³²⁻³³ have been widely exploited for biosensing and diagnostic applications. Despite these successes, glycan conjugated GNPs (GNP-glycans) have not been exploited as new structural and mechanistic probes for multivalent lectin-glycan interactions.

Recently, we have shown that CdSe/ZnS quantum dots (QDs) displaying a dense layer of mannose glycans are powerful structural probes for multivalent lectin-glycan binding.³⁴⁻³⁶ The QD probes can not only quantify the binding affinity *via* a ratiometric FRET readout but also dissect the different binding modes between a pair of closely-related, almost identical tetrameric lectins, DC-SIGN³⁷⁻³⁸ and DC-SIGNR³⁹ (collectively abbreviated as DC-SIGN/R hereafter). DC-SIGN/R are important lectin receptors which play a key role in facilitating the HIV, Ebola virus and Zika virus infections.^{34-35, 40-41} Moreover, DC-SIGN is also key to immune regulation,^{1, 5, 42-43} making it an highly attractive target for developing immunotherapies against important human diseases such as cancer, allergy, and autoimmune diseases.^{1, 42, 44} However, its tetrameric structure remains unknown, making it difficult to develop novel glycoconjugates that can potentially and specifically target DC-SIGN for therapeutic interventions, especially when the overlapping glycan specificity of various lectins is considered.¹ Using the QD-glycans, we have found that DC-SIGN's 4 CRDs bind simultaneously to one QD and give an impressive affinity enhancement factor (β) of up to 1.5 million fold over the corresponding monovalent binding. The QD-glycans also potently inhibit DC-SIGN-mediated augmentation of Ebola virus entry into host cells with sub-nM IC₅₀ values.³⁵ Despite such success, the cytotoxicity and long term toxicity of the CdSe QD scaffold has significantly limited its potential use as DC-SIGN targeting therapeutic agents, especially under *in vivo* conditions.

To address this issue, here we have constructed polyvalent glycan-nanoparticle probes on a 5 nm (diameter) GNP scaffold to take the advantages of GNPs' excellent biocompatibility, low/non-toxicity, and robust gold-thiol surface chemistry for easy control of the glycan density and surface presentation.²⁸ Moreover, after coating with lipoic acid (LA) based glycan ligands containing a terminal α -mannose (Man) or α -mannose- α -1,2-mannose (DiMan), the GNP-glycans are of the similar size and mannose glycan coating to gp120 trimer,⁴⁵⁻⁴⁶ the HIV surface densely glycosylated glycoprotein which is responsible for initiating HIV-DC-SIGN interaction to facilitate HIV infection. Thus, the GNP-glycans are good mimics of gp120 for probing its interaction with DC-SIGN. Using DC-SIGN/R as

model lectins, we show that the GNP's strong fluorescent quenching property⁴⁷ can be exploited as a reliable method to quantify multivalent GNP-glycan-lectin binding for the 1st time. Moreover, its nanoscale size, and high contrast under electron microscopy imaging have been integrated into a multimodal readout to reveal the different binding modes and affinity enhancement mechanisms for DC-SIGN/R (*e.g.* simultaneous binding for DC-SIGN, but inter-crosslinking for DC-SIGNR). Furthermore, the GNP-lectin binding modes are found to be directly linked to the GNP's ability to block lectin mediated virus infection of host cells: only a GNP-glycan which binds simultaneously to all binding sites of the target lectin can potently and completely block virus infection, but not that showing a cross-linking binding mode.

Results and Discussion

Ligand design and synthesis. The schematic structures of the GNP-glycan conjugates and the chemical structures of the glycan ligands used in this study are shown in **Figure 1**.

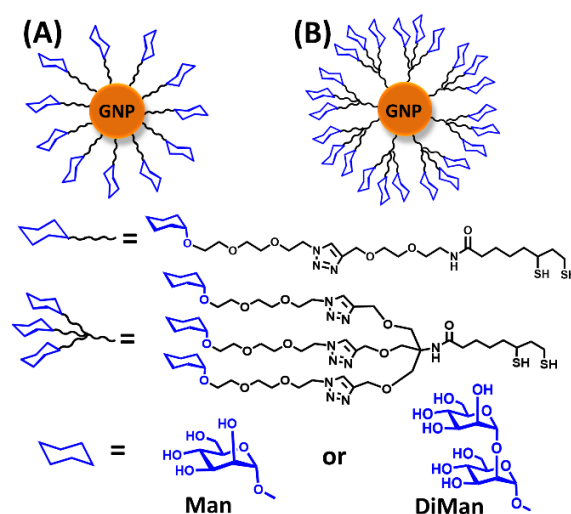
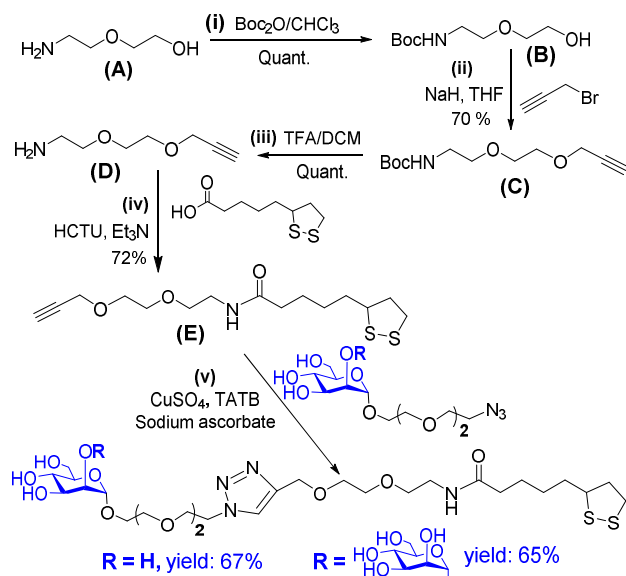


Figure 1. Schematic of the GNP-glycans used in this study. The GNP is coated with DHLA-based glycan ligand containing either one (A) or three (B) terminal α -1-mannose or α -1-mannose- α -1,2-mannose to tune its surface glycan valency. The chemical structures of glycan ligands are depicted underneath.

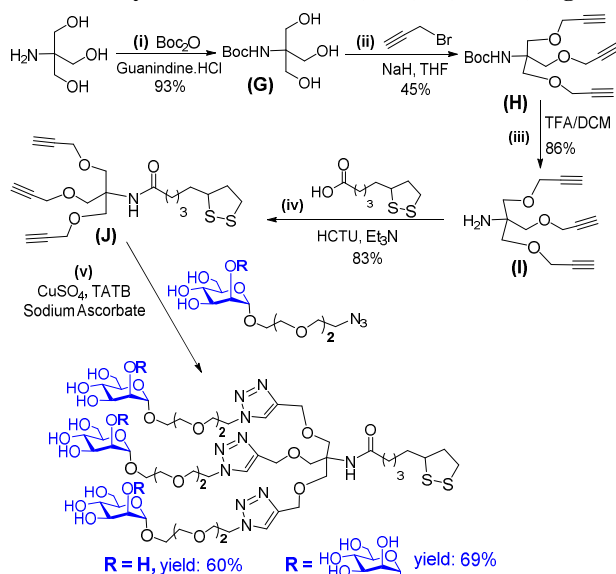
Each ligand contains three unique functional domains, a dihydro-lipoic acid (DHLA) for strong binding to the GNP surface *via* the formation of 2 strong Au-S bonds to impose excellent stability,⁴⁸ a short, flexible oligo-(ethylene glycol) linker to enhance water solubility, and to resist non-specific adsorption,^{35, 49} and a terminal an α -1-mannose or α -1-mannose- α -1,2-mannose (abbreviated as DHLA-Man or DHLA-DiMan, respectively, hereafter) for specific binding to DC-SIGN/R.³⁴⁻³⁵ We have found previously that DC-SIGN binds more efficiently to QDs capped with higher mannose densities.³⁴ Moreover, glycoconjugates with higher glycan valency have often shown to exhibit better virus inhibition potencies.^{9, 14, 16, 21, 50} Thus we are interested to study whether GNP-glycans' DC-SIGN/R binding affinity and virus inhibition potency can be further enhanced by increasing its surface glycan density. Thus, a branched version of the DHLA-glycan ligand, each containing three terminal glycans, are also synthesized (denoted as DHLA-(Man)₃ or DHLA-(DiMan)₃, respectively).⁵⁰ The branched ligands have the same DHLA anchoring group for GNP binding as the monomeric glycans. Thus, a similar number of ligands are expected to coat

each GNP, allowing us to prepare more densely glycosylated GNPs as shown schematically in **Figure 1**.

Scheme 1. Synthetic route to LA-Man-/DiMan ligands



Scheme 2. Synthetic route to LA-(Man)₃-(DiMan)₃ ligands



The synthetic routes to the monomeric LA-glycan and branched trimeric LA-(glycan)₃ ligands are shown in Schemes 1 and 2, respectively. First, the LA based linker molecules each containing one or three terminal acetylene groups were synthesized in good yields using the standard BOC protection and amide coupling chemistries. Details of the synthetic procedures and spectroscopic data of the intermediate compounds were provided in the Supporting Information (SI). Second, α -mannose and α -mannose- α -1,2-mannose appending a hydrophilic di(ethylene glycol)-azide linker (N_3 -EG₂-Man-/DiMan) to provide some flexibility to the terminal glycans were synthesized using our established procedures.³⁴⁻³⁵ Third, the LA-acetylene linkers were coupled efficiently to N_3 -EG₂-Man/DiMan *via* the Cu-catalyzed click chemistry¹⁶ to give the desired LA-Man/DiMan or LA-(Man)₃/(DiMan)₃ ligands (see Experimental Section for the general procedures and spectroscopic data, and SI for their ¹H

and ¹³C NMR spectra). Finally, the LA-glycan ligands were reduced quantitatively to their corresponding DHLA forms by tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl)⁵¹⁻⁵² before they were used to prepare the GNP-glycans.

GNP-glycan preparation. GNP-glycans (GNP capped with the DHLA-glycan ligands) were prepared by incubation of a 5 nm citrate stabilized GNP with the above DHLA-glycan ligands in water at a ligand:GNP molar ratio of 1000:1 for 24 h. Any free unbound ligands were removed by ultra-filtration using 30K MWCO filter tubes and washing with pure water. GNP-glycans prepared using LA-glycans or their reduced DHLA-glycan forms were found to be identical in terms of hydrodynamic sizes and stability. Thus the air-stable LA-glycan ligands could be directly used to make the GNP-glycans, eliminating the need of reduction and handling air-sensitive DHLA-glycan ligands that were essential in QD-glycan preparation *via* cap-exchange.^{34, 40, 48} This made the GNP-glycan preparation simple and straightforward. This result was fully consistent with earlier literature reports that dialkyldisulfides were cleaved upon binding to gold surfaces, forming identical self-assembled monolayers to their alkylthiol counterparts.⁵³ The GNP-glycans were highly stable, no changes of physical appearance or precipitation were observed after prolonged storage (>6 months) in a fridge. They were uniform and monodisperse in both water and in a binding buffer (20 mM HEPES, 100 mM NaCl, 10 mM CaCl₂, pH7.8) with hydrodynamic diameters (D_h s) of ~11 nm (see SI, Figure S1 and Table S1). Such D_h values matched well to the size of gp120 trimer (~12 nm).⁴⁵ Moreover, the GNPs were densely coated with mannose containing glycans similar to those found on gp120 surface. Using the ligand amount difference between that used and that remained in the supernatant after conjugation (measured by a phenol-sulfuric acid assay),³⁵ the numbers of ligands bound on each GNP were estimated as ~490, ~690, 720, and ~650 for LA-Man, LA-DiMan, LA-(Man)₃ and LA-(DiMan)₃, respectively. Using the D_h values and the method reported by the Mirkin group,⁵⁴ the average inter-glycan distances were estimated as ~0.97, ~0.83, ~0.46, and ~0.49 nm for GNP-Man, GNP-DiMan, GNP-(Man)₃ and GNP-(DiMan)₃, respectively (SI, Table S2). The average inter-glycan distances of GNP-Man/DiMan fell well within the majority of inter-glycan sequon distances (*e.g.* 0.7-1.3 nm) found on gp120,⁴⁶ but those of GNP-(Man/DiMan)₃ were considerably smaller. These results suggested that the GNP-Man/DiMan could act as good mimics of gp120 for probing its DC-SIGN interaction.

Quantifying GNP-glycan-DC-SIGN/R binding affinity. Besides strong plasmonic absorption, GNPs are well-known for their strong and universal fluorescence quenching property.⁴⁷ Moreover, its quenching has shown to follow the nano-surface energy transfer (NSET),⁵⁵⁻⁵⁶ rather than Förster resonance energy transfer (FRET) mechanism. As a result, its quenching is more superior and covers a longer distance range than most organic quenchers relying on the FRET mechanism.^{47, 55} Indeed, the GNP's superior quenching ability has been widely exploited for biosensing applications,⁵⁷⁻⁵⁸ but not as a readout method for lectin-glycan binding quantification. Here the GNP's outstanding fluorescence quenching was exploited as a new readout for multivalent lectin-glycan binding quantification for the first time (**Figure 2A**). To do this, we first introduced a cysteine site-specifically on the CRD (outside its glycan binding pocket so not affecting its glycan binding properties) of DC-SIGN/R extracellular segments by mutagenesis. The mutant proteins have shown to retain faithfully the tetrameric structure and glycan binding properties of wildtype proteins.³⁴⁻³⁵ We then labeled the

proteins using a maleimide modified Atto-594 and confirmed the labeling by high resolution mass spectroscopy (SI, Figure S2). We then recorded the fluorescence spectra of labeled DC-SIGN/R without and with each GNP-glycan under a fixed protein:GNP molar ratio of 1:1 over a concentration range of 0-64 nM. All binding studies were carried out in a binding buffer containing a large excess of bovine serum albumin (BSA, 1 mg/mL final concentration). The inclusion of large excesses of BSA, a non-target serum protein of high abundance *in vivo*, in binding studies made the conditions resemble more closely to real biological situations. Moreover, this also greatly reduced non-specific interactions and adsorption of proteins and GNPs to surfaces (a main source of experimental errors at low concentrations, *e.g.* < 10 nM).⁵⁹

The typical fluorescence spectra showing the binding between DC-SIGN and GNP-DiMan were given in **Figure 2B**. In the absence of GNP-DiMan, DC-SIGN's fluorescence intensity was found to increase linearly with concentration. While in the presence of GNP-DiMan, its fluorescence was greatly reduced and increasingly deviated from the linear relationship with the increasing concentration (SI, Figure S3). This result was fully consistent with that, with the increasing concentration, an increasing proportion of DC-SIGN were bound to and quenched by GNP-DiMan in close proximity. The specificity of DC-SIGN-GNP-DiMan binding was verified by two controls: (1) mixing DC-SIGN with a GNP capped with a control DHLA-EG₂-OH ligand lacking the terminal glycan produced negligible quenching; (2) free mannose could effectively compete with GNP-DiMan binding to DC-SIGN, leading to a significant, dose-dependent fluorescence recovery (SI, Figure S4).

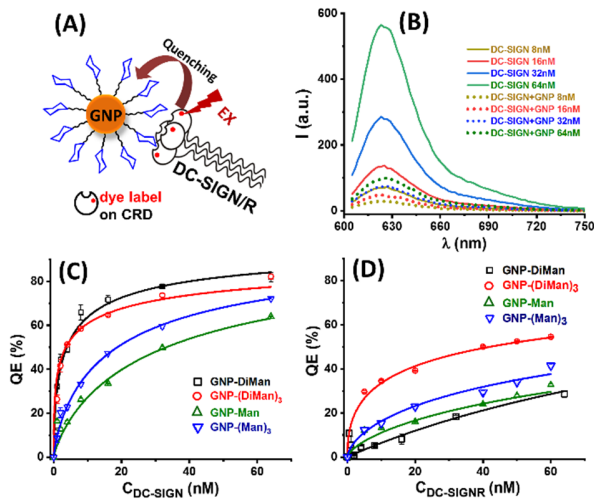


Figure 2. (A) Schematic showing the quenching of DC-SIGN/R (dye-labeled) fluorescence upon binding to a GNP-glycan, which acts as a readout for binding quantification. (B) Typical fluorescence spectra of varied concentrations of DC-SIGN without (solid lines) and with (broken lines) 1 molar equivalent of GNP-DiMan ($\lambda_{EX} = 590$ nm). (C, D) The quenching efficiency (QE%-)concentration relationship for DC-SIGN (C) or DC-SIGNR (D) binding to the various GNP-glycans fitted by the Hill's equation.

To analyze the binding data quantitatively, we first calculated the quenching efficiency (QE) at each concentration (C) *via*:

$$QE\% = (IF_0 - IF)/IF_0 \times 100\% \quad (1)$$

Where IF_0 and IF are the integrated fluorescence of the protein in the absence and presence of each GNP-glycan, respectively. Given that the fluorescence of DC-SIGN/R increases linearly with C in the absence of GNP-DiMan (SI, Figure S3), and GNPs can completely quench various fluorophores at close proximity (*e.g.* > 99.9%),⁴⁷ the QE% thus faithfully represents the percentage of proteins bound to the GNP-glycan. Thus, the apparent binding dissociation constant (K_d) can be derived from the QE- C relationship by fitting with Hill's equation:

$$QE = QE_{max} \times C^n / (K_d^n + C^n) \quad (2)$$

Where QE_{max} , K_d , C , and n are the maximum QE (fixed at 100), apparent binding dissociation constant, protein concentration, and Hill coefficient, respectively.

Table 1. Summary of the fitting parameters of DC-SIGN/R binding to various GNP-glycans derived from Figures 2C/D.

GNP-glycan + Protein	K_d (nM)	n	R^2
GNP-Man + DC-SIGN	33.1 ± 2.1	0.82 ± 0.07	0.997
GNP-(Man) ₃ + DC-SIGN	18.7 ± 0.3	0.77 ± 0.01	0.999
GNP-DiMan + DC-SIGN	3.9 ± 0.3	0.59 ± 0.03	0.999
GNP-(DiMan) ₃ + DC-SIGN	3.6 ± 0.1	0.42 ± 0.01	1.00
GNP-Man + DC-SIGNR	214 ± 68	0.68 ± 0.10	0.994
GNP-(Man) ₃ + DC-SIGNR	133 ± 20	0.63 ± 0.07	0.996
GNP-DiMan + DC-SIGNR	152 ± 37	0.99 ± 0.14	0.954
GNP-(DiMan) ₃ + DC-SIGNR	42 ± 2	0.48 ± 0.03	0.999

Figures 2B and **2C** showed the best fits of the QE- C relationships for DC-SIGN/R binding to each GNP-glycan. The resulting fitting parameters were summarized in Table 1. Four key points could be drawn from the binding data given in Table 1. (1) DC-SIGN bound more strongly to GNP-glycans than DC-SIGNR did. This effect was particularly pronounced for GNP-DiMan where the binding affinity difference was as high as ~40 fold. As GNP-DiMan here presented a good mimic of gp120, its stronger binding affinity with DC-SIGN over DC-SIGNR could thus help explain why DC-SIGN was found more effective than DC-SIGNR in transmitting infections for some HIV strains.⁶⁰ This result also agreed well with those reported previously with QD-glycans where the binding K_d s were measured ratiometrically *via* FRET.³⁴ Given that the CRDs in DC-SIGN/R had the same mannose binding motifs,⁶¹ the difference here indicated that DC-SIGN/R might adopt different modes in binding to GNP-glycans, similar to those observed with QD-glycans.³⁵ (2) Further increasing the GNP surface glycan density *via* capping with the dendritic LA-(glycan)₃ ligands significantly improved the binding affinity with DC-SIGNR (*e.g.* $K_d \sim 42$ v.s. ~ 152 nM, or 3.6 fold enhancement for GNP-(DiMan)₃ v.s. GNP-DiMan), but not much with DC-SIGN, suggesting that DC-SIGNR may prefer binding to GNPs with even higher glycan densities. (3) Low nM K_d (*e.g.* 3.9 nM) for DC-SIGN binding with GNP-DiMan was obtained, which was ~250,000 fold tighter than the corresponding monomeric binding between DiMan and DC-SIGN CRD ($K_d = 0.9$ mM),²⁵ suggesting that a polyvalent display of the glycans on the GNP greatly enhanced its multivalent binding affinity with DC-SIGN. These results were fully consistent with those reported for other multivalent lectin-glycoconjugate interactions.^{10, 14, 18, 22} (4) A higher degree of affinity enhancement was observed for DC-SIGN binding to GNP-DiMan over GNP-Man, possibility due to the former being able to exploit more efficiently the CRD's secondary glycan

bind sites than the later.⁶¹ Overall, these results agreed well with those reported previously with the QD-glycans.³⁵

Probing DC-SIGN/R-GNP-glycan binding mode and affinity enhancing mechanism. First we monitored the D_h s of GNP-DiMan before and after binding to wildtype DC-SIGN/R (unlabeled) by dynamic light scattering (DLS).⁶² GNP-DiMan displayed a single D_h species of ~ 11 nm with a narrow distribution (full width at half maximum, FWHM = 3.5 nm, **Figure 3A1**) in the binding buffer. After mixing with DC-SIGN (protein: GNP

molar ratio = 20:1), the size of the dominate species was significantly increased ($D_h = \sim 41$ nm) and broadened (FWHM ~ 28 nm, **Figure 3A2**). This result was consistent with that expected for each GNP-glycan being bound by a limited number of DC-SIGN molecules, forming a protein shell on the GNP. In contrast, mixing DC-SIGNR with GNP-DiMan produced a dominant species whose D_h was gradually increased from *ca.* 300 to >2000 nm over a period of 4 h (**Figure 3A3**). Such sizes were clearly too big to be individual GNP-DC-SIGNR assemblies, a strong indication of macroscopic assembly, arising presumably from DC-SIGNR and GNP-DiMan inter-crosslinking.

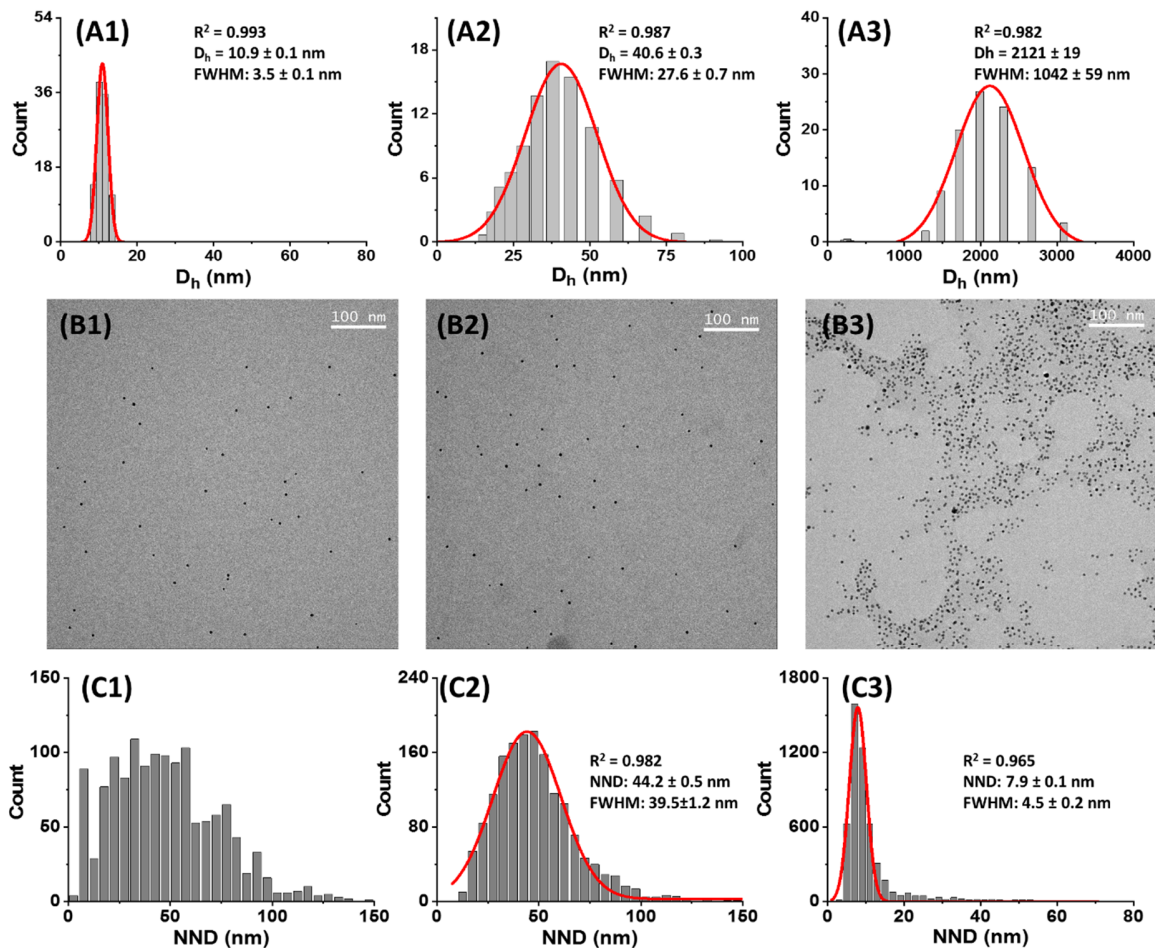


Figure 3. Typical D_h distribution histograms of GNP-DiMan only (10 nM, **A1**); GNP-DiMan (10 nM) + wildtype DC-SIGN (200 nM, **A2**) or GNP-DiMan (16 nM) + wildtype DC-SIGNR (640 nM, **A3**). TEM images of cryo-prepared samples of GNP-DiMan only (40 nM, **B1**), GNP-DiMan (40 nM) + DC-SIGN (1.5 μ M, **B2**) or GNP-DiMan (40 nM) + DC-SIGNR (1.5 μ M, **B3**), and their corresponding GNP nearest neighbor distance (NND) histograms (**C1**, **C2**, **C3**). The red curves show the Gaussian fits of the histograms.

This assumption was verified by transmission electron microscopy (TEM) imaging of cryo-prepared GNP-DiMan-protein samples *via* rapid plunge freezing followed by vacuum drying which we had shown previously to preserve the native dispersion states of nanoparticle solutions.⁶³ In the absence of DC-SIGN/R, GNP-DiMan appeared as isolated single particles (**Figure 3B1**) and gave a randomly distributed nearest neighbor distance (NND) over a range of 10 to 100 nm (**Figure 3C1**). Binding of DC-SIGN with GNP-DiMan also gave isolated single particles (**Figure 3B2**), although the resulting NND distribution histogram could be fitted well ($R^2 = 0.982$) by a single

Gaussian distribution to yield an average NND of ~ 44 nm (**Figure 3C2**). Interestingly, this NND value matched well to the D_h size of GNP-DiMan-DC-SIGN assembly (~ 41 nm), suggesting that the formation of a layer of DC-SIGN on the GNP prevented individual GNPs from coming close to each other. In contrast, binding of DC-SIGNR with GNP-DiMan produced large scale, closely-packed GNP assemblies of hundreds of nm cross (**Figure 3B3**) with a rather small NND of ~ 7.9 nm (**Figure 3C3**). This result agreed well with the DLS measurement, confirming that binding of DC-SIGNR led to macroscopic assemblies of GNPs *via* DC-SIGNR-GNP-DiMan intercross linking. These

results revealed that the binding modes of DC-SIGN/R to GNP-DiMan were clearly different: DC-SIGN must have bound simultaneously to one GNP-DiMan *via* all of its four binding sites and formed a layer of proteins surrounding each GNP, giving rise to isolated single particles. In contrast, DC-SIGNR and polyvalent GNP-DiMan inter-crosslinked each other, and formed large scale but closely-packed GNP-DC-SIGNR assemblies. Therefore, by harnessing GNPs' nanoscale size and high contrast under TEM imaging, we have developed a new multimodal readout which successfully dissected the distinct modes of GNP-DiMan in binding to DC-SIGN/R. This result agreed fully with those reported previously with QD-glycans.³⁵

The distinct binding modes exhibited by DC-SIGN/R could also help explain their different affinities with GNP-glycans. For DC-SIGN, its simultaneous binding with one GNP *via* all 4 of its CRDs should yield strong affinity enhancement from both the favorable enthalpy and entropy terms. For DC-SIGNR, whilst its inter-crosslinking mode of binding with GNP-glycans could maximize the binding enthalpy, the resulting large scale assemblies would incur significant entropy penalty. As a result, DC-SIGN should bind more strongly to GNP-glycans than DC-SIGNR did, matching well to the apparent K_d data measured by fluorescence quenching (Table 1).

Correlation between lectin-GNP-glycan affinity and assembly. The credibility of this GNP quenching based affinity measurement method was further verified by the DLS data. For example, binding of an increasing amount of DC-SIGN to a fixed amount of GNP-DiMan (10 nM) gave a more rapid increase of D_h than that to GNP-Man (Figure 4A), consistent with the former being a stronger binding partner than the latter (apparent K_d : 3.9 *v.s.* 33 nM). Moreover, mixing an increasing amount of DC-SIGNR with GNP-(Man)₃ yielded no noticeable D_h changes throughout the concentration range studied (20-200 nM), indicating no measurable binding (Figure 4B). While mixing DC-SIGNR with GNP-(DiMan)₃ also produced no apparent D_h changes initially (≤ 100 nM); as DC-SIGNR concentration was increased further, a dramatic increase of D_h was observed, particularly at 200 nM, a clear indication of the formation of large scale GNP-DC-SIGNR assemblies (see Figure 4B, and SI, Figures. S5-S8).

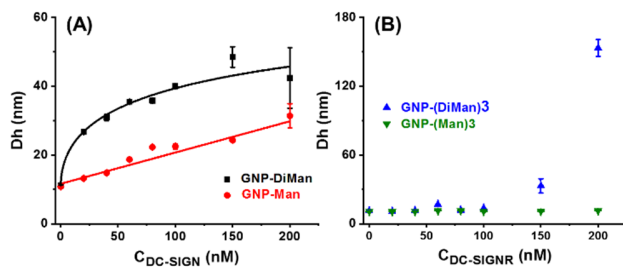


Figure 4. D_h - C_{protein} relationships for GNP-glycan (10 nM) binding to wildtype DC-SIGN/R at different concentrations. (A) DC-SIGN binding to GNP-DiMan (black squares, data fitted by Hill's equation: $Y = a + (b-a) \times X^n / (k^n + X^n)$; where $a = 11.1 \pm 0.0$ nm; $b = 80 \pm 99$ nm; $k = 200 \pm 990$ nM and $n = 0.53 \pm 0.36$, $R^2 = 0.997$) or GNP-Man (red dots, data fitted by a linear relationship, $Y = a + b \times X$; where $a = 11.6 \pm 0.7$ nm; $b = 0.091 \pm 0.010$, $R^2 = 0.928$); (B) DC-SIGNR binding to GNP-(DiMan)₃ (blue triangles) or GNP-(Man)₃ (green triangles).

To provide a more quantitative explanation of the data, here we introduced a new term: the potential of assembly formation (PAF):

$$\text{PAF} = C_{0(\text{GNP})} \times C_{0(\text{protein})} / (K_d)^2 \quad (3)$$

Where $C_{0(\text{GNP})}$, $C_{0(\text{protein})}$ and K_d are GNP-glycan and protein starting concentrations and their apparent binding K_d , respectively. As K_d indicates 50% binding at equilibrium, the PAF thus represents the ratio of a reaction quotient to equilibrium of a reversible binding reaction. Where $\text{PAF} = 1$ would indicate equilibrium (50%) binding, while $\text{PAF} > 1$ indicates binding being favorable and $\text{PAF} > 10$ being strongly favorable (>90%). Similarly, $\text{PAF} < 1$ indicates binding being unfavorable and $\text{PAF} < 0.1$ being strong unfavorable (<10% binding).

Using this definition and the apparent K_d s given in Table 1, the PAFs for DC-SIGN binding to GNP-Man and GNP-DiMan were in the range of 0.18-1.8, and 13-131, respectively. While those for DC-SIGNR binding to GNP-(Man)₃ and GNP-(DiMan)₃ ranged from 0.011 to 0.11, and 0.11 to 1.1. Combining the PAF value and binding mode would allow us to predict the assembly outcome. For example, with a PAF of >10 and simultaneous binding, most of the added DC-SIGN molecules should readily bind to GNP-DiMan to saturate its surface binding capacity, giving rise to a rapidly increasing and quickly saturated D_h with the increasing protein concentration. This was exactly what was measured from DLS. In contrast, the PAF of DC-SIGN-GNP-Man binding span across 1, which would indicate a gradually increased binding, again matching well to a positive, linear D_h -concentration relationship (Figure 4A). For inter-crosslinking binding between DC-SIGNR and GNP-glycans, large-scale assemblies were only observed for GNP-(DiMan)₃ mixed with the highest protein concentration (200 nM) which had a PAF of 1.1 and indicated the binding became favorable. While all other conditions where PAFs were < 1, no significant binding (assembly) was observed (Figure 4B). Together, these results established PAF as a useful indicator for predicting the binding (assembly) outcome of multivalent lectin-glycan binding partners. Furthermore, the excellent agreement between the predicted outcome based on PAFs (derived from K_d s measured *via* the GNP quenching method) and those observed from DLS implied that this new fluorescence quenching based binding affinity method was highly credible and reliable. Interestingly, the ability of lectins to bind and inter-crosslink with virus surface glycoproteins had shown to play an important role in virus neutralization.⁶⁴⁻⁶⁶ Thus, we anticipate that the PAF criteria proposed here could act as a useful tool to predict lectin-virus interactions and neutralization.

Blocking DC-SIGN/R-mediated augmentation of Ebola virus glycoprotein-driven transduction. The distinct binding modes and affinities of GNP-glycans with two important viral receptors, DC-SIGN/R, should result in different abilities in blocking DC-SIGN/R-mediated virus infection of host cells. To investigate this potential and any possible correlation, human embryonic kidney 293T cells transfected to express DC-SIGN or DC-SIGNR on their membrane were used as described previously.³⁵ Murine leukemia virus (MLV) vector particles bearing the Ebola virus surface glycoprotein (EBOV-GP) and encoding the luciferase gene were used to model Ebola virus entry into cells. The virus particles can bind efficiently to DC-SIGN/R (*via* their surface EBOV-GP) and binding results in increased transduction as determined by luciferase expression in host cells.³⁴⁻³⁵ Binding of high affinity GNP-glycans to host cell surface DC-SIGN/R could block these lectin receptors from further binding to EBOV-GP, reducing luciferase gene transduction. Indeed, pretreatment of 293T cells with GNP-glycans ef-

ficiently inhibited DC-SIGN-mediated augmentation of transduction in a dose-dependent manner (see SI, Figure S10). GNP-DiMan/(DiMan)₃ were found to be more potent in blocking the transduction than GNP-Man/(Man)₃. This result was consistent with the former being stronger DC-SIGN binders than the latter. The dose-dependent inhibition data were fitted well by a simple competition model:

$$NA = IC_{50}/[IC_{50} + C] \quad (4)$$

Where NA , IC_{50} and C were normalized luciferase activity, GNP concentration giving 50% inhibition, and GNP concentration, respectively. Whilst DC-SIGNR-mediated augmentation of transduction was also inhibited by GNP-glycans, the resulting

dose-dependent inhibition data were best fitted by a modified competition model:

$$NA = EC_{50}^n/[EC_{50}^n + C^n] \quad (5)$$

Where the inhibition coefficient, n , was found to be in the range of 0.4 to 0.6 (Table 2). Moreover, GNP-glycans could not completely inhibit DC-SIGNR-mediated transduction, even at high concentrations (see **Figure 5A/B**, and also SI, Figure S11). This result revealed that GNP-glycans were much less effective in blocking DC-SIGNR-mediated augmentation of viral gene transduction as compared to that of DC-SIGN-dependent transduction.

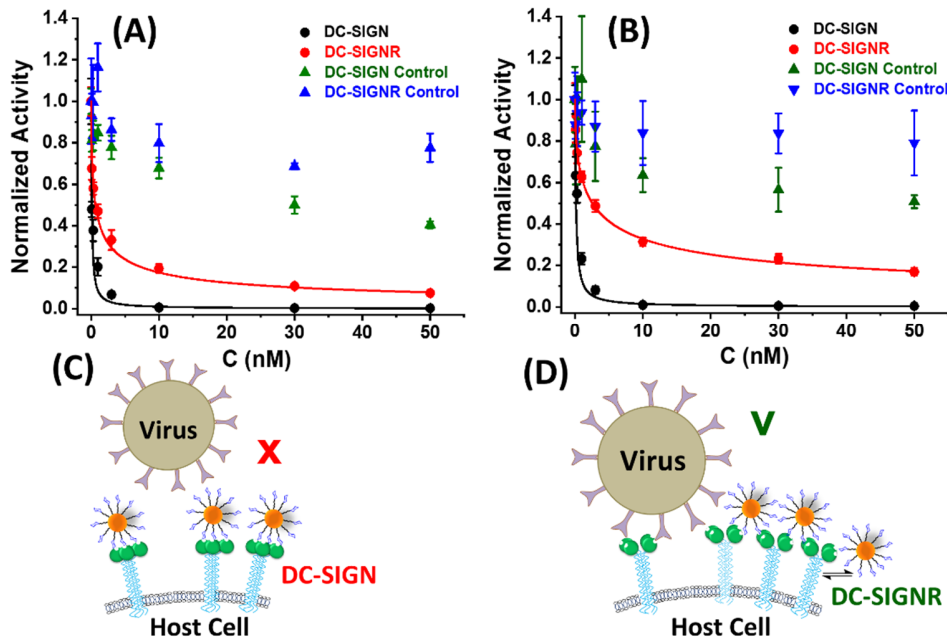


Figure 5. Plot of normalized luciferase activities of DC-SIGN- or DC-SIGNR- expressing 293T cells as a function of the concentration of GNP-DiMan (A) or GNP-(DiMan)₃ (B) inhibitors. Data for virus particles bearing the EBOV-GP are shown in dots while those of control virus particles bearing the VSV-G are shown in triangles. Schematics beneath showing the different inhibition efficiencies of GNP-glycans against DC-SIGN (C) or DC-SIGNR (D) mediated infections. (C) For DC-SIGN expressing host cells, all of the binding sites of its surface DC-SIGNs are bound and blocked by GNP-glycans, making them unavailable for further binding to virus surface EBOV-GPs to initiate infection. (D) For DC-SIGNR expressing host cells, GNP-glycans cross-link with some surface DC-SIGNRs but the bindings are weak and dynamic at the edges, allowing the virus to access the unblocked DC-SIGNR binding sites to initiate infection.

Table 2. Summary of inhibition data for GNP-glycans against DC-SIGN/R-mediated EBOV-GP driven infection of 293T host cells. * shows the EC_{50} values for DC-SIGNR.

GNP-glycan	Receptor	IC/EC ₅₀ (nM)	n	R ²
GNP-Man	DC-SIGN	0.26±0.08	1	0.889
GNP-(Man) ₃	DC-SIGN	1.57±0.25	1	0.937
GNP-DiMan	DC-SIGN	0.095±0.017	1	0.921
GNP-(DiMan) ₃	DC-SIGN	0.15±0.03	1	0.890
GNP-Man	DC-SIGNR	7.3±1.2*	0.42±0.04	0.980
GNP-(Man) ₃	DC-SIGNR	53±17*	0.61±0.14	0.902
GNP-DiMan	DC-SIGNR	0.63±0.09*	0.56±0.02	0.990
GNP-(DiMan) ₃	DC-SIGNR	2.6±0.1*	0.53±0.02	0.997

We believe this result is reasonable from both the binding affinity (GNP-glycans bind more weakly to DC-SIGNR than to DC-

SIGN) and binding mode points of view. Each GNP-glycan could bind simultaneously to all 4 of DC-SIGN's binding sites, completely blocking them from further binding to virus surface EBOV-GPs to initiate cell entry (Figure 5C). In contrast, the inter-crosslinking mode of binding between GNP-glycans and DC-SIGNR meant that each GNP could only bind to 2 of the 4 binding sites in DC-SIGNR. It would be very difficult for GNP-glycans to cross-link all cell surface DC-SIGNRs to fully block their binding sites. Even if this was possible, the DC-SIGNRs at the cluster edge would still only bind divalently (*via* 2 outward facing binding sites) to GNP-glycans, where the binding would be weak and dynamic. These, together with any unbound binding sites, could act as toe-holds for binding to viral surface EBOV-GPs to initiate cell uptake and infection (**Figure 5D**). Therefore, an inhibitor that inter-crosslinks with target receptors would be much less effective in blocking virus infection than its simultaneous binding counterpart, making it almost impossible to achieve complete inhibition (**Figure 5C/D**). Moreover, luciferase activities of control virus particles bearing the

vesicular stomatitis virus glycoprotein (VSV-G), which cannot use DC-SIGN/R for augmentation of cell entry, were not affected significantly by GNP-glycan treatment, confirming that the observed inhibitory effects were specific (SI, Figure S10). Importantly, the IC₅₀ value of GNP-DiMan in blocking DC-SIGN-mediated augmentation of EBOV-GP-dependent entry was measured as 0.095±0.017 nM. This value was considerably lower (by almost an order of magnitude) than those measured for some of the most potent glycoconjugate inhibitors of Ebola virus infection reported previously (*e.g.* the giant globular multivalent glycofullerenes, IC₅₀: 0.67 nM²¹, the virus-like glycodendrimer nanoparticles, IC₅₀: 0.91 nM,¹⁶ and our previous QD-EG₃-DiMan, IC₅₀: 0.70±0.2 nM³⁵). This result demonstrated an outstanding potency of the GNP-glycans in blocking DC-SIGN-mediated EBOV-GP driven infection of host cells. Importantly, as virus surface glycans are maintained by host cell's glycosylation apparatus, they are mostly conserved and unaffected by virus mutation.⁶⁷ Therefore, the potential advantages of developing potent glycoconjugate viral inhibitors over other anti-viral strategies include two folds: (1) it can reduce the chances of virus mutation by blocking its entry to host cells, and (2) its treatment potency is unlikely affected by virus mutation, allowing us to provide a potentially long lasting solution.

Conclusion

In summary, we have developed polyvalent GNP-glycans as new powerful structural and mechanistic probes for multivalent lectin-glycan interactions. By exploiting GNPs' outstanding fluorescence quenching property, we have developed a new method for quantifying multivalent lectin-glycan interaction, revealing that a polyvalent display of mannose containing glycans on GNPs greatly enhances their binding affinities with two vitally important viral receptors, DC-SIGN/R. Moreover, by exploiting GNPs' nanoscale size and high TEM contrast, we have revealed the distinct binding mode and affinity enhancing mechanisms for GNP-glycans binding with DC-SIGN/R. Importantly, the assembly outcomes of the dose-dependent GNP-lectin binding are in perfect agreement with those predicted from their respective binding modes and affinities, verifying the credibility of our new affinity method. Furthermore, we have found that GNP-glycans can potentially block DC-SIGN-mediated EBOV-GP driven viral infections of host cells with IC₅₀ values down to 95±17 pM, making it the most potent glycoconjugate inhibitor against EBOV-GP driven infection. Notably, its inhibition potency will unlikely be affected by virus mutation, allowing us to provide potentially a long lasting solution. Finally, we have revealed that only a GNP-glycan inhibitor showing a simultaneous-, but not a crosslinking-, binding mode is able to completely block the target lectin mediated virus infection. Together, these data provide a useful guidance in designing polyvalent glycoconjugates for potent, specific inhibition of virus infection. Given their low-/non- toxicity and excellent biocompatibility, the GNP-glycans are perfectly placed for a wide range of applications, from probing fundamental structural mechanisms of glycobiology, developing novel biophysical and biomedical assays, to developing novel therapeutics against deadly virus infections and immune dysregulation diseases such as cancer, allergy and autoimmune diseases.

Experimental Section

Materials. Gold nanoparticle (nominal diameter 5 nm, λ_{max} ~520 nm) was purchased from Sigma Aldrich as a stabilized suspension in citrate buffer or synthesized in house. 2-(2-aminoethoxy)-ethanol, Di-*tert*-butyl decarbonate, sodium sulfate, sodium hydride

(60% dispersion in mineral oil), 3-bromo-1-propyne, potassium hydroxide, trifluoroacetic acid, triethylamine, sodium bicarbonate, *O*-(6-Chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexa fluorophosphate (HCTU), lipoic acid (LA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP.HCl), copper sulfate, sodium ascorbate, Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA), tris(hydroxymethyl)aminomethane (Tris base), guanidine hydrochloride, anhydrous DMF and other chemicals were purchased from Sigma Aldrich, Alfa Aesar, Fluorochem, Thermo Scientific, VWR International or Acros organics with >99% impurity and used as received without further purification unless specified elsewhere. All the solvents were obtained in >99% purity from Fischer Scientific and used as received. When used as reaction solvents in anhydrous reactions, THF and CH₂Cl₂ were dried and deoxygenated using an Innovative Technology Inc. PureSolv® solvent purification system. Ultra-pure water (resistance >18.2 MΩ.cm) purified by an ELGA Purelab classic UVF system, was used for all experiments and making buffers.^{59, 62}

Glycan-ligand synthesis by click chemistry. The general protocols employed in preparing the LA-glycan ligands *via* click chemistry were as follow.¹⁶ To a 1:1 (v:v) mixed THF:H₂O solution (2.0-5.0 mL) containing the glycan-EG₂-N₃ (1.1 mole equiv.) and LA-linker (1 mole equiv.) were added CuSO₄.5H₂O (0.036 mole equiv.), TBTA (0.063 mole equiv.) followed by sodium ascorbate (0.135 mole equiv.) and the resulting solution was stirred at RT. After 3 h, TLC confirmed the consumption of all starting materials. The solvent was then evaporated and the crude product was purified by size exclusion chromatography using Biogel P2 column using water as eluent to afford the desired pure LA-glycan product.

LA-Man: Yield = (67%, 0.18 mmol). ¹H NMR (500 MHz, CD₃OD) δ (ppm): 8.04 (s, 1H, triazole-H), 4.8 (d, 1H, *J* 1.6 Hz, Man H-1), 4.65 (s, 2H), 4.6 (t, 2H, *J* 5 Hz), 3.9 (t, 2H, *J* 5.1 Hz), 3.86-3.81 (m, 3H), 3.8 (dd, 1H, *J* 1.7 Hz, 3.4 Hz, Man H-2), 3.72 (d, 1H, *J* 5.5 Hz), 3.69-3.67 (m, 3H), 3.66 (s, 2H), 3.65-3.63 (m, 2H), 3.62-3.61 (m, 4H), 3.60-3.57 (m, 2H), 3.53 (t, 2H, *J* 5.5 Hz), 3.39-3.34 (m, 3H), 3.20-3.15 (m, 1H), 3.12-3.07 (m, 1H), 2.49-2.42 (m, 1H), 2.20 (t, 2H, *J* 7.4 Hz), 1.92-1.85 (m, 1H), 1.73-1.59 (m, 4H), 1.50-1.40 (m, 2H). ¹³C NMR (125 MHz, CD₃OD) δ: 176.1 (C=O), 145.7 (C=C), 126.0 (C=C), 101.7 (Man C-1), 74.6, 72.6, 72.1 (Man C-2), 71.6, 71.5, 71.4, 71.3, 70.8, 70.6, 70.4, 68.6, 67.7, 65.0, 63.0 (Man C-6), 57.6, 51.5, 41.3, 40.3, 39.3, 36.8, 35.7, 29.8, 26.7. HRMS: calculated *m/z* for C₂₇H₄₉N₄O₁₁S₂ (M+H)⁺ 669.2834; found 669.2838.

LA-DiMan: Yield = (65%, 0.080 mmol). ¹H NMR (500 MHz, CD₃OD) δ (ppm): 8.05 (s, 1H, triazole-H), 5.11 (d, *J* 1.7 Hz, 1H), 4.96 (d, *J* 1.8 Hz, 1H), 4.66 (s, 2H), 4.60 (t, 2H, *J* 5.1 Hz), 3.97 (dd, 1H, *J* 3.3, 1.8 Hz), 3.91 (dd, 2H, *J* 5.5, 4.7 Hz), 3.88 – 3.78 (m, 6H), 3.72 – 3.65 (m, 8H), 3.62 (dt, 6H, *J* 5.0, 1.3 Hz), 3.58 (dt, 3H, *J* 11.2, 1.6 Hz), 3.53 (t, 2H, *J* 5.5 Hz), 3.43 – 3.32 (m, 3H), 3.17 (ddd, 1H, *J* 10.9, 7.1, 5.4 Hz), 3.10 (dt, 1H, *J* 11.0, 6.9 Hz, 1H), 2.46 (dtd, 1H, *J* 12.1, 6.7, 5.4 Hz), 2.20 (t, 2H, *J* 7.4 Hz), 1.89 (dq, 1H, *J* 12.7, 6.9 Hz), 1.77 – 1.57 (m, 4H), 1.45 (qt, 2H, *J* 9.4, 5.9 Hz). ¹³C NMR (125 MHz, CD₃OD) δ: 176.1 (C=O), 145.7 (C=C), 126.0 (C=C), 104.2 (Man C-1), 100.1 (Man C-1'), 80.6, 75.1, 74.7, 72.4, 72.1, 71.9, 71.6, 71.5, 71.4, 71.3, 70.8, 70.6, 70.5, 69.0, 68.8, 67.9, 65.0, 63.2, 63.1, 57.6, 51.5, 41.3, 40.4, 39.3, 36.8, 35.7, 29.8, 26.7. HRMS: calculated *m/z* for C₃₃H₅₈N₄O₁₆S₂ (M+H)⁺ 831.3233; found 831.3242.

LA-(Man)₃: Yield = (60%, 0.060 mmol). ¹H NMR (500 MHz, D₂O) δ (ppm): 8.03 (s, 3H, triazole-H), 4.87 (d, 3H, *J* 1.8 Hz, H-1), 4.64 – 4.61 (m, 6H), 4.60 (s, 6H), 3.98 – 3.96 (m, 6H), 3.95 (dd, 3H, *J* 3.5, 1.8 Hz, H-2), 3.89 (d, 1H, *J* 2.0 Hz), 3.87 (d, 2H, *J* 1.7 Hz), 3.84 – 3.79 (m, 6H), 3.77 (d, 2H, *J* 5.6 Hz), 3.74 (d, 7H, *J* 3.1 Hz), 3.67 (d, 3H, *J* 9.8 Hz), 3.65 – 3.61 (m, 25H), 3.61 – 3.53 (m, 3H), 3.21 – 3.12 (m, 2H), 2.42 (dq, 1H, *J* 12.3, 6.1 Hz), 2.18 (t, 2H, *J*

7.0 Hz), 1.91 (dt, 1H, J 13.6, 6.8 Hz), 1.71 – 1.64 (m, 1H), 1.58 – 1.48 (m, 4H), 1.33 (q, 3H, J 7.7 Hz). ^{13}C NMR (125 MHz, D_2O) δ : 176.5 (C=O), 144.0, 125.3, 99.9 (C-1), 72.7, 70.5, 69.9, 69.5, 69.4, 68.8, 67.5, 66.7, 66.3, 63.5, 60.9, 59.7, 56.5, 50.0, 40.2, 38.0, 35.9, 33.7, 27.5, 24.9. HRMS: calculated m/z for $\text{C}_{57}\text{H}_{99}\text{N}_{10}\text{O}_{28}\text{S}_2$ (M+H) $^+$ 1435.6066; found 1435.6096.

LA-(DiMan) $_3$: Yield = (69%, 0.030 mmol). ^1H NMR (500 MHz, D_2O) δ (ppm): 8.03 (s, 3H, triazole-H), 5.11 (d, 3H, J 1.8 Hz, H-1), 5.03 (d, 3H, J = 1.9 Hz, H-1'), 4.64 – 4.62 (m, 6H), 4.60 (d, 6H, J 2.9 Hz), 4.08 (dd, 3H, J 3.4, 1.8 Hz, H-2), 3.97 (td, 9H, J = 4.5, 2.5 Hz), 3.92 – 3.68 (m, 40H), 3.66 – 3.61 (m, 29H), 3.58 – 3.53 (m, 3H), 3.21 – 3.13 (m, 2H), 2.42 (dq, 1H, J 12.3, 6.1 Hz), 2.18 (t, 2H, J 7.0 Hz), 1.91 (dd, 1H, J 13.1, 6.8 Hz), 1.67 (m, 1H), 1.59 – 1.49 (m, 4H), 1.32 (p, 3H, J 7.7 Hz). ^{13}C NMR (125 MHz, D_2O) δ : 179.0 (C=O), 146.5, 127.9, 104.8 (C-1), 100.9 (C-2), 81.2, 75.8, 75.3, 74.2, 72.8, 72.7, 72.5, 72.2, 72.0 (2), 71.9, 71.3 (2), 70.1, 69.4 (2), 69.0, 66.1, 63.7, 63.4, 62.9, 62.3, 59.0, 52.6, 42.8, 40.6, 38.4, 36.2, 30.1, 27.4. HRMS: calculated m/z for $\text{C}_{75}\text{H}_{130}\text{N}_{10}\text{O}_{43}\text{S}_2$ (M+H) $^{2+}$ 961.3862; found 961.3858.

GNP-glycan preparation. 10 mL of 5 nm GNP stock was concentrated to 250 μL using a 30 kDa MWCO spin column and washed with H_2O ($3 \times 200 \mu\text{L}$) to remove any impurities. Then DHLA-glycan ligands dissolved in H_2O were added to the GNP solution in a molar ratio of GNP:DHLA-glycan = 1:1000. The resulting solution was mixed and stirred at RT in dark for overnight. The resulting mixture was passed through a 30 kDa MWCO spin column by centrifugation at $15,000 \times g$ for 2 min and the residues were washed with H_2O ($3 \times 200 \mu\text{L}$) to give the GNP-glycan stock. The filtrate and washing through liquids were combined and used to evaluate the glycan loading on GNPs as described previously.³⁴ The concentration of the GNP-glycans was determined from its absorbance at 520 nm using the Beer-Lambert law and a GNP molar extinction coefficient of $1.1 \times 10^7 \text{ M}^{-1}\text{cm}^{-1}$.

Protein production and labeling. The soluble extracellular segments of DCSIGN (DC020) and DCSIGNR (DSR034) were expressed as inclusion bodies in *E. coli* and purified by Man-Sepharose affinity column followed by Superdex size exclusion column as reported previously.³⁴ The mutant proteins, DC020 Q-274C and DSR034 R278C were constructed by site directed mutagenesis and labelled with atto594-maleimide as described previously.^{35, 40} The labeled proteins were purified by mannose-Sepharose affinity column. All the proteins obtained were characterized by high resolution mass spectrometry (HRMS) and their corresponding spectra were shown in SI, Figure S1. The dye labeling efficiency (per protein monomer) for DC-SIGN and DC-SIGNR was determined to be 87 % and 82 %, respectively, based on the relative intensity of the labeled and unlabeled protein peaks measured from MS.

Fluorescence spectra. All fluorescence spectra were recorded on a Cary Eclipse Fluorescence Spectrophotometer using a 0.70 mL quartz cuvette under a fixed λ_{EX} of 595 nm over a range of 605–750 nm. All measurements were performed in a binding buffer (20 mM HEPES, 100 mM NaCl, 10 mM CaCl_2 , pH 7.8) containing 1 mg/mL of BSA to minimize any nonspecific absorption of the GNPs and proteins on surfaces. For apparent K_d measurement, the concentrations of labeled DC-SIGN or DC-SIGNR protein and GNP-glycans were varied simultaneously in a fixed protein:GNP molar ratio of 1:1. The samples were incubated at RT for 20 min before recording the fluorescence spectra. The fluorescence spectra of the protein in the absence of the GNP-glycans, recorded under identical experiment conditions, were used to determine the quenching efficiency. The instrument PMT voltages were adjusted to compensate the low fluorescence signals at low concentrations. The quenching efficiency (QE) of DC-SIGN/R binding to each GNP-glycan was calculated *via* Eq. 1 and the resulting QE-concentration relationship

was fitted by Hill's equation (Eq. 2) to derive the apparent binding K_d values.

Dynamic light scattering. The hydrodynamic diameters (D_h) of wildtype DC-SIGN/R and GNP-glycans in pure water and in a binding buffer (20 mM HEPES, 100 mM NaCl, 10 mM CaCl_2 , pH 7.8) were recorded on a Malvern ZETASizer-Nano using disposable polystyrene cuvettes as reported in our previous papers.^{35, 68} For monitoring GNP-DiMan binding to wildtype DC-SIGN/R, a series of samples, each containing GNP-DiMan (10 nM) and a varying amount of wildtype DC-SIGN/R, were mixed in a binding buffer at RT for 20 mins before DLS measurement was carried out. 10 consecutive scans were performed for each sample and the resulting volume D_h distribution histograms for each sample were combined and fitted by Gaussian distributions to estimate their D_{hs} .³⁵

TEM imaging. Three samples: (1) GNP-DiMan, (2) GNP-DiMan + wildtype DC-SIGN, and (3) GNP-DiMan + wildtype DC-SIGNR were incubated (final $C_{\text{GNP}} = 16 \text{ nM}$ and $C_{\text{protein}} = 640 \text{ nM}$) in a binding buffer overnight. The samples were prepared by plunge-freezing into liquid ethane followed by warming under vacuum to capture the GNP dispersions in their native dispersed state as demonstrated in our previous paper.⁶³ Briefly, 3.5 μL of suspension was placed onto a plasma-cleaned TEM grid with a continuous carbon support film, blotted, and plunge frozen into liquid ethane. The TEM grids were then warmed to RT over several minutes by placing the specimens in the liquid nitrogen cooled storage container in a rotary pumped vacuum desiccator.

The samples were then analyzed using an FEI Titan³ Themis 300 G2 S/TEM equipped with FEI SuperX energy dispersive X-ray (EDX) spectrometers and a Gatan OneView CCD. Images were collected for each sample, with EDX spectroscopy used to confirm that the small nanoparticles imaged were indeed GNPs as Au species were detected together with other peaks (carbon, oxygen, silicon, and copper) due to the microscope, TEM grid or support film. A series of images at the same magnification were recorded for each sample, allowing easy comparison of the nanoparticle dispersion state of the three samples. The TEM images were analyzed automatically by MATLAB scripts to derive the nearest neighbor distances (NNDs) as reported previously.³⁵

Virus Inhibition. The effects of GNP-glycans (glycan = Man, DiMan, (Man) $_3$ or (DiMan) $_3$) on Ebola virus glyco-protein (EBOV-GP) driven entry into 293T cells were assessed by using our established procedures.³⁴⁻³⁵ Briefly, 293T cells seeded in 96-well plates were transfected with plasmids encoding DC-SIGN or DC-SIGNR or control transfected with empty plasmid (pcDNA). The cells were washed at 16 h post transfection and further cultivated at 37°C, 5% CO_2 in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS). At 48 h post transfection, the cells were exposed to twice the final concentration of GNP-glycan inhibitor in DMEM supplemented with 10% FBS for 30 min in a total volume of 50 μL . Thereafter, the resulting cells were inoculated with 50 μL of preparations of MLV vector particles encoding the luciferase gene and bearing either EBOV-GP (which can use DC-SIGN/R for augmentation of host cell entry) or the vesicular stomatitis virus glycoprotein (VSV-G, which cannot use DC-SIGN/R for augmentation of host cell entry). Under these conditions, binding of GNP-glycan nanoparticles to 293T cell surface DC-SIGN/R receptors can block EBOV-GP interactions with these lectin receptors, resulting in reduced transduction efficiency of the virus particles and hence reducing the cellular luciferase activity. At 6 h post inoculation, 100 μL of fresh DMEM culture medium was added and the cells incubated for another 72 h. Thereafter, luciferase activities in cell lysates were determined using a commercially available kit (PKJ), following the manufacturer's instructions, as described in our previous publication.

ASSOCIATED CONTENT

Supporting Information. Experimental details including materials and methods, protein production, labeling and MS characterization, LA-glycan ligands syntheses and their ¹H and ¹³C NMR spectra, and supporting figures showing fluorescence spectra of GNP-DiMan binding to DC-SIGN, mannose competition studies, D_h distribution histograms of the GNP-glycans, DC-SIGN/R, and GNP-glycan binding to DC-SIGN/R at different concentrations, TEM images of cryo-prepared GNP-DiMan + DC-SIGN/R samples, and the original data of GNP-glycans inhibiting DC-SIGN/R-mediated EBOV-GP driven infections.

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Author Contributions

D.Z., Y.G. and S.P. conceived the idea. D.B. and E.P. conducted ligand synthesis, GNP conjugation and GNP-lectin binding studies. I.N. conducted virus inhibition assays; D.B., Y.L. and B.W.T. synthesized glycans; J.H. and E.K. performed some protein purification, characterization and DLS studies. U.S.A. provided some GNPs. N.H. performed TEM imaging studies. D.Z., Y.G. and D. B. analyzed data and wrote the paper. All authors have given approval to the final version of the manuscript.

‡These authors contributed equally to this work.

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Notes

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ABBREVIATIONS

GNP, gold nanoparticle; DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; DC-SIGNR, DC-SIGN related lectin found on endothelial cells; TLC, thin layer chromatography; HPLC: high performance liquid chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry.

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