Communication

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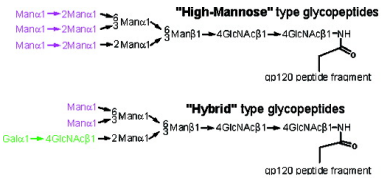

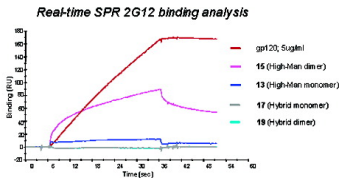

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Toward Fully Synthetic Carbohydrate-Based HIV Antigen Design: On the Critical Role of Bivalency

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Until very recently, extensive glycosylation of HIV envelope proteins had been considered to be one of the major impediments to the development of an HIV vaccine.[1] Indeed, this "glycan shield" was perceived to confer protection from antibodies which would recognize the peptide backbone of the gp120 trimer surface.[2] The envelope glycoprotein gp120 interacts sequentially with the cellular receptor CD4 and a member of the chemokine receptor family, thus initiating HIV entry into the T-cell.[3] The gp120 peptide chain is heavily glycosylated, typically bearing 24 Asn-linked glycans.[4]

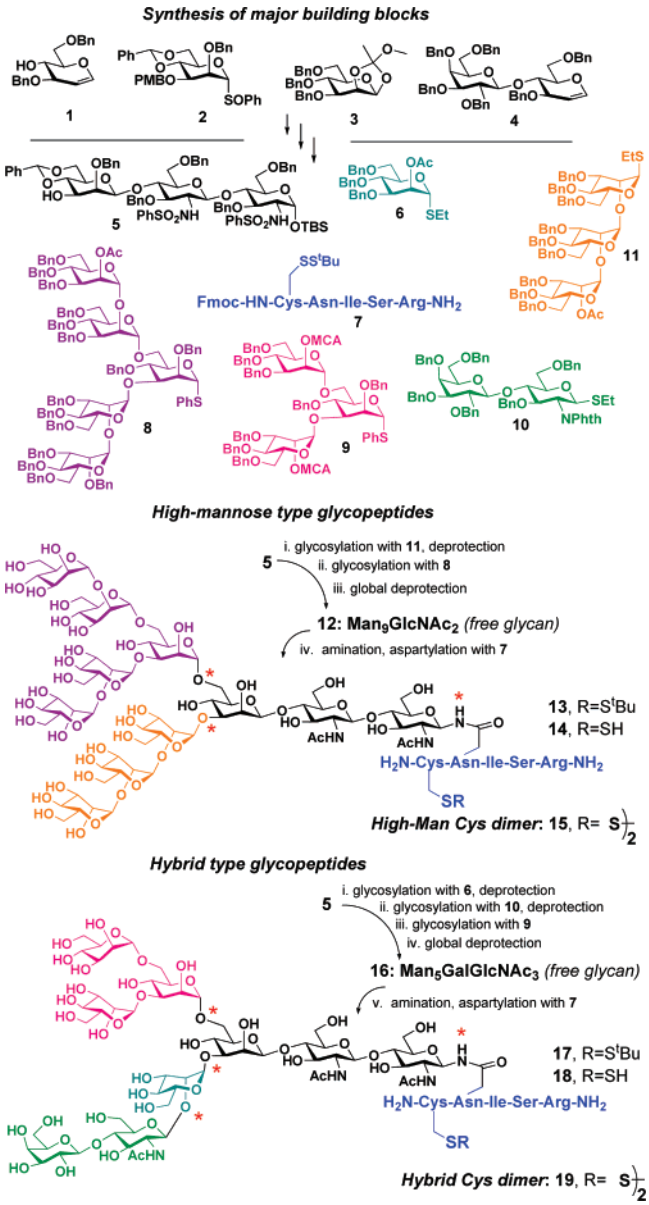
The idea of utilizing gp120 carbohydrates as antigens for eliciting broadly neutralizing immune responses gained recognition only when the structure of the 2G12 antibody epitope was unveiled.[5] This antibody, isolated from a long-term survivor of infection, was shown to efficiently neutralize a wide spectrum of different HIV isolates in vitro and to protect macaques from simian−human immunodeficiency virus challenge.[6,7]

Alanine scanning mutagenesis and glycosidase digestion studies suggested that 2G12 recognizes either high-mannose[8] or hybrid-type[9] glycans modifying Asn 332, 339, and 392 residues of gp120. With this in mind, we set out to develop fully synthetic constructs mimicking the 2G12 carbohydrate epitope as potential antigen candidates for application in HIV vaccine formulations.[10,11] We sought to test such compounds as probes in binding 2G12. While such data may not necessarily serve to establish construct immunogenicity, binding studies could provide insights into the real structure of the gp120 antigenic surface, thereby allowing for optimization of synthetic constructs directed to induction of neutralizing immune response. These syntheses were enabled by synthetic methodology and synthetic logic previously developed in our laboratory for building glycopeptide ensembles containing highly complex glycan domains.[12,13]

Our program commenced with the preparation of the major oligosaccharide building blocks including the core β-mannose/chitobiose trisaccharide **5**.[14] The "D1 arm" saccharides **10** and **11** of the high-mannose and hybrid glycans respectively, and the upper domains, i.e., pentasaccharide **8** and trisaccharide **9** branches, were also synthesized (Scheme 1). These fragments were appropriately assembled to provide free **12** and **16**. The reducing termini of these fully synthetic oligosaccharides were then aminated as previously described,[12] building on earlier protocols of Kochetkov[15] and Lansbury.[16] Each glycosylamine was coupled to a Cys-protected gp120$^{331-335}$ pentapeptide.

Finally, the resulting Cys-blocked glycopeptides **13** and **17** were reduced to liberate the Cys sulfhydryl function, thereby affording compounds **14** and **18**, respectively.[17,18] We describe here the first real-time analyses of 2G12 binding to these gp120-targeted constructs.

Scheme 1. Synthesis of gp120$^{331-335}$ Glycopeptides Carrying High-Mannose and Hybrid-Type Fragments[a]



[a] Red asterisks denote assembly points.

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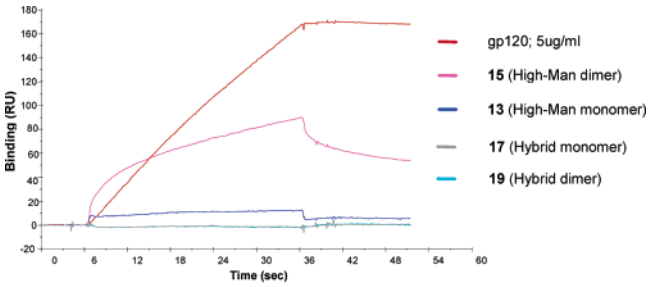


Figure 1. Analyses of substrates' binding to 2G12 (signals for **17** and **19** overlap at the baseline).

Table 1. Qualitative Assessment of 2G12 Binding

cmpd (concn)	carbohydrate type	Cys SH state	binding, RU
12 (40 μM)	high-mannose	none	<1
13 (20 μM)	high-mannose	blocked	5
14 (10 μM)	high-mannose	free	75
14 (10 μM)+DTT	high-mannose	free	9.5
15 (10 μM)	high-mannose dimer	dimer	78
16 (40 μM)	hybrid	none	<1
17 (20 μM)	hybrid	blocked	<1
18 (20 μM)	hybrid	free	<1
19 (20 μM)	hybrid dimer	dimer	<1

Binding analyses utilized the surface plasmon resonance (SPR) technology, and were carried out using the Biacore 3000 system (Figure 1/Table 1).[19,20] 2G12 and a human IgG1 isotype-control antibody were immobilized by the amine coupling method to a CM5 sensor chip, generating the active and reference surfaces. A single injection of the tested material resulted in its successive exposure first to the reference surface and then to the active surface. Each binding profile represents an automatic subtraction of the reference surface signal from the 2G12 surface signal. Binding experiments were performed at 25 °C in HBS-P buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.005% Surfactant P20). The sensor surface was regenerated with a short pulse of 3.5 M $MgCl_2$. Recombinant HIV-1_{JR-FL} gp120[21] was tested for comparison.

With the synthetic gp120 glycopeptides in hand, we could probe their binding to 2G12. In the high-mannose series, free glycan **12** binding was below detection threshold; however, glycan/pentapeptide conjugate with free Cys SH **14** demonstrated significant binding with 2G12. At the same time, the conjugate with the protected sulfhydryl function (**13**) showed only a very low level of binding (Table 1). The high sensitivity of binding of 2G12 to the apparent state of the sulfur atom in the N-terminal cysteine was initially puzzling, given the perception that binding is in either case directed to the glycan domain.

An important clue arose on examination of the H_2O stock solution of the presumed thiol **14**. Liquid chromatography/mass spectroscopy (LC/MS) analysis indicated that this material was now actually a mixture of the monomeric and the oxidized disulfide forms, with a prevalence of the latter. Moreover, treatment of the compound **14** stock (0.5 mM as per compound monomer) with dithiotreitol (DTT) at 25 mM (50-fold molar excess as calculated per compound monomer) resulted in significantly reduced binding (Figure 2).

In control experiments, it was confirmed that after the passage of the DTT-pretreated sample, the 2G12 surface retains the ability to bind the unreduced compound. In another control, we similarly used 25 mM DTT to pretreat the gp120 stock (5 μM) and detected no significant effects on 2G12 binding despite an even greater (5000-fold) excess of DTT over gp120.

These experiments, in the aggregate, suggested that the dimeric form of the glycopeptide is responsible for observed 2G12 binding. Indeed, when dimer **15** (Scheme 2) was then prepared, in

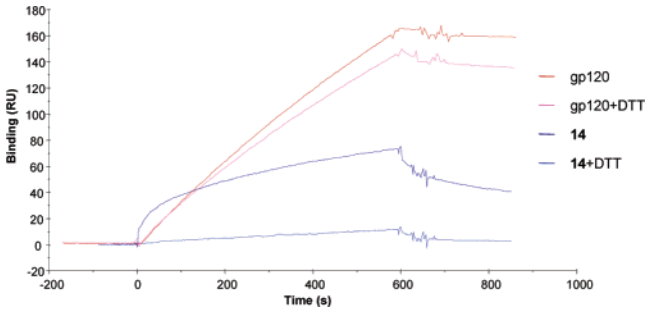
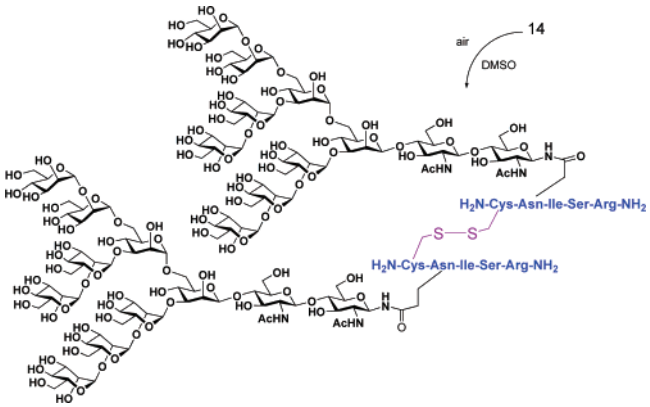


Figure 2. Analysis of DTT effect on binding.

Scheme 2. Formation of the High-Mannose Dimer **15**



homogeneous form, by DMSO oxidation of **14**, it exhibited strong binding to 2G12 (Figure 1).

We then evaluated the corresponding set of gp120 constructs, but now carrying hybrid-type carbohydrates, which lack the lower trimannose (D1) arm present in the high-mannose glycans. In hybrid compounds **16**−**19** this sector is replaced by an *N*-acetyllactosamine residue (Figure 1). Additionally, the upper pentasaccharide branch in hybrids **16**−**19** is trimmed to the trisaccharide level. *It was found that none of the constructs possessing the hybrid-type glycan pattern, including the dimeric structure (see **19**), showed any detectable binding.* Initial glycosidase digestion studies had suggested the possibility that hybrid elements in gp120 may be responsible for 2G12 binding, since treatment of recombinant gp120 with Endo F2 glycosidase, which cleaves mannose residues from high-mannose, but not hybrid chains, had no effect on binding.[9] However, our findings demonstrate that hybrid glycans are not recognized by 2G12. The sensitivity of binding to the multimeric character of the glycan is certainly in keeping with the structural notions offered by Wilson.[5]

To probe whether the dimeric high-mannose compound **15** and gp120 recognize the same site on 2G12, competition binding experiments were performed (Figure 3). Compound **15** was injected into the flow cell at concentrations up to 10 μM, followed by an injection of gp120 at a constant concentration of 12.5 nM. Increasing the amount of pre-bound **15** resulted in progressive inhibition of gp120 binding. The monomeric form of the glycopeptide **14** did not block gp120 binding, as expected. In reciprocal experiments, prebound gp120 (0−100 nM) also progressively inhibited the binding of the compound **15** (2.5 μM) to 2G12. These results indicate that gp120 and glycopeptide **15** compete for binding to 2G12, *supporting the idea that the dimeric glycopeptide binds to 2G12 by mimicking the clustered gp120 epitope*.

The observed binding profile of dimer **15** points to a rather complex dynamics (Figure 4) that does not fit into a simple 1:1 Langmuir model.[22] The observed profile can be viewed as including

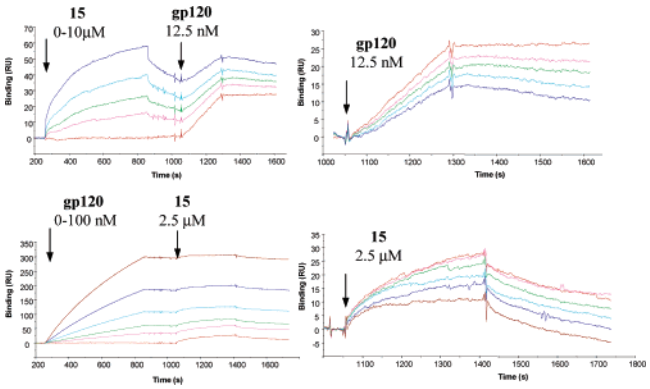


Figure 3. Competition binding data for glycopeptide **15** and gp120. Sensograms on the right top and bottom are normalized before injections of gp120 and compound **15**, respectively.

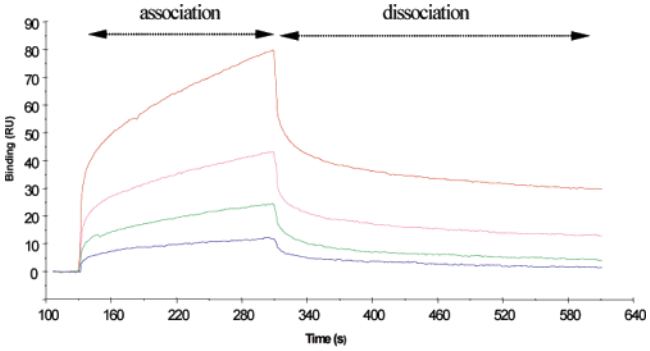


Figure 4. Glycopeptide **15** binding profiles at 1.25, 2.5, 5, 10 μM concentration.

two association (fast and slow) and two dissociation (fast and slow) components, and may indicate a required conformational adjustment for the binding of the second glycan. Our finding that the clustered construct demonstrates significantly stronger binding than the monomeric glycopeptide is in agreement with the cocrystal structure of the 2G12/high-mannose sugar complex, where at least two polysaccharides bind to spacially adjacent pockets on the surface of the antibody. These data are in agreement with ELISA binding studies of high-mannose constructs with artificial spacer system reported by Wang and co-workers.[11] These studies also demonstrated increased binding in multivalent systems.

At present, we are unable to characterize the precise nature of the bivalent effect on binding. Studies designed to obtain more detailed structural insights are well under way. Further optimization of the linker between the polysaccharides is another promising direction for design of antigens intended for use in HIV vaccines. The constructs described above will be evaluated as part of our HIV vaccine quest. At the same time, designs of later-generation vaccine candidates are moving forward. These ongoing investigations build upon the key observations described above and are enabled by the major advances in the synthesis of complex glycopeptides.

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Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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