Virus-like glycodendrnanoparticles displaying quasi-equivalent nested polyvalency upon glycoprotein platforms potently block viral infection

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Ligand polyvalency is a powerful modulator of protein–receptor interactions. Host–pathogen infection interactions are often mediated by glycan ligand–protein interactions, yet its interrogation with very high copy number ligands has been limited to heterogenous systems. Here we report that through the use of nested layers of multivalency we are able to assemble the most highly valent glycodendrimeric constructs yet seen (bearing up to 1,620 glycans). These constructs are pure and well-defined single entities that at diameters of up to 32 nm are capable of mimicking pathogens both in size and in their highly glycosylated surfaces. Through this mimicry these glyco-dendri-protein-nano-particles are capable of blocking (at picomolar concentrations) a model of the infection of T-lymphocytes and human dendritic cells by Ebola virus. The high associated polyvalency effects ($\beta >10^6$, $\beta/N \sim 10^2-10^3$) displayed on an unprecedented surface area by precise clusters suggest a general strategy for modulation of such interactions.

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he initial stages of an infectious process are crucial for subsequent immune response and elimination of pathogens. The innate immune system comprises mechanisms and specialized cells responsible for first contact with external biological agents. Detection of invaders via pathogen recognition receptors and subsequent activation of antimicrobial defences triggers specific antigen responses. DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) receptor is one of the most important pathogen recognition receptor. It is expressed mainly on the surface of dendritic cells (DCs), and some subtypes of macrophages. DC-SIGN recognizes in a multivalent manner mannose and fucose and specializes these artificial systems face is achieving adequate size and valency beyond 32 (refs 9,18) has not been possible before with full control (Indeed, valencies > 100 are rare in any glycodendrimeric structure. See the following references André et al 25 and Camponovo et al.23 for examples of 128-mer lactoside and 243-mer xyloside display, respectively.22,23)

Several studies have been directed towards the preparation of synthetic carbohydrate systems able to block or stimulate DC-SIGN with the focus on mimicking the glyco-epitopes found on pathogens. By using DC-SIGN as an entry point some viruses are capable of escaping from the processing and degradation events carried out by the immune defence machinery at antigen-presenting cells. Therefore, the inhibition of pathogen entry through the blockade of this receptor at early stages of infection is one strategy for new antiviral agents.

We have previously demonstrated that symmetrically multivalent glycan ligands mounted on protein platforms (glycodendriproteins) are useful tools to study carbohydrate-protein interactions and are able to control or modulate a desired response.24–27. Other dendrimers displays on proteins have also been explored subsequently.28–30. However, to date, this approach has provided only limited carbohydrate valency levels on single protein platforms. We envisaged that a controlled design for a highly polyvalent protein display of sugars might be achieved through a novel strategy of ‘nested polyvalency’ (polyvalent display of polyvalency) (Fig. 1a).

We describe here the realization of this approach through the multivalent assembly of protein monomers themselves carrying polyvalent glycan display motifs (glycodendrons). The resulting glycodendriproteinfominomultimers display many glycans in a precise manner. Consistent with associated guiding physical principles, these constructs therefore display symmetry and pseudosymmetry at both the level of protein assembly and glycodendron. These synthetic glycoprotein assemblies display the highest known number of glycans (n = 1,620) yet presented in a homogeneous manner. We have applied this idea (Fig. 1) here to the inhibitory immunomodulation of a DC-SIGN-pathogen glycoprotein interaction (Fig. 1b).

Results

Construction of glyco-dendri-protein-nano-particles. We reasoned that assembly of an entity with similar dimensions (in the form of a self-assembled protein sphere-likeicosahedron QB34, ~28 nm in diameter) might mimic the display in target pathogens (Ebola pseudotyped virus particles have ~90 nm in diameter). Using a tag-and-modify27 strategy, we chose to selectively introduce a non-natural amino acid (tag) on the protein that could be used for the attachment (modify) of the selected glycodendrons appropriately functionalized at the focal position. As a stringent test of this approach we chose to mimic the highly glycosylated pathogen envelopes of Ebola virus. We selected a monomer QB34,35 protein as carrier, which assembles into a 180-copy multimer. QB multimer has been used by us and others to display glycans for other purposes but in these prior experiments only partial levels of nonspecific modification were achieved (see Supplementary Discussion). The result of these prior incomplete reactions was a formation of mixtures and not the desired homogeneous and (pseudo)symmetrical display required for this study.36,37. This proteic platform provided the necessary viral mimic scaffold (core diameter, ~28 nm) to construct the desired multivalent systems.

The designed glycodendrons were prepared in a straightforward manner by the Cu(I)-catalysed modification38,39 of the Huisgen cycloaddition as depicted in Fig. 2. α-α-Mannose was chosen to be introduced onto the dendritic scaffolds as a relevant ligand that is recognized by DC-SIGN. Synthesis of glycodendron was accomplished using a modular strategy that advantageously avoided the need for carbohydrate protection. First, unprotected dodecyl mannosyl 3 (see Supplementary Methods) was coupled to the trialkynyl pentaerythritol core 2 using CuSO4 and sodium ascorbate in a 1:1 mixture of water:THF (tetrahydrofuran) at room temperature (RT).40 The focal azido group required for subsequent site-specific conjugation to the protein tag, was introduced by reaction with sodium azide to give 5. The designed modular strategy also allowed use of 5 in the construction of higher generation glycodendron. Thus, coupling of 5 with the trialkynyl core 6 gave 7 (Fig. 2), which was similarly converted to an azide 8 for protein modification.

Unnatural alkyne-containing amino acid L-homopargylylglycine (Hpg) was site-specifically introduced into the protein that would make up the proteic scaffold (QB) to serve as an alkyn ‘tag’ through the expression of corresponding gene sequences in an auxotrophic strain of E. coli (B834(DE3)).41 Gene sequences were designed to create a protein displaying alkyn at a site on the outer surface of the eventual icosaehedral platform (Hpg16) for which the position could simply be controlled by the Met triplet codon ATG. Replacement of wildtype methionine (Met) residues, with near-isosteric amino acid isoleucine allows reassignment of the codons in the gene sequence to allow incorporation instead of Hpg as a ‘tag’ (see Supplementary Methods for full details). The resulting QB- (Hpg16) was characterized, including by mass spectrometry and dynamic light scattering (Fig. 3 and see Supplementary Methods and Supplementary Fig. S1), demonstrating the introduction of the Hpg amino acid into the sequence. On the basis of previous results,42,43, QB- (Hpg16) was modified using a reaction mixture of Cu(I)Br complexed by tris(1-ethylacetate)-IH,1,2,3-triazol-4-yl) methylamine in acetonitrile (Fig. 3a). It should be noted that the presence of Hpg on the protein results in slower Huisgen cycloaddition reaction rates in comparison with those obtained when azide in the form of azidohomoalanine (Aha) is present in the protein.42,43. Optimization of the reaction conditions by varying catalyst loading and stepwise addition, afforded the desired glycodendron-bearing virus-like particles. Thus, reaction of QB- (Hpg16) with trivalent glycodendron 5 afforded QB- (Man3) bearing 540 terminal mannosyl residues and reaction with second-generation nonavalent glycodendron 8 gave QB- (Man3) 8. Both reactions proceeded with > 95% conversion; consistent with greater bulk a longer reaction time (up to 7.5 h) was required for reaction of 8 to form 1,620-mer QB- (Man3) 8. Increasing particle diameter was observed, Q (27.6 nm) → QB- (Man3) 8 (29.8) → QB- (Man3) 8 (32.0), consistent with the controlled introduction of ‘shells’ of glycosylation ~1.1 nm thick (Fig. 3b and see Supplementary Table S1 for rough estimate of dendrimer length).
for each dendron generation. To the best of our knowledge, this is the first synthesis reported of such highly functionalized monodisperse glycodendriproteins (bearing up to 1,620 terminal sugar moieties) using a convergent approach.

**Inhibition of DC-SIGN in vitro and on T lymphocytes.** The inhibitory function of these glycodendriproteins was tested in several ways. Competition ELISA assay (See Supplementary Methods and Supplementary Figs S2 and S3) revealed that Qβ-(Man3)180 could completely inhibit the binding of DC-SIGN (as an Fc chimera) to a synthetically mannosylated glycoprotein (albumin bearing Man2–3(Man2–6)Man) with an estimated IC50 ~ 35–40 nM. A complete lack of inhibition by control, non-glycosylated Qβ confirmed dependence of this promisingly potent inhibition upon glycan.

Next, an Ebola viral infection model44, was explored using mammalian T-lymphocyte (Jurkat) cells displaying DC-SIGN. Recombinant viruses were produced in HEK 293 T cells; the viral construction was pseudotyped with Ebola virus envelope GP (EboGP) or the vesicular stomatitis virus envelope glycoprotein (VSV-G) and expressed luciferase as a reporter of the infection 45. The inhibition of DC-SIGN-dependent infection of T-lymphocyte Jurkat cells (examined in at least three independent experiments) demonstrated that unglycosylated Qβ reduced infection minimally (Fig. 4a). In contrast, Qβ-(Man9)180 showed strong dose-dependent inhibition of the infection process (Fig. 4a–c). Indeed, Qβ-(Man9)180 presented a notable antiviral activity, inhibiting infection by ~ 80% at 5 nM; estimated IC50 = 9.62 nM for β-(Man3)180 and = 910 pM for Qβ-(Man9)180.

VSV-G is able to infect T-lymphocyte Jurkat cells independently of DC-SIGN44 and provided a positive control in infection experiments; consistent with the proposed model of inhibition (Fig. 1) this glycan-independent pathway for VSV-G was completely uninhibited (Fig. 4b,c). In this model, the Ebola infection process is absolutely dependent on the presence of DC-SIGN on the cell surface. Jurkat cells not expressing DC-SIGN were used as a negative control in the infection studies and showed no infection by Ebola pseudovirus (See Supplementary Fig. S4). The ratio of infection in cis between Jurkat DC-SIGN + and Jurkat cells was ~ 2,600 for Ebola virus infection and 0.96 for VSV infection.

**Blocking of DC infection by pseudotyped Ebola.** Having shown such potent inhibition of infection of a stable cell line that displays DC-SIGN, we next evaluated inhibition in the perhaps more relevant and more demanding context of inhibition of primary cells. DCs are a primary target of Ebola infection; these display multiple C-type lectins that could provide a different or modulated route for infection with potentially higher affinity for virus. Accordingly, DCs were generated from isolated human peripheral blood mononuclear cells (PBMCs) and tested. We were pleased to find that as for the stable Jurkat cell line presenting DC-SIGN alone, Qβ-(Man9)180 displayed potent activity (Fig. 4d), inhibiting infection by ~ 80% at 5 nM and > 95% at 25 nM (estimated IC50 ~ 2 nM for Qβ-(Man9)180). Excitingly, these data indicate that the mode of inhibition of these synthetic glycodendrinanoparticles translates into cellular contexts relevant to human infection and are consistent with the mode of action suggested in Fig. 1.

**Discussion**

Although the evaluation of the number of monomer units interacting during these inhibitory processes is complicated by quasi-equivalence32, the data obtained indicate that these systems afford a clear polyvalency effect (β)46 when compared with the
Figure 2 | Creation of polyvalent mannose-terminated glycodendrons. Synthesis of the glycondendron reagents 5 and 8 used in the assembly of virus-like glycodendri-nano-particles (see Fig. 3). TBTA, tribenzyl(tris)triazoylamine; DMF, N,N-dimethylformamide.
monomer methyl α-D-mannopyranoside) (see Supplementary Table S2). The inhibitory properties of each mannoside monomer unit can therefore be considered to be ~250-fold and ~860-fold (as judged by β/N, see Supplementary Table S2) more potent when displayed in Qβ(Man3)180 and Qβ(Man9)180, respectively, than when displayed alone. The efficiency of this system therefore

Figure 3 | Controlled assembly and characterization of virus-like glycodendri-nano-particles. (a) A tag-and-modify strategy allowed the generation of the second nested layer of multivalence from Qβ(Hpg16)180 using glycondendron reagents 5 and 8. R = corresponding glycondendron. (b) Dynamic light scattering histograms showing the hydrodynamic radius of Qβ (radius 13.8 nm), Qβ(Man3)180 (radius 14.9) and Qβ(Man9)180 (radius 16.0). (c) Mass spectrometric analysis of monomer proteins of the particles. Pi, phosphate.
relied upon a vital combination of not only a high number of displayed ligands but also display size and geometry. To date, no homogeneous polyvalent systems have been described that can generate such a large surface area (solvent-accessible surface area ~725,000 Å²) as the systems we have described here. The system we describe here is based on an icosahedral scaffold (T = 3, triakis icosahedral) that generates several underlying quasi-symmetry elements including those that relate the displayed glycodendrons in a two-fold, three-fold and six-fold manner (see Supplementary Movie 1). It is tempting to speculate that this, in turn, allows the simultaneous display of many distinct putative global glycan clusters where the glycan ‘tips’ are all quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent). It is tempting to speculate that this, in turn, allows the simultaneous display of many distinct putative global glycan clusters where the glycan ‘tips’ are all quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent). It is tempting to speculate that this, in turn, allows the simultaneous display of many distinct putative global glycan clusters where the glycan ‘tips’ are all quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent). It is tempting to speculate that this, in turn, allows the simultaneous display of many distinct putative global glycan clusters where the glycan ‘tips’ are all quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent). It is tempting to speculate that this, in turn, allows the simultaneous display of many distinct putative global glycan clusters where the glycan ‘tips’ are all quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent). It is tempting to speculate that this, in turn, allows the simultaneous display of many distinct putative global glycan clusters where the glycan ‘tips’ are all quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent). It is tempting to speculate that this, in turn, allows the simultaneous display of many distinct putative global glycan clusters where the glycan ‘tips’ are all quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent). It is tempting to speculate that this, in turn, allows the simultaneous display of many distinct putative global glycan clusters where the glycan ‘tips’ are all quasi-equivalent (for example, 6-fold-related faces displaying a putative global glycan cluster where the glycan ‘tips’ are also quasi-equivalent).

In summary, a novel nested polyvalency approach combined with tag-and-modify site-selective protein synthesis has allowed the creation of homogeneous protein platforms bearing glycodendrons. These well-defined polyvalent glycoprotein assemblies present on their surface up to 1,620 copies of glycan, a remarkably high valency never obtained before using a fully controlled strategy. These glycodendrimer protein particles show exciting antiviral activity, preventing mammalian cell infection by Ebola pseudotyped virus through competitive blockade of the DC-SIGN receptor in the nanomolar to picomolar range. These results clearly indicate the efficiency of these systems to interact with this pattern recognition receptor and to compete with pathogens during their entry into target cells. Their in vivo activity remains to be tested and it is possible that such constructs, if used in this context, could elicit humoral responses that might potentially neutralize some of the interactions studied here. The high activity and fascinating (quasi)symmetric, high surface area morphology of these new glycoconjugates provides promising candidates for the development of both new antiviral agents as well as probes of larger-scale biological events.
Methods

Synthesis of glycodendrons. Glycodendron 4: 2-Azidoethyl α-D-mannopyranoside (306 mg, 1.2 mmol), 2-(2-chloroethoxy)ethylidene (2-propynyloxymethyl)methane (128 mg, 0.37 mmol), CuSO₄·5H₂O (9 mg, 0.04 mmol), and tribenzylic(tri)azolylamine (39 mg, 0.07 mmol) and sodium ascorbate (29 mg, 0.15 mmol) were dissolved in 2 ml of THF/H₂O (1:1). After consumption of the starting material (3 h), the solvent was evaporated and the crude was purified by size-exclusion chromatography (Sephadex LH-20 MeOH 100%), furnishing the glycodendron 4 as a white foam (347 mg, 86%).

Glycodendron 5: Glycodendron 4 (80 mg, 0.07 mmol) and sodium azide (47 mg, 0.70 mmol) were dissolved in N,N-dimethylformamidem (2 ml). The mixture was stirred at 60 °C for 4 days. After consumption of the starting material, the solution was concentrated by size-exclusion chromatography (Sephadex G-25 H₂O/Methanol 9:1), furnishing glycodendron 5 (76 mg, 95%) as a white foam.

Glycodendron 7: Glycodendron 5 (30 mg, 0.03 mmol), 2-[2-(2-chloroethoxy)ethylidene (2-propynyloxymethyl)methane (3.6 mg, 0.008 mmol), CuSO₄·5H₂O (0.5 mg, 0.002 mmol), tri[tribenzylic(tri)azolylamine (1.7 mg, 0.003 mmol) and sodium azide (1.3 mg, 0.006 mmol) were dissolved in THF/H₂O (1:1, 1 ml). After consumption of the starting material, the solution was evaporated and the crude was purified by size-exclusion chromatography (Sephadex LH-20 MeOH 100%), furnishing glycodendron 7 (22 mg, 75%) as a white foam.

Glycodendron 8: Glycodendron 7 (25 mg, 0.007 mmol) and sodium azide (4 mg, 0.07 copper (I) bromide (99.999%) in acetonitrile (32.6 g, 100 ml of water. Next, 1.5 ml of TCEP (1 M) to allow the protein to denature and 25 nM. As a control, an experiment of inhibition of infection of Ebola-GP was performed in the presence of antibody anti-DCl/SIGN and mannann at concentration of 25 μg ml⁻¹.

The values of percentage of inhibition correspond to the mean of two independent experiments (error bars describe the range between the two values obtained).

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Author contributions

R.R.-V. and M.S.N. synthesized the glycodendrons and the glycodendriproteins, J.L. carried out the Ebola infection experiments. J.R.K. expressed and purified Qb. M.S.N., R.D., J.R. and B.G.D. designed the experiments. All the authors discussed results and analysed the data. M.S.N. and B.G.D. wrote the manuscript. Correspondence and requests for materials should be addressed to R.D., J.R. and B.G.D.

Additional information

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Erratum: Virus-like glycodendrinanoparticles displaying quasi-equivalent nested polyvalency upon glycoprotein platforms potently block viral infection

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During the production of this Article, the y axis of the bar chart in Fig. 4d was mislabelled. The correct version of Fig. 4 appears below.