Alma Mater Studiorum – Università di Bologna

Ph.D. program in Cellular, Molecular and Industrial Biology

XXII cycle

Scientific discipline: **BIO/11**

Evaluation of glycoconjugate antigens as vaccine candidates against group A *Streptococcus* and human immunodeficiency virus infections

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2010

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Abbreviations

CHO, carbohydrate

ConA, Concanavalin A

EU, ELISA units

GAS, group A streptococcus

GAS-PS, GAS polysaccharide

GNL, Galantus Nivalis Lectin

HA, hemagglutinin

HSA, human serum albumin.

i.m., intramuscular

i.p., intraperitoneal

Man, mannose

RT, room temperature

sc., subcutaneous

vol, volume

wt, weight

Acknowledgements

I would like to express my sincere gratitude to Dr. Paolo Costantino and prof. Vincenzo Scarlato, who drove my scientific education and gave me an opportunity to present the results of the work at conferences and meetings and participate in external collaborations.

I would like to thank all the members of the Vaccine Chemistry Research group from Novartis V&D in Siena - Francesco Berti, Roberto Adamo, Daniela Proietti, Marta Tontini, Maria Rosaria Romano, Emilia Cappelletti, Gabriele Costantini, Vittoria Pinto, Tommaso Niccolai, Elisa Danieli, and Barbara Brogioni who helped with experiments and discussion of the present thesis.

I would like to acknowledge and thank for an essential contribution: Paola Fontani, Emiliano Chiarot, Sabrina Capo, Giuliano Bensi, Daniele Casini, Immaculada Margarit Y Ros, Guido Grandi and Rino Rappuoli from the Research Center of Novartis V&D in Siena.

I was a recipient of a Novartis fellowship from the current PhD program of the University of Bologna. Research on HIV glycoconjugate vaccine received financial support from European Union (MUVAPRED project, FP6).

Introduction

Carbohydrate-based vaccines

Glycoconjugate vaccines, in which a cell surface carbohydrate from a micro-organism is covalently attached to an appropriate carrier protein, have been proved to be one of the most effective means to generate protective immune responses to prevent a wide range of diseases. The technology appears to be generic and applicable to many pathogen agents, as long as antibodies against surface carbohydrates help protect against infection.

The cell surfaces of bacteria, parasites and viruses exhibit oligosaccharides that are often distinct from those of their hosts. Carbohydrates are virulence factors commonly used by pathogens to establish an infection and escape the host immune response. The polysaccharide capsules of bacteria prevent complement activation (Roitt 1997) and inhibit phagocytosis. Carbohydrates form protective shields over conserved viral protein epitopes (Baghian *et al.* 2000; Scanlan *et al.* 2002; Sanders *et al.* 2002; Mori *et al.* 2003; Han *et al.* 2004; Barrientos, Gronenborn 2005; Goffard *et al.* 2005; Helle *et al.* 2006), serve for interaction with host cell surface receptors leading to enhancement of viral spread (Pohlmann *et al.* 2001; Alvarez *et al.* 2002; Halary *et al.* 2002; Klimstra *et al.* 2003; Lozach *et al.* 2005; Davis *et al.* 2006) or induction of immunosuppressive response (Shan *et al.* 2007). Thus, carbohydrate antigens represent an attractive target for vaccine development.

Pneumococcal capsular polysaccharide appeared the first carbohydrate antigen of a pathogen to be investigated for vaccine application. Immunogenic and

protective properties of pneumoccocal polysaccharide had been investigated starting from as early as 1920s. In 1980s two formulations of anti-pneumococcal vaccine have been introduced containing capsular polysaccharide derived from 14 and 23 *pneumonia* serotypes, respectively. However, the vaccine was poorly immunogenic in infants under the ages of two, elderly and immunocompromised persons (Vliegenthart 2006). The same limitation was revealed for meningococcal polysaccharide vaccines (Broker *et al.* 2009).

Carbohydrates themselves do not represent ideal vaccines due to the fact that, with a fewer exceptions, they cannot not be presented in the context of MHC-antigen complex to the T-helper cells and therefore are poor immunogens (Avci, Kasper 2009). However, carbohydrate conjugation to carrier proteins results in the induction of T-cell-dependent arm of the adaptive immunity. The age of glycoconjugate vaccines started in 1931, when Avery and Goedel discovered that covalent attachment of carbohydrates to a suitable protein induced an enhanced immunogenicity compared to the polysaccharides as such (Avery, Goedel 1931). Carbohydrate-protein conjugates therefore are capable of eliciting a long-lasting response to vaccination and are effective in adults and young children.

Polysaccharides have been successfully conjugated to a variety of proteins, such as tetanus toxoid, diphtheria toxoid and its non-toxic mutant CRM_{197}, the outer membrane complex from *Neisseria meningitidis* (Giannini *et al.* 1984; Verez-Bencomo *et al.* 2004; Jones 2005; Pace 2009). Using these protein carriers effective conjugate vaccines against *Haemophilus influenza* type b, meningococcus of the serogroups A, C, W, Y, and pneumococcus (13-valent

formulation) have been developed (Jones 2005; Lucas *et al.* 2005). Novel findings proposed short peptides and proteins containing specific CD4+ T cell epitopes as potential candidates with improved carrier properties for the future applications (Falugi *et al.* 2001; Liakatos, Kunz 2007).

Isolated bacterial carbohydrates for conventional vaccines

Vaccines prepared with bacterial polysaccharides have been widely used against a host of diseases for several decades (Goldblatt 1998). The carbohydrate antigens for those vaccines were traditionally isolated from biological sources. A potent set of analytical tools as NMR, mass spectrometry and others have helped to investigate the structure of bacterial carbohydrate antigens. Native polysaccharides are either homopolymers or made up of between two and six repeating sugar units, the chemical nature of which defines the serotype of the organism. For example, there are 91 different types of polysaccharides associated with pneumococci, 13 polysaccharides of meningococci, and 9 polysaccharides of group B streptococci (Paoletti and Madoff 2002; Sabharwal *et al.* 2006; Pollard *et al.* 2009).

Protective immunity against encapsulated bacteria has been correlated with anti-polysaccharide antibodies. Usually immune response is serotype-specific, and multivalent formulations containing several types of polysaccharides are preferred for vaccination e.g. 14- and 23-valent pneumococcus vaccine (Vliegenthart 2006), unless the only type of polysaccharide is ascribed for the circulating virulent strains as in case of group A *Streptococcus* (Cunningham 2000). Commercially available vaccines based on purified capsular polysaccharides or glycoconjugates include those against *Neisseria*

meningitidis, *Streptococcus pneumoniae*, *Haemophilus influenza* type b and *Salmonella typhi* (Ada, Isaacs 2003).

Synthetic oligosaccharides as new targets for vaccine development

In contrast to conventional polysaccharide isolation, chemical synthesis provides access to pure, homogeneous oligosaccharides of well-defined structure that contain single reactive groups for covalent conjugation. Additionally, in some cases synthetic oligosaccharides were found to have better immunogenic properties than native polysaccharides (Pozsgay *et al.* 1999). Design of synthetic oligosaccharides is usually based on the core antigenic determinants of long polysaccharide chains and may help to evaluate how particular structural features e.g. length, conformation, or non-reducing end residue, influence carbohydrate immunogenicity. Conjugate vaccines containing synthetic oligosaccharides have generated a protective immune response for a growing number of infectious diseases including *Streptococcus pneumonia* type 3, 6A, 6B and 14, *Shigella dysenteriae* type 1 and *Haemophilus influenzae* type b (Chong *et al.* 1997; Benaissa-Trouw *et al.* 2001; Jansen *et al.* 2001; Verez-Bencomo *et al.* 2004; Pozsgay *et al.* 2007; Safari *et al.* 2008). Until recently, the major obstacle to the development of synthetic carbohydrate vaccines were the challenges associated with the chemical synthesis of oligosaccharides. Automated oligosaccharide synthesis promises now ready access to synthetic antigens and tools such as glycan microarrays to assess an immune response and to map antibody epitopes. Synthetic antigens formed the basis for tumor vaccines candidates as well as *Bacillus anthracis, Plasmodium falciparum* and *Leishmania* conjugate vaccines (Schofield *et al.* 2002; Liu *et al.* 2006; Ragupathi

et al. 2006; Liakatos, Kunz 2007; Galonic, Gin 2007; Kamena *et al.* 2008; Robbins *et al.* 2009).

Focus of the doctorate study

This PhD thesis discusses the rationale for design and use of synthetic oligosaccharides for the development of glycoconjugate vaccines and the role of physicochemical methods in the characterization of these vaccines. The study concerns two infectious diseases that represent a serious problem for the national healthcare programs: human immunodeficiency virus (HIV) and Group A Streptococcus (GAS) infections. Both pathogens possess distinctive carbohydrate structures that have been described as suitable targets for the vaccine design.

The Group A *Streptococcus* cell membrane polysaccharide (GAS-PS) is an attractive vaccine antigen candidate based on its conserved, constant expression pattern and the ability to confer immunoprotection in a relevant mouse model. Analysis of the immunogenic response within at-risk populations suggests an inverse correlation between high anti-GAS-PS antibody titres and GAS infection cases. Recent studies show that a chemically synthesized core polysaccharide-based antigen may represent an antigenic structural determinant of the large polysaccharide. Based on GAS-PS structural analysis, the study evaluates the potential to exploit a synthetic design approach to GAS vaccine development and compares the efficiency of synthetic antigens with the long isolated GAS polysaccharide. Synthetic GAS-PS structural analogues were specifically designed and generated to explore the impact of antigen length and terminal residue composition.

For the HIV-1 glycoantigens, the dense glycan shield on the surface of the envelope protein gp120 was chosen as a target. This shield masks conserved protein epitopes and facilitates virus spread via binding to glycan receptors on susceptible host cells. The broadly neutralizing monoclonal antibody 2G12 binds a cluster of high-mannose oligosaccharides on the gp120 subunit of HIV-1 Env protein. This oligomannose epitope has been a subject to the synthetic vaccine development. The cluster nature of the 2G12 epitope suggested that multivalent antigen presentation was important to develop a carbohydrate based vaccine candidate. Hereafter I will describe the development of neoglycoconjugates displaying clustered HIV-1 related oligomannose carbohydrates and their immunogenic properties.

Chapter 1

Preparation, characterization and immunogenicity of HIV-1 related high-mannose oligosaccharides-CRM_{197} glycoconjugates

Introduction

Worldwide human immunodeficiency virus (HIV) pandemic involves approximately 33 millions of people with 2.7 millions of new infections and 2 millions of deaths each year (WHO, 2007). It is generally believed that an effective prophylactic weapon against HIV-1 could be a vaccine capable of eliciting both neutralizing antibodies and T-cell responses. However, numerous defense mechanisms help HIV-1 to evade host immune attacks directed against HIV envelope (Env) neutralization epitopes by means of frequent mutations, structural occlusions achieved by protein complex formation and heavy glycosylation (Kwong *et al.* 2002; Pantophlet, Burton 2006; Wei *et al.* 2003). The latter leads to formation of so-called “glycan shield” that masks conserved protein epitopes (Burton *et al.* 2005; Calarese *et al.* 2003). This shield provides to the virus an additional source of antigen heterogeneity due to the numerous glycoforms in which proteins can exist and, being produced by the host glycosylation machinery, is expected to induce immune tolerance. Nevertheless, a unique carbohydrate epitope mapped by the human broadly neutralizing monoclonal antibody 2G12 was discovered on the surface of Env gp120 giving to HIV glycans potential to be considered as candidates for an anti HIV-1 vaccine (Burton *et al.* 2004).

The neutralizing carbohydrate epitope of gp120 consists of a cluster of terminal α-D-Man-(1,2)-α-D-Man residues (Manα1,2-Man) on the D1 and D3 arms of $Man_9GlcNAc_2$ residues (Calarese *et al.* 2005; Scanlan *et al.* 2002). An extended antibody binding surface is formed by a unique heavy chain variable domain-swapped configuration which favors the possibility of multiple interactions with mannose surface (Calarese *et al.* 2003). Man_4, Man_6 and Man_9 derivatives of natural $Man_9GlcNAc_2$ oligosaccharide (Fig. 1) were proposed as possible "building blocks" of a future glycoconjugate vaccine because they possess Manα1,2-Man units essential for 2G12 recognition and have been proved to interact with 2G12 in binding and inhibition assays (Adams *et al.* 2004; Calarese *et al.* 2005; Lee *et al.* 2004; Wang *et al.* 2004).

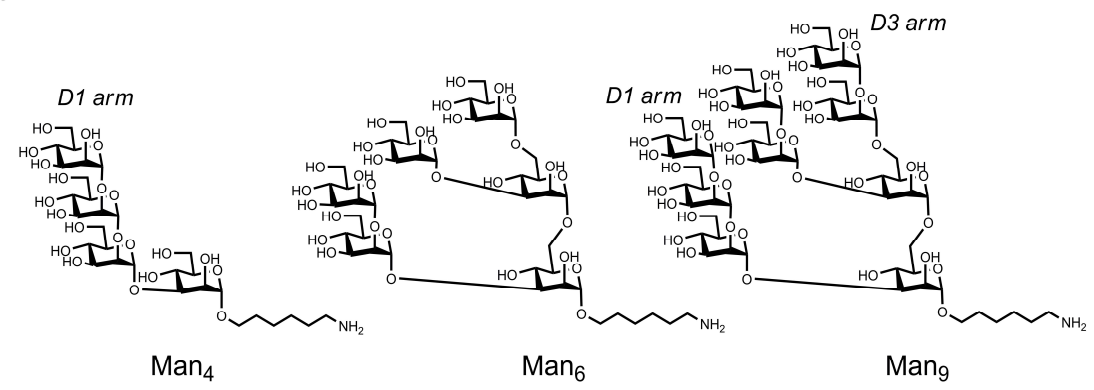

Figure 1. Structures of oligomannoses.

The cluster nature of the 2G12 epitope suggests the importance of multivalent presentation of oligomannoses in developing glycoconjugate molecules as possible candidate vaccines. Synthetic high-mannose clusters of 2-, 4- and higher valence, compared to monovalent sugars, showed enhanced binding to 2G12 and up to 110 times lower IC_{50} when used as inhibitors of the interaction

between antibody and gp120 (Krauss *et al.* 2007; Wang J. *et al.* 2007; Wang *et al.* 2008). Two types of clustering scaffold have been investigated so far: in one case high mannoses were randomly oriented by a flexible linker around a galactose core (Wang *et al.* 2004; Ni *et al.* 2006); in a different study a semirigid cyclic peptide scaffold served to position the carbohydrate moieties at the correct distance as defined by the crystal structure of gp120 (Krauss *et al.* 2007; Wang J. *et al.* 2007). The latter strategy seemed to provide a better mimic of the native epitope, but the sterical constraints of the model led to incorporation of a lower number of carbohydrate chains (Joyce *et al.* 2008). In summary, up to now three HIV-related glycoconjugates have been used for *in vivo* studies: the monovalent Man_4 conjugated to BSA (Astronomo *et al.* 2008), the bivalent $Man_9GlcNAc_2$ on the cyclic peptide scaffold conjugated to *Neisseria meningitides* outer membrane protein complex (OMPC) (Joyce *et al.* 2008), and the galactose-based tetravalent $Man_9GlcNAc_2$-cluster conjugated to *Tetanus toxoid* T-helper peptide (Ni *et al.* 2006). None of them elicited “2G12-like” response that cross-reacted with HIV Env proteins.

During my thesis I have investigated polyamidoamine (PAMAM) dendrons as possible scaffolds to make clusters with HIV-1 related high-mannose oligosaccharides (Fig. 2). PAMAM dendrons appeared attractive due to their potential low immunogenicity (Chabre, Roy 2008; Boas *et al.* 2006) and built-in surface functionalities which provide multiple sites for sugar incorporation. The high-mannose oligosaccharide clusters have been coupled to CRM_{197}, a non-toxic mutant of diphtheria toxin already extensively used as carrier for glycoconjugate vaccines in humans (Broker *et al.* 2009; Giannini *et al.* 1984;

Jackson *et al.* 2009). Formulated with the human acceptable adjuvant MF59 (Burke *et al.* 2009; Galli *et al.* 2009; Seubert *et al*. 2008), the glycoconjugates were tested in rabbits and mice. I report here preparation, structural characterization, antigenic and immunogenic properties of these oligomannose-PAMAM-CRM_{197} conjugates.

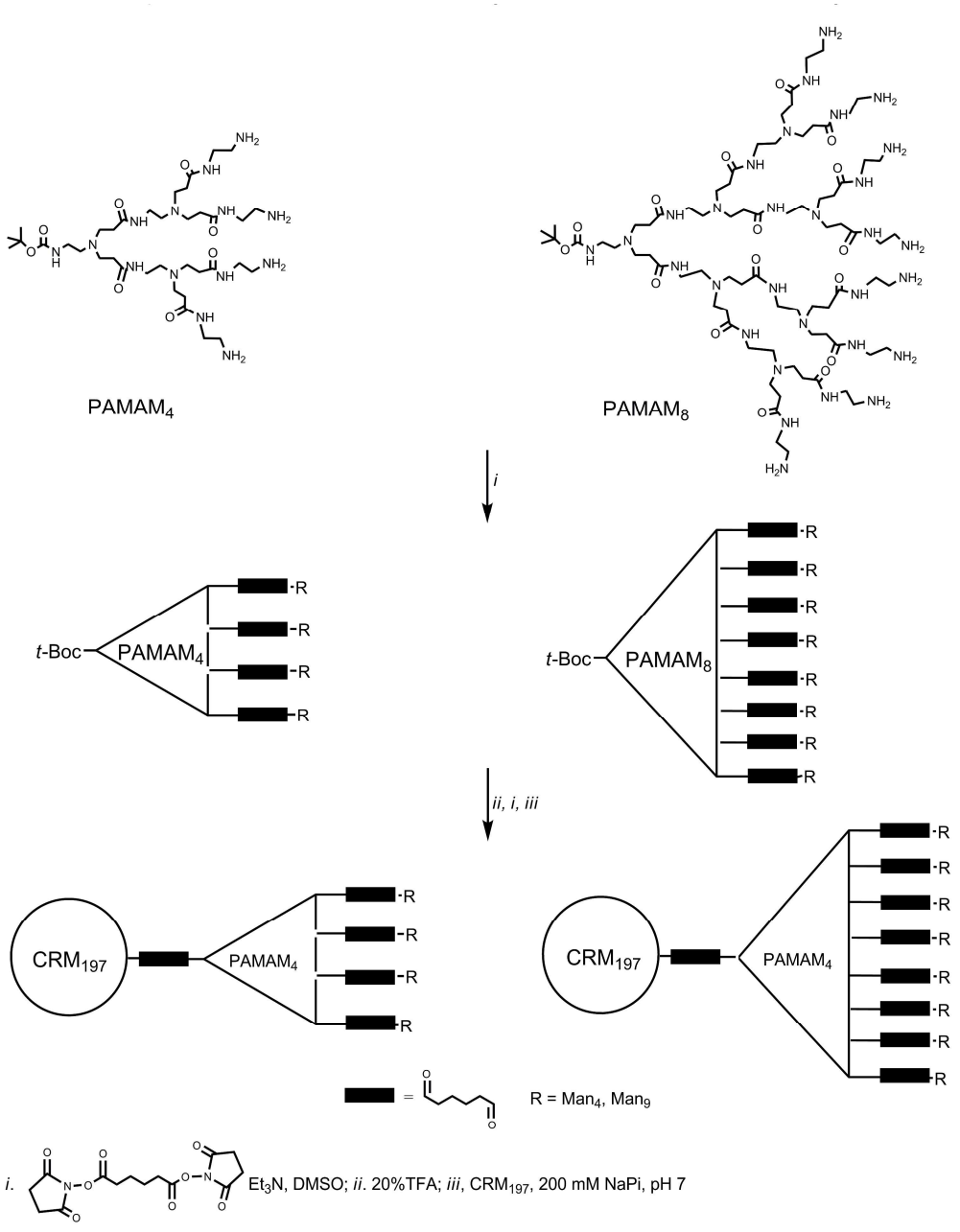

Figure 2. Synthesis of PAMAM-oligosaccharide clusters.

Results

Oligomannose cluster synthesis and characterization

High-mannose oligosaccharides equipped with a six-carbon amino linker at the reducing end were first converted into the corresponding succinimidyl adipate esters and then reacted with $PAMAM_4$ or $PAMAM_8$ (Fig. 2). The glycodendrons were purified by C4 hydrophobic interaction cartridge where the excess of unreacted oligosaccharides eluted in the flow through. Fractions containing the fully derivatized PAMAM were identified by ESI Q-TOF MS.

Glyco-$PAMAM_4$ dendrons were analyzed by direct infusion of the sample into Q-TOF system, glyco-$PAMAM_8$ dendrons were analyzed by UPLC paired (coupled) to Q-TOF. The general pattern of the ESI-MS spectra showed molecular ion peaks related to the fully derivatized PAMAMs as major species and a slight fragmentation of molecules due to the loss of Man units starting from the molecular ion peak, as better evidenced in the deconvoluted spectra.

The ESI-MS analysis of the Man_4PAMAM_8 UPLC peak (Fig. 3) showed multiple molecular ions corresponding to the Man_4PAMAM_8 molecule (8760.14 Da) bearing different charges, the most abundant of which was the 5+ molecular ion, although 3+, 4+ and 6+ ions were present as well.

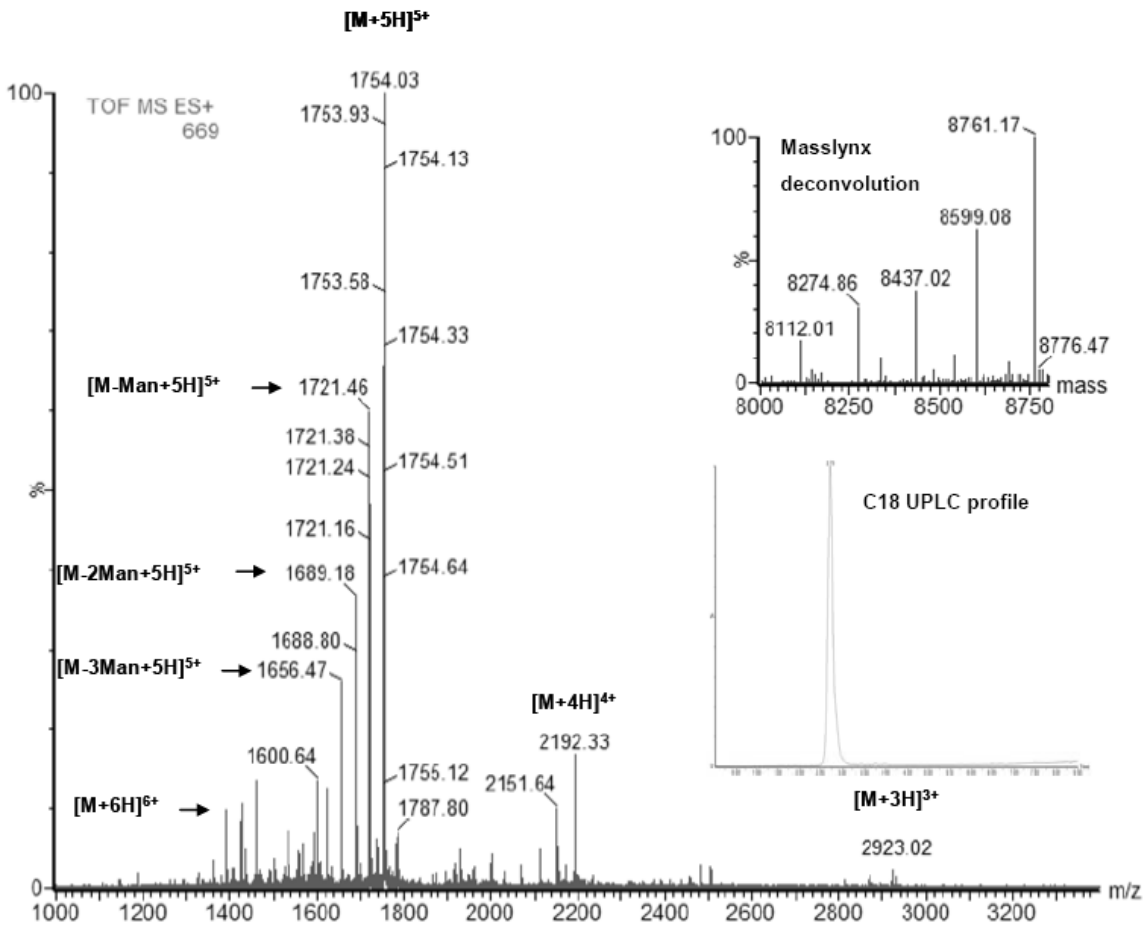

Figure 3. LC-MS profile of Man_4PAMAM_8.

Man_4PAMAM_4 (4346.05 Da) was characterized by the presence of the 3+ and the 4+ molecular ions as most intense peaks (Fig. 4).

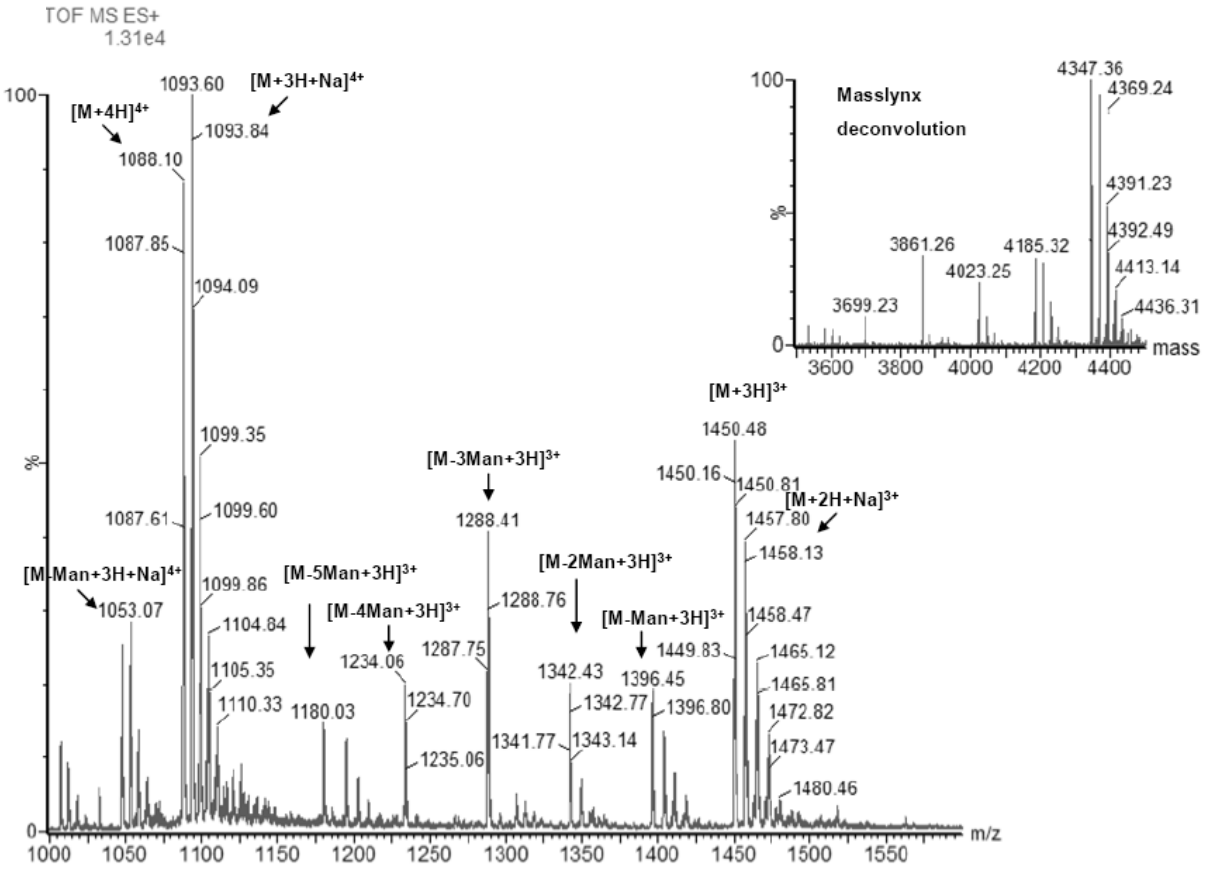

Figure 4. LC-MS profile of Man_4PAMAM_4.

Prominent ions in the spectrum of Man_9PAMAM_4 (7587.11 Da) were the 4+ charged (Fig. 5). The presence of a molecular ion with a mass of 4157.13 Da was attributed to the reaction of activated Man_9 with a $PAMAM_4$ minor contamination, where an ethylenediamine unit (60 Da) had been lost. This contamination is most likely a side product from incomplete reaction during the synthesis of PAMAM dendrimer as reported by others (Schwartz *et al.* 1995).

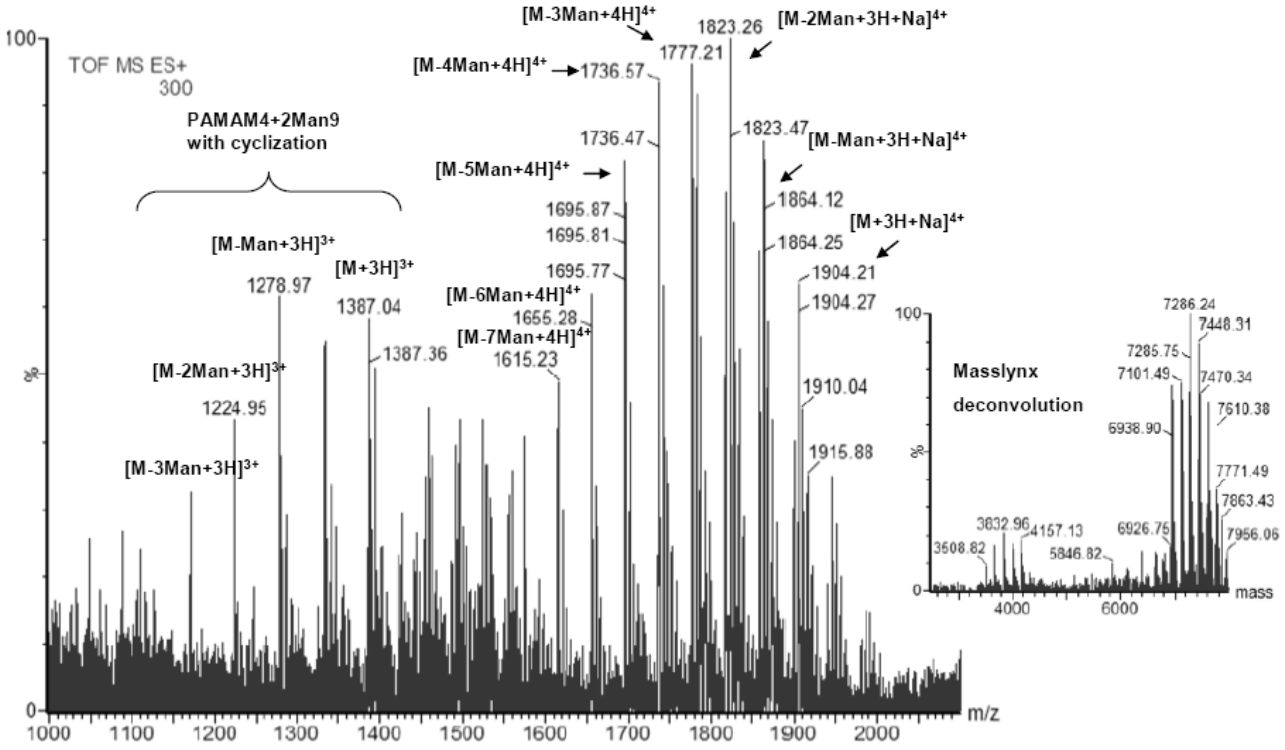

Figure 5. LC-MS profile of Man_9PAMAM_4.

The ESI spectrum of Man_9PAMAM_8 (15242.25 Da) (Fig. 6) presented the 6+ ion as prominent peaks and two minor contaminations. The first was attributed to the $PAMAM_8$ derivatized with solely 2 Man_9 units. The second corresponded to the $PAMAM_8$ conjugated to 6 Man_9 moieties and presenting a bridging between two ethylendiamines through an adipate molecule, very likely due to a contamination of activated Man_9. However, we deemed the desired Man_9PAMAM_8 cluster the most abundant product

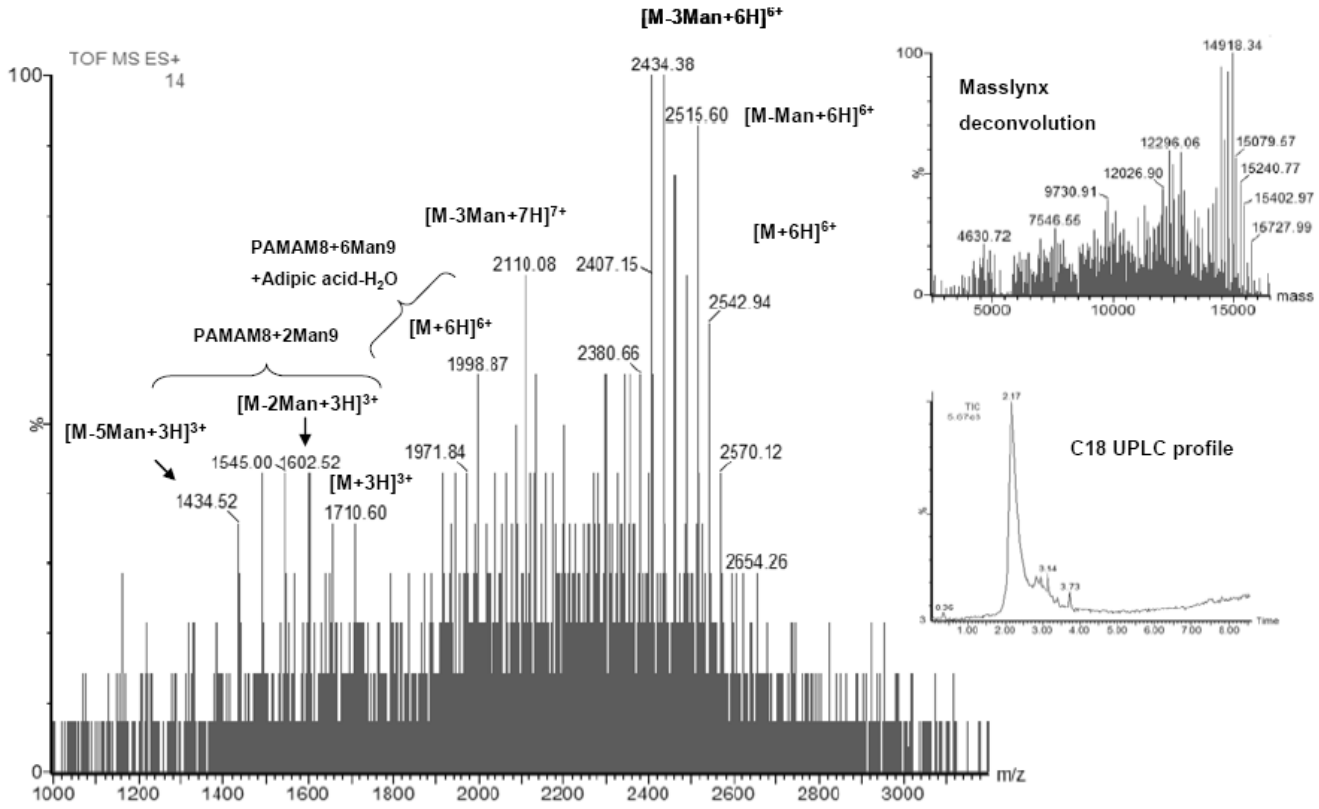

Figure 6. LC-MS profile of Man_9PAMAM_8.

Immunochemical characterization of oligomannose antigens

In order to determine the relative ability of the different oligomannose systems to bind 2G12, I performed competitive experiments using surface plasmon resonance (SPR). HIV protein gp140 UG37 was immobilized on a Biacore CM5 chip, and 2G12 with and without inhibitors was injected over it. Initial screening of monovalent oligomannoses showed inferior inhibitory capacity of Man_6 as compared to Man_4 and Man_9. In fact, 1.2 mM Man_4 and 0.54 mM Man_9 inhibited gp140-2G12 interaction by 84.0% and 68.8%, respectively, while 0.81 mM Man_6 showed only 12.4%. I therefore concentrated attention on Man_4 and Man_9 antigens and explored if clustering influences the binding ability to 2G12.

SPR inhibition assay evidenced lower IC_{50} values for $PAMAM_4$ and $PAMAM_8$ clusters, as compared to their respective monovalent oligosaccharides (Fig. 7). An example of the inhibition assay sensorgrams is presented on Figure 8.

IC_{50} of Man_4PAMAM_4 and Man_9PAMAM_4 clusters were 13 and 11 times lower than IC_{50} of Man_4 and Man_9, respectively; moreover IC_{50} of Man_4PAMAM_8 and Man_9PAMAM_8 clusters were 2 and 2.6 times lower than IC_{50} of Man_4PAMAM_4 and Man_9PAMAM_4, respectively. The absolute IC_{50} values were comparable for both Man_4 and Man_9 clusters. Thus multivalent presentation of oligomannose increased their avidity to 2G12, and the smaller D1-armed Man_4 competed for 2G12 at the same level as D1D3-armed Man_9.

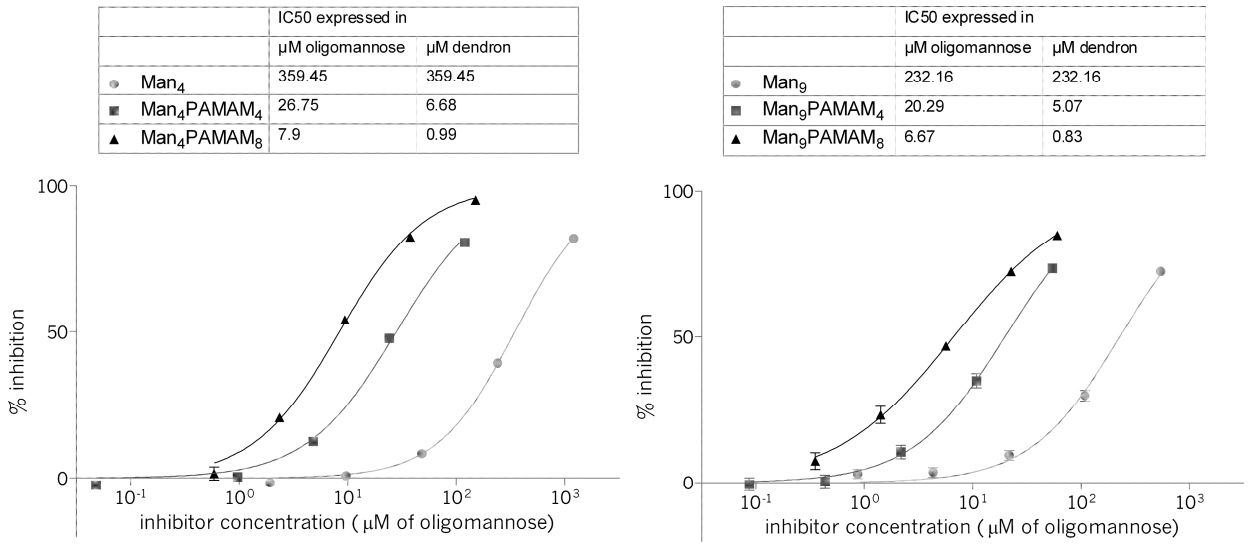

Figure 7. Biacore inhibition of 2G12-gp140 interaction by monovalent and clustered oligomannoses. Inhibition was calculated as difference in maximal binding.

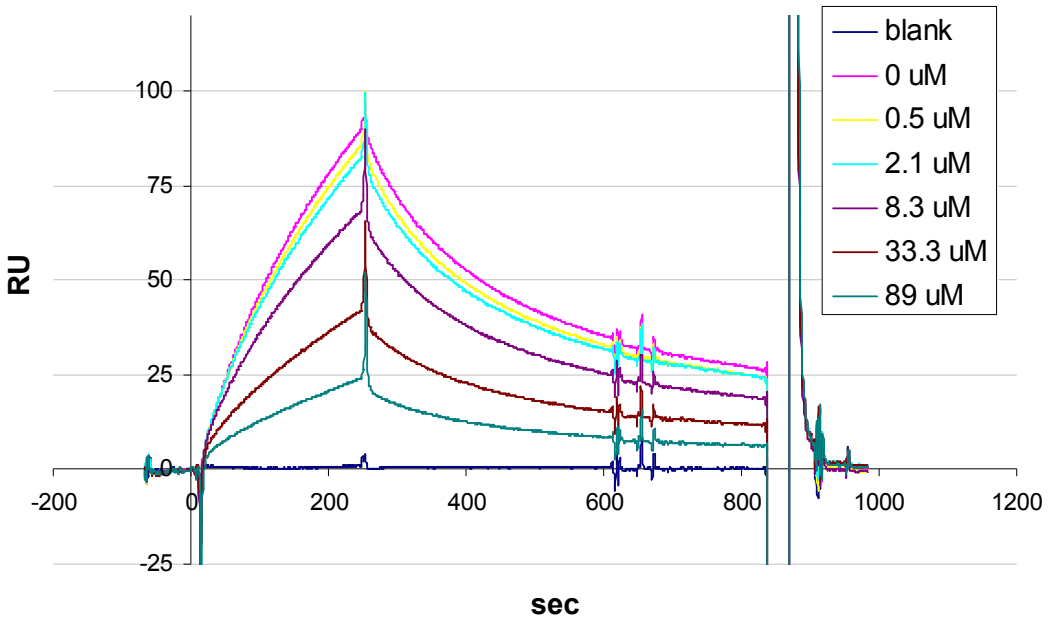

Figure 8. Sensorgrams of the surface plasmon resonance inhibition assay on the example of Man_9PAMAM_4. Legend of each figure indicates inhibitor concentrations.

Synthesis of CRM_{197} glycoconjugates

A well-established way to improve poor immunogenicity of carbohydrate antigens is the conjugation to a protein carrier which provides T cell epitopes. Therefore, high-mannose oligosaccharides have been coupled, plain or PAMAM-clustered, to the lysine residues of CRM_{197}. Using disuccinimidyl adipate linker chemistry (Fig. 2) I have synthesized a panel of glycoconjugates that have been characterized by carbohydrate/protein ratio (wt/wt) and SDS-PAGE. The glycosylation degree ranged from 39.0 to 58.4% (Table I).

Gel line (Fig. 8)	Antigen	Mw of the hapten portion (Da)	Average molar ratio of CHO units[a] or clusters[b] to protein	CHO/Protein % wt/wt	Expected glycoconjugate average Mw (Da)
1	**Man_9-CRM_{197}**	1659	16[a]	43.8	84544
2	**Man_4PAMAM_4-CRM_{197}**	4356	10[b]	43.6	101560
3	**Man_9PAMAM_4-CRM_{197}**	7600	6[b]	58.4	103600
4	**Man_4PAMAM_8-CRM_{197}**	8770	4[b]	39.0	93080
5	**Man_9PAMAM_8-CRM_{197}**	15258	2[b]	44.1	88516

Table I. Chemical characteristics of high-mannose glycoconjugates.

In SDS-Page (Fig. 9) the glycoconjugates migrated with diffuse bands which cover a region consistent with the expected increase of Mw as compared to CRM_{197} and suggesting certain heterogeneity of the glycoconjugate molecules due to the multiple conjugation sites on CRM_{197} represented by 39 lysine residues in its structure (Bardotti *et al.* 2008).

Additional characterization verified neoglycoconjugate recognition by 2G12 in ELISA assay (Fig. 10), in which equivalent amounts of glycoproteins were captured to microplate wells and then detected with 2G12 and GNL. The latter has specificity for α1-3/α1-6 mannoses and served as a positive control (Hester, Wright 1996; Krishnamoorthy *et al.* 2009). No 2G12 recognition was observed for Man_6PAMAM_4-CRM_{197} neither in ELISA, nor in western-blot.

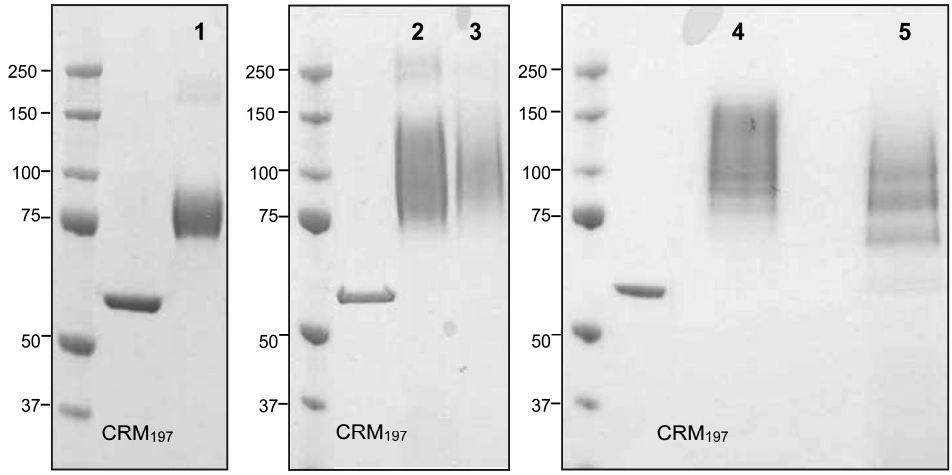

Figure 9. CRM_{197} glycoconjugates with synthetic oligosaccharides and clusters analyzed by coomassie-stained SDS-PAGE in 4-12% Bis-Tris gel. Line numbers indicate Man_9-CRM_{197} (1), Man_4PAMAM_4-CRM_{197} (2), Man_9PAMAM_4-CRM_{197} (3), Man_4PAMAM_8-CRM_{197} (4) and Man_9PAMAM_8-CRM_{197} (5).

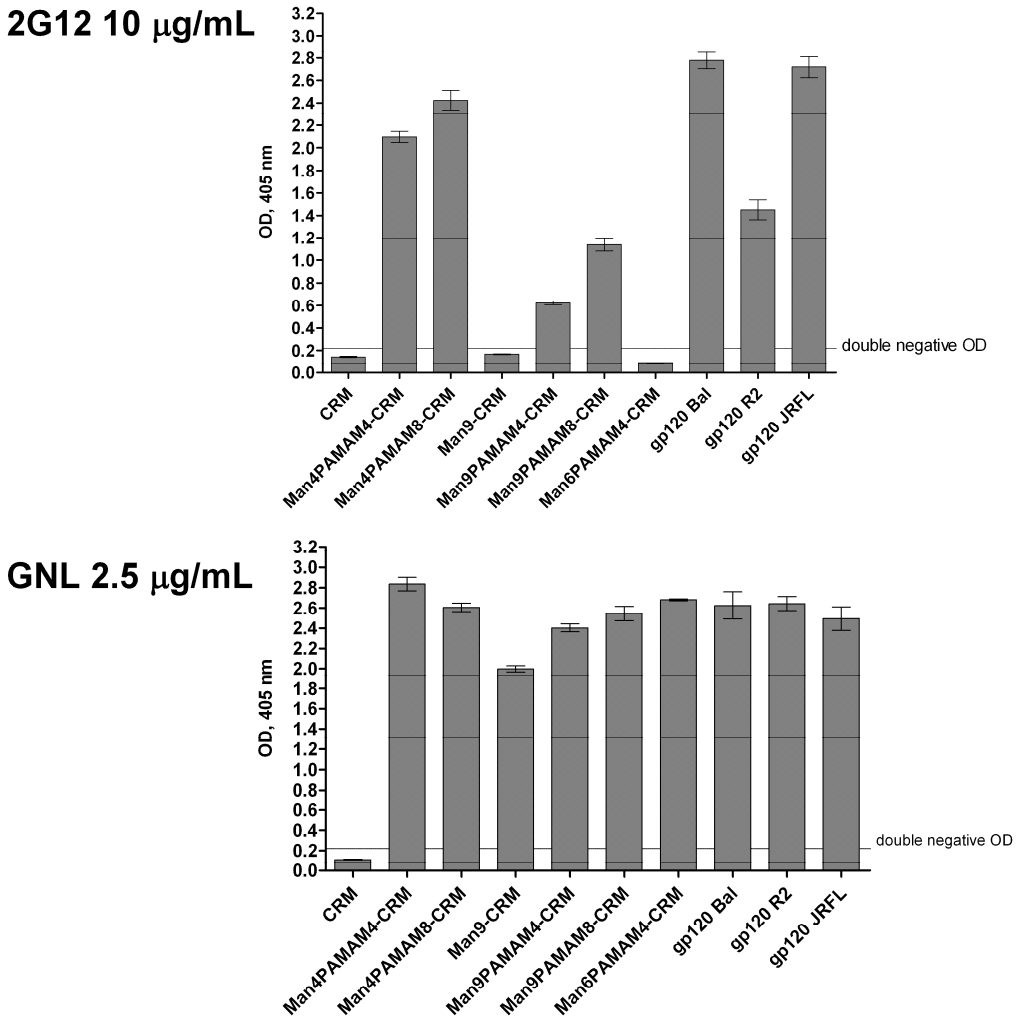

Figure 10. Binding of oligomannose glycoconjugates to 2G12 and GNL lectin. Oligomannose glycoconjugates were coated on ELISA microplates at 100 ng/well. CRM_{197} was used as negative control. Each value is the mean±SD of four replicates.

Glycoconjugates immunogenicity in rabbits and mice

Initially we tested the immunogenicity of Man_4- and Man_9-PAMAM glycoconjugates compared to Man_9-CRM_{197}. Man_4/Man_9-$PAMAM_4$ and Man_4/Man_9-$PAMAM_8$ glycoconjugates were tested at 20 and 5 μg carbohydrate dose, respectively, in group of 2-4 rabbits. In all cases the antigens were

formulated with MF59, an oil-in-water buffered emulsion of 5% squalene, 0.5% Tween 80 and 0.5% Span 85 (Seubert *et al*. 2008).

First, the antibody response was assessed by means of ELISA with Man_9 conjugated to HSA via squarate linker as coating reagent. Since different carrier and coupling chemistry was used, this ELISA revealed only oligomannose-specific antibodies. As seen in Fig. 11 a,b,c all glycoconjugate antigens induced Man_9-specific IgG titer, and in particular it was manifested that Man_9-glycoantigens, clustered or plain, elicited a stronger antibody response in comparison to Man_4 antigens. No significant carbohydrate-specific IgM titers were detected (data not shown).

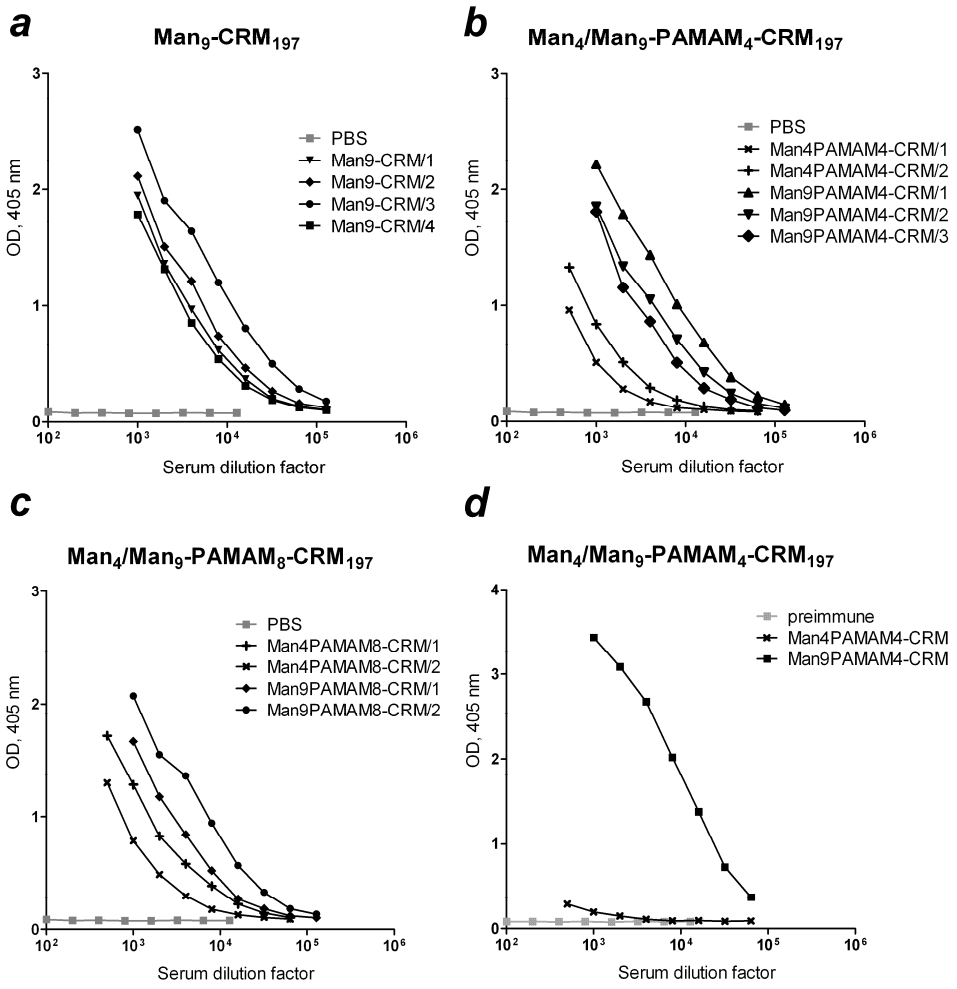

Figure 11. Detection of anti-Man_9 antibodies for single rabbit (a, b, c) and pooled mice (b) antisera. Sera are matched with corresponding immunization antigens. Coating antigen has been synthesized with different conjugation chemistry in order to detect antibodies specific only for synthetic oligomannose part.

Following the main goal of this study, the cross-reactivity of the rabbit sera against HIV-1 gp120 proteins was examined then. Several clade B gp120 proteins were coated onto ELISA microplates and tested against pools of rabbit post immunization sera. None of the gp120 proteins showed cross-reactivity with antiserum panel, meanwhile as expected 2G12 and GNL did recognize

everyone (Fig. 12).

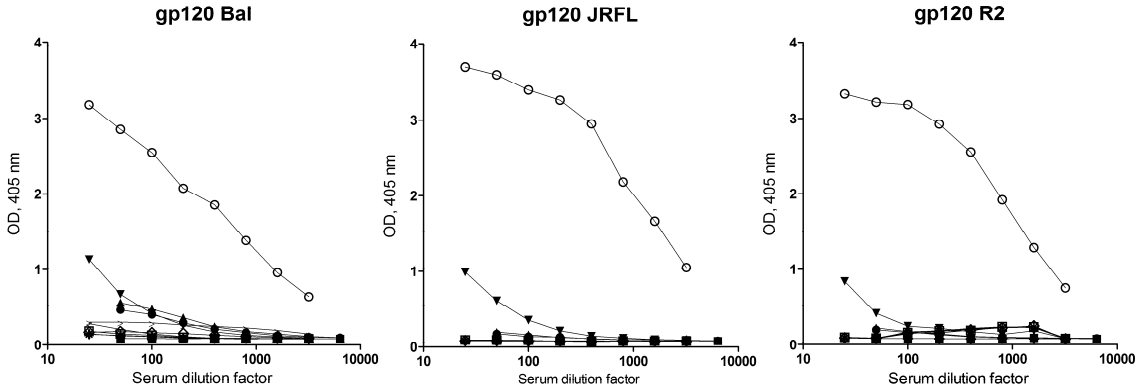

Figure 12. Binding of pooled animal antisera to HIV-1 gp120 glycoproteins. Rabbit antisera: PBS-MF59 (+), Man_9-CRM_{197} (□), Man_4PAMAM_4-CRM_{197} (<), Man_9PAMAM_4-CRM_{197} (>), Man_4PAMAM_8-CRM_{197} (◊) and Man_9PAMAM_8-CRM_{197} (*); mice antisera: preimmune (■), Man_4PAMAM_4-CRM_{197} (▲), Man_9PAMAM_4-CRM_{197} (●); GNL (○) and 2G12 (▼). GNL and 2G12 have 2 and 10 μg/mL at first graph point, respectively; two-fold dilution scheme was applied.

In order to collect data with a different animal model Man_4PAMAM_4-CRM_{197} and Man_9PAMAM_4-CRM_{197} were additionally tested in mice. Anti-Man_9 antibodies were clearly elicited by the Man_9 conjugate, while the response of Man_4PAMAM_4-CRM_{197} was weak (Fig. 11 d). Also in this case, when the antisera were tested against HIV-1 gp120, no cross reaction was observed (Fig. 12).

Anti-carrier antibody response

The presence of anti-CRM_{197} antibodies in antiserum pools of rabbit and mice immunized with the different conjugates was examined, and it turned out that in all cases anti-carrier antibodies have been induced. Interestingly, in rabbit PAMAM-based conjugates seemed to induce a lower anti-carrier response as

compared to Man_9-CRM_{197} which could be explained considering a certain shielding of relevant T- or B-cell epitopes of CRM_{197} by the glycodendron haptens (Fig. 13).

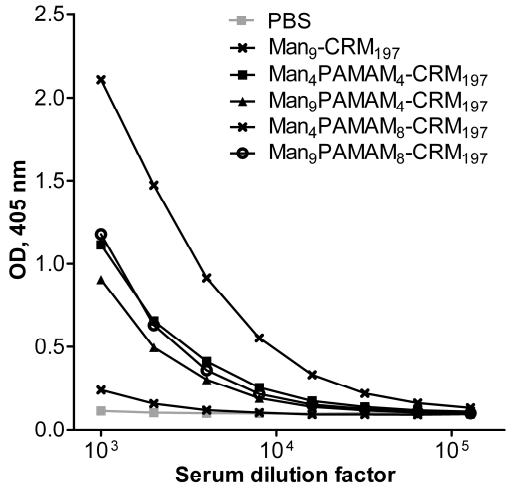

Figure 13. Binding of pooled rabbit antisera to CRM_{197} glycoproteins.

Discussion

HIV is characterized by a densely glycosylated surface, which enhances the effectiveness of immune escape and is implicated in viral dissemination (Pohlmann *et al.* 2001; Shan *et al.* 2007). Human broadly neutralizing antibody 2G12 and mannose-binding lectin cyanovirin-N were found to recognize high-mannose oligosaccharides on the surface of HIV-1 gp120, and both demonstrated anti-HIV activity at nanomolar level (Bewley *et al.* 2001; Binley *et al.* 2004). Moreover, both 2G12 or cyanovirin-N have shown so far no autoimmune property, probably due to their strict specificity to dense oligomannose surfaces that have not been observed among human glycoproteins (Scanlan *et al.* 2002; Scanlan *et al.* 2007). This suggested that

high-mannose oligosaccharides are feasible targets for a vaccine aiming at eliciting “2G12”-like antibodies. Up to now various synthetic approaches have been applied to prepare clusters with the aim of mimicking the 2G12 epitope (Astronomo *et al.* 2008; Joyce *et al.* 2008; Ni *et al.* 2006), however, none of the obtained molecules reported gp120 cross-reactive immune response virtually due to initial low antigen affinity to 2G12. The affinity increase that can be achieved by multivalent presentation of carbohydrate ligands prompted us to explore PAMAM dendrons that offer high coupling valence and are low-immunogenic *per se* (Chabre, Roy 2008; Boas *et al.* 2006). Utilization of such scaffolds could provide more control in spatial presentation of sugars than conjugation to Lys residues of carrier protein does, allowing a better emulation of the dense arrangement of oligomannoses on the gp120 glycan. In a recent study nine- and 27-valent oligomannose dendrons showed similar affinity and inhibition capacity in binding 2G12 to gp120 (Wang *et al.* 2008), suggesting that rising cluster valence above nine moieties would not necessarily lead to further increase in the 2G12 affinity.

In my thesis I report the first *in vivo* study with glycoconjugates containing four- and eight-valent high-mannose oligosaccharide dendrons. Obtained glycoconjugates consisted of the HIV-1 related carbohydrate antigens clustered onto the PAMAM dendrons and subsequently conjugated to CRM_{197}, which is well known for its excellent properties as carrier for bacterial oligo- and polysaccharides and is widely used in licensed glycoconjugate vaccines (Bardotti *et al.* 2008; Broker *et al.* 2009; Mawas *et al.* 2004; Safari *et al.* 2008; Torosantucci *et al.* 2005). The antigens were formulated with the potent MF59

adjuvant which was shown to be effective in boosting both cellular and humoral immune response and being of common use for seasonal flu vaccination (Burke *et al.* 2009; Galli *et al.* 2009; Seubert *et al.* 2008). The combination of mentioned above factors was designed to confer to the glycoantigens improved immunogenic features.

Oligomannoses for the development of our glycoantigens were chosen on the basis of biochemical, biophysical and crystallographic evidences available in the literature (Adams *et al.* 2004; Calarese *et al.* 2005; Calarese *et al.* 2003; Pashov *et al.* 2005; Sanders *et al.* 2002; Wang *et al.* 2008). Man_4, Man_6 and Man_9 candidates possessed terminal Manα1,2-Man units that were shown to be essential for 2G12 recognition being involved into binding in the antibody combining site (Calarese *et al.* 2005; Scanlan *et al.* 2002). Although it was expected that all three oligosaccharide candidates would demonstrate 2G12 reactivity, Man_6 antigen showed low potency as inhibitor compared to Man_4 and Man_9 oligosaccharides in SPR studies; moreover Man_6PAMAM_4-CRM_{197} did not demonstrate 2G12 binding in ELISA assay (Fig. 10). This may indicate that trisaccharide α-D-Man-(1,2)-α-D-Man-(1,2)-α-D-Man, present in both Man_4 and Man_9, is required for the affinity interaction. This observation is in line with the structural requirements of ligand binding for high-mannose-specific lectin cyanovirin-N (Bewley *et al.* 2001). 2G12 recognition of Man_6 observed in the glycoarray studies indicates that this oligosaccharide benefits from dense multivalent display on the surface of microarray slide (Adams *et al.* 2004). Nevertheless, its structural features might be not sufficient to provide enough 2G12 affinity in case of a lower density carbohydrate presentation.

Having this information at disposal, I focused only on Man_4 and Man_9 antigens. The preparation of high-mannose glycodendrons was based on the activation of the amino groups present in the oligosaccharide linkers with an excess of disuccinimidyl adipate, followed by the reaction of activated oligosaccharides with t-Boc-protected PAMAM (Yi *et al.* 1998). The effect of oligosaccharide multivalent presentation was evidenced by the enhancement of glycodendron capacity to inhibit 2G12-gp140 interaction (Fig. 7). After hydrolysis of the t-Boc group and activation of the amino function again with disuccinimidyl adipate, the clusters with four and eight oligomannose antennae were conjugated to CRM_{197}. As a result I was able to synthesize glycoconjugates with 39-58% carbohydrate content, which is significantly higher than previous studies reporting a 15-19% range (elaborated from Astronomo *et al.* 2008; Joyce *et al.* 2008; Ni *et al.* 2006). Glycodendron conjugation to CRM_{197} did not seem to effect their conformation that was evidenced by maintenance of 2G12 recognition (Fig. 10).

Immunization of rabbits and mice with MF59-formulated CRM_{197} glycoconjugates of Man_4 and Man_9 antigens induced specific anti-Man_9 antibodies (Fig. 11). In all cases Man_9-conjugates induced stronger response as compared to the Man_4-conjugates, which can be easily explained considering the structural differences between Man_4 and Man_9 However, neither the four- nor the eight-valent flexible PAMAM dendron antigens induced gp120 cross-reactive antibodies (Fig. 12), indicating that the presentation of oligomannose sugars was not sufficient to mimic the native carbohydrate epitopes. Previous studies conducted with glycoconjugates prepared from high-mannose oligosaccharides clustered onto scaffolds and then linked to diverse carriers,

such as BSA, OMPC or *Tetanus toxoid* T-helper peptide, have also failed to induce antibodies cross reactive with HIV-1 gp120 (Astronomo *et al.* 2008; Joyce *et al.* 2008; Ni *et al.* 2006).

In the present work the different model based on the use of PAMAM dendrons as a way to display the HIV-1 related oligomannoses in a clustered form on the surface of CRM_{197} confirms the significant difficulties in the identification of a suitable carbohydrate-based anti-HIV vaccine candidate. The failure of high-mannose-PAMAM-CRM_{197} conjugates to provide antibodies with affinity for gp120 could be explained with inappropriate spacing of oligomannose antennae in the synthesized clusters, too much flexibility introduced by the presence of two subsequent six-carbon spacer chains between the oligomannoses and the PAMAM core, or too wide separation among the cluster molecules on the carrier protein surface. Those issues should be definitively addressed in the future work for a synthetic anti-HIV vaccine.

Recently reported data on the rabbit "2G12"-like serum response to immunization with Man_8-reach mutant yeast cells gives a hint that the oligomannose density exposure is likely to be one of the dominating factors for designing HIV glycoantigens (Luallen *et al.* 2008; Luallen *et al.* 2009; Luallen *et al.* 2009 a). This outcome, which might be due to the abundant high-mannose glycosylation of yeast proteins comprising approximately 100% of protein weight (elaborated from ref. Luallen *et al.* 2009 and Luallen *et al.* 2009 a), suggests that finding the right density and exhibition of oligomannose surface could be the key for the search of best mimics of the native 2G12 epitope.

Chapter 2

Rational design and evaluation of a synthetic carbohydrate-based Group A Streptococcus vaccine candidate

Introduction

Group A *Streptococcus* (GAS) infections represent a significant healthcare concern throughout the world. GAS is responsible for a broad spectrum of diseases ranging from asymptomatic colonization, uncomplicated pharyngeal and skin infections to life-threatening invasive illnesses including sepsis, necrotizing fasciitis and toxic shock syndrome (Mitchell 2003). Epidemiology reveals correlations between bacterial strains responsible for GAS pharyngitis, a frequent childhood illness, and invasive streptococcal disease in adults (Haukness et al. 2002; Linder et al. 2005). Pharyngitis may lead to delayed sequela as rheumatic fever. In developing countries rheumatic fever remains endemic and causes hundreds of thousands of deaths every year (WHO 1992). Currently, no vaccine to prevent GAS infections exists although GAS has been on the WHO priority prevention list for decades.

The diversity of GAS strains is the major challenge for the development of an anti-GAS vaccine. GAS bacteria contain a surface polysaccharide consisting of repeating [→3)α-L-Rha*p*(1→2)[β-D-Glc*p*NAc(1→3)]α-L-Rha*p*(1-]$_n$ units (Fig. 1). These long polysaccharide chains adopt a helical conformation where the rhamnoses form the helix core and the immunodominant *N*-acetylglucosamine residues are exposed on the periphery (Pitner et al. 2000; Johnson et al. 2002). The GAS polysaccharide (GAS-PS) is conserved and constantly expressed in

the vast majority of M+ GAS strain serotypes (Cunningham et al. 2000; Lancefield et al. 1933; Mc, Lancefield et al. 1955). The conserved nature of GAS-PS renders a potentially attractive conjugate vaccine. Purified GAS-polysaccharide has been conjugated to tetanus toxoid carrier and elicited a protective immune response in a challenge model in mice (Sabharwal et al. 2006). Serum anti-GAS-PS antibodies confer protection against GAS throat colonization and promote bacterial phagocytosis (Sabharwal et al. 2006; Salvadori et al. 1995).

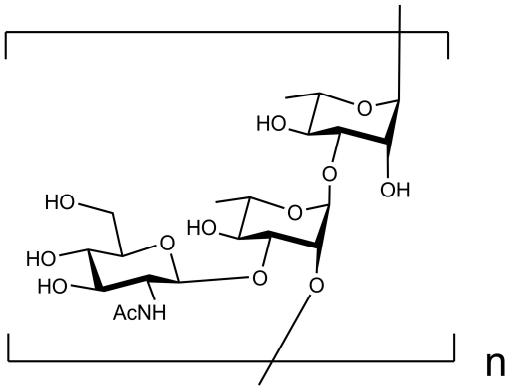

Figure 1. Structures of the repeating unit of the cell-wall polysaccharide of GAS.

Efforts to determine the antibody-binding epitope of GAS-PS revealed a core antigenic determinant – a hexamer structure of two repeating units (Michon et al. 2005). The human anti-GAS humoral is believed to recognize hexasaccharide (Michon et al. 2005). To date no evidence that a minimal GAS-PS core antigen determinant can elicit an immunoprotective response has been obtained.

Up to now synthetic approaches applied to the development of carbohydrate vaccines has been evaluated in a very limited set of infection disease areas but, nevertheless, has shown its efficacy in the generation of a protective response

(Benaissa-Trouw et al. 2001; Chong et al. 1997; Jansen et al. 2001; Pozsgay et al. 2007; Robbins et al. 2009; Safari et al. 2008; Verez-Bencomo et al. 2004). Compared to the conventional polysaccharide isolation, the synthetic approach has an advantage of generating pure, homogeneous oligosaccharides of well-defined structure, rationally planned to contain immunodominant saccharide composition and single-site reactive groups for covalent conjugation. Present study directly compares *in vivo* efficacy of CRM_{197} glycoconjugate of the native GAS-PS material versus that of the synthetic oligosaccharides designed to evaluate optimal oligosaccharide length and terminal molecule residues, as both parameters may influence carbohydrate immunogenicity (Benaissa-Trouw et al. 2001; Jansen et al. 2001; Michon et al. 2005; Ragupathi et al. 2006; Safari et al. 2008). Purified, native GAS-PS antigen (Fig. 1) and synthetic oligosaccharide structures **1**-**4** (Fig. 2) were coupled to CRM_{197} and tested in a relevant mouse model.

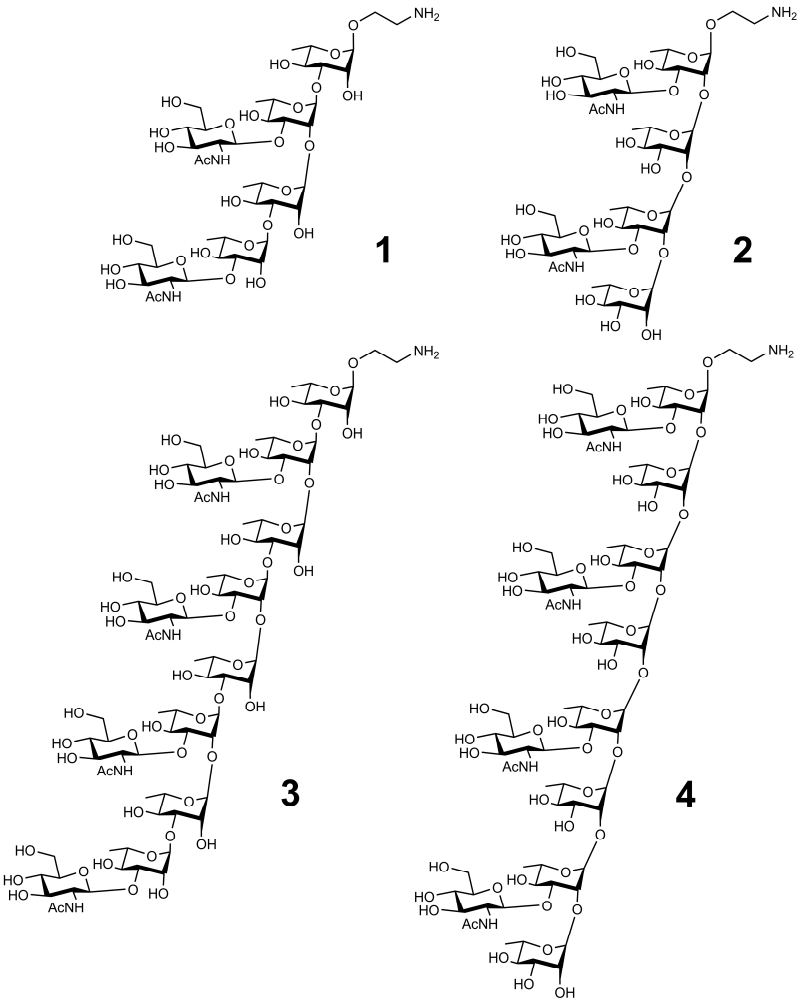

Figure 2. Structures of the synthetic oligosaccharides **1**-**4** designed for present work.

Results

GAS-PS isolation and purification

Native GAS-PS was extracted from GAS SF370 M1 strain. Final GAS-PS preparation was characterized with <0.3% of DNA and <0.005% hyaluronic acid. MicroBCA assay detected 2-3% protein contamination; however, this value was confirmed neither by Bradford method, nor silver stain SDS-PAGE. This suggests that the MicroBCA detection was likely influenced by reducing groups of GAS-PS (Waffenschmidt, Jaenicke 1987; Franco-Fraguas et al. 2003). As

determined by multiple laser light scattering (MALLS), the average molecular weight of GAS-PS was 8.9 ± 1.0 kDa which corresponded to 18 repeating units; dn/dc value was 0.168 mL/g.

Purified GAS-PS was additionally characterized by ^{1}H NMR analysis. The ^{1}H NMR spectrum was consistent with the published structure (Johnson, Pinto 2002) (Fig. 3). It was possible to detect the so-called variant GAS polysaccharide, representing a polyrhamnose [→3)α-L-Rha*p*(1→2)α-L-Rha*p*(1-]$_n$ without branching GlcNAc residues. In certain cases this variant carbohydrate substitutes typical GAS-PS in the bacteria cell wall after passage in mice (Mc, Lancefield 1955; Michon et al. 2005). In the ^{1}H-NMR spectrum the signal of poly-rhamnose H_1^{RhaB} was shifted in comparison to conventional GAS CHO H_1^{RhaB}, while both variant and conventional H_1^{RhaA} signals were merged (Fig. 3). Integration of the variant H_1^{RhaB} and the merged H_1^{RhaA} peaks allowed us to calculate a molar ratio between poly-rhamnose and GlcNAc-containing species of GAS-PS: % polyrhamnose = $[H1^{RhaB}{}_{VAR}/(H1^{RhaA} + H1^{RhaA}{}_{VAR})]$ x 100. Polyrhamnose content for purified GAS-PS lots was <10% for typical polysaccharide preparations.

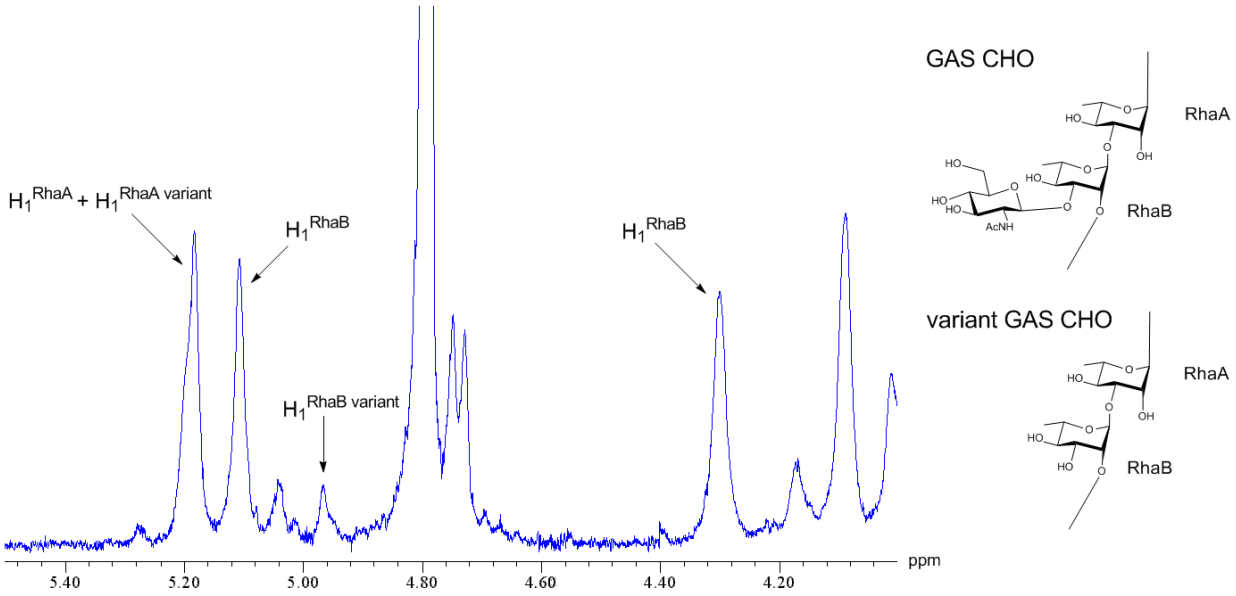

Figure 3. Expansion of the region of the ^{1}H NMR spectrum of purified GAS cell wall polysaccharide. Assigned are the peaks crucial for the determination of residual polyrhamnose content.

Generation and characterization of glycoconjugates

A well-established way to improve poor immunogenicity of carbohydrate antigens is through conjugation to a protein carrier which provides T-cell epitopes. Therefore, the carbohydrate structures were coupled to lysine residues of CRM_{197}, a non-toxic mutant of diphtheria toxin already extensively used as a carrier for glycoconjugate vaccines in humans (Broker et al. 2009; Giannini et al. 1984; Jackson et al. 2009). Depending on whether the native or the synthetic saccharide was used, the conjugation of carbohydrate haptens to the lysines of the carrier protein was accomplished by two approaches (Fig. 4). In the case of synthetic oligosaccharides the amino groups on the linker were derivatized with disuccinimidyl adipate and subsequently coupled to CRM_{197}. Native GAS-PS was instead conjugated to CRM_{197} via direct reductive amination.

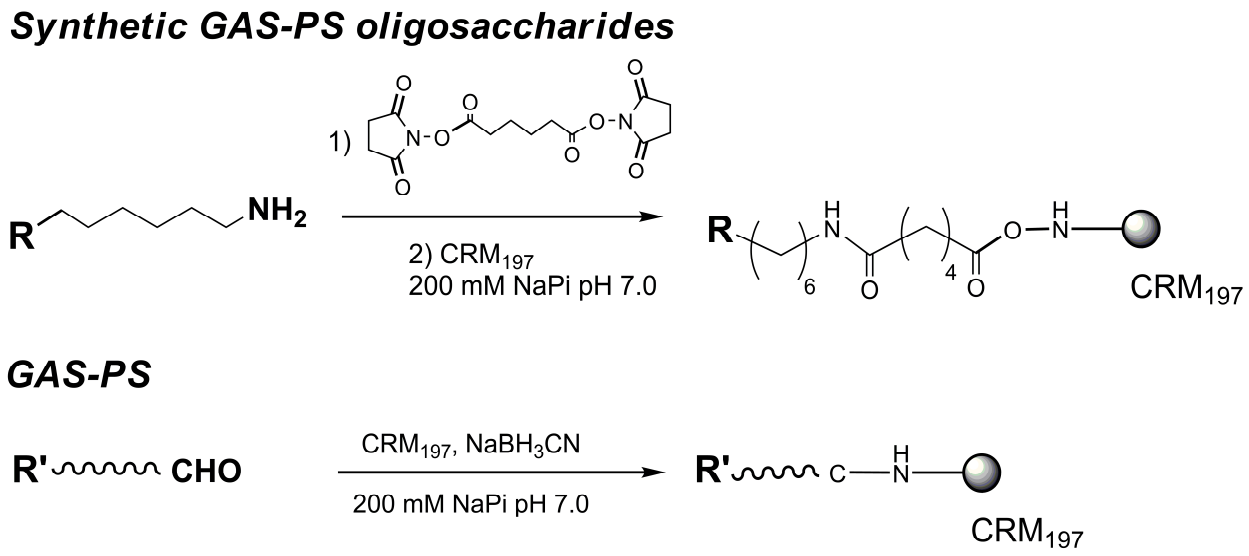

Figure 4. Conjugation of GAS carbohydrates to CRM_{197}.

The conjugates were characterized by SDS/PAGE, carbohydrate/protein ratio, free saccharide, size exclusion HPLC, and MALDI-TOF mass spectrometry. In Fig. 5 the SDS-PAGE profile of different GAS conjugates provides evidence that glycosylation with the synthetic oligosaccharides produced conjugates with a more homogeneous pattern as compared to CRM_{197}-GAS-PS, which had a significant polydispertion in the molecular size due to the larger carbohydrate hapten.

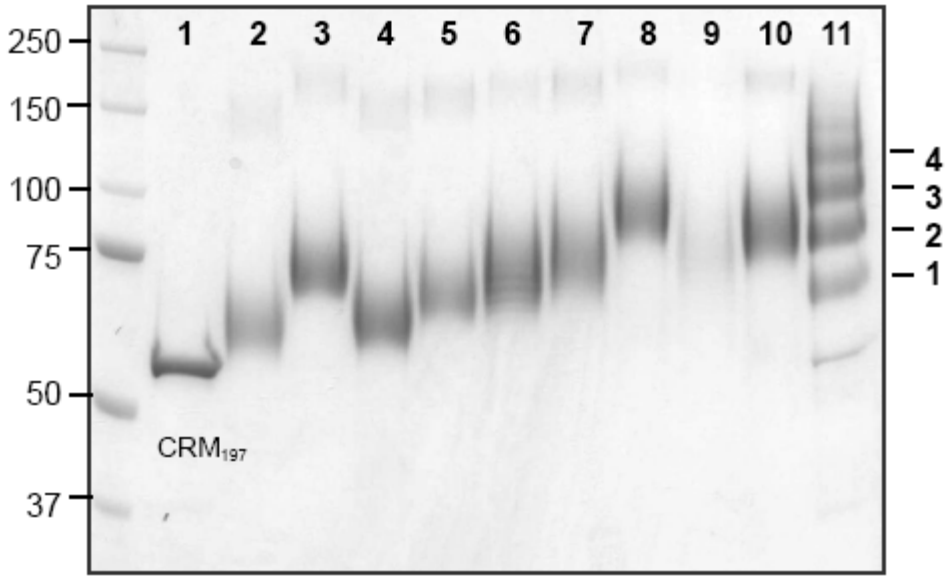

Figure 5. CRM_{197} glycoconjugates analyzed by coomassie-stained SDS-PAGE in 7% Tris-Acetate gel. The glycoconjugates migrate with diffuse bands which cover a region consistent with the expected increase of Mw as compared to CRM_{197}. Line numbers indicate CRM_{197} (1), CRM_{197}-**1**/1 (2), CRM_{197}-**1**/2 (3), CRM_{197}-**2**/1 (4), CRM_{197}-**2**/2 (5), CRM_{197}-**3**/1 (6), CRM_{197}-**3**/2 (7), CRM_{197}-**3**/3 (8), CRM_{197}-**4**/1 (9), CRM_{197}-**4**/2 (10), CRM_{197}-GAS-PS (11). The numbers on the right indicate the numbers of GAS-PS chains attached to CRM_{197}.

The carbohydrate/protein ratio of the conjugates made with native GAS-PS ranged from 40 to 50% (wt/wt) corresponding to 2-3 on molar basis; the molar carbohydrate/protein ratio of conjugates made with synthetic GAS oligosaccharides ranged from 3 to 12 as determined by chemical methods (Table I).

Conjugate code	Average CHO/Protein Mol/mol		Total IgG GMT	AI[a], M NaSCN
	Chemical assays	MALDI-TOF		
CRM_{197}-1/1	3,4	nd	2911	1,29
CRM_{197}-1/2	11,6	14,2	5980	0,87
CRM_{197}-2/1	5,1	nd	1895	1,08
CRM_{197}-2/2	7,6	7,5	2465	0,64
CRM_{197}-3/1	5,8	nd	10262	0,81
CRM_{197}-3/2	7,5	8,8	17780	1,24
CRM_{197}-3/3	12,2	13,6	639	0,68
CRM_{197}-4/1	7,6	nd	4063	0,92
CRM_{197}-4/2	9,2	11,5	2108	0,85
GAS-PS	2-3	3-4	12086	0,86

Table I. Glycoconjugate composition and geometric mean titers (GMT) of serum anti-GAS-PS antibodies induced after mice immunization. The number of carbohydrate chains per protein was calculated from CHO/protein content ratio derived from chemical characterization (HPAEC-PAD, MicroBCA) and MALDI-TOF. The avidity index (AI) is expressed as the concentration of NaSCN needed to reduce the OD_{450} by 50%. [a] Pooled sera was analyzed.

Both synthetic and native glycoconjugates were analyzed by MALDI-TOF. As an example, a representative mass spectrum of the glycoconjugate CRM_{197}-**3**/2 and CRM_{197}-GAS-PS is shown in Fig. 6. The profile of CRM_{197}-**3**/2 is composed of a polydispersion of Mw centered at 77922.9 corresponding to nine oligosaccharide chains per CRM_{197} molecule; the profile of CRM_{197}-GAS-PS is composed of a polydispersion of Mw centered at 87356.4 and 96778.3 corresponding to three

and four GAS-PS chains per CRM_{197} molecule, respectively. A comparison between the average glycosylation degree obtained by chemical methods and MALDI-TOF is reported in Table I.

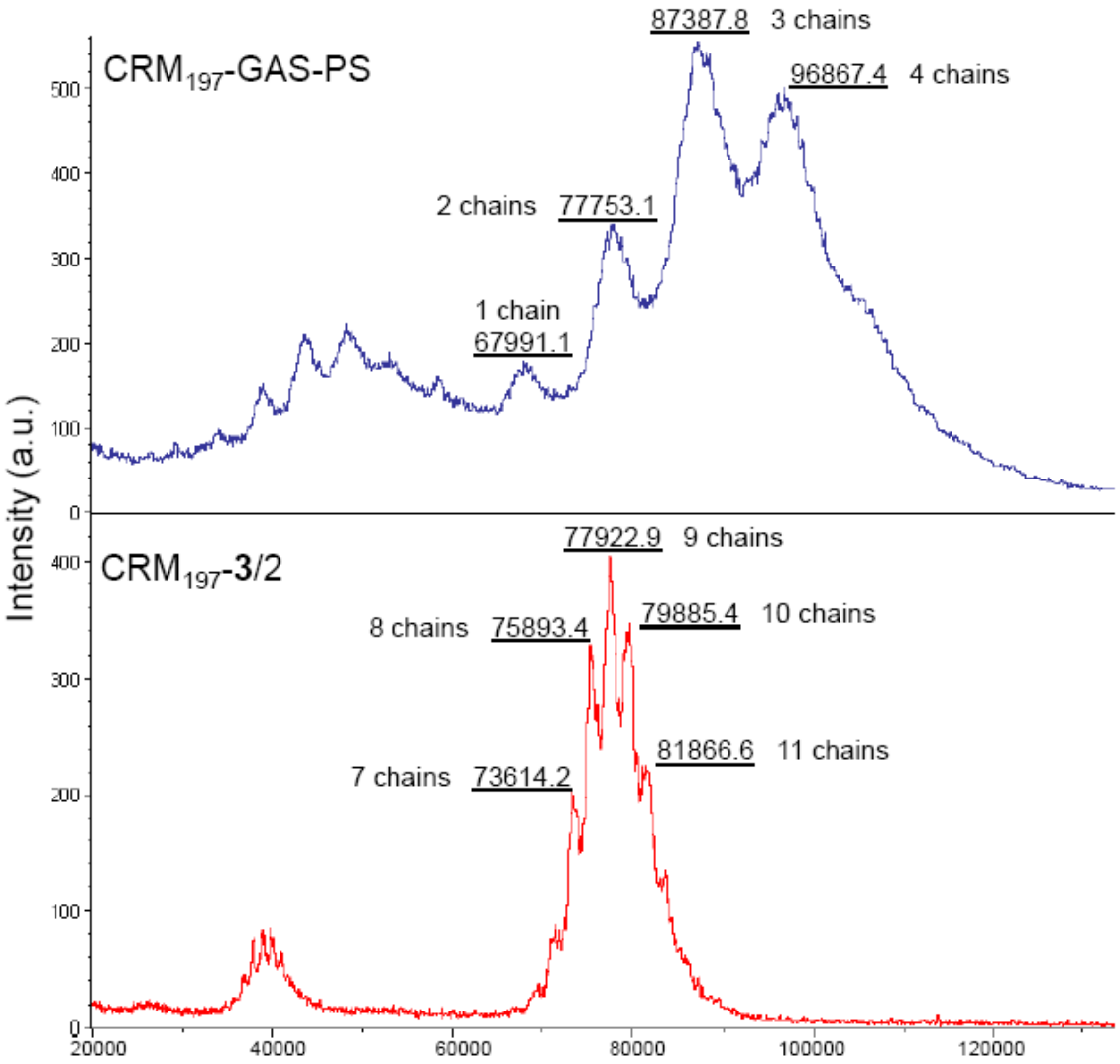

Figure 6. MS spectra of the glycoconjugate CRM197-**3**/2 and CRM_{197}-GAS-PS.

Size-exclusion HPLC analysis of the different conjugates confirmed the SDS-PAGE results evidencing higher Mw dispersion of CRM_{197}-GAS-PS as compared to the synthetic GAS oligosaccharide glycoconjugates (Fig. 7). The

glycoconjugates were purified by ultrafiltration, and amounts of unconjugated sugar was <10%.

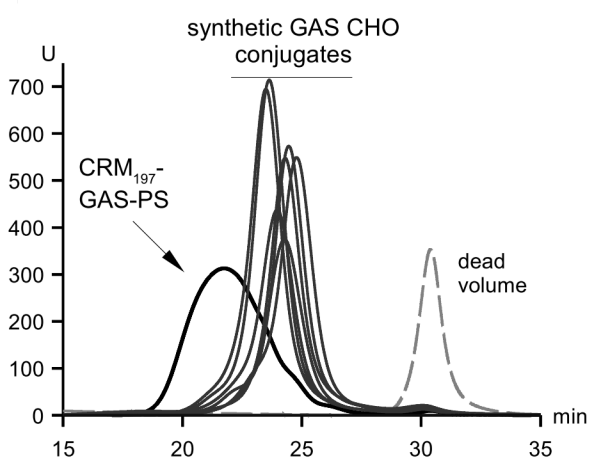

Figure 7. Size-exclusion HPLC profile of the glycoconjugates.

Anti-GAS-PS serum response induced by glycoconjugates

CD-1 mice received one prime and two boosting doses of the carbohydrate antigens protein conjugates (10 μg each). Control groups were immunized either with alum only or unconjugated GAS-PS. Sera were collected on day 49, two weeks after the third immunization to assess antibody response by ELISA, using GAS-PS coupled to HAS as coating reagent.

All synthetic conjugates regardless saccharide chain length, carbohydrate loading and nature of the terminal sugar, were able to induce GAS-PS specific IgG in mice (Fig. 8). In general, the antibody response was statistically-lower than the one elicited by the CRM_{197}-GAS-PS (P<0.05). A notable exception was represented by the conjugates CRM_{197}-**3**/1 and CRM_{197}-**3**/2 with a carbohydrate loading of 5.8 and 7.5 respectively (Table I), which induced a level of anti-GAS-PS IgG comparable to that obtained with the native GAS-PS glycoconjugate. On

the other hand, CRM_{197}-**3**/3 with a carbohydrate loading of 12 induced significantly lower titres (P<0.05). This observation seems to indicate that for a given chain length differences in the glycosylation degree are crucial for the induction of an optimal antibody response.

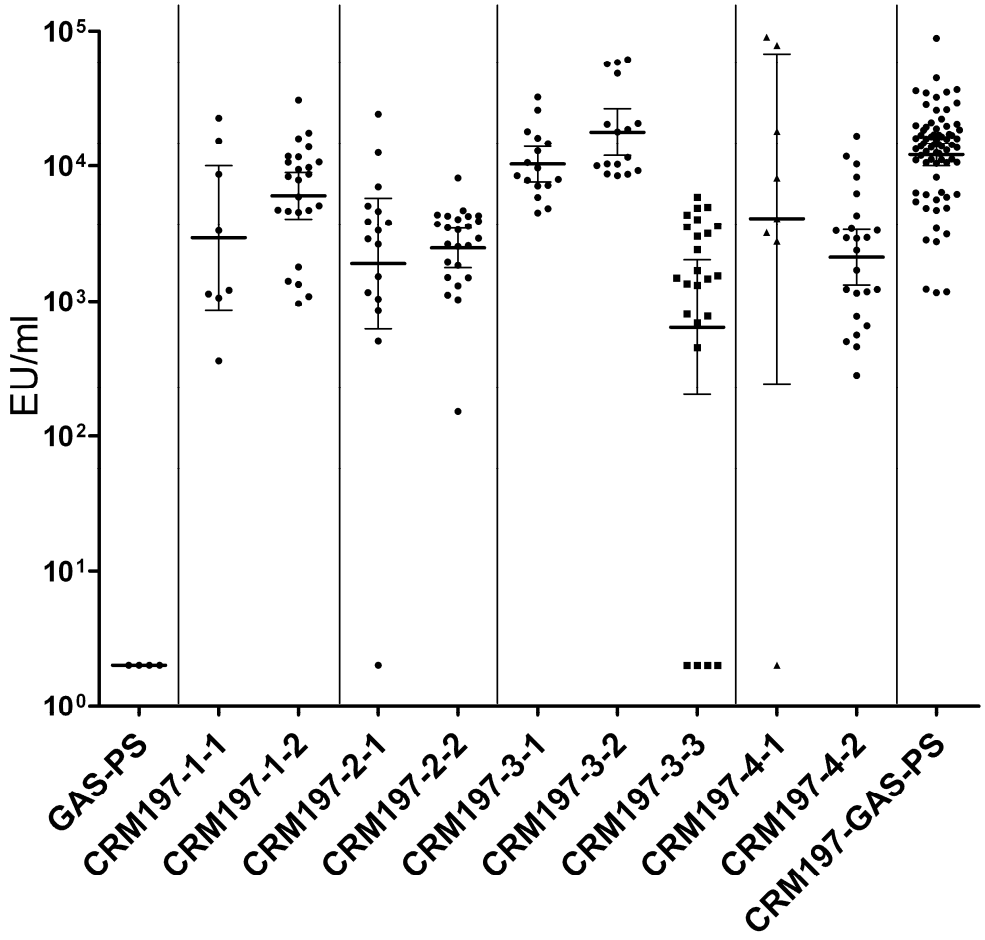

Figure 8. Total IgG antibody titres against GAS-PS. Mice were immunized with unconjugated GAS-PS, CRM_{197}-GAS-PS and CRM_{197}-glycoconjugates of synthetic oligosaccharides. The graph shows the measurements for one-three groups of 8-16 mice immunized independently; conjugates have been injected in seven experiments. Each dot represents single mice sera. Horizontal bars indicate GMT meaning of group with 95% confidence interval statistical bars. GMT values are shown in Table I.

Concerning the influence of the terminal non-reducing sugar on the ability to induce a specific antibody response against the native GAS-PS, a direct comparison between CRM_{197}-**1**/1 and CRM_{197}-**2**/1 (same length, similar loading, but different terminal non-reducing sugar) did not reveal a significant difference, although GMT induced by the conjugates which contain the oligosaccharides terminating with GlcNAc appeared higher (2911 EU/mL vs 1895 EU/mL). Similar observation could be made for the pair of the dodecasaccharide glycoconjugates CRM_{197}-**3**/2 and CRM_{197}-**4**/1 (GMT 17780 EU/mL vs 4063 EU/mL, respectively).

Analysis of IgG subclasses revealed that both synthetic oligosaccharides and GAS-PS glycoconjugates primarily induced IgG1 (Fig. 9), showing a bias toward a Th2 response that could have been an influence of Alum chosen as an adjuvant [41, 42]. Avidity of specific IgG detected with tiocyanate elution ELISA was not significantly different between the immunization groups (Table I).

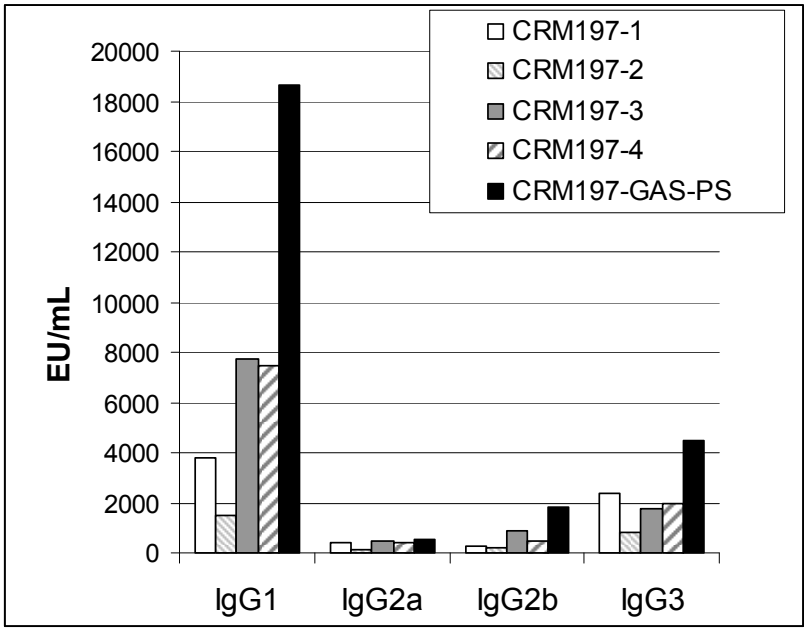

Figure 9. Distribution of the anti-GAS-PS IgG subclasses on the example of one immunization group.

Specificity of anti-GAS-PS polyclonal antibodies

In order to determine whether carbohydrate chains longer than hexasaccharides represent any additional epitopes for antibody recognition, sera from a group of mice immunized with CRM_{197}-**3** were pooled and depleted for anti-**1** antibodies by affinity chromatography. Loss of serum reactivity against **1** in ELISA assay verified the effectiveness of affinity resin. The depleted serum pool was tested further against an HSA conjugate of **3**. No response was observed against **3,** or it was less than 1% of primary anti-**3** specific IgG and was not detected due to limited sensitivity of the ELISA test. This suggests that although **3** represents an elongated structure and contains more repeating units than the hexasaccharide **1**, it did not elicit any antibodies specific for larger epitopes. The same procedure was repeated for the serum pool from a group of mice immunized with GAS-PS

glycoconjugate. No serum anti-GAS-PS reactivity was observed after depletion of antibodies specific for the hexasaccharide **1** indicating equal IgG specificity.

Protection against i.p. challenge with GAS

Mice immunized with the three doses of Alum-formulated glycoconjugates (10 μg) or strain-corresponding M protein (5 μg) were challenged three weeks after the last immunization with an appropriate LD90 dose of GAS strain. Mice survival was monitored up to 14 days after challenge, but a six day observation was optimal to obtain statistically significant protection data. Additionally, with the same purpose of the optimization of statistical comparison, protection data for different glycoconjugate lots were merged, so that the protection level was attributed to the type of carbohydrate antigen but not to the particular loading (Table II).

Immunization with strain-relevant M protein conferred over 80% protection. Mice immunized with conjugated carbohydrate antigens had significantly lower mortality than control mice immunized with unconjugated GAS-PS (P<0.001). CRM_{197} glycoconjugates conferred similar protection level against M1 strain challenge, ranging from 33% to 53% survival. Proof-of-concept of cross-protection properties was verified for the hexasaccharide **1**. CRM_{197}-**1** conferred 56% of protection against the M23 strain challenge, whereas CMR_{197}-GAS-PS rescued 34% of animals.

Formulated antigen	Mice number	**% of survivals**	P value
M1 serotype challenge			
Adjuvant alone	64	15	
GAS-PS	40	13	0,7784
M1 protein	64	91	<0,0001
CRM_{197}-GAS-PS	**64**	**47**	**0,0002**
CRM_{197}-1	**40**	**53**	**0,0001**
CRM_{197}-2	**48**	**42**	**0.0026**
CRM_{197}-3	**64**	**33**	**0.0381**
CRM_{197}-4	**40**	**53**	**0,0001**
M23 serotype challenge			
PBS	64	11	
M23 protein	32	84	<0.001
CRM_{197}-GAS-PS	**64**	**34**	**0,0027**
CRM_{197}-1	**16**	**56**	**0.0233**

Table II. Active immunization studies in mice after i.p. challenge with different M1 and M23 GAS serotypes. Three weeks after the final antigen dose, mice were infected with inoculum dose of 50 cfu/mouse for M23 strain and $2.5x10^6$ cfu/mouse for M1 strain. Survival level indicated corresponds to day 6 after the challenge.

Discussion

The presence of anti GAS-PS antibodies in human sera has been evidenced since 1965 (Schmidt, Moore 1965; Karakawa et al. 1965). Subsequently it was demonstrated that the level of human GAS-PS-specific antibodies peaks at the age of 17 in correspondence to the reduced incidence of GAS infection (Zimmerman et al. 1971; Paul 1957), and that these antibodies might play a

significant role in the protection from GAS infection (Salvadori et al. 1995). Sabharwal *et al.* (2005) showed that colonization of GAS in the human throats inversely correlated with the levels of anti-GAS-PS antibodies, and that mice immunized with a GAS-PS-TT conjugate were protected against GAS challenge. The above findings in conjunction with the high diversity of M+ GAS serotypes encountered in the endemic regions of developing countries, makes GAS-PS, which is conserved in most, if not all, strains of this pathogenic bacterium, an attractive molecule for the development of a vaccine against Group A Streptococcus infections. Some concerns about GlcNAc-containing vaccines have been voiced due to a possible role of anti-GAS-PS antibodies in the development of GAS infection sequela, like acute rheumatic fever or Sydenham's chorea (Malkiel et al. 2000; Kirvan et al. 2006; Shikhman et al. 1993). However the antibodies induced in mice by GAS-PS-TT conjugates did not react with several types of human tissues (Sabharwal et al. 2006).

In the present thesis I have described the synthesis, conjugation, antigenic and immunogenic properties of synthetic GAS core oligosaccharides. The study was designed to address several fundamental questions: i) can minimal GAS-PS core antigen determinants to elicit a protective antibody response? ii) what structural features influence the immunogenicity of synthetic antigens? iii) how do oligosaccharide-protein conjugates compare to isolated GAS-PS conjugates as far as immunogenicity and immunoprotection *in vivo* are concerned? Synthetic carbohydrate-based anti-GAS vaccine candidate was directly compared to the immunogenic and protective properties of a conjugate made with the native GAS-PS.

The synthetic approach for the production of carbohydrate antigens provided pure material of well-defined structure in contrast to isolated GAS-PS that varies in molecular weight and is contaminated with the poly-rhamnoses. Conjugation of the carbohydrates with a single reactive group per molecule allowed for efficient preparation of the glycoconjugates with one configuration and did not lead to the formation of high molecular weight aggregates with intermolecular linkages (Fig. 4-7). The composition of these glycoconjugates was detected by physico-chemical methods, and facilitated the development of standardized criteria for reliable prediction of their immunogenic properties.

CRM_{197} conjugates with the native GAS-PS and with four different synthetic GAS oligosaccharides varying in length and composition of the reducing end were prepared and characterized by physico-chemical methods (Fig. 4-7, Table I). CRM_{197} conjugates of both synthetic oligosaccharides and native GAS-PS elicited high levels of serum GAS-PS-specific IgG with a bias towards IgG1 production (Fig. 8, 9). Carbohydrate antigen formulation with Alum was suitable, as it favored a Th2 response that is preferred for a vaccination against extracellular pathogens (Bloom, Lambert 2003; Kalinski, Moser 2005; Shikhman et al. 1993). The synthetic oligosaccharides and GAS-PS were the same antigenically and induced antibodies with hexasaccharide paratope. Subsequently, presence of these antibodies were found to be a correlate of the protective immunity against GAS M1 serotype, one of the common agents of invasive and toxic streptococcal diseases (Courtney et al. 2009; Cunningham 2000; Klenk et al. 2007; Stanley et al. 1995). The conjugates made with synthetic GAS oligosaccharides were able to induce in mice a significant level of

protection against M1 strain challenge ranging from 33 to 53% which is comparable to the protection of 47% obtained by the conjugate with the native polysaccharide (Table II). Cross-protection paradigm was revealed with GAS M23 serotype challenge which resulted in significant protection level of 56% induced by the synthetic conjugate CRM_{197}-**1** as compared to the 34% induced by CRM_{197}-GAS-PS.

Trying to establish a few variables related to the immunogenicity of the synthetic GAS oligosaccharides conjugates, the attention was focused onto the saccharide chain length, the glycosylation degree and the non-reducing sugar. Our results show that a saccharide chain length of six, corresponding to the minimal size of the epitope, is sufficient to elicit a protective response characterized by antibodies which recognize the native polysaccharide. Concerning the glycosylation degree, a loading between 6 and 8 carbohydrate chains has been found optimal for the glycoconjugates with dodecasaccharide **3**, while higher loading of 12 chains led to a decrease in the immunogenicity (Table I). Pozsgay *et al.* (Pozsgay et al. 1999), studying the immunogenicity of glycoconjugates containing synthetic Shigella dysenteriae type 1 oligosaccharides of different length and at various loading, concluded that the saccharide length and the glycosylation degree do not play independent roles and that, for each chain length, the maximum antibody response is obtained with a given combination of the two variables. Accordingly, it may be supposed that the carbohydrate should not be overloaded onto the protein, because it can “shield” T/B-cells epitopes essential for efficient immunogenicity of the carrier, but still should be present in a sufficient amount to promote B-cell receptor

clustering. It is likely that the above observation is valid also in the case of the GAS synthetic conjugates presented in this study, and it might be a general rule. Therefore, in order to compare the immunogenicity of conjugates with different chain lengths it seems appropriate determining for each of them the best combination “chain length/loading”.

The non-reducing terminal sugar may also play a role in the immunogenicity of synthetic antigens since recent study on *Shigella dysenteriae* type 1 O-specific oligosaccharide and *Streptococcus pneumonia* type 14 capsular polysaccharide demonstrated that an increase in carbohydrate-specific antibody response was associated with the exposure of GlcNAc and galactose, respectively, on the nonreducing end of the oligosaccharide antigen (Safari et al. 2008; Pozsgay et al. 2007). GlcNAc residue on the non-reducing terminus contributed to the immunogenicity of the GAS-PS core epitope as evidenced by the relatively higher GMT induced by glycoantigens **1** and **3** (Table I).

In conclusion, it was possible to establish the conditions in which glycoconjugates with synthetic GAS-PS structures have equal or better immunogenicity than native GAS-PS. The glycoconjugate CRM_{197}-**3**/1 and CRM_{197}-**3**/2, exposing optimal number of dodecasaccharides containing GAS-PS antigenic core and immunodominant GlcNAc sugar on the non-reducing terminus, elicited in mice specific IgG titres of 10262 and 17780 EU/mL, respectively, as compared to 12082 EU/mL induced by CRM_{197}-GAS-PS, demonstrating that by means of rational design it is possible to design an optimized glycoantigen.

In conclusion this study has enlarged the previous knowledge on the immunogenicity and protective properties of GAS carbohydrate antigens (Michon et al. 2005; Reimer et al., 1992; Sabharwal et al. 2006) by demonstrating that synthetic oligosaccharides conjugated to CRM_{197} as protein carrier could elicit in animal model similar level of antibodies and protection to those induced by a conjugate with the purified GAS cell wall polysaccharide. The synthetic approach toward the development of a conjugate vaccine against Group A *Streptococcus* infection appears therefore a viable option which deserves further studies and optimization.

Conclusion

Vaccination represents an efficient, safe and cost-effective way for prevention and prophylaxis of devastating human diseases. Carbohydrate-based conjugate vaccines are included into the children immunizations schedule worldwide, and due to an active research in the field new vaccine candidates are being generated continuously (Hecht *et al.* 2009). A major contribution to such success was made by the detailed investigation of the structure of natural carbohydrates of pathogenic agents and progress in the development of conjugation methods. During the past decade, advances in the chemical synthesis of oligosaccharides have brought synthetic approach applied to the carbohydrate-based vaccines to a leading position in the field. The prior-art knowledge on the structure of protective carbohydrate epitopes of group A *Streptococcus* and human immunodeficiency virus and accessibility of the synthetic oligosaccharides containing the antigenic determinants of those epitopes provided basis for the present work.

Conventionally, carbohydrate antigens for vaccine development have been isolated from biological sources. Application of synthetic approach procures pure, homogeneous oligosaccharide antigens of homogenous structure, rationally planned to contain immunodominant saccharide composition and single-site incorporated reactive groups for covalent conjugation. Such oligosaccharides represent single molecular species and thereby being good starting compounds for the preparation of well-defined vaccines. A large set of physicochemical methods served to assess the conjugation outcome and

characterize the composition of obtained neoglycoconjugates. This analysis facilitated the development of standardized criteria for reliable prediction of their immunogenic properties. In the present work mentioned advantages of synthetic oligosaccharides and analytical tools have been applied to the research on the structural requirements of the protective carbohydrate epitopes of efficient group A *Streptococcus* and HIV glycoconjugate vaccines. The focus of the study was on the generation, characterization and immunogenic evaluation of the carbohydrate antigens of the mentioned pathogens.

Flexible polyamidoamine (PAMAM) scaffold have been exploited to generate four- and eight-valent sugar clusters of HIV-1-related oligomannose antigens Man_4, Man_6 and Man_9. Oligomannoses for the development of our glycoantigens were chosen on the basis of biochemical, biophysical and crystallographic evidences available in the literature (Calarese et al. 2003; Calarese et al. 2005; Pashov et al. 2005; Sanders et al. 2002; Wang et al. 2008). The multivalent presentation of oligomannoses aimed to mimic the native epitope of the mannose-specific broadly neutralizing antibody 2G12. Clusterization indeed increased the avidity of Man_4 and Man_9 to 2G12. The synthetic glycodendrons covalently coupled to the protein carrier CRM_{197} and formulated with the adjuvant MF59 were used to immunize two animal species. Oligomannose-specific IgG antibodies were generated, however, the antisera failed to recognize recombinant HIV-1 gp120 proteins. This study made contribution to the global knowledge on carbohydrate-based HIV-related antigens, structural requirements of the oligomannoses to be used for their construction and using of PAMAM scaffolds as suitable multivalent carriers. The result indicates that

further structural vaccinology work is needed to identify an antigen presentation that closely matches *in vivo* the structure of the epitope mapped by 2G12.

The study on GAS synthetic glycoconjugate vaccine demonstrates the value of synthetic oligosaccharides for carbohydrate conjugate vaccine development along with other published studies (Benaissa-Trouw et al. 2001; Chong et al. 1997; Jansen et al. 2001; Liu et al. 2006; Pozsgay et al. 1999; Pozsgay et al. 2007; Ragupathi et al. 2006; Robbins et al. 2009; Safari et al. 2008; Saksena et al. 2007; Schofield et al. 2002; Verez-Bencomo et al. 2004). A series of hexa- and dodecasaccharides were designed based on the GAS-PS structure and prepared by chemical synthesis. The synthetic oligosaccharide-CRM_{197} conjugates served to explore the impact of antigen length and the nature of the residue on the non-reducing end on immunogenicity. The synthetic oligosaccharides conjugated to CRM_{197} elicited a similar level of immune response when compared to isolated GAS-PS conjugated to CRM_{197} and conferred comparable levels of immunoprotection in an established challenge system in mice using two of the main circulating GAS strains. The obtained immunoprotection results lay the foundation for the future clinical evaluation of oligosaccharide conjugate vaccine candidates. The use of oligosaccharides representing the conserved GAS polysaccharide is a promising approach to address the highly diverse nature of M+ GAS serotypes encountered in the endemic regions of developing countries.

This thesis demonstrates that rational design of carbohydrate-conjugate vaccines is a potent tool and is beginning to impact vaccine development and regulatory processes.

Material and Methods

Chapter 1

Materials

Synthetic high-mannose oligosaccharides, equipped with an amino linker, were purchased from Ancora Farmaceuticals (MA, USA); their characterization data can be found in Online resource 3. $PAMAM_4$ and $PAMAM_8$ were kindly provided by professor G. Catelani (University of Pisa, Italy). CRM_{197} were internally produced in Novartis V&D, Siena, Italy. The 2G12 antibody, HIV proteins gp140 UG37 (clade A strain 92/UG/037, a.a. 32-662, NCBI protein database No. AAC97548, catalog no. ENV001) was purchased from Polymun Scientific (Vienna, Austria). HIV gp120 Bal (a.a.32-518, GenBank No. M68893, catalog no. IT-001-002p), gp120 R2 (a.a.41-520, GenBank No. AF128126, catalog no. IT-001-0029p), and gp120 JRFL (a.a.34-518, GenBank No. U63632, catalog no. IT-001-0024p) were purchased from Immune Technology Corp. (New York, US). Polymun and Immune Technology recombinant proteins are expressed in CHO and 293T cells, correspondingly. Biotinylated Galantus Nivalis Lectin (GNL) was purchased from Vector Laboratories (CA, US).

Analytical methods

Total saccharide concentratio*n* was determined by HPAEC-PAD analysis (ICS-3000 Dionex system). Briefly, oligomannose carbohydrate preparation was hydrolyzed in 2 M trifluoracetic acid for 2h at 100°C, dried and then dissolved in water. 20 µL of sample were injected into CarboPac PA1 analytical column (250 mm x 4 mm i.d., Dionex) with CarboPac PA1 guard column (50 mm x 4 mm i.d.,

Dionex). Isocratic separations were performed using a 30-min 16 mM NaOH followed by a 5-min 500 mM NaOH regeneration step and 15-min re-equilibration, set to a flow rate of 1.0 mL min^{-1}. Monosaccharide peaks were detected directly by using quadruple-potential waveform pulsed amperometry on a gold working electrode and an Ag/AgCl reference electrode. Raw data were elaborated on Chromeleon 6.8 chromatography software (Dionex) with application of 0.5-10 µg/mL mannose calibration curve. Rapid hexose quantification was performed by Phenol-H_2SO_4 method (Scott RW *et al.* 1967). Protein concentration was determined by Micro BCA kit (Thermo Fisher Scientific).

ESI Q-TOF MS analyses

Analyses by direct sample injection were performed in a Micromass Q-Tof Micro system (Waters MS Technologies, UK) diluting the samples 1:200 (v/v) or less in 0.1% formic acid, 1:1 (v/v) acetonitrile:water. For LC-Mass analyses the Q-tof Micro system was coupled to an UPLC system (ACQUITY UPLC System, Waters, UK). Chromatographic separations of samples diluted in water were performed on 2.1 mm i.d. x 50 mm ACQUITY BEH C18 1.7 µm column (Waters Corp., USA). Elution was performed with a linear gradient of 2-50% B for 8 min, then 50%-100% B for 1.5 min, reconditioned 2% B for 2 min each cycle, where A = water with 0.1% formic acid and B = acetonitrile with 0,1% formic acid. Each cycle duration was 13 min at a flow rate 0.4 mL/min. 10 µL aliquots of sample were loaded. HPLC peak detection was performed by total ion current and best peak intensity measurement.

TOF MS analysis was performed operating in positive ion mode (ESI). The

nebulization gas was set to 800 L/h at a temperature of 250°C, the cone gas set to 50 L/h and the source temperature set to 100°C. The capillary and cone voltages were 3500 V and 30 V, respectively. The Q-Tof Micro was operated with collision energy of 5 V. The data acquisition rate was set to 0.1 s with a 0.1 s inter-scan delay. The raw data were analyzed by the Micromass MassLynx applications manager Version 1.0, using Maxent3 for deconvolution (Waters, UK). The general strategy for assigning peaks to glycodendrons involved: 1) identification of a pair or series of ions in the spectra separated by the mass of a mannose saccharide (162 Da); 2) assigning individual peaks of these distributions.

PAMAM cluster synthesis and purification

In a typical experiment Man_4, Man_6 or Man_9 synthetic oligosaccharide with a six-carbon amino linker at the reducing end (20 μmol) were treated with disuccinimidyl adipate (200 μmol) in 0.3 mL DMSO containing 43 μmol of triethylamine. After 2 hours of vigorous stirring the activation of sugar was checked by TLC performed on aluminium plates coated with silica gel 60 Å F_{254} (Merck) with detection by charring with 10% ethanolic H_2SO_4. The activated oligosaccharide was purified by precipitation in 9 volumes of ethylacetate; the pellet obtained by centrifugation was washed two times with 1 mL of ethylacetate and vacuum dried.

The succinimidyl-activated oligosaccharides were then coupled to $PAMAM_4$ and $PAMAM_8$ with stoichiometry of 8:1 and 20:1 mol/mol, respectively. The reaction was carried out in 0.1 mL DMSO containing 20 μL/mL triethylamine at RT.

Cluster formation was monitored in process by HPLC-ESI MS analysis and in some cases a further addition of activated oligosaccharide was performed in order to maximize the formation of the desired cluster. The reaction mixture was then lyophilized and dissolved in water. The excess of unreacted oligosaccharide was removed by hydrophobic interaction on a C4 column (0.5 mL resin, Bioselect, Grace Vydac) activated with methanol and preconditioned with water and eluted with a stepwise gradient of methanol (0-80% in water). Fractions of 2 mL were analyzed by TLC and ESI Q-TOF MS; and those containing fully-substituted PAMAM were dried to remove methanol. The yields for cluster formation and purification varied from 46-77% for $PAMAM_4$-based dendrons and 28-30% for $PAMAM_8$-based dendrons

$PAMAM_4$-Boc ESI MS *m/z* ($C_{37}H_{76}N_{14}O_8$): found 845.56 $((M+H)^+$, calc. 845.60), 423.27 $((M+2H)^{2+}$, calc. 423.31). *$PAMAM_8$-Boc* ESI MS *m/z* ($C_{37}H_{76}N_{14}O_8$): found 879.70 $((M+2H)^{2+}$, calc. 879.62), deconv. 1758.37 $((M+H)^+$, calc. 1758.24). *Man_4PAMAM_4-Boc* ESI MS *m/z* ($C_{181}H_{320}N_{18}O_{100}$): found 1450.48 $((M+3H)^{3+}$, calc. 1450.54), 1458.80 $((M+3H+Na)^{3+}$, calc. 1457.87), 1396.45 $((M+3H-Man)^{3+}$,calc. 1396.49), deconv. 4347.36 $((M+H)^+$, calc. 4347.06), 4369.24 $((M+Na)^+$, calc. 4369.04), 4185.32 $((M+H-Man)^+$, calc. 4185.01). *Man_9PAMAM_4-Boc* ESI MS *m/z* ($C_{301}H_{520}N_{18}O_{200}$): found 1898.62 $((M+4H)^{4+}$, calc. 1898.87), 1904.21 $((M+3H+Na)^{4+}$, calc. 1904.36), 1858.09 $((M+4H-Man)^{4+}$,calc. 1858.33), deconv. 7587.53 $((M+H)^+$, calc. 7588.12), 7610.38 $((M+Na)^+$, calc. 7610.10), 7425.80 $((M+H-Man)^+$, calc. 7426.06). *Man_4PAMAM_8-Boc* HPLC t_R=2.728 min; ESI MS *m/z* ($C_{365}H_{644}N_{38}O_{200}$): found 1754.03 $((M+5H)^{5+}$, calc. 1754.06), 1721.46 $((M+5H-Man)^{5+}$, calc. 1721.63), 1689.18

((M+5H-2 Man)$^{5+}$, calc. 1689.20), 2192.33 ((M+4H)$^{4+}$, calc. 2192.32), 2152.64 ((M+4H-Man)$^{4+}$, calc. 2151.79), 2111.22 ((M+4H-2Man)$^{4+}$, calc. 2111.25), deconv. 8761.17 ((M+H)$^{+}$, calc. 8761.15), 8599.08 ((M+H-Man)$^{+}$, calc. 8599.09), 8437.02 ((M+H-2 Man)$^{+}$, calc. 8437.04). *Man_9PAMAM_8-Boc* HPLC t_R=2.172 min; ESI MS *m/z* ($C_{605}H_{1044}N_{38}O_{400}$): found 2179.85 ((M+7H)$^{7+}$, calc. 2179.72), 2156.65 ((M+7H-Man)$^{7+}$, calc. 2156.55), 2133.57 ((M+7H-2 Man)$^{7+}$, calc. 2133.39), 2542.94 ((M+6H)$^{6+}$, calc. 2542.83), 2515.60 ((M+6H-Man)$^{6+}$, calc. 2515.81), 2488.67 ((M+6H-2Man)$^{6+}$, calc. 2488.79), deconv. 15240.77 ((M+H)$^{+}$, calc. 15243.26), 15079.57 ((M+H-Man)$^{+}$, calc. 15081.21), 14918.34 ((M+H-2 Man)$^{+}$, calc. 14919.15).

Competitive Surface Plasmon resonance

The experiments were carried out with a BiaCore X100 system in a HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 0,005% surfactant Tween 20, pH 7.4). For coupling two flow cells of a CM5 chip (GE Healthcare) were activated by injection of EDC/NHS mixture for 7 min at 10 µL/min, followed by injection of 10 µg/mL gp140 UG37 in sodium acetate pH 4.5 over the channel two until the target level was reached; both were then blocked with 1.0 M ethanolamine pH 8.5 for 7 min at 10 µL/min. Final immobilization level of gp140 was 6800 RU. 2G12 solution with and without carbohydrate inhibitors was injected over both channels, and the binding profile was obtained by subtraction of the blank signal in channel one from the gp140 UG37 signal in channel two. 2 µg/mL 2G12 was incubated with 0-1200 µM carbohydrate inhibitor for 15 minutes at 37°C before the analysis. Analyte was injected at 10 µL/min for 4 min, followed by 6 min dissociation and 30 sec of regeneration with 10 mM glycine, 3M NaCl pH 2.0.

Sensorgrams were elaborated on the Biacore X100 software package. Inhibition percentage was calculated as ($S_{no\ inhibitor} - S_{inhibitor}$)*100/$S_{no\ inhibitor}$, where S is the stability level with reference subtraction (buffer sample). The stability level corresponds to the RU signal at 270 sec and was chosen for the calculation as less influenced by the analyte bulk effect in comparison to the binding level (measured 240 sec) [46]. Inhibition curve was fitted on the Graphpad Prism software using variable slope model (Graphpad Prism Inc.). Two data points, (x_1, y_1) and (x_2, y_2), adjacent to the 50% inhibition titer were chosen, where x is inhibitor concentration and y is corresponding inhibition level. IC50 value was calculated according to the equation $IC50=10^{\frac{\log x_1-(y_1-50)\cdot\log(x_1-x_2)}{y_1-y_2}}$.

Conjugation of oligomannose and oligomannose glycodendrons to CRM_{197}

t-Boc protecting groups in glycodendrons were cleaved by reaction in 20% trifluoracetic acid (TFA) for two hours at RT. The removal of t-Boc was verified by MS analysis, and the samples were extensively dried under vacuum to remove TFA. Monovalent oligosaccharides or deprotected glycodendrons were then activated with disuccinimidyl adipate and purified according to the procedure reported above. The activated oligosaccharides were then conjugated in 200 mM sodium phosphate pH 7.2 to CRM_{197} (10-20 mg/mL) with a stoichiometry of 30:1 or 40:1 glycodendron:protein (mol/mol). After overnight incubation at 37°C, conjugates were then purified from the excess of unconjugated carbohydrate using ultrafiltration spin columns with 30 kDa or 50 kDa cut-off (Vivaspin, Sartorius). The purified glycoconjugates were analyzed for their protein and carbohydrate content and by SDS-PAGE.

Conjugation of Man_9 to HSA via diethyl squarate chemistry

The synthetic oligosaccharide (20 mmol) was treated with 3,4-diethoxy-3-cyclobuten-1,2-dione (150 mmol) in 0.1 mL 1:1 vol ethanol:100 mM sodium phosphate pH 7.0. After overnight incubation with vigorous stirring activation of sugar was checked by TLC. The excess of linker was removed by hydrophobic interaction C18 column (C18-E, Strata, Phenomenex) after 3 CV water and 3 CV ethylacetate washing steps with final methanol elution. Target fractions were dried to remove methanol. The activated oligosaccharides were conjugated in 200 mM sodium borate pH 9.2 to CRM_{197} (10-20 mg/mL) with a stoichiometry of 30:1 (mol/mol). Purification and characterization was performed as described above. This glycoconjugate has been used in ELISA as coating reagent for anti Man9 antibodies determination.

Animal immunizations

Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in the conduct of all animal studies. Groups of 2-4 female white Zealand rabbits (2 kg weight) were immunized on days 1, 21 and 35 with 5 or 20 µg of carbohydrate antigens or with PBS both formulated 1:1 (v/v) with MF59 and delivered in a final volume of 250 µL , intramuscularly into both quadriceps. Sera were collected on days 20, 34 and 42.

Groups of 8 female Balb/c mice were immunized on days 1, 14 and 28 with 1 µg of carbohydrate antigens or PBS both formulated with MF59 and delivered in a volume of 150 µL by subcutaneous injection. Sera were collected on day 0, 27 and 42.

ELISA

a) *Determination of anti Man_9-specific antibodies.* 96-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with 100 µL/well of a 1 µg/mL solution of Man_9-squarate-HSA in PBS. Plates were incubated overnight at +4°C, then washed three times with TPBS (PBS with 0,05% Tween 20, pH 7.4) and blocked with 100 µL/well of 2% BSA (Sigma-Aldrich) for 1 hour at 37°C. Subsequently each incubation step was followed by triple TPBS wash. Sera, prediluted 1:25-1:1000 in 2 % BSA-TPBS, were transferred into coated- plates (200 µL) and then serially two-fold diluted followed by 2h incubation at 37°C. Then 100 µL/well of 1:10000-1:20000 diluted appropriate alkaline phosphatase-conjugated secondary antibody (Sigma Aldrich) were added and plates incubated for 1h at 37°C. Subsequently 100 µL/well of 1 mg/mL pNPP disodium hexahydrate (Sigma Aldrich) in 1M diethanolamine (pH 9.8) was distributed onto plates. After 30 minutes of development at RT plates were read at 405 nm with a microplate spectrophotometer. Antibody titres were defined as the reciprocal of those dilutions that gave an optical density (OD) higher than three times the average OD of preimmune or mock-immunized sera.

b) Evaluation of anti HIV-1 gp120 and anti-CRM specific antibodies.

In order to evaluate the ability of rabbit or mouse immune sera to recognize HIV-1 gp120 glycoproteins or CRM_{197}, the ELISA protocol described above has been repeated but using as coating 100 ng/well of three different HIV-1 clades Bal, JRLF and R2, and CRM_{197}.

Chapter 2

Materials

Synthetic GAS oligosaccharides, equipped with an amino linker, were purchased from Ancora Pharmaceuticals (Medford, USA). CRM_{197} was internally produced in Novartis V&D, Siena, Italy.

Bacterial strains and culture conditions

Streptococcal strain GAS SF370 M1 was obtained from University of Siena, Italy. Streptococcal strain M1 3348 was obtained from Istituto Superiore di Sanità, Rome, Italy. GAS M23 2071 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures). Bacterial strains were stored at −80°C and routinely grown in Todd-Hewitt broth (Difco) at 37°C until mid-log phase.

Analytical methods

Total saccharide concentration was determined by HPAEC-PAD analyses (ICS-3000 Dionex system). Briefly, the GAS-PS preparation was hydrolyzed in 4 M trifluoracetic acid for 2 h at 100°C, dried and dissolved in water. 20 µL samples were injected into CarboPac PA1 analytical column (250 mm x 4 mm i.d., Dionex) with CarboPac PA1 guard column (50 mm x 4 mm i.d., Dionex). Isocratic separations were performed using a 15-min 50 mM NaOH followed by a 5-min 500 mM NaOH regeneration step and 10-min re-equilibration, set to a flow rate of 1.0 mL min^{-1}. Monosaccharide peaks were detected directly by using quadruple-potential waveform pulsed amperometry on a gold electrode and an Ag/AgCl reference electrode. Raw data were elaborated on a Chromeleon 6.8 chromatography software (Dionex) with application of 0.5-10

µg/mL GlcNAc calibration curve. Rapid hexose quantification was achieved by Phenol-H_2SO_4 method (Scott RW et al. 1967). Unconjugated saccharide concentration was analyzed by passing 1 mL of purified neoglycoconjugate preparation at 10 µg/mL saccharide concentration through C4 hydrophobic interaction column (0.5 mL resin, Bioselect, Grace Vydac). Unconjugated carbohydrate was eluted with 1 mL of 20% acetonitrile in water and subsequently estimated by HPAEC-PAD analysis. Protein concentration was determined by MicroBCA and Bradford protein assay kit (Thermo Fisher Scientific). Hyaluronic acid concentration was determined using test kit (Corgenics). DNA concentration was determined by spectrophotometric OD method (Haque et al. 2003). Size-exclusion HPLC for glycoconjugate profiling was done with TSK Gel G4000SW column on Ultimate-3000 HPLC system (Waters). All the samples were eluted at 0.5 mL/min flow with a mobile phase buffer containing 0.1 M sodium phosphate, 0.1 M sodium sulfate, 5% acetonitrile, pH 7.2.

Spectroscopy and chromatography

NMR analyses: Lyophilized CHO samples were dissolved in deuterium oxide (D_2O, 99.9% atom D, Aldrich) to produce a uniform solution. ^1H NMR experiments were recorded at 25°C on a Bruker Avance™ 600 MHz spectrometer, using a 5-mm broadband probe (Bruker). The XWINNMR™ software package (Bruker) was used for data acquisition and processing. 32k data points were collected over a 10 ppm spectral width for the proton spectra. The transmitter was set at the HDO frequency, which was also used as reference signal (4.79 ppm). ^1D proton NMR spectra were collected using a

standard one-pulse experiment. *Determination of dn/dc and SEC-MALLS analysis:* SEC-MALLS analysis was performed using a TSK-Gel® G2000SWXL column (Tosoh Bioscience) coupled with an Alliance 2695 solvent delivery module (Waters, Millipore). The polysaccharide sample was injected at the concentration of 11.6 mg/mL in 10 mM NaPi pH 7.0 and eluted at the flow rate of 0.5 mL/min. Scattered light intensities were measured using a Dawn EOS multi-angle light scattering photometer (Wyatt Technology Corp.). Data were collected and processed using the software ASTRA™ (Wyatt technology Corp.). The absolute molecular mass was determined according to the equation $R(\theta) = K^*McP(\theta)[1-2A2McP(\theta)]$, where $R(\theta)$ is the excess Rayleigh ratio, K* the polymer constant for a particular scattering system, M the molecular mass, c the solute concentration (g/mL), $P(\theta)$ the form factor related to the mean square radius r_g of the particle and A2 is the second virial coefficient, a measure of solute-solvent interaction, which to a first approximation can be taken as zero (Tanford 1961). dn/dc value of GAS polysaccharide was measured using an interferometric refractometer Optilab DSP (Wyatt Technology Corp.) pre-calibrated with NaCl samples. A series of six different polysaccharide samples with concentration ranging from 0.039 to 1.158 mg/mL in 10 mM NaPi pH 7.0 was injected through the refractometer, starting with the lowest concentration. The dn/dc value was calculated using the software DNDC™ (Wyatt Technology Corp.). *MALDI-TOF* mass spectra of CRM_{197} and glycoconjugates were recorded by an UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics) in linear mode with positive ion detection. All the samples for analysis were

prepared by mixing 1 µL product and 1 µL of sinnapinic acid matrix in 0.1% TFA and 30% acetonitrile. 1 µL of each mixture has been deposited on samples plate, dried at room temperature for 10 min and subjected to the spectrometer.

Isolation of native GAS-PS

GAS-PS was released from bacterial culture by reductive acidic treatment as previously described (Sabharwal et al. 2006) and purified by several steps of ultrafiltration and chromatography. Briefly, acid-treated cell pellet suspension was clarified by orthogonal filtration using 0.65 µm pore glass fiber filter (Sartopure GF2 capsule, Sartorius). Then permeate was diafiltrated with 30K cut-off membrane (Hydrosart, Sartorius) against 1M NaCl and water. Obtained material was additionally purified by anion exchange chromatography (Q-Sepharose FF resin, AKTA systems, GE Healthcare). PS-containing fractions were concentrated by TFF using 5K membrane (Sartorius).

Preparation and characterization of glycoconjugates

In a typical experiment synthetic oligosaccharide (1 µmol) was treated with disuccinimidyl adipate (10 µmol) in 1.5 mL DMSO containing 0.01 mL triethylamine. After 2 h of vigorous stirring, chromatography of the crude mixture in silica gel (0.035-0.70 mm, 60 Å, Sigma-Aldrich) in a gradient of 0-70% methanol in ethylacetate yielded activated oligosaccharide. Fractions were analyzed by TLC (ethylacetate-methanol 1:1) with detection by charring with 10% ethanolic H_2SO_4 and ninhydrin (1.5 mg/mL in 38:1.75:0.25 1-BuOH/H_2O/HOAc). Fractions containing pure activated CHO were merged with subsequent determination of active ester groups (yield 13-39%) (Pitner et al. 2000). Alternative method of the activated oligosaccharide purification consisted

in precipitation of the reaction mixture in 9 volumes of ethylacetate; the pellet obtained by centrifugation was washed two times with 1 mL of ethylacetate and vacuum dried (yield 42-51%). A conjugation stoichiometry of 30:1 active ester group mol/ protein mol was applied. A solution of CRM_{197} in 100 mM NaPi buffer pH 7.0 (10-20 mg/mL) was added to the dried activated oligosaccharide. The mixture was incubated ON at RT, mixing very gently with a magnetic stirrer. Native GAS-PS was conjugated to lysine residues of CRM_{197} by reductive amination in the presence of $NaBH_3CN$ (Jennings, Lugowski 1981). The reaction was carried out in 200 mM NaPi pH 8.0 with stoichiometry 4:2:1 (wt:wt:wt) of CHO:$NaBCNH_3$:protein. The solution was 0.22 µm filtered and kept at 37°C for 2 days. Conjugates were purified from excess of unconjugated carbohydrate using ultrafiltration spin columns with 30K or 100K cut-off (Vivaspin, Sartorius).

Active immunizations

Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in the conduct of all animal studies. Groups of 8-16 female CD-1 mice (5-6 week old) were immunized on days 1, 21 and 35 with 10 µg of carbohydrate antigen (100 µL final volume). Adjuvant alone or non conjugated polysaccharide (10 µg) were used for negative control groups, while strain-specific M protein (10 µg) was used for positive control groups. Antigens were formulated with aluminium hydroxide before injection. Sera samples were collected before the first immunization and two weeks after the third immunization (day 49).

Serum ELISA

Titration of carbohydrate-specific antibodies was performed on individual sera 2

weeks after the last immunization. For that purpose 96-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with 100 µL/well of PBS-diluted 1 µg/mL HSA conjugates of GAS-PS. Plates were incubated for 3 hours at room temperature, then washed three times with TPBS (PBS with 0.05% Tween 20, pH 7.4) and blocked with 100 µL/well of 2% BSA (Sigma-Aldrich) for 1 h at 37°C. Subsequently each incubation step was followed by triple TPBS wash. Serum samples were initially diluted 1:500-1:1000 in 2 % BSA in TPBS, transferred into coated-blocked plates (200 µL) and serially two-fold diluted followed by 2 h incubation at 37°C. Then 100 µL/well of 1:2000-1:5000 diluted alkaline phosphatase-conjugated goat anti-mouse IgM (µ-chain specific, Sigma Aldrich), IgG or given IgG subclass (whole molecule, Sigma Aldrich) antibody were added and left for 2 h at 30°C. 100 µL/well of 3 mg/mL pNPP disodium hexahydrate (Sigma Aldrich) in 1M diethanolamine (pH 9.8) was distributed onto plates to visualize the amount of bound alkaline phosphatase. After 10 minutes of development at RT plates were read with a microplate spectrophotometer at 405 nm. Antibody titres are those dilutions that gave an optical density (OD) higher than the mean plus five times the standard deviation of the average OD obtained in the preimmune sera. The titres were normalized with respect to the reference serum and expressed in ELISA units (EU) per mL. Reference serum was obtained from GAS CHO glycoconjugate immunized mice. The thiocyanate elution ELISA for affinity studies is described elsewhere (McCloskey et al. 1997). Absorbance value of sera without NaSCN was at least 1.0.

Immunoadsorption of anti-hexasaccharide antibodies from mice sera

HSA conjugate (100 µg) of oligosaccharide **1** was coupled to 200 µL NHS-

activated sepharose resin (GE Healthcare) following the manufacturer instructions. Excess of active groups on the resin was blocked with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3. Operations with small amount of resin were performed in clarification spin column (Vivapure, Sartorius) with 0.22 µm membrane filter. Resin was equilibrated in PBS, incubated with 1:20 PBS-diluted mice sera for 10 min and then spun. The flow through was cycled five times through the column. Between cycles resin was regenerated by 100 mM glycine, pH 2.7 and re-equilibrated with PBS. 1:100 diluted sera from flow-through fraction was tested for its reactivity against HSA conjugates of **1**, **3** and GAS-PS in ELISA assay. Reference anti-CRM197 titration was performed to allow the recalculation of antibody titers due to unspecific absorption.

In vivo protection assays

Immunized animals were intraperitoneally (i.p.) challenged on day 56, three weeks after the last immunization with a bacterial dose ranging from 50 (for M23 2071 strain) to $2.5x10^6$ (for M1 3348 strain) colony forming units (cfu) per mouse (lethal dose 90, LD90). Animals were monitored on a daily basis and euthanized when they exhibit defined humane endpoints that were pre-established for the study in agreement with Novartis Animal Welfare Policies. Bacterial cultures for infection experiments were grown in Todd-Hewitt broth until mid-log phase, washed twice, appropriately diluted in fresh medium; and 200 µL were administered to each mouse by i.p. injection. Samples of the inoculum were plated on Todd-Hewitt broth plates (Difco) supplemented with 0.5% yeast extract and 0.5% sheep blood to verify the infectious dose. The plates were incubated at 37°C overnight, and the number of colonies was counted next day.

Statistics

Unpaired t-test was applied to natural logarithm values of serum ELISA titres to determine the differences between GMT of the immunization groups. Fisher's exact test was used to determine the differences in protection level. Comparison was performed between negative control group (adjuvant only) and immunization group of interest.

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