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Investigating the immunodominance of carbohydrate antigens in a bivalent unimolecular glycoconjugate vaccine against serogroup A and C meningococcal disease

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Abstract Multicomponent constructs, obtained by coupling different glycans to the carrier protein, have been proposed as a way to co-deliver multiple surface carbohydrates targeting different strains of one pathogen and reduce the number of biomolecules in the formulation of multivalent vaccines. To assess the feasibility of this approach for anti-microbial vaccines and investigate the potential immunodominance of one carbohydrate antigen over the others in these constructs, we designed a bivalent unimolecular vaccine against serogroup A (MenA) and C (MenC) meningococci, with the two different oligomers conjugated to same molecule of carrier protein (CRM_{197}). The immune response elicited in mice by the bivalent MenAC construct was compared with the ones induced by the monovalent MenA and MenC vaccines and their combinations. After the second dose, the bivalent construct induced good levels of anti-MenA and anti-MenC antibodies with respect to the controls. However, the murine sera from the MenAC construct exhibited good anti-MenC bactericidal activity, and very low anti-MenA functionality when compared to the monovalent controls. This result was explained with the diverse relative avidities against MenA and MenC polysaccharides, which were measured in the generated sera. The immunodominant effect of the MenC antigen was fully overcome following the third immunization, when sera endowed with higher avidity and excellent bactericidal activity against both MenA and MenC expressing strains were elicited. Construction of multicomponent glycoconjugate vaccines against microbial pathogens is a feasible approach, but particular attention should be devoted to study and overcome possible occurrence of immune interference among the carbohydrates.

Keywords Glycoconjugates · Immunodominance · CRM_{197} · Meningococcoccal vaccines · Multicomponent vaccines

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Introduction

Glycoconjugate vaccines have been demonstrated a very efficacious instrument to prevent many infectious diseases from encapsulated pathogens like *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* [1, 2]. To ensure protection against the different carbohydrate structures expressed by some bacteria, different polysaccharides need to be included in the formulations of these vaccines. Based on the diverse polysaccharide structure, over 90 different pneumococcal serogroups have been identified [3], while 13 serogroup polysaccharides are associated with meningococci (although only five serogroups of meningococcal polysaccharide - A, B, C, Y and W135, and the emerging serotype X, commonly cause disease) [4]. The need of wider serotype coverage is leading to formulation of glycoconjugate vaccines including a growing number of polysaccharides and, therefore, increasingly complex and with an increased amount of associated carrier protein. For example a 13-valent anti-pneumococcal vaccine is currently available, while a 15-valent is under clinical development [5]. Five serogroups, A, B, C,

W-135 and Y, cause the majority of meningococcal meningitis, and four of them (A, C, W-135 and Y) have been targeted to develop monovalent or multivalent carbohydrate-based vaccines [6]. In theory the complexity of a multivalent glycoconjugate vaccine could be decreased applying the concept of multicomponent unimolecular constructs, in which different carbohydrate antigens are linked to the same carrier molecule.

An early report highlighted the feasibility of this strategy to obtain an immune response against two different microbial polysaccharides [7]. In this case oligosaccharide haptens derived from *S. pneumoniae* type 6A and *N. meningitidis* group C CPSs covalently linked through active ester intermediates to CRM_{197} were shown to induce specific and functional anti-carbohydrate IgG antibodies. However, the immunological responses were compared to the unconjugated oligosaccharide, but not to the corresponding monovalent glycoconjugates [7]. Recently, a strategy based on tailored conjugation via squarate chemistry has been used to bind to Tetanus Toxoid (TT) synthetic hexaglycosyl antigens from the two LPSs of *Vibrio cholerae* serogroup Inaba and Ogawa or, alternatively, a glycan antigen and a carbohydrate-based immunomodulator to obtain a multicomponent vaccine candidate against this microbial pathogen [8]. The immunological evaluation of such multicomponent conjugates was not detailed. In another example, the β-glucan laminarin covalently linked to TT in conjunction with a synthetic β-mannan disaccharide, has been described to function as ligand for the Dectin-1 receptor, thereby increasing the immunological response against the β-mannan antigen [9].

Multicomponent unimolecular constructs, including different tumor associated carbohydrates (TACAs) along a peptide backbone, which was coupled to a single carrier protein providing T cell help, have been proposed for anti-cancer therapy [10–12]. TACAs are usually mono- or short length oligosaccharides that are poorly immunogenic even after conjugation to the carrier protein own to their character of self-antigens. Therefore multicomponent unimolecular constructs were designed for the co-delivery to antigen presenting cells, which are responsible for the initial processing of glycoconjugates [1] of multiple glycans from one particular cancer type and/or adjuvants in order to potentiate their immune responses.

We envisaged that the conjugation onto the carrier protein of two or more carbohydrate antigens targeting different strains of the same pathogens would be a possible strategy to co-deliver simultaneously the glycans to the antigen presenting cells. This would reduce the number of biomolecules composing the formulation of a multivalent vaccine.

However, it is known that when the immune system is exposed to complex microorganisms or antigens, the cellular immunity focuses toward one or just a few antigenic determinants, which effect the immune response [13, 14]. This phenomenon, which is known as immunodominance, could potentially impact the immunogenicity of multicomponent constructs.

Therefore, we became interested in assessing the feasibility of conjugation onto the carrier protein of two structurally different medium length oligosaccharide haptens targeting diverse strains/serogroups, and understanding if this would result in the immunodominance of any of the two antigens.

In order to ascertain the efficacy of multicomponent constructs we focused our attention on the two relevant polysaccharides from *N. meningitidis* serogroup A and C (MenA and MenC, respectively), which are the components of well-established carbohydrate-based vaccines [6]. *N. meningitidis* is the major cause of meningococcal meningitis world-wide, with a particularly high incidence of epidemics in children [15]. Among the different serogroups, A accounts for a high incidence in the regions of Africa extending from very high Senegal to Ethiopia, that are known as the'African Meningitis Belt' [16, 17]. Meningococcal infections due to serogroup C have been described with particularly high incidence in Australia [18]. This serogroup causes one third of meningococcal cases reported with US, with a significant high case fatality ratio (14.6 %) [19]. Outbreaks of meningococcal disease related to serogroup C have been identified also in Brazil [20].

Different tetravalent A, C, W-135 and Y conjugate vaccines are now available to prevent this terrible disease [21, 22], and a monovalent polysaccharide-tetanus toxoid conjugate vaccine against serogroup A meningococcal disease, specifically designed for Africa, has been recently licensed [23].

In this paper we describe the preparation of a bivalent unimolecular glycoconjugate vaccine against meningococcal serogroup A and C by conjugation to the carrier protein of MenA and MenC oligosaccharide haptens through active ester and thiol-maleimide coupling chemistry, respectively (Fig. 1). Following immunological evaluation in mice, we examined the proficiency of the synthesized glycoconjugate in inducing bactericidal antibodies against the co-delivered MenA and MenC polysaccharides as compared to the monovalent conjugates and to their physical mixtures.

Materials and methods

*Preparation of MenA and MenC oligomers **5** and **4** for conjugation with CRMemcs **6**.* End terminal aminated MenA oligomer with avDP 15 and MenC oligomer avDP 20 were prepared as reported in literature [24, 25]. The aminated oligosaccharides were dried under vacuum, dissolved in 1:9 H_2O:DMSO at $[NH_2]$=40 μmol/ml, and then reacted with 12 equiv. of di-*N*-hydroxysuccinimidyl adipate (sidea) and 5 equiv. of triethylamine as compared with primary amino groups. The reaction mixture was kept at room temperature for 2 h with gentle stirring. The activated oligosaccharides were separated from the reagents by precipitation with 1:4 of

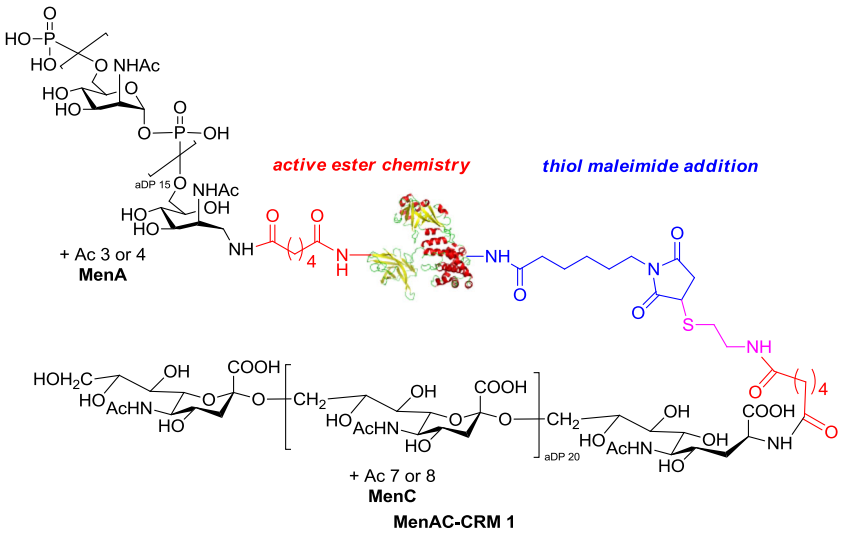


Fig. 1 Chemical structure of the bivalent unimolecular MenAC-CRM_{197} **1** vaccine

acetone, followed by washing of the precipitate with 1:4 acetone, and drying under vacuum. Content of *N*-hydroxysuccinimide ester groups (ae) introduced in the activated MenA and MenC oligosaccharides **5** and **2**, respectively, was determined by colorimetric assay [24].

$MenC_{cystamine}$ oligomers **3** were obtained by reaction of $MenC_{sidea}$ oligosaccharide **2** with cystamine (10 equiv.) at 20 mg/ml concentration in 1:9 H_2O:DMSO (5 ml) containing 10 % v/v of triethylamine. After stirring for 3 h at room temperature, the oligosaccharide was precipitated by addition of 9 volumes of ethylacetate. The solid was purified on a G15 Sephadex column (GE Healthcare), eluting with 20 mM NaCl. Fractions containing the oligomers were combined and concentrated to a volume of 20 mg/ml in 10 mM sodium phosphate (NaPi) pH 7.2, based on sialic acid quantification. To release the free thiol groups, the mixture was treated with 3 equiv of 0.5 M TCEP and stirred for 3 h. After purification on a G15 Sephadex column (GE Healthcare), the thiol groups of MenC-SH **4** were estimated before conjugation.

Preparation of CRMemcs ***6***. In a typical experiment, 1.52 mg of EMCS linker (Pierce, Thermo) were dissolved in 50 µl of DMSO, and 44 µl of the prepared mixture (10 equiv.) were added to a solution of CRM_{197} (obtained from Novartis Vaccines Manufacturing) (1 ml of 32.5 mg/ml stock solution) in 400 µl of 100 mM NaPi, 1 mM EDTA pH 8.1. The solution was stirred for 3 h, and then washed in a 30 kDa Vivaspin filter (Sartorius), dialyzing against 10 mM NaPi, 1 mM EDTA pH 7.2. The protein was reconstituted with 10 mM NaPi, 1 mM EDTA and the protein content was quantified. The linker/protein molar ratio was determined by MALDI-TOF mass spectrometry analysis run in an UltraFlex III MALDI-TOF/TOF instrument, (Bruker Daltonics) in linear mode and with positive ion detection. The samples for analysis were prepared by mixing 2.5 µl of product and 2.5 µl of sinapinic acid matrix (Sigma); 2.5 µl of each mixture was deposited on a samples plate, dried at room temperature for 10 min, and subjected to the spectrometer (see Supporting Information). An average of 6.5 linkers was introduced.

Preparation of MenAC conjugate ***1*** $MenA_{sidea}$ oligomers **5** (25 mol sugar/mol of protein) were incubated overnight with CRM_{emcs} **6** at a protein concentration of 20 mg/ml in 200 mM NaPi pH 7.2. Formation of the glycoconjugated was monitored by 4–12 % SDS page in MOS buffer. The glycoconjugate was purified by ammonium sulfate precipitation and used for the next step after quantification of the protein content.

MenA-CRM_{emcs} **7** was incubated overnight with MenC-SH 4 (1:30 protein/thiols) in 10 mM NaPi pH 7.2, 1 mM EDTA. The MenAC-CRM glycoconjugate **1** was firstly purified on a S300 Sephadex column (GE Healthcare) with 10 mM NaPi, 150 mM NaCl pH 7.2 as eluent to remove protein with lower glycosylation degree, and secondly chromatographed on a High Trap Phenyl sepharose resin (GE Healthcare) with a gradient 3 M NaCl to water to separate the unconjugated oligomers. Fractions containing the glycoconjugate were pooled and concentrated on a 30 kDa Vivaspin filter (Sartorius) to be reconstituted with 10 mM NaPi pH 7.2.

The protein content was determined by colorimetric assay micro-BCA, while the saccharide content and free saccharide % by HPAEC-PAD analysis [6].

Preparation of control conjugates ***8–10*** Glycoconjugates **8** and **9** were prepared as reported in literature [24].

Glycoconjugate **10** was prepared by incubating MenSH **4** with CRM_{emcs} (conjugates **9** and **10**, respectively) in 10 mM NaPi pH 7.2, 1 mM EDTA. The crude mixture was precipitated from aqueous ammonium sulfate, reconstituted in 3 M NaCl and purified on a High Trap Phenyl sepharose resin with a gradient 3 M NaCl to remove the unconjugated oligomers. Fractions containing the glycoconjugate were pooled and concentrated on a 30 kDa Vivaspin filter (Sartorius) to be reconstituted with 10 mM NaPi pH 7.2. The protein content of conjugates **8**–**10** was determined by colorimetric assay micro-BCA, while the saccharide content and free saccharide % by HPAEC-PAD analysis [6]. Endotoxin levels were measured for all the prepared glycoconjugates and resulted below 4 EU/μg.

Mice immunization Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in the conduct of all animal studies.

Vaccines were formulated in PBS pH 7.2 buffer in a volume of 200 μl per dose. An injection volume of 200 μl per mouse was used. Aluminium phosphate ($AlPO_4$) was used as adjuvant in an amount of 0.12 mg (expressed as Al^{3+}) per dose. MenAC vaccine **1** was administered to mice in 2 μg per dose based on MenA saccharide content (corresponding to 2.5 μg in term of MenC saccharide). $MenA_{sidea}$-CRM_{197} **8** was used at a saccharide based dosage of 2 μg as control for MenA antigen. $MenC_{sidea}$-CRM_{197} **9** and Men-CRMemcs **4** were used at a saccharide based dosage of 2.5 μg as controls for MenC antigen. Physical mixtures of **8** and **9** or **10** were used as additional controls. BALB/c mice were immunized subcutaneously at day 1, 14 and 28. Bleedings were performed at day 0 (pre immune), day 28 (post 2) and day 42 (post 3). A group received PBS with adjuvant for negative control.

ELISA analysis The antibody response induced by the glycoconjugates against the MenA and MenC polysaccharides were measured by ELISA. Plates were coated with the polysaccharide by adding 100 μl/well of a 5 μg/ml polysaccharide solution in pH 8.2 PBS buffer, followed by incubation overnight at 4 °C. Coating solutions were removed from the plates by washing each well three times with PBS buffer containing 0.05 % of Tween 20 (Sigma) (TPBS). A blocking step was performed by adding 100 μl of BSA solution at 3 % in TPBS and incubating the plates 1 h at 37 °C. Blocking solution was removed from the plates by washing three times per well with TPBS. 200 μl of pre-diluted serum (1:25 for pre immune, 1:200 for a reference serum, 1:50–1:100 for test sera) was added to the first well of each column of the plate, while 100 μl of TPBS was dispensed into the remaining wells. Eight twofold serial dilutions along each column were then performed by transferring from well to well 100 μl of sera solutions. After primary Abs dilution, plates were incubated for 2 h at 37 °C. After three washings with TPBS, 100 μl TPBS solutions of secondary antibody alkaline phosphates conjugates (anti mouse IgG 1:10000 Sigma-Aldrich) were added and the plates incubated 1 h at 37 °C. Three more washes with TPBS were performed, when 100 μl/well of a 1 mg/ml of p-NPP (Sigma) in a 0.5 M di-ethanolammine buffer pH 9.6 were added. After 30 min of incubation at room temperature, plates were read at 405 nm using a Biorad plate reader. Raw data acquisition was performed by Microplate Manager Software (Biorad). Sera titers were expressed as the reciprocal of sera dilution corresponding to a cut-off OD=1. Each immunization group is represented as the geometrical mean (GMT) of the single mouse titers.

For avidity ELISA the procedure described above was followed, with the inclusion of an additional step. After the plate was washed following incubation of the dilution of pooled polyclonal serum from each immunized group, ammonium thiocyanate (NH_4SCN) phosphate buffer, pH 6.0, was added to the appropriate wells in triplicate, in a final concentration of 6 M and 2.5-fold dilutions thereof [26]. The plates were allowed to stand for 15 min at room temperature before triple washing with TPBS and proceeding with the assay as described above. For the data analysis, the $\log_{10}$ of binding % was plotted vs the molar concentrations of NH_4SCN [26, 27]. The data, which all presented an OD >1, were fitted by linear regression. Sets of data having a linear fitting with a correlation coefficient ≥0.88 M were accepted [27]. The NH_4SCN concentration able to produce a 50 % reduction in the initial IgG level was calculated (avidity index, AI). Standard deviation for the triplicate sets of data was calculated (Tables 2 and 3).

For the determination of IgG1, IgG2a and IG2b subclasses, pool of sera were analyzed as described above, except for the use of anti IgG1, IgG2a and IgG2b secondary antibody alkaline phosphates conjugates (anti mouse IgG 1:10000 Sigma-Aldrich), respectively.

The graphical analysis of ELISA data was performed by GraphPad 5.0 software, using Mann Withney statistical analysis.

Rabbit serum bactericidal assay (rSBA) Functional antibodies of pooled sera induced by vaccine immunization were analyzed by measuring the complement-mediated lysis of *N. meningitidis* with an *in vitro* bactericidal assay described in the literature [28]. SBA titers were conventionally assigned when two successive sera dilutions lead to at least 90 % bacteria killing and were expressed as the reciprocal serum dilution, which results in 50 % of bacteria killing. F8238 and MenC11 were used as reference strain for MenA and MenC, respectively.

Results

Preparation and characterization of glycoconjugates MenA and MenC CPSs consists of (1→6)-linked 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate repeating units, predominantly O-acetylated at 3-OH, and (2→9)-linked polysialic acids partially acetylated at 7- or 8-OH, respectively (Fig. 1) [24]. Our strategy to synthesize the MenAC construct **1** was based on the insertion of these two different glycan haptens onto the lysine residues of CRM_{197}, using amide bond formation and thiol-maleimide addition as coupling chemistries. CRM_{197} is a protein widely used in vaccines development and manufacturing that presents 39 lysine residues [29], 19 of which are available for conjugation [6].

Preliminary experiments using MenA and MenC oligomers **5** and **2**, respectively, as both *N*-hydroxysuccinimide (NHS) derivatives showed that the insertion of one oligosaccharide onto the protein rendered the second coupling very challenging. This behavior could be due to the length and/or the presence of negative charges in the two oligomers. Thus we predicted that insertion of spacers bearing maleimide moieties onto few of the more exposed lysine residues would preserve some of them for the conjugation of a second oligosaccharide after insertion of the first one by NHS mediated coupling. To apply this strategy, the active esters of $MenC_{sidea}$ **2** were condensed with an excess of cystamine, in order to obtain the protected mercapto derivative $MenC_{cystamine}$ **3** (Scheme 1). Insertion of the cystamine was assessed by the presence of the ^{1}H NMR signal of $NHCH_2CH_2S$ appearing as triplets at 3.00 ppm correlating with a triplet at 3.38 for the $NHCH_2$ and a triplet signal of $SCH_2CH_2NH_2$ at 2.88 ppm correlating with a signal underneath the sugar CH region at 3.44 ppm for the CH_2NH_2 (Fig. 1S-b). The covalent linkage of the cystamine moiety with the oligomer was confirmed by DOSY NMR proton experiments (data not shown). Reduction of the thioether linkage by reaction with TCEP released the thiols for the conjugation step of the oligomer **4**. At ^{1}H NMR the installed cysteamine appeared as two triplets at 3.36 and 2.85 ppm, which were attributed to the $HNCH_2$ and CH_2SH signals, respectively (Fig. 1S-c).

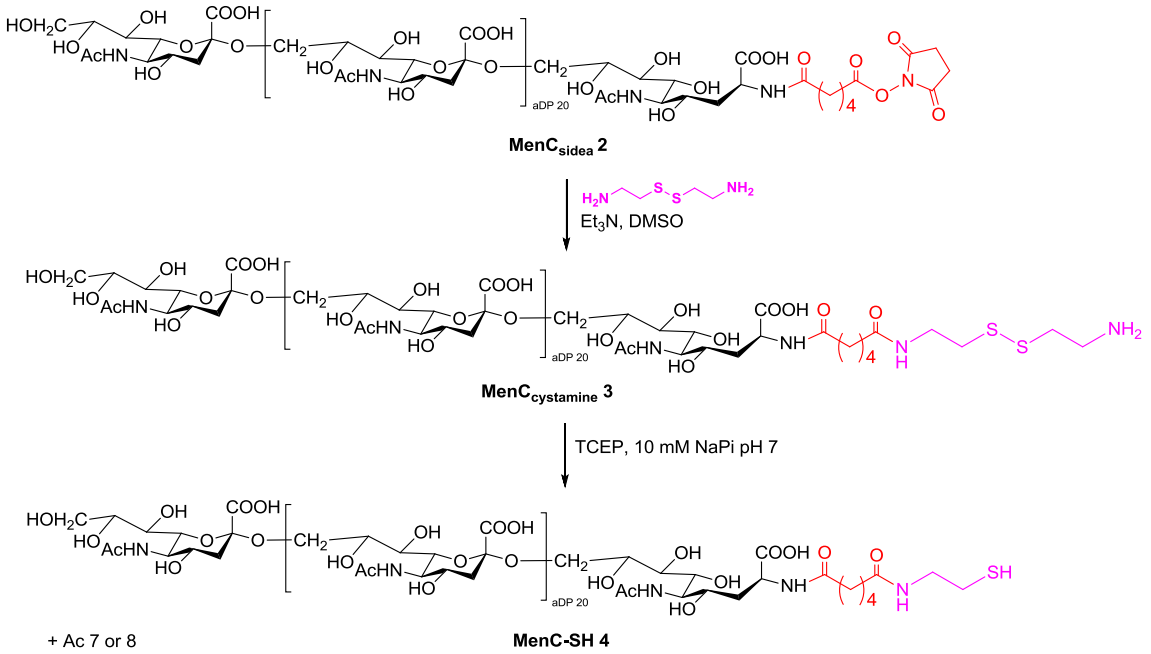
Reaction of CRM_{197} with 10 equiv. of the linker EMCS allowed insertion of 6.5 maleimide moieties per mol of protein, as estimated by MALDI TOF spectrometry (see SI). Interestingly, we have recently shown that among all the available lysine residues of CRM_{197}, there are six more surface accessible sites which are prone to react with NHS linkers [30]. After dialysis against a membrane with cut off 30 kDa, the obtained CRM_{emcs} **6** was conjugated to the NHS form of MenA oligomer **5** at 25:1 carbohydrate-protein ratio (Scheme 2). The MenA-CRM_{emcs} **7** conjugate was purified by precipitation with ammonium sulfate and subjected to thiol maleimide addition with MenC-SH **4**. The presence of the linker enabled the successful orthogonal coupling of the second carbohydrate antigens. A purification step by size exclusion chromatography on a S300 Sephadex column allowed removal of the product with lower glycoconjugation degree. Finally, the unconjugated saccharide was separated from the MenAC-CRM conjugate **1** by purification on Phenyl Sepharose resin. MenA and MenC saccharides were quantified by HPAEC-PAD analysis. SDS page electrophoresis gel and HPLC chromatogram of the synthesized glycoconjugate are depicted in Fig. 2.

A glycoconjugate of MenA antigen as control for *in vivo* studies was prepared by conjugation of $MenA_{sidea}$ **5** to CRM_{197} [24, 25]. In the case of MenC, both active ester activated oligomer **2** and thiol derivative **4** were conjugated to CRM_{197} and CRM_{emcs} to study the effect of the thiol-maleimide coupling chemistry in comparison to the known condensation by amide bond formation [6]. As it can be seen from Table 1, equivalent amounts of MenA and MenC antigens could be loaded onto CRM_{197} by our conjugation approach.

Furthermore, condensation via active esters occurred with higher efficiency than thiol-maleimide addition, and conjugate **9** prepared by the first chemistry showed a higher glycoconjugation level than the counterpart **10** obtained by the latter reaction.

Immunochemical evaluation To assess the capability of the synthesized MenAC-CRM conjugate **1** to raise antibodies recognizing the MenA and MenC capsular polysaccharides, groups of 8 BALB/c mice were immunized subcutaneously with three doses, 2 weeks apart, of 2 μg on saccharide base of MenA antigens, which corresponded in the bivalent structure to a dosage of 2.5 μg on saccharide base of MenC antigen. The conjugates were formulated with aluminum phosphate, an adjuvant commonly used for vaccines in the market or in preclinical development [31]. With the aim of studying any possible influence of the conjugation strategy on the immunogenicity of the glycoconjugates, the physical mixtures of MenA-CRM conjugate **8** obtained by active esters chemistry (sidea), with MenC conjugates **9** or **10**, prepared via amide bond formation (sidea) and Michael addition to the maleimide (emcs), respectively, were used as controls. As additional controls, the monovalent conjugates **8**, **9** and **10** were also utilized, to study the effect of the monovalent versus bivalent formulation of the two meningococcal antigens. In order to reveal any discrepancy in the immune response towards construct **1**, and reduce the *plateau* effect usually observed after three doses, sera were analyzed after the second and third vaccination for their anti-carbohydrate IgG content and the antibody functionality [24].

After the first boost, the bivalent conjugate **1** elicited anti-MenA polysaccharide IgG titers comparable to the monovalent $MenA_{sidea}$-CRM **8** and to the bivalent formula-



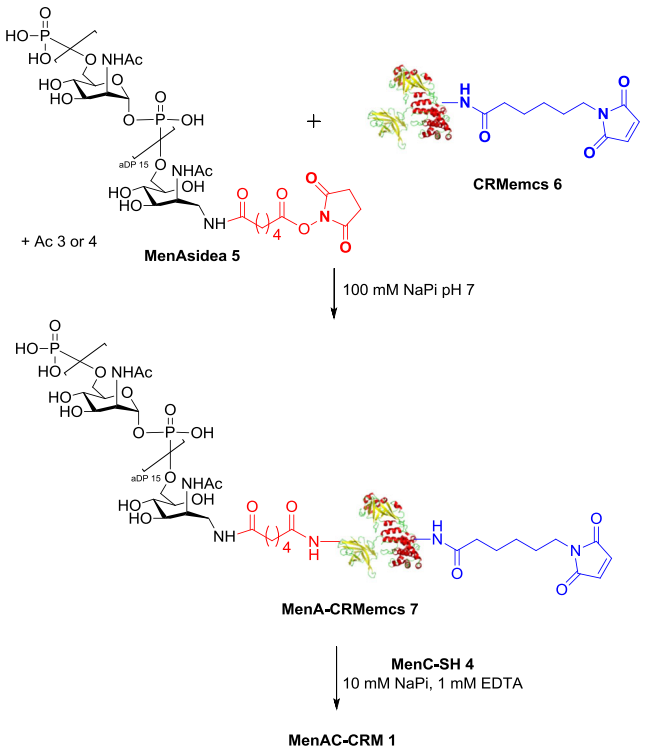
Scheme 1 Insertion of thiol groups in MenC oligomers

tions of **8** with MenC$_{sidea}$-CRM 9 or MenC$_{emcs}$-CRM **10** (Table 2, Fig. 3S). The construct **1** elicited anti-MenC polysaccharide IgG levels comparable to the monovalent vaccine **9** and to the physical mixtures of **8** and **9** or **10** (Table 3, Fig. 3S). However, the IgG titers induced by **1** were lower than the monovalent conjugate 10 (p 0.016), where a MenC sulfhydryl antigen was coupled to the protein similarly to the construct 1, suggesting that the concomitant linkage of the MenA hapten might affect the anti-MenC antibody level.

Following the second boost, the anti-MenC IgG titers elicited by the MenAC-CRM conjugate **1** were significantly lower than those obtained with the monovalent conjugates MenC$_{sidea}$-CRM **9** and MenC$_{emcs}$-CRM **10** (p 0.009 and 0.018, respectively), but comparable to levels induced by **9** and **10** in mixture with MenA$_{sidea}$-CRM **8**. Together these evidences indicated that the co-delivery of the MenA antigen could play a role in decreasing the anti-MenC antibody production.

To measure the functionality of the murine sera obtained by immunization with the set of glycoconjugates, rSBA titers were estimated. This model is usually applied as a surrogate of protection for meningococcal antigens [32, 33].

After the second immunization all the conjugates induced good anti-MenC functional activity (Table 3 and Fig. 3b). In particular, the bactericidal titer elicited by sera from mice immunized with the bivalent conjugate **1** (4096) was comparable to the titer of the monovalent vaccine **10** (4096), slightly higher than the mixture of **10** with **8** (2048), and lower than conjugate **9** (8192) and its mixture with **8** (8192>t >16384). As it can be observed from Fig. 3a, all the analyzed sera induced a decrease in the group A meningococcal growth in comparison to the negative control (PBS). However, the MenA positive control **8** alone and in mixture with **9** elicited sera with a titer of 1024 and 512, respectively, whereas the antibodies elicited by **1** and mixture of **10** and **8** lead to a



Scheme 2 Preparation of the bivalent MenAC-CRM$_{197}$ conjugate **1**

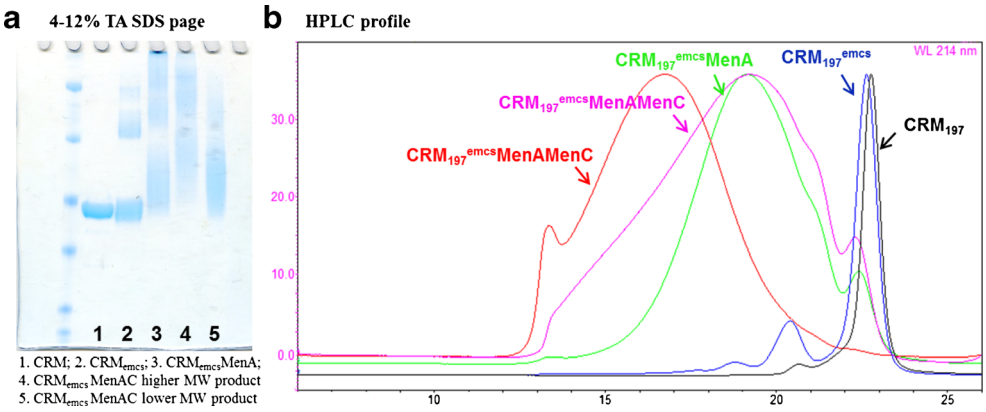


Fig. 2 Comparison of (**a**) SDS page gel electrophoresis and (**b**) HPLC chromatogram of the bivalent glycoconjugate MenAC-CRM$_{197}$ and related intermediates of preparation. HPLC was run on a Tosoh Bioscience LLC TSgel G3000SW column, using 100 mM NaPi- 100 mM Na_2SO_4, containing 5 % acetonitrile, pH 7.2 for elution and applying a 0.5 ml/min flow

reduction in the bacterial population at an extent that was not sufficient to assign a bactericidal titer (NA, Table 2).

Overall these findings suggested that, after the second immunization, the antibody bactericidal activity induced by the MenA antigen would be reduced by the co-administration with MenC, but not *vice versa*. This effect was particularly remarkable in the MenAC-CRM construct **1**, where the two saccharide haptens are installed concomitantly onto the same molecule of carrier protein.

Therefore, at this stage, MenC appeared as the immunodominant antigen in construct **1**.

We interrogated whether the different behavior of the anti-MenA sera, which possessed comparable anti carbohydrate IgG titers but different functionality, was determined by the diverse avidity of the generated antibodies [34]. To answer this question, the pools of polyclonal sera obtained by immunization with our set of glycoconjugates were analyzed at different concentrations of NH_4SCN. This chaotropic salt enables to roll out the antibodies with lower avidity, thus the thiocyanate concentration which produced a 50 % reduction in the effective antibody binding (avidity index, AI) can be used to compare the avidity of the different sera (Tables 2 and 3) [26, 27]. A clear dependence of the antibody functionality on the corresponding avidity was not observed, as reported in literature for other antigens [35]. However, the avidity indices towards the MenA antigen of all the analyzed sera were comprised in a narrow range (0.15–0.55 M). The serum against the MenA-CRM conjugate **8**, which exhibited the highest functionality, presented also the highest AI (0.55 M, Table 2). Sera endowed with lower avidity (0.15–0.19 M) presented a reduced functionality in comparison to control **8**.

By contrast, the anti-MenC avidity indices were distributed over a larger range (0.16–0.88 M), but even sera with the lowest avidity (0.16 M) showed a good functionality. This evidence indicates that a lower avidity would be sufficient to confer to the polyclonal antibodies a good bactericidal activity against the serogroup C with respect to the serogroup A meningococcus. Since a comparable AI was measured in the serum from the MenAC-CRM **1** construct towards both the MenC (0.16 M) and the MenA antigen (0.19 M), this would translate into an immunodominant character of MenC over MenA antigen in term of functionality.

After the third immunization, polyclonal sera elicited by the bivalent conjugate **1** exhibited very high anti-MenA bactericidal titers, which were comparable to those induced by the monovalent vaccine **8**, and its combinations with MenC conjugates **9** or **10**. Importantly, the construct **1** gave a bactericidal anti-MenA titer of 8192, while a titer of 4096 was obtained for the corresponding physical mixture prepared by combination of MenA$_{sidea}$-CRM **8** with MenC-CRM$_{emcs}$ **9**, in which the same conjugation chemistries as in **1** were applied.

The sera from immunization with MenAC-CRM conjugate **1**, although with significantly lower IgG titer (Table 3), showed an excellent anti-MenC bacteridical activity in comparison to the monovalent formulations of MenC$_{sidea}$-CRM **9** and MenC-CRM$_{emcs}$ **10**. Additionally, the functionality of this serum was comparable to that from the combinations of MenA$_{sidea}$-CRM **8** with MenC$_{sidea}$-CRM **9** or MenC-CRM$_{emcs}$ **10**. Analysis of the AIs showed a general increase

Table 1 Physicochemical characteristics of conjugate **1** and controls determined by HPAEC-PAD analysis

Glycoconjugate	Saccharide/protein ratio (*w/w*)	mol MenA/ mol protein	Saccharide/protein ratio (*w/w*)	mol MenC/ mol protein
MenAC-CRM **1**	0.11	1.5	0.14	1.4
MenA$_{sidea}$-CRM **8**	0.42	6.0		
MenC$_{sidea}$-CRM **9**			0.57	5.6
MenC-CRM$_{emcs}$ **10**			0.18	1.8

Table 2 Anti-MenA IgG titers, affinity index and bactericidal titers of sera from mice immunized with tricomponent conjugate **1** and controls. *t*=titer; NA=not assigned

	Post II			Post III		
Glycoconjugate	GMT (95 %)	AI±STD[a]	rSBA titer	GMT (95 %)	AI±STD[a]	rSBA titer
PBS	2	-	<16	2	-	<16
$MenA_{sidea}$-CRM **8**	1337 (761; 3514)	0.55±0.13	1024	4800 (3063; 7737)	2.30±0.58	8192>t >16384
MenAC-CRM **1**	1648 (1162; 2738)	0.19±0.02	NA	3701 (2209; 6491)	0.58±0.13	8192
$MenA_{sidea}$-CRM **8**+$MenC_{sidea}$-CRM **9**	912 (583; 2564)	0.19±0.08	512	2713 (1893; 6532)	0.84±0.09	8192>t >16384
$MenA_{sidea}$-CRM **8**+MenC-CRM_{emcs} **10**	2169 (1405; 3395)	0.15±0.03	NA	3394 (1925; 6475)	1.38±0.05	4096

[a] Avidity index ± standard deviation from triplicate experiments

of the avidity towards the two polysaccharide antigens (Tables 2 and 3), since AIs measured towards the MenA and MenC antigens were in the range 0.58–2.30 M and 0.31–1.76 M, respectively. This was in agreement with the enhanced bactericidal activity achieved by the third immunization.

To further investigate whether differences in term of antibody functionality could be determined by a variation in the Th1/Th2 balance among the anti-carbohydrate immune responses, the antibody subclasses of the pooled sera were evaluated (see Fig. 2S, SI). A prevalence of IgG1 with respect to IgG2a was found after both the second and third dose of the vaccines, thus indicating a Th2 driven response which is typical of Alum formulated antigens [36]. Thus, the immunodominance of MenC antigen over MenA in the construct **1** was not correlatable to differences in the IgG1/IgG2a ratio in comparison to the controls.

Discussion

The assembling of multicomponent conjugates from poly- or oligosaccharide antigens derived from different serogroups of one pathogen or from different pathogens can be an interesting approach to reduce the number of biomolecules administered during vaccination. To assess the feasibility of this approach and investigate whether conjugation of different carbohydrate

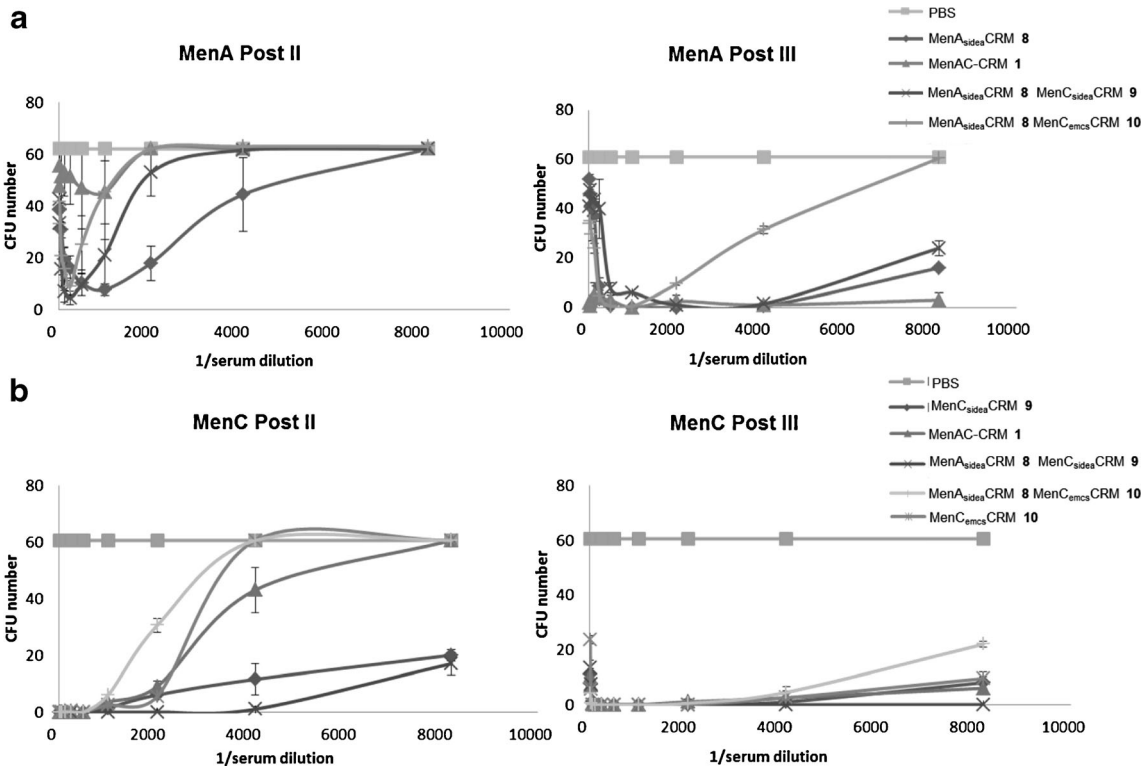


Fig. 3 Analysis of the reduction of meningococci strains **a**) F8238 and **b**) C11 growth in presence of the murine polyclonal antibodies elicited by the construct **1**, and by negative and positive controls

Table 3 Anti-MenC IgG titers, affinity index and bactericidal titers of sera from mice immunized with tricomponent conjugate **1** and controls. *t*=titer

	Post II			Post III		
Glycoconjugate	GMT (95 %)	AI±STD[a]	rSBA titer	GMT (95 %)	AI±STD[a]	rSBA titer
PBS	2	-	<16	2	-	<16
$MenC_{sidea}$-CRM **9**	3112 (1578; 6222)	0.26±0.04	8192	24899 (17596; 37604)	0.64±0.19	8192>t >16384
MenC-CRM_{emcs} **10**	3467 (2526; 5474)	0.16±0.05	4096	22499 (12705; 39295)	0.31±0.13	8192>t >16384
MenAC-CRM **1**	1511 (995; 2305)[b]	0.33±0.03	4096	9600 (6125; 15475)[c]	0.45±0.25	8192>t >16384
$MenA_{sidea}$-CRM **8**+$MenC_{sidea}$-CRM **9**	2016 (1512; 4125)	0.88±0.21	8192>t >16384	11416 (6934; 30416)	0.97±0.15	8192>t >16384
$MenA_{sidea}$-CRM **8**+MenC-CRM_{emcs} **10**	2365 (1640; 3460)	0.44±0.06	2048	12906 (6357; 25243)	1.76±0.47	8192>t >16384

[a] Avidity index ± standard deviation from triplicate experiments; b. p 0.016 vs conjugate **10**; c. p 0.009 and 0.018 vs **9** and **10**, respectively

antigens to the carrier protein could result in immunodominance of any of them, we prepared a bivalent unimolecular MenAC-CRM vaccine employing two carbohydrate antigens and a carrier protein from vaccines currently in the market, and for which robust read-out of the *in vivo* evaluation can be achieved.

To this end we have developed a strategy for the conjugation of two different medium length oligosaccharides, MenA and MenC, onto the carrier protein CRM_{197} based on functionalization of the protein with maleimide groups, insertion of one antigen through a chemical arm bearing an active ester linker and subsequent coupling of the second antigen via thiol-maleimide addition. To ascertain differences in the immune response against the novel construct and the controls, murine sera were assayed for the antibody level, avidity and functionality after the first and second boost. The bivalent unimolecular glycococonjugate demonstrated to possess a good immunogenicity in comparison to the MenA and MenC control vaccines and their combinations. The murine sera obtained after the second and third immunization with the bivalent construct exhibited good anti-MenC bactericidal activity as compared to the controls. Conversely, the antibodies elicited by the conjugate after the first boost were endowed of lower anti-MenA functionality in comparison to the anti-MenC bactericidal activity. The co-administration of MenA and MenC antigens was also demonstrated to reduce the anti-MenA but not the anti-MenC bactericidal activity, particularly in the bivalent construct. Avidity indices measured on the murine sera unraveled that a lower avidity towards MenC in comparison to MenA polysaccharide would be sufficient to confer to the antibodies a good bactericidal activity. Since after the second dose comparable relative avidities were determined for both the MenC and the MenA antigen in the bivalent vaccine, this would translate into an immunodominant character of MenC over MenA antigen in term of functionality.

These results highlight that conjugation of two microbial polysaccharides onto the same carrier protein could give rise to immune interference among the carbohydrate antigens. This event can be better evidenced after the first boost, when lower levels of antibodies, with lower avidity and bactericidal activity, are elicited. After the second boost, which is typically administered for the evaluation of glycoconjugates in mice model [37], a higher level of antibodies endowed with enhanced avidity and excellent functionality enable to fully overcome the immunodominance of one of the two carbohydrate haptens. Thus, the choice of the vaccination schedule may be relevant in determining and surmounting the occurrence of immunodominance in these complex biomolecules. Further exploration is needed to clarify whether the carbohydrate immune interference is dependent on the conjugated haptens or the conjugation chemistries used in this type of constructs. The impact of this effect could be different for anti-cancer therapeutic vaccines, where a larger number of doses is usually administered [38].

Noteworthy, the effect of increased carrier protein load following the use of multivalent or combination vaccines on the immune response to both the polysaccharide hapten and the carrier protein is still poorly delineated [39, 40]. In some cases pre-existing immunity against the carrier protein can induce reduction of anti-polysaccharide antibody levels (carrier epitope suppression) [41], although increased anti-glycan responses have been also reported [42].

In the present study the content of each carbohydrate antigen in the bivalent MenAC construct was lower than that in the corresponding monovalent conjugates (Table 1), consequently a 4-fold larger amount of carrier protein was administered for the bivalent glycoconjugate in comparison to the controls. We expect that conjugation of different carbohydrate haptens to the one single carrier protein and fine tuning of the sugar/protein ratio may be helpful to study and circumvent the carrier epitope suppression.

In conclusion, this study highlights that the construction of multicomponent glycoconjugate vaccines against microbial pathogens can be a feasible approach provided that the risk of inducing immunodominance is diligently evaluated.

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