

Combined chemical synthesis and tailored enzymatic elongation provide fully synthetic and conjugation-ready *Neisseria meningitidis* serogroup X vaccine antigens

Davide Oldrini, Timm Fiebig, Maria Rosaria Romano, Daniela Proietti, Monika Berger, Marta Tontini, Riccardo De Ricco, Laura Santini, Laura Morelli, Luigi Lay, Rita Gerardy-Schahn, Francesco Berti, and Roberto Adamo

ACS Chem. Biol., **Just Accepted Manuscript** • DOI: 10.1021/acscchembio.7b01057 • Publication Date (Web): 26 Feb 2018

Downloaded from <http://pubs.acs.org> on February 28, 2018

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.

1
2
3 **Combined chemical synthesis and tailored enzymatic elongation provide**
4 **fully synthetic and conjugation-ready *Neisseria meningitidis* serogroup X**
5 **vaccine antigens**
6
7

8 Davide Oldrini^{a†}, Timm Fiebig^{b†}, Maria Rosaria Romano^a, Daniela Proietti^a, Monika Berger,^b
9 Marta Tontini^a, Riccardo De Ricco^a, Laura Santini^a, Laura Morelli^{c‡}, Luigi Lay^c,
10 Rita Gerardy-Schahn^b, Francesco Berti^a, Roberto Adamo^{*a}
11
12
13

14
15 ^a*GSK, Via Fiorentina 1, 53100 Siena, Italy*

16 ^b*Institute of Clinical Biochemistry, Hannover Medical School, 30625 Hannover, Germany*

17 ^c*University of Milan, Department of Chemistry, via Golgi 19, 20133, Milan, Italy*

18 [†]*These authors contributed equally to the study*

19 ^{*}corresponding author: roberto.x.adamo@gsk.com

20
21
22 [‡]Present address: University of Milan, Department of Medical Biotechnology and Translational Medicine,
23 Via Saldini 50, 20133 Milan, Italy
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Abstract

Studies on the polymerization mode of *Neisseria meningitidis* serogroup X capsular polymerase CsxA recently identified a truncated construct that can be immobilized and used for length controlled on-column production of oligosaccharides.

Here we combined the use of a synthetic acceptor bearing an appendix for carrier protein conjugation and the on-column process to a novel chemo-enzymatic strategy. After protein coupling of the size optimized oligosaccharide produced by the one-pot elongation procedure, we obtained a more homogeneous glycoconjugate compared to the one previously described starting from the natural polysaccharide. Mice immunized with the conjugated fully synthetic oligomer elicited functional antibodies comparable to controls immunized with the current benchmark MenX glycoconjugates prepared from the natural capsule polymer or from fragments of it enzymatically elongated.

This pathogen-free technology allows the fast total *in vitro* construction of predefined bacterial polysaccharide fragments. Compared to conventional synthetic protocols, the procedure is more expeditious and drastically reduces the number of purification steps to achieve the oligomers. Furthermore, the presence of a linker for conjugation in the synthetic acceptor minimizes manipulations on the enzymatically produced glycan prior to protein conjugation. This approach enriches the methods for fast construction of complex bacterial carbohydrates.

Introduction

Neisseria meningitidis (Men) is a Gram-negative bacterium responsible for bacterial meningitis and sepsis. Of the 12 different serogroups, the most common pathogenic strains are serotypes A, B, C, W and Y. Over the last 10 years, outbreaks of MenX occurred in Niger¹, Uganda, Kenya², Togo and Burkina Faso³, and rising incidence was also reported in America, Europe⁴ and China⁵. Monovalent carbohydrate-based vaccines to combat MenC infections and multivalent formulations targeting MenACWY serogroups have been licensed⁶, and proven to be cost-effective preventive therapies⁷.

Although Men is diffused worldwide, a large prevalence is particularly observed in a region of Sub-Saharan Africa, the so called meningitis belt⁸. In major African epidemics, attack rates range from 100 to 800 per 100,000 population, but individual communities have reported rates as high as 1%, caused almost entirely by serogroup A⁹. To decrease Men epidemics in Africa, in 2010 a MenA conjugate vaccine (MenAfriVac) was rolled out in a mass vaccination program in the meningitis belt¹⁰. While the introduction of MenAfriVac has substantially reduced the incidence of serogroup A infections, the occurrence of other serotypes, such as C, W and X still remains relevant¹¹. Increasing concern has also been raised by possible serogroup displacement, already described for A and C Men serogroups in China^{12, 13}. As a consequence, a clinical trial testing vaccination with a pentavalent MenACWXY Tetanus Toxoid-conjugate (including MenX) has been lately completed¹⁴.

Additionally, over the last 5 years different reports have disclosed strategies for conjugation of MenX CPS related fragments. Micoli et al.¹⁵ identified proper bacterial growth conditions for the optimal production and release of MenX PS. A glycoconjugate vaccine was prepared by CPS hydrolysis with a final average degree of polymerization (avDP) of 15-20 repeating units, showing its effectiveness in protecting against MenX disease.

As an alternative to the polysaccharide isolation from pathogen cultures, chemically synthesized MenX oligomers with a maximal length of DP4 have been tested after coupling to a carrier protein^{16,17,18}. However, the size of the oligomers was not sufficient to induce a proper immune response. In contrast, *in vitro* production of the MenX polymer by use of the recombinant MenX polymerase CsxA and introduction of this polymer (CPSX) into the established glycoconjugate production procedure, delivered a vaccine that in mouse immunization studies induced a robust immune response identical to the benchmark¹⁹. Importantly, in this earlier protocol the enzyme catalyzed synthesis of CPSX was started with acceptors obtained by hydrolysis of the natural polysaccharide. Similarly to the approach used for other meningococcal vaccines²⁰, the polymer chains were then hydrolyzed, sized to a length of avDP10 and, after appropriate functionalization, conjugated to a carrier protein^{15, 21}.

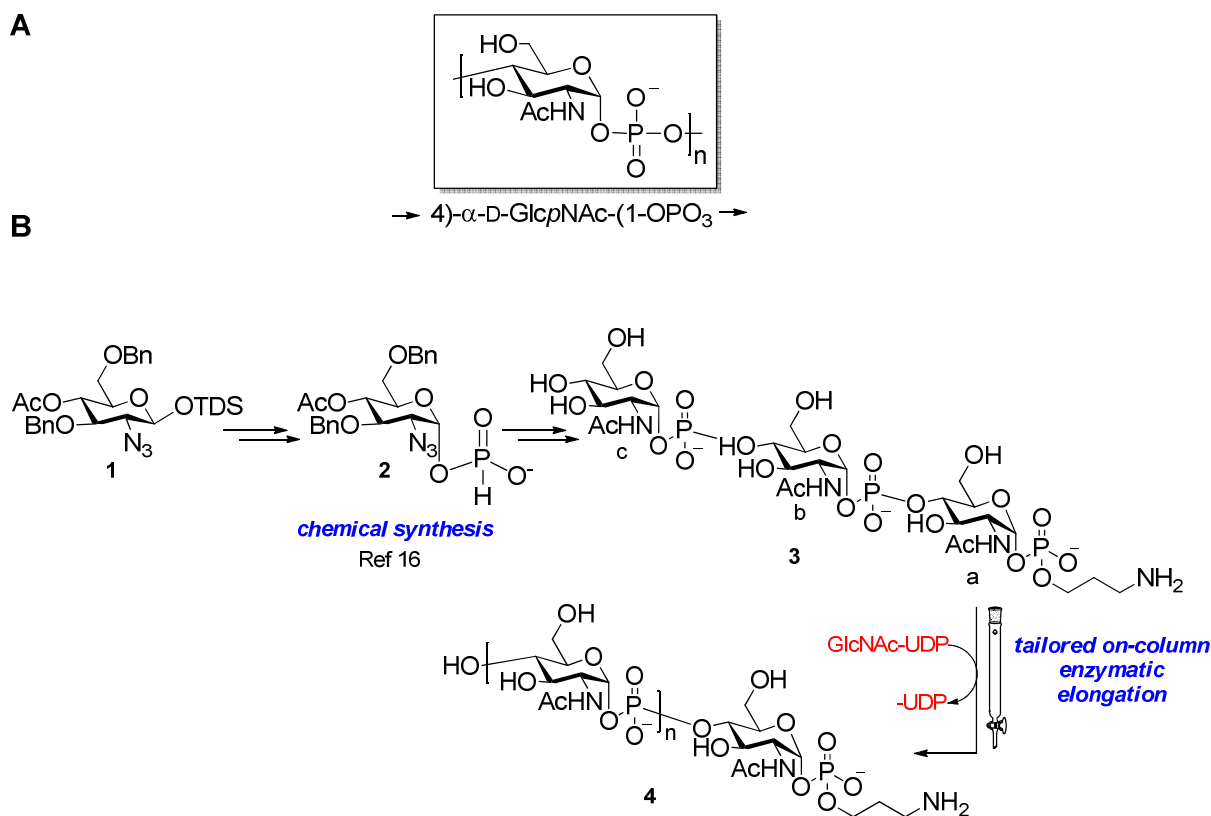


Figure 1. (A) Structure of the repeating unit of MenX capsule polysaccharide. (B) Proposed approach based on on-column elongation of a synthetic MenX trisaccharide acceptor¹⁶. TDS = thexyldimethylsilyl. Letters a-b in structure 3 are used for NMR assignments.

32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

In the last decade, methods for accelerating the production of biologically relevant carbohydrates have encountered fast development²²⁻²⁵. The availability of *in vitro* prepared defined glycans is key for the production of glycoconjugates devoid of pathogenic contaminants, with higher standards for characterization and more robust correlation between structure and immune activity²⁶. Approaches combining synthetic and enzymatic steps have also been successfully developed to get access to a large number of carbohydrates²⁷. Despite chemo-enzymatic synthesis has found its major use for the synthesis of mammalian glycans^{28, 29}, examples are also reported for the production of complex bacterial sugars from Group B *Streptococcus*, *Neisseria meningitidis* and *Shigella flexneri*³⁰⁻³². In this context, the use of heterologous bacterial enzymes has been exploited for the challenging incorporation of 1,2-cis linkages and α -sialylation, since it offers indisputable advantages in the stereochemical control of the reaction compared to chemical methods. Bacterial enzymes have also been used to assemble mammalian glycans³³.

Here we describe a chemo-enzymatic approach based on the enzyme catalyzed one-pot elongation of a short synthetic MenX trisaccharide acceptor, bearing a chemical appendix already in place for conjugation. By use of a recently described distributive variant of the capsule polymerase CsxA³⁴, we synthesized oligomers with predefined average length which were directly conjugated to the genetically detoxified diphtheria toxin CRM₁₉₇. The enzyme was coupled to a solid-phase to facilitate downstream processing of the reaction mixture. The chemo-enzymatically made glycoconjugate was immunologically evaluated in comparison to a glycoconjugate prepared using a polysaccharide fraction isolated from bacterial culture.

Results and Discussion

Chemical synthesis of MenX trisaccharide acceptor

MenX capsular polysaccharide consists of N-acetylglucosamine-4-phosphate residues held together by α -(1 \rightarrow 4) phosphodiester bonds (Figure 1A). Taking in consideration that the elongation cascade of CsxA polymerization was commenced in a previous report from a short acceptor with avDP 3.2¹⁹, we chemically synthesized the CPSX trisaccharide acceptor **3** (Figure 1B), as previously reported¹⁶. Briefly, 2-azido-2-deoxy silyl glucoside **1** was prepared in multigram scale in 9 steps from D-glucosamine hydrochloride (18% overall yield), and converted in two steps into the α -hydrogenphosphonate **2**, which was employed as key building block (Figure 1B).¹⁶ Elongation of intermediate **2** was carried out by iteration of a two-steps sequence, comprising pivaloyl chloride-mediated hydrogenphosphonate coupling followed by Zemplén de-O-acetylation, to eventually afford trisaccharide **3** after global deprotection. On the whole acceptor **3** was obtained in 18 steps from D-glucosamine hydrochloride with a 0.3% overall yield, without taking into account that some intermediates could be recycled during the process¹⁶. NMR spectra of the trimer were in agreement with the published data¹⁶. The presence of a phosphodiester-linked amino propyl linker provides a chemical handle for coupling to a carrier protein^{15, 35}.

N-terminus CsxA mapping and on-column attachment

CsxA used for the production of long CPSX is able to elongate short CPSX fragments with an avDP of \sim 3¹⁹. CsxA works following two different elongation mechanisms: in the initial phase of the reaction, short DPs are elongated through a distributive mechanism, and the nascent chain is released after each transfer until an intermediate chain length of \sim DP 18 is reached. Subsequently, intermediate length fragments (<DP18) bind to a separate binding site located in the C-terminus and are elongated in a processive manner, undergoing multiple rounds of monomer addition to reach the final polysaccharide length³⁴. The interplay of the two mechanisms leads to skewed or even

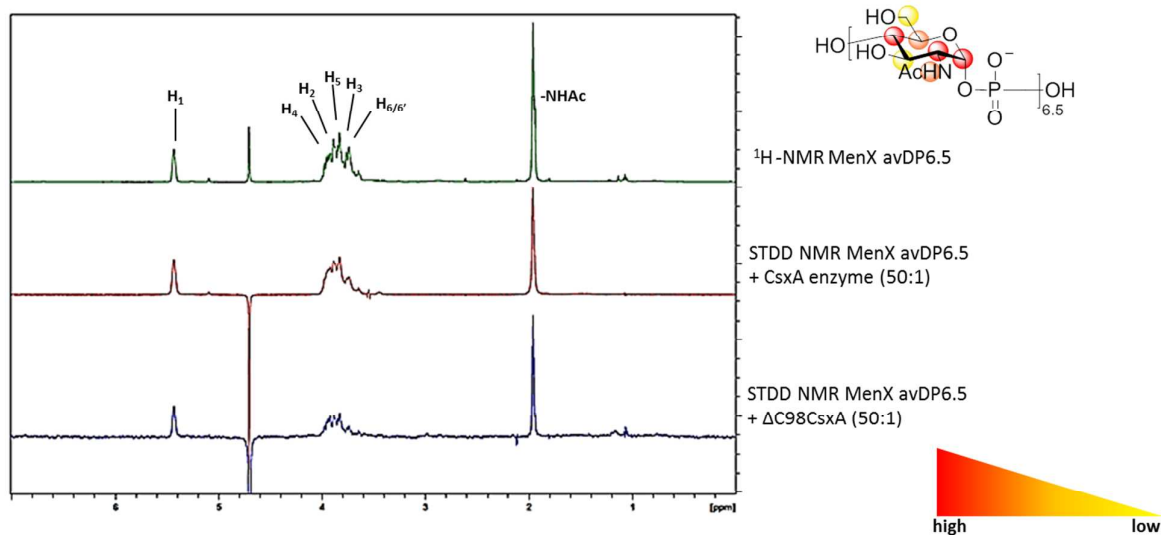
1
2
3 separated product pools when using CxsA and prevents the generation of oligosaccharides suitable
4 for the generation of a glycoconjugate vaccine³⁴.

5
6 Recently, a truncated version of CsxA was generated (Δ C98-CsxA) lacking the C-terminal binding
7 site and thus allowing tailored synthesis of oligosaccharides with a length suitable for
8 glycoconjugate synthesis³⁴. While Δ C98-CsxA was shown to elongate DP>2, it had not been
9 investigated if the missing C-terminus changed the overall binding pattern of the oligosaccharide
10 acceptors. Consequently, we first analyzed the binding of short acceptors (avDP6.5) to wild type
11 CsxA in comparison to Δ C98-CsxA.
12
13

14
15 Saturation Transfer Difference (STD) NMR experiments were undertaken in order to qualitatively
16 map the average positions of the MenX oligomers closer to binding pockets of the two constructs.
17 An evaluation of the STDD-NMR spectrum (STD-NMR with blank subtraction) of the complex
18 formed by CsxA and the avDP of 6.5 fragments exhibited the strongest signal enhancement for H-
19 1, H-2 and H-4, followed by H-5 and the NHAc moiety and, with minor intensity, H-3 and H-6
20 (Figure 2A). This suggests that the attack of the GlcNAc-UDP donor to the hydroxyl at position 4
21 might occur from the bottom face of the oligomer. Most importantly, the same pattern of signals
22 was observed between Δ C98-CsxA and the avDP6.5 mixture, with the only difference that the
23 STD-NMR intensity was lower if compared to full-length CsxA, indicating a weaker binding to the
24 tested oligomers, as expected for a distributive enzyme.
25
26

27
28 Following our main purpose to use the synthetic CPSX trimer as priming oligosaccharide for the
29 enzymatic elongation, the interaction between this molecule and both CsxA constructs was mapped
30 (Figure 2B). Again, the STD-NMR intensity in the presence of Δ C98-CsxA was lower, but the
31 binding pattern was comparable to CsxA and, most importantly, in good agreement to the overall
32 binding epitope of the natural avDP6.5. As anticipated, no STD-NMR signal was recorded for the
33 linker, demonstrating that it does not interact with the enzyme in a specific way. Interestingly, both
34 constructs have contact to H-1a and H-1b,c, demonstrating that CsxA is able to accommodate the
35 synthetic trimer in its active site pocket and this interaction is not altered by the missing C-terminal
36 binding site. Although we cannot exclude that a shorter fragment could bind to the enzyme, these
37 findings confirmed that the synthetic trisaccharide was engaged by a C-terminally truncated CsxA
38 construct with sufficient interaction for further elongation. A more in depth structural study of the
39 interaction between the enzyme and different length oligomers will deserve further exploration.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

A MenX avDP 6.5



B MenX synthetic DP3

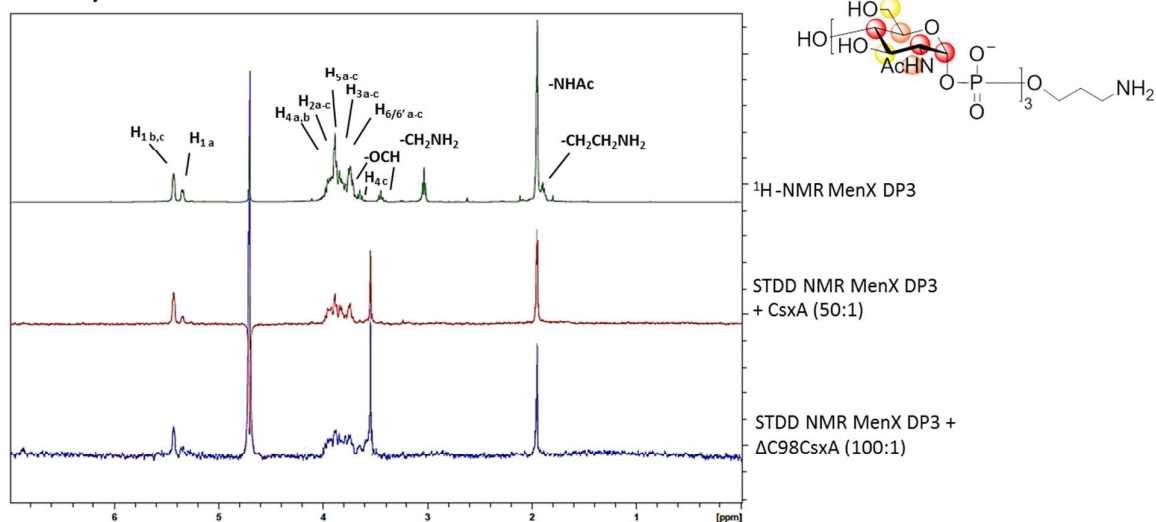


Figure 2. STDD-NMR spectra of the two MenX oligomers in complex with (A) full length CsxA and (B) truncated version ΔC98-CsxA at the specified molar ratio. Chemical shifts of the linker (at 3.4, 3.8, 2.9 and 1.9 ppm) disappear at the STD NMR showing no engagement with the enzyme.

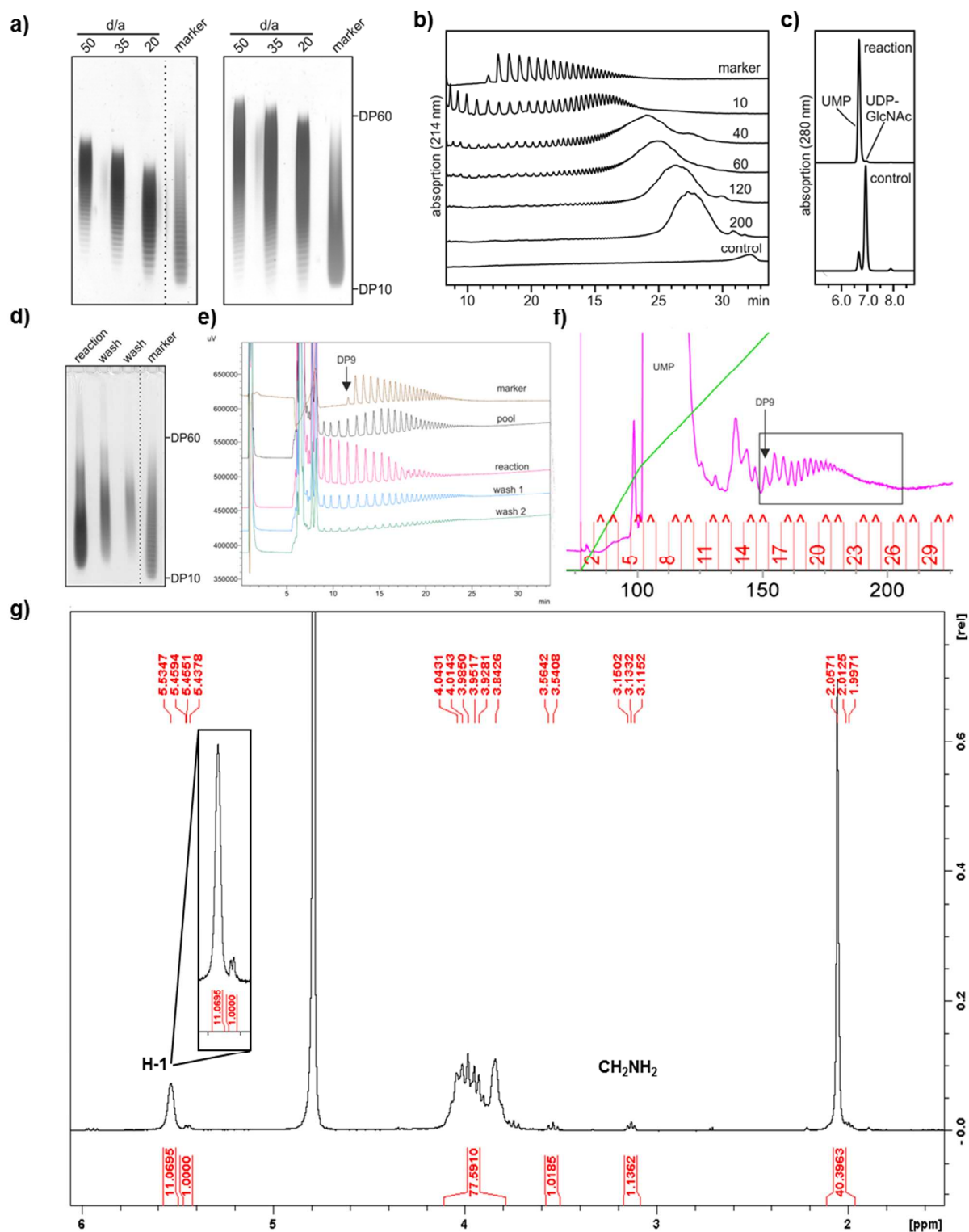


Figure 3. (A) Elongation of DP2-10 acceptor by $\Delta N58\Delta C98$ -CsxA (left) and $\Delta C98$ -CsxA (right) at different donor/acceptor (d/a) ratios. (B) HPLC-AEC (214 nm) of synthetic DP3 elongated by $\Delta N58\Delta C98$ -CsxA at the indicated d/a ratios. (C) HPLC-AEC (280 nm) demonstrates that the donor sugar UDP-GlcNAc is completely converted to UMP in a representative trimer elongation reaction. A reaction in the absence of acceptor was used as control. (D) Alcian blue/silver stained gel showing the reaction products after one column passage and two wash fractions in comparison to the marker $\Delta vDP15$. The gel shows an almost

1
2
3 overlapping profile between the targeted length and the formed oligomer after one column passage. (E)
4 HPLC-AEC (214 nm) showing the reaction mixture after one column passage, two wash fractions and all
5 fractions pooled (pool pre MonoQ) in comparison to avDP15 marker material. (F) Preparative AEC
6 (MonoQ) was used to separate UMP from the pool. Calibration of the column using DPs of known size
7 enabled the removal of DPs<9 to allow a maximal resemblance of the pooled fraction (pool post MonoQ)
8 to the marker material. (G) ¹H NMR spectrum (400 MHz, D₂O, 25°C) of the final product (pool post MonoQ)
9 indicating an avDP of 12.
10

11 12 13 **MenX oligosaccharide production**

14
15 During the last years, on-column enzyme immobilization came out as a novel tool to reduce the
16 high cost of enzyme production³⁶. The main advantage of immobilized enzymes is the possibility to
17 recover it at the end of the reaction for re-utilization and, consequently, an easier purification of the
18 products from residual enzyme³⁷.
19

20
21 In a previous study, ΔN58ΔC98-CsxA, a recombinant N- and C-terminally truncated CsxA
22 construct has already been proven suited for on-column immobilization and tailored synthesis of
23 CPSX oligosaccharides³⁴. ΔN58ΔC98-CsxA was reported to be superior to ΔC98-CsxA with regard
24 to expression level due to the missing, disordered N-terminal part of the protein, and was therefore
25 chosen also in this study for the solid-phase elongation of the synthetic trimer³⁴. First, by STD-
26 NMR the binding of MenX fragments with ΔN58ΔC98-CsxA was assessed to be over impossible
27 with that of ΔC98-CsxA (Figure S1, Supplemental information). Then, conditions for the
28 elongation were set up on a natural acceptor with DP2-10, monitoring the reaction with the Alcian
29 blue/silver stained PAGE analyses (Figure 3A)³⁸. As previously shown for ΔC98-CsxA³⁴, the
30 dispersity of the products generated at different donor to acceptor ratios (d/a) revealed a distributive
31 elongation. Notably, ΔN58ΔC98-CsxA allowed controlling the polymerization up to a size similar
32 to the marker material containing oligomers with DP10-60.
33

34
35 To determine optimal reaction conditions, ΔN58ΔC98-CsxA reactions at varying ratios between
36 UDP-GlcNAc (10 mM) and the trimer were performed and the products were analyzed by HPLC-
37 Anion Exchange Chromatography (AEC) (Figure 3B). The fact that the chain length of the
38 produced polysaccharides decreases with decreasing UDP-GlcNAc/trimer ratio demonstrates that
39 the linker does not impair the enzymes previously reported distributive elongation mechanism
40 (Figure 3B). Moreover, the high efficacy of the reaction remains unaltered as shown by the
41 complete conversion of UDP-GlcNAc to UMP (Figure 3C).
42

43
44 In a next step, the ΔN58ΔC98-CsxA construct was coupled to a HisTrap column via the C-terminal
45 His6-tag as recently described (Figure 4)³⁴. In our previous reports, we showed that 27 mg of
46 dN58dC98-CsxA are purified from 1 L of expression culture¹⁹, and 62.5 μg or 250 μg of enzyme
47
48
49
50
51
52

are enough to convert 12.4 mL of reaction mixture (containing 10 mM of UDP-GlcNAc) into the final product in an overnight or 4 h reaction²¹, respectively. In order to ensure the highest degree of conversion of the synthetic acceptor into the target oligomer in one column passage, we decided to load 100 mL of expression culture onto the column to transform 1.2 mL of reaction mixture into product, corresponding to more than a 100 fold excess of enzyme compared to the previous studies. The column was washed with binding buffer before the reaction mixture, containing the synthetic trimer and GlcNAc-UDP, was pumped through once (Figure 3C). According to the preceding tests (Figure 3B), a d/a ratio of ~15:1 was selected to target a size of avDP15. One passage through the column was sufficient to convert the acceptor into a product population similar to the target material, as verified by Alcian blue/silver stained PAGE (Figure 3D) and HPLC-AEC (Figure 3E). The material was purified by anion exchange chromatography (MonoQ) using a linear sodium chloride gradient³⁴. To allow maximal resemblance to the target material typically used in glycoconjugate preparations²⁰ (shown as marker in Figure 3E), only fractions containing DP>9 (eluting between 320-550 mM of NaCl) were collected, dialyzed and further characterized (Figure 3E-F).

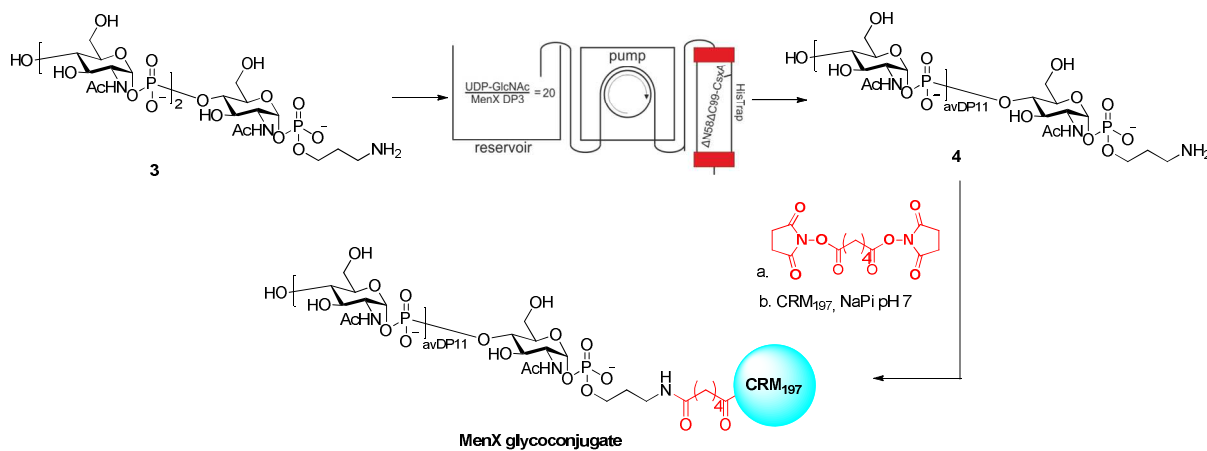


Figure 4. Reactions leading from trimer **3** to MenX glycoconjugate, which include on-column enzymatic elongation using GlcNAc-UDP as donor to give **4** and formation of an active ester for protein conjugation.

The average length of the obtained oligomers was established using NMR analysis (Figure 3F and Figure S1 Supplemental information). The ¹H NMR clearly showed a ratio of 1:11 between the H-1 of the downstream end modified with the linker, appearing as a doublet of doublets at 5.45 ppm ($J_{1,2} = 1.4, 7.3$ Hz), and the H-1 of the other anomeric positions at 5.53 ppm, indicating that an avDP of 12 was achieved with complete stereochemical control. In addition, the triplet at 3.13 ppm ($J = 6.7$ Hz) assigned to the CH_2NH_2 of the linker unambiguously confirmed the incorporation of the synthetic acceptor in the enzymatically elongated oligomer (Figure 3G). The avDP was confirmed

1
2
3 in the ^{31}P NMR spectrum of the obtained oligomer by the 1:11 ratio of the linker modified
4 phosphodiester signal compared to the signals of the other interchain phosphodiesters (Figure S3).
5 The discrepancy between the targeted avDP of 15 and the experimentally obtained avDP of 12
6 indicates that the incorporation of GlcNAc-UDP into the oligomer proceeded with a yield >80%,
7 when only one passage on the column was applied. Based on the FPLC (Figure 3F) profile the
8 avDP 12 contained a distribution from 9 up to 20 repeating units.
9
10
11
12
13
14
15

16 **Conjugation to CRM₁₉₇**

17 The presence of propyl-amine linker at the reducing end terminus renders the synthesized oligomer
18 ready for conjugation through a reported procedure based on the active ester chemistry (Figure 4)³⁹,
19 ⁴⁰. Briefly, the oligomer was reacted with an excess of the N-hydroxysuccinimidyl adipate in
20 DMSO:H₂O containing triethylamine. The half ester, quantitatively obtained by precipitation with
21 ethyl acetate and lyophilization, was then incubated overnight with the CRM₁₉₇ at a 25:1
22 saccharide/protein molar ratio.
23
24
25

26 SDS-Page gel electrophoresis showed occurrence of conjugation with no detectable presence of
27 unconjugated CRM₁₉₇ (Figure 5A). Western blot analysis with anti MenX serum confirmed that the
28 sugar was covalently linked to the protein.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Characteristics of glycoconjugates

| Glycoconjugate | Saccharide/protein (mol/mol) | Saccharide/protein (w/w) | Conjugation efficiency (%) |
|---|------------------------------|--------------------------|----------------------------|
| MenX-CRM ₁₉₇ <i>chemo-enzymatic</i> | 3.1 | 0.2 | 12 |
| MenX-CRM ₁₉₇ <i>enzymatic</i> | 3.2 | 0.2 | 32 |
| MenX-CRM ₁₉₇ <i>bacterial derived</i> | 4.1 | 0.3 | 34 |

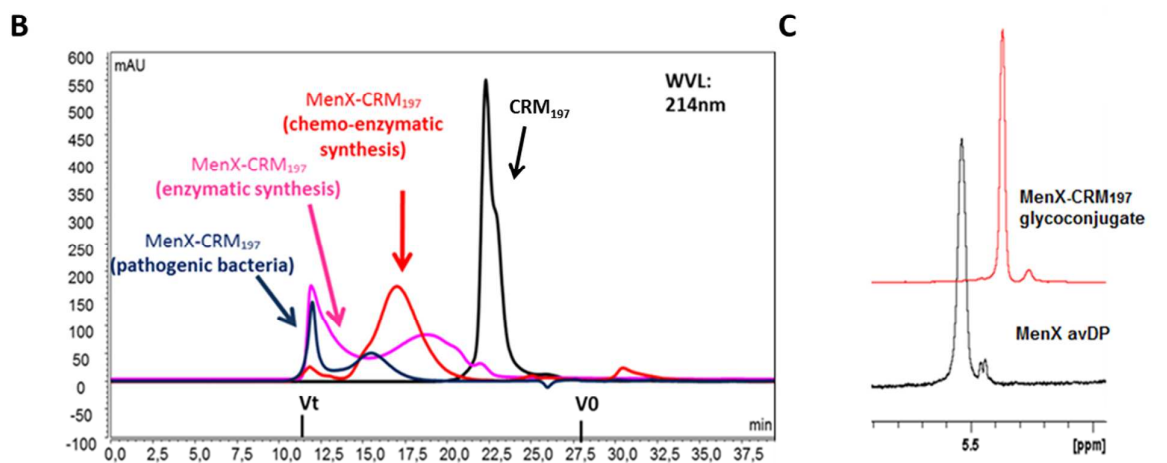


Figure 5. Characterization of the glycoconjugates: (A) SDS-PAGE gel electrophoresis and Western-Blot analysis of the generated glycoconjugate in comparison to the two previously reported controls^{15, 21}. (B) HPLC profile of the conjugates in size exclusion chromatography using absorption at 214 nm for detection. (C) Zoom of the H-1 signals of MenX oligosaccharide detected at ¹H NMR before (400 MHz, D₂O) and after conjugation (600 MHz, D₂O).

The formed glycoconjugate was purified by precipitation with ammonium sulfate, and characterized by microBCA and anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analyses for quantification of the protein and saccharide components respectively. The final product was characterized by a 0.2 w/w saccharide to protein ratio, corresponding to a 3.1 mol/mol ratio, that was similar to the conjugates previously reported (Table 1)^{15, 21}. The resulting conjugation efficiency of 12%, however, was lower than that achieved for the other MenX glycoconjugates (32-34%), where a 10-12:1 saccharide:protein ratio was used at higher protein concentrations. At the applied protein concentration (~5 mg/mL) an efficiency ranging from 20 to 50% has been achieved for a number of glycans^{39, 40}, and a glycosylation level similar to that obtained in this work has been reached also with lower (10-15:1) saccharide:protein ratios⁴¹. This suggests that scale up of the process under more concentrated conditions could improve the conjugation efficiency, minimizing the hydrolysis of the active ester which is in competition with the amide formation.

The HPLC size exclusion chromatography profile of the purified glycoconjugate showed a peak at lower retention time than the unconjugated protein and with a more homogeneous profile in

comparison with our previously produced glycoconjugates, presumably as result of the more defined length selected for conjugation and lower occurrence of cross linking in the coupling step (Figure 5B).

Moreover, ^1H NMR of the final glycoconjugate showed 1:11 ratio between H-1 of the modified downstream end and the H-1 of the other anomeric positions, comparable to the one of the starting oligomer (Figure 5C and Figure S2 Supplemental information). This result indicated that there was no discrimination of oligosaccharide lengths during the conjugation reaction.

Immunogenicity in mice

To test the immunogenicity of the novel glycoconjugate vaccine, a mouse immunization study was carried out. For this purpose, 1 μg saccharide dose was injected subcutaneously to eight female BALB/c mice (5-6 wk old) per group. Two positive controls were used in this experiment: a conjugate of MenX oligomers obtained from pathogenic bacteria (avDP15-20) and a conjugate prepared by enzymatic elongation of an acceptor produced from polysaccharide sizing (avDP10).

Three immunizations were conducted with two week intervals, whereby the vaccines were formulated in phosphate buffered saline, pH 7.2, in the presence of aluminum phosphate as adjuvant. After the second and third vaccination, anti MenX IgG levels were measured by ELISA. CPSX-CRM₁₉₇ glycoconjugate induced an immunogenic response comparable to the positive control with a significant booster effect after the third dose (Figure 6).

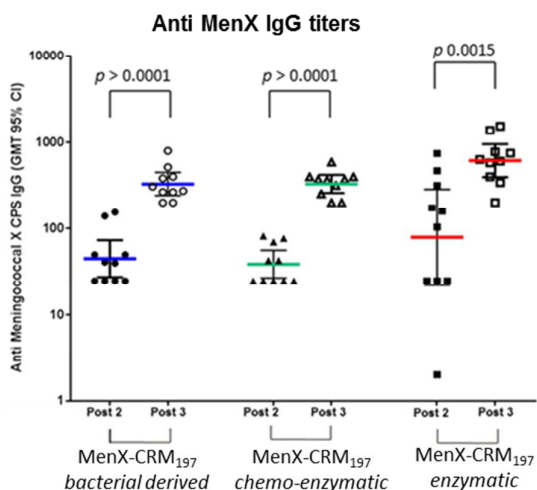


Table 2. Serum Bactericidal Assay

| Glycoconjugate | SBA Titers |
|---|------------|
| MenX-CRM ₁₉₇ <i>bacterial derived</i> | 8192 |
| MenX-CRM ₁₉₇ <i>chemo-enzymatic</i> | 4096 |
| MenX-CRM ₁₉₇ <i>enzymatic</i> | 4096 |

Figure 6. (A) Anti-MenX IgG ELISA titers after the second and third dose of glycoconjugates, using CPSX for coating. Each dot represents an individual animal. The horizontal bars indicate the geometric mean titer and 95% confidence intervals. Statistical analysis between the indicated groups was performed according to Mann–Whitney test; SBA mediated by rabbit complement on pooled sera are shown in Table 2.

1
2
3 The functional activity of the elicited antibodies against MenX strain after the third immunization
4 was evaluated determining the complement mediated bactericidal activity of the sera (Table 2)¹⁵.
5 Rabbit serum was used as complement source. The pooled serum obtained after immunization with
6 the chemo-enzymatically synthesized hapten showed a strong bactericidal activity (4096) against
7 MenX. This bactericidal effect was comparable to the previously reported effects induced with the
8 control glycoconjugates (8192 and 4096 for the glycoconjugate composed of polysaccharide from
9 the pathogen and from *in vitro* polymerization of a pathogenic derived acceptor, respectively)^{15, 21}.
10
11
12
13
14

15 **Conclusions**

16 Possible emergence of *N. meningitidis* serogroup X in Africa renders urgent the development of a
17 conjugate vaccine. Methods for fast, pathogen free production of the vaccine are desirable. Here we
18 describe a simple on-column procedure for the total synthesis of tailored and ready for coupling
19 MenX fragments. Starting with a functionalized synthetic trisaccharide acceptor and GlcNAc-UDP
20 as donor, the engineered capsule polymerase CsxA delivered oligomers with defined average
21 length, bearing at the downstream end a linker for conjugation to the carrier protein.
22
23
24
25
26

27 By qualitative mapping of the interactions between the enzyme and the trisaccharide with STD-
28 NMR, we demonstrated that the short synthetic molecule was engaged by the N-terminal portion of
29 the polymerase (Δ C98-CsxA) similarly to a longer fragment obtained by sizing of the natural
30 polymer. This portion is responsible for the distributive elongation mechanism. The optimized
31 construct Δ N58 Δ C98-CsxA was then immobilized on-column and utilized for controlled elongation
32 of the synthetic trisaccharide up to an avDP12. The produced oligomer was conjugated to the carrier
33 protein CRM₁₉₇ and tested in a mouse animal model resulting in strong production of bactericidal
34 antibodies.
35
36
37
38
39

40 The production of oligosaccharides composed of repeating units linked to each other through
41 phosphodiester bonds for vaccine manufacturing has been shown feasible by chemical methods,
42 such as polycondensation via H-phosphonate intermediates⁴². These approaches present some
43 important drawbacks: during the polycondensation reaction the formation of a linear product is in
44 competition with cyclization⁴³; the control of the stereochemistry is challenging when 1,2-cis bonds
45 are formed⁴⁶; finally the yield in the generation of phosphodiester bridges in some cases has been
46 observed to decrease at the increase of the length⁴⁴.
47
48
49
50

51 The described technology in a simple and cost efficient way generates fully synthetic
52 oligosaccharide antigens with controlled average length, thus avoiding the dangerous handling of
53 pathogens. Compared to conventional synthetic protocols, the number of steps needed to obtain the
54 oligomers in stereoselective manner are remarkably reduced. Furthermore, the presence of a linker
55
56
57
58
59
60

1
2
3 for conjugation in the synthetic acceptor minimizes the manipulations on the enzymatically
4 produced glycan, such as hydrolysis, sizing and reductive amination steps, which are typically
5 needed before coupling to a carrier protein. A variety of enzymes involved in bacterial capsular
6 biosynthesis are known^{45, 46} and enzymatic in vitro elongation has been shown feasible for both
7 homopolymers^{27, 47} and heteropolymers⁴⁴ from different meningococcal serogroups. Therefore this
8 approach is in principle applicable for the preparation of a number of glycans currently used to
9 produce vaccines against bacteria and other pathogens.
10
11

12
13
14 There are reports highlighting that small well-defined synthetic carbohydrate epitopes selected on
15 the basis of their binding with protective antibodies are sufficient to elicit a robust immune response
16 in an animal model^{24, 48, 49}. In absence of structural information on the polysaccharide antigenic
17 determinant, the described approach provided oligomers with a distribution optimized to induce a
18 protective immune response comparable to longer structures and higher than the trimer and tetramer
19 so far reported, indicating that relevant polysaccharide epitopes are fully covered.
20
21

22
23 Along with one-pot/automated solid-phase/enzymatic syntheses, this on-column elongation chemo-
24 enzymatic synthesis enriches the methods for expeditious construction of complex bacterial
25 carbohydrates.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Materials and methods

STD-NMR experiments. NMR experiments were carried out on a Bruker 500 MHz NMR instrument equipped with a TBI cooled probe at controlled temperature (± 0.1 K). Data acquisition and processing were performed using TOPSPIN 1.3 and 3.1 softwares, respectively. Suppression of water signal was achieved by excitation sculpting (2 msec selective square pulse). Proton-Carbon Saccharides resonances were assigned collecting both 1D (^1H and ^{13}C) and 2D (COSY, HSQC) experiments, using standard pulse sequences. STD-NMR experiments were acquired with 96 scans over 96 accumulations and spectral width of 6000 Hz (12 ppm) at 298 K; a saturation transfer of 2/4 sec was applied to enhance the saturation transfer effect, irradiating at a frequency of 8.0 and -2.0 ppm (3 different spectra recorded for each sample). No differences were observed in the STD spectra irradiating at 8.0 or -2.0 ppm (4000 and -1000 Hz respectively) for all samples, confirming that the saturation transfer effect was not irradiation dependent. To avoid pitfalls in the interpretation of STD-NMR spectra, a negative control spectrum was always recorded in absence of enzyme (ligand and buffer) at the very same condition (concentration and pH) of the enzyme-saccharide samples. The STD negative controls were always subtracted to the relative enzyme-saccharide STD spectrum, obtaining the STDD experiments.

For sample preparation, the enzymatic constructs were exchanged in the working buffer (Tris d-11 50 mM, 20 mM MgCl_2 , in D_2O at pH 8.0 ± 0.1) through 2 mL Zeba Spin desalting column saturated with 3 cycles of working buffer, enzymes were finally eluted at the same starting concentration of 1 mg/mL. The 100% of recovery was assessed through microBCA spectrophotometric assay. Trisaccharide acceptor was dissolved in the working buffer too. Ligand (saccharides): enzyme ratios were set from 1:50 up to 1:100 mol/mol, depending on the sample.

Chemo-enzymatic synthesis of MenX oligosaccharide.

Small scale reactions (Fig. 3b) using $\Delta\text{N58}\Delta\text{C98}$ -CsxA were performed as previously described³⁴. Briefly, 50-200 nM of enzyme was incubated in 75 μL of reaction buffer (50 mM Tris pH 8.0, 20 mM MgCl_2 , 1 mM DTT) in the presence of 10 mM UDP-GlcNAc and varying concentrations of the synthetic acceptor. Separation of the generated products by HPLC-AEC was performed on a Prominence UFLC-XR (Shimadzu) equipped with a CarboPac PA-100 column (2 x 250 mm, ThermoFisher) using an established method⁴⁷.

For solid-phase synthesis, $\Delta\text{N58}\Delta\text{C98}$ -CsxA was expressed in M15 (pREP4) over night at 15°C, as previously described³⁴. 50-100 mL of expression culture were pelleted, lysed, coupled to a HisTrap (GE Healthcare) column and thoroughly washed with (i) binding buffer (50 mM Tris pH 8.0, 500 mM NaCl, 35 mM imidazole, 1 mM DTT) and (ii) equilibration buffer (50 mM Tris pH 8.0, 1 mM

1
2
3 DTT) on an ÄKTA FPLC (GE Healthcare) until a stable base line was established. The HisTrap
4 column was connected to a peristaltic pump P1 (GE Healthcare), and 1.2 mL of reaction mixture
5 (50 mM Tris pH 8.0, 20 mM MgCl₂, 10 mM UDP-GlcNAc, containing 1.2 mg of synthetic trimer at
6 0.7 mM concentration) was pumped through with a flow-rate of 0.25-0.5 mL/min and fractions
7 were collected. Samples were analyzed by Alcian blue/silver stained PAGE and HPLC-AEC as
8 previously described^{34, 45} and saccharide containing fractions were pooled and further analyzed as
9 described below to give the final avDP 12 oligomer (with a conversion > 80%).
10
11
12
13
14

15
16 **CRM₁₉₇ conjugation of chemo-enzymatic obtained MenX oligosaccharide.** A solution of di-N-
17 hydroxysuccinimidyl adipate (10 eq) and triethylamine (0.2 eq) in DMSO was added to oligomer
18 (1.6 mg, 0.4 μmol). The reaction was stirred for 3h, then the product was precipitate at 0°C by
19 adding ethyl acetate (9 volumes). The solid was washed 10 times with ethyl acetate (2 volumes
20 each) and lyophilized. The activated sugar was conjugated to CRM₁₉₇ (1 mg, 0.017 μmol) in
21 sodium phosphate 100 mM at a protein concentration of 5 mg/ml. The glycoconjugate was dialyzed
22 against 10 mM sodium phosphate pH 7.2 and then precipitated with ammonium sulfate providing
23 450 μg of glycoconjugate (45%).
24
25
26
27
28
29

30
31 **Alcian blue/silver PAGE and SDS-PAGE gel electrophoresis.** SDS-Page has been performed on
32 pre-casted polyacrylamide gels (NuPAGE Invitrogen) that can be used with different
33 polyacrylamide concentration, from 3-8% gradient to 7%. The electrophoretic runs have been
34 performed in Tris-Acetate SDS running buffer (NuPAGE Invitrogen) loading 2.5-5 μg of protein
35 each sample, using the electrophoretic chamber with a voltage of 150V for about 40 minutes. After
36 electrophoretic running, the gel has been washed in H₂O for 3 times and then with dye comassie.
37
38
39
40

41
42 **Western Blot analysis.** SDS-Page was run as described before. Gel was transferred on cellulose
43 with iBlot gel transfer stacks nitrocellulose kit and washed 5 times with PBS 1x pH 7.2 + 0,05%
44 tween and blocked with PBS 1x pH 7.2 + BSA 1%. Pooled serum from MenX-TT conjugate was
45 used as primary antibody (1:500 dilution, 1h) followed by 5 washes with PBS + tween 0,05%
46 buffer. Anti-mouse IgG alkaline phosphatase was used as secondary antibody (1:2000 dil., 30min)
47 followed by 5 washes with PBS + tween 0.05% buffer. Western-Blot was finally developed with
48 AP conjugate substrate kit (BIORAD).
49
50
51
52
53

54
55 **HPAEC-PAD analysis.** As previously described⁵⁰, standard samples of MenX PS at five increasing
56 concentrations ranging between 0.2 and 8.0 μg/mL were prepared to build the calibration curve.
57
58
59
60

1
2
3 MenX samples were prepared targeting the calibration curve midpoint. The reference and analytical
4 samples were prepared in 2 M trifluoroacetic acid, incubated at 100°C for 2.5h, followed by 30min
5 at 4°C and finally quenched with 2 M sodium hydroxide. All analytical samples were filtered with
6 0.45 µm Phenex-NY (Phenomenex) filters before analysis. HPAEC-PAD analysis was performed
7 with a Dionex ICS-5000 equipped with a CarboPac PA1 column (4 × 250 mm; Dionex) coupled
8 with a PA1 guard to column (4 × 50 mm; Dionex). Samples (50 µL injection volume) were run at 1
9 mL/min, using isocratic elution with 14 mM NaOH, followed by a washing step with 0.5 M NaOH.
10 In both HPAEC-PAD analyses, the effluent was monitored using an electrochemical detector in the
11 pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A
12 quadruple-potential waveform for carbohydrates was applied. The resulting chromatographic data
13 were processed using Chromeleon software 6.8 (Dionex). GlcNAc-4-phosphate⁵¹ concentration
14 were determined and converted in µmol/mL to estimate the relative mol/mol ratio.
15
16
17
18
19
20
21
22
23

24 **Size Exclusion High Performance Liquid Chromatography (SEC-HPLC).** SEC-HPLC has been
25 performed on UltiMate 3000 HPLC system (Dionex part of Thermo Fisher Scientific) equipped
26 with a PDA, RF2000 Fluorescence Detector and RI-101 Shodex Detector. Chromatography has
27 been performed in 0.1 M NaSO₄, 0.1 M NaPi, 5% CH₃CN, pH 7.2 on TSK 3000SWXL (Tosoh
28 Bioscience) analytical column at flow rate of 0.5 ml/min, with 50 µL of injection volume loading
29 15-25 µg of sample in protein content. The resulting chromatographic data were processed using
30 Chromeleon 6.8 software.
31
32
33
34
35

36 **Immunization of mice.** Protocols were approved by the Italian Ministry of Health (Approval
37 number n. 804/2015-PR). All mice were housed under specific pathogen-free conditions at the GSK
38 Vaccines Animal Resource Center in compliance with the relevant guidelines (Italian Legislative
39 Decree n 26/2014). Three groups of eight female BALB/c mice were immunized by subcutaneous
40 injection of glycoconjugates at 1 µg dose in saccharide content using alum phosphate as an
41 adjuvant. Mice received the vaccines at days 1, 14 and 28. Sera were bled at days 0, 27 and 42.
42
43
44
45
46
47

48 **ELISA of sera.** Microtiter plates (96 wells, NUNC, Maxisorp) were coated with 100 µL of 5µg/mL
49 of MenX CPS in Phosphate Buffered Saline (PBS) pH 8.2. Plates were incubated overnight at 2-
50 8°C, washed three times with PBST (0.05% Tween-20 in PBS pH 7.4) and saturated with 100
51 µL/well of PBST-B (3% Bovine Serum Albumin-BSA in PBST) for 60 min at 37°C. The plates
52 were then aspirated to remove the solution and re-washed three times with PBST. 200 µl/well of
53 pre-diluted serum (1:25 for pre immune (negative control), 1:100-1:200 for a reference serum and
54
55
56
57
58
59
60

1
2
3 from 1:50 to 1:500 for test sera) was added in the first well of each column of the plate, while on the
4 other wells 100 μ l of TPBS has been dispensed. Eight two-fold serial dilutions along each column
5 were then performed by transferring from well to well 100 μ l of sera solutions. Following primary
6 antibody dilution, plates were incubated for 2 h at 37° C. After three washes with TPBS, 100 μ l/well
7 TPBS solutions of secondary antibody alkaline phosphates conjugates (anti mouse IgG 1:10000,
8 Sigma-Aldrich) were added, and the plates incubated 1 h at 37° C. Three more washes with TPBS
9 were done, then 100 μ l/well of a 1 mg/ml of p-NPP (Sigma) in a 0.5 M di-ethanolamine buffer pH
10 9.6 was added. After 30 min of incubation at room temperature, plates were read at 405 nm using a
11 Molecular Devices plate reader. Raw data acquisition has been performed by Softmax Pro 5.4. Sera
12 titers were expressed as the reciprocal of sera dilution corresponding to a cut-off OD = 1. Each
13 immunization group was represented as the geometrical mean (GMT) with 95% CI of the single
14 mouse titers. The statistical and graphical analysis was performed by GraphPad Prism 6.0 software.
15
16
17
18
19
20
21
22
23

24 **Serum bactericidal assay (rSBA).** A commercial lot of baby rabbit complement was used as
25 source of active complement. *N. meningitidis* strain was grown overnight on chocolate agar plates at
26 37°C in 5% CO₂. Colonies were inoculated in Mueller-Hinton broth, containing 0.25% glucose to
27 reach an OD₆₀₀ of 0.05-0.08 and incubated at 37°C with shaking. When bacterial suspension
28 reached OD₆₀₀ of 0.25-0.27, bacteria were diluted in the assay buffer (Dulbecco's Phosphate-
29 Buffered Saline, DPBS) with MgCl₂ and CaCl₂ with 1% BSA) at the working dilution (ca. 10⁴
30 CFU/mL). The total volume in each well was 50 μ L with 25 μ L of serial two-fold dilutions of the
31 test serum, 12.5 μ L of bacteria at the working dilution and 12.5 μ L of baby rabbit complement. All
32 sera to test were heat-inactivated for 30 minutes at 56°C. Negative controls included bacteria
33 incubated, separately, with the complement serum without the test serum and with test sera and the
34 heat-inactivated complement. Immediately after the addition of the baby rabbit complement,
35 negative controls were plated on Mueller-Hinton agar plates (time 0). Plates were incubated for 1
36 hour at 37°C, then each sample was spotted in duplicate on Mueller-Hinton agar plates (time 1).
37 Agar plates were incubated overnight at 37°C and the colonies corresponding to time 0 and time 1
38 (surviving bacteria) were counted. The serum bactericidal titer was defined as the serum dilution
39 resulting in 50% decrease in colony forming units (CFU) per mL, after 60 min incubation of
40 bacteria in the reaction mixture, compared to control CFU per mL at time 0. The reference strain
41 meningococcal serotype X was Z9615.
42
43
44
45
46
47
48
49
50
51
52
53
54
55

56 Supporting Information

57
58
59
60

The Supporting Information, including additional STD NMR spectra and characterization of the synthesized MenX oligomer, is available free of charge on the ACS Publications website.

References

1. Boisier, P., Nicolas, P., Djibo, S., Taha, M. K., Jeanne, I., Mainassara, H. B., Tenebray, B., Kairo, K. K., Giorgini, D., and Chanteau, S. (2007) Meningococcal meningitis: unprecedented incidence of serogroup X-related cases in 2006 in Niger, *Clin. Infect. Dis.* *44*, 657-663.
2. Mutonga, D. M., Pimentel, G., Muindi, J., Nzioka, C., Mutiso, J., Klena, J. D., Morcos, M., Ogaro, T., Materu, S., Tetteh, C., Messonnier, N. E., Breiman, R. F., and Feikin, a. D. R. (2009) Epidemiology and risk factors for serogroup X meningococcal meningitis during an outbreak in Western Kenya, 2005–2006, *Am. J. Trop. Med. Hyg.* *80*, 619-624.
3. Delrieu, I., Yaro, S., Tamekloe, T. A., Njanpop-Lafourcade, B. M., Tall, H., Jaillard, P., Ouedraogo, M. S., Badziklou, K., Sanou, O., Drabo, A., Gessner, B. D., Kambou, J. L., and Mueller, J. E. (2011) Emergence of epidemic Neisseria meningitidis serogroup X meningitis in Togo and Burkina Faso, *PLoS One* *6*, e19513.
4. Castillo, C. M. d., Vazquez, J. A., Romero, J., and Pascual, A. (2003) Infections by Neisseria meningitidis serogroup X in Spain, *Clin. Microbiol. Infect.* *9*, 964-965.
5. Chen, C., and Huang, Y. (2008) A first meningococcal meningitis case caused by serogroup X Neisseria meningitidis strains in China, *Chin. Med. J.* *7*, 664-666.
6. Costantino, P., Rappuoli, R., and Berti, F. (2011) The design of semi-synthetic and synthetic glycoconjugate vaccines, *Expert Opin. Drug Discov.* *6*, 1045-1066.
7. Delea, T. E., Weycker, D., Atwood, M., Neame, D., Alvarez, F. P., Forget, E., Langley, J. M., and Chit, A. (2017) Cost-effectiveness of alternate strategies for childhood immunization against meningococcal disease with monovalent and quadrivalent conjugate vaccines in Canada, *PLoS One* *12*, e0175721.
8. Stephens, D. S., Greenwood, B., and Brandtzaeg, P. (2007) Epidemic meningitis, meningococcaemia, and Neisseria meningitidis, *Lancet* *369*, 2196-2210.
9. LaForce, M. F., Ravenscroft, N., Djingarey, M., and Viviani, S. (2009) Epidemic meningitis due to Group A Neisseria meningitidis in the African meningitis belt: a persistent problem with an imminent solution, *Vaccine* *27 Suppl 2*, B13-19.
10. Frasc, C. E., Preziosi, M. P., and LaForce, F. M. (2012) Development of a group A meningococcal conjugate vaccine, MenAfriVac(TM), *Hum. Vaccin. Immunother.* *8*, 715-724.
11. Trotter, C. L., Lingani, C., Fernandez, K., Cooper, L. V., Bitá, A., Tevi-Benissan, C., Ronveaux, O., Préziosi, M.-P., and Stuart, J. M. (2017) Impact of MenAfriVac in nine countries of the African meningitis belt, 2010–15: an analysis of surveillance data, *Lancet Infect. Dis.* *17*, 867-872.
12. Ni, J. D., Jin, Y. H., Dai, B., Wang, X. P., Liu, D. Q., Chen, X., Zheng, Y., and Ye, D. Q. (2008) Recent epidemiological changes in meningococcal disease may be due to the displacement of serogroup A by serogroup C in Hefei City, China, *Postgrad Med J* *84*, 87-92.
13. Ji, X., Yao, P. P., Zhang, L. Y., Li, Y., Xu, F., Mei, L. L., Zhu, S. R., Zhang, Y. J., Zhu, H. P., and van der Veen, S. (2017) Capsule switching of Neisseria meningitidis sequence type 7 serogroup A to serogroup X, *J Infect.* *75*, 521-531.
14. La Force, M. (2017) Progress on MenACWYX vaccine: Phase 1 trial results, *MRF Conference*. , www.meningitis.com.
15. Micoli, F., Romano, M. R., Tontini, M., Cappelletti, E., Gavini, M., Proietti, D., Rondini, S., Swennen, E., Santini, L., Filippini, S., Balocchi, C., Adamo, R., Pluschke, G., Norheim, G., Pollard, A., Saul, A., Rappuoli, R., MacLennan, C. A., Berti, F., and Costantino, P. (2013) Development of a glycoconjugate vaccine to prevent meningitis in Africa caused by meningococcal serogroup X, *Proc. Natl. Acad. Sci. U.S.A.* *110*, 19077-19082.
16. Morelli, L., and Lay, L. (2013) Synthesis of Neisseria meningitidis X capsular polysaccharide fragments, *Arkivoc*, 166-184.
17. Morelli, L., Cancogni, D., Tontini, M., Nilo, A., Filippini, S., Costantino, P., Romano, M. R., Berti, F., Adamo, R., and Lay, L. (2014) Synthesis and immunological evaluation of protein conjugates of neisseria meningitidis X capsular polysaccharide fragments, *Beilstein J. Org. Chem.* *10*, 2367-2376.
18. Harale, K. R., Dumare, N. B., Singh, D., Misra, A. K., and Chhikara, M. K. (2015) Synthesis of a tetrasaccharide and its glycoconjugate corresponding to the capsular polysaccharide of Neisseria meningitidis serogroup X and its immunochemical studies, *RSC Adv.* *5*, 41332-41340.

19. Fiebig, T., Berti, F., Freiberger, F., Pinto, V., Claus, H., Romano, M. R., Proietti, D., Brogioni, B., Stummeyer, K., Berger, M., Vogel, U., Costantino, P., and Gerardy-Schahn, R. (2014) Functional expression of the capsule polymerase of *Neisseria meningitidis* serogroup X: a new perspective for vaccine development, *Glycobiology* *24*, 150-158.
20. Broker, M., Dull, P. M., Rappuoli, R., and Costantino, P. (2009) Chemistry of a new investigational quadrivalent meningococcal conjugate vaccine that is immunogenic at all ages, *Vaccine* *27*, 5574-5580.
21. Fiebig, T., Romano, M. R., Oldrini, D., Adamo, R., Tontini, M., Brogioni, B., Santini, L., Berger, M., Costantino, P., Berti, F., and Gerardy-Schahn, R. (2016) An efficient cell free enzyme-based total synthesis of a meningococcal vaccine candidate, *npj Vaccines* *1*, 16017.
22. Seeberger, P. H. (2015) The logic of automated glycan assembly, *Acc Chem Res* *48*, 1450-1463.
23. Hahm, H. S., Schlegel, M. K., Hurevich, M., Eller, S., Schuhmacher, F., Hofmann, J., Pagel, K., and Seeberger, P. H. (2017) Automated glycan assembly using the Glycoeer 2.1 synthesizer, *Proc. Natl. Acad. Sci. U.S.A.* *114*, E3385-E3389.
24. Schumann, B., Hahm, H. S., Parameswarappa, S., G., Reppe, K., Wahlbrink, A., Govindan, S., Kaplone, P., Pirofski, L.-a., Witzentrath, M., Anish, C., Pereira, C. L., and Seeberger, P. H. (2017) A semisynthetic *Streptococcus pneumoniae* serotype 8 glycoconjugate vaccine, *Sci. Transl. Med.* *9*, eaaf5347
25. Classen, T., and Pietruszka, J. (2017) Complex molecules, clever solutions - Enzymatic approaches towards natural product and active agent syntheses, *Bioorg Med Chem.*
26. Adamo, R. (2017) Advancing homogeneous antimicrobial glycoconjugate vaccines, *Acc Chem Res* *50*, 1270-1279.
27. McCarthy, P. C., Saksena, R., Peterson, D. C., Lee, C. H., An, Y., Cipollo, J. F., and Vann, W. F. (2013) Chemoenzymatic synthesis of immunogenic meningococcal group C polysialic acid-tetanus Hc fragment glycoconjugates, *Glycoconj. J.* *30*, 857-870.
28. Li, L., Liu, Y., Ma, C., Qu, J., Calderon, A. D., Wu, B., Wei, N., Wang, X., Guo, Y., Xiao, Z., Song, J., Sugiarto, G., Li, Y., Yu, H., Chen, X., and Wang, P. G. (2015) Efficient Chemoenzymatic Synthesis of an N-glycan Isomer Library, *Chem. Sci.* *6*, 5652-5661.
29. Wang, Z., Chinoy, Z. S., Ambre, S. G., Peng, W., McBride, R., de Vries, R. P., Glushka, J., Paulson, J. C., and Boons, G. J. (2013) A general strategy for the chemoenzymatic synthesis of asymmetrically branched N-glycans, *Science* *341*, 379-383.
30. Yan, F., Wakarchuk, W. W., Gilbert, M., Richards, J. C., and Whitfield, D. M. (2000) Polymer-supported and chemoenzymatic synthesis of the *Neisseria meningitidis* pentasaccharide: a methodological comparison, *Carbohydr. Res.* *328*, 3-16.
31. Pozsgay, V., Gaudino, J., Paulson, J. C., and Jennings, H. J. (1991) Chemo-enzymatic synthesis of a branching decasaccharide fragment of the capsular polysaccharide of type III Group B *Streptococcus*, *Bioorg. Med. Chem. Lett.* *1*, 991-994.
32. Salamone, S., Guerreiro, C., Cambon, E., Andre, I., Remaud-Simeon, M., and Mulard, L. A. (2015) Programmed chemo-enzymatic synthesis of the oligosaccharide component of a carbohydrate-based antibacterial vaccine candidate, *Chem. Commun.* *51*, 2581-2584.
33. Johnson, K. F. (1999) Synthesis of oligosaccharides by bacterial enzymes, *Glycoconj. J.* *16*, 141-146.
34. Fiebig, T., Litschko, C., Freiberger, F., Bethe, A., Berger, M., and Gerardy-Schahn, R. (2018) Efficient solid-phase synthesis of meningococcal capsular oligosaccharides enables simple and fast chemoenzymatic vaccine production, *J. Biol. Chem.* *293*, 953-962.
35. Costantino, P., Viti, S., Podda, A., Velmonte, M. A., Nencioni, L., and Rappuoli, R. (1992) Development and phase 1 clinical testing of a conjugate vaccine against meningococcus A and C, *Vaccine* *10*, 691-698.
36. Datta, S., Christena, L. R., and Rajaram, Y. R. (2013) Enzyme immobilization: an overview on techniques and support materials, *Biotech* *3*, 1-9.
37. Karav, S., Cohen, J. L., Barile, D., and de Moura Bell, J. M. (2017) Recent advances in immobilization strategies for glycosidases, *Biotechnol. Prog.* *33*, 104-112.
38. Min, H., and Cowman, M. K. (1985) Combined alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: applicati onto electrophoretic of molecular weight distribution, *Anal. Biochem.* *155*, 275-285.
39. Gao, Q., Tontini, M., Brogioni, G., Nilo, A., Filippini, S., Harfouche, C., Polito, L., Romano, M. R., Costantino, P., Berti, F., Adamo, R., and Lay, L. (2013) Immunoactivity of protein conjugates of carba analogues from *Neisseria meningitidis* a capsular polysaccharide, *ACS Chem. Biol.* *8*, 2561-2567.
40. Adamo, R., Romano, M. R., Berti, F., Leuzzi, R., Tontini, M., Danieli, E., Cappelletti, E., Cakici, O. S., Swennen, E., Pinto, V., Brogioni, B., Proietti, D., Galeotti, C. L., Lay, L., Monteiro, M. A., Scarselli, M., and

- 1
2
3 Costantino, P. (2012) Phosphorylation of the synthetic hexasaccharide repeating unit is essential for the
4 induction of antibodies to Clostridium difficile PSII cell wall polysaccharide, *ACS Chem Biol* 7, 1420-1428.
- 5 41. Tontini, M., Romano, M. R., Proietti, D., Balducci, E., Micoli, F., Balocchi, C., Santini, L., Masignani,
6 V., Berti, F., and Costantino, P. (2016) Preclinical studies on new proteins as carrier for glycoconjugate
7 vaccines, *Vaccine* 34, 4235-4242.
- 8 42. Verez-Bencomo, V., Fernandez-Santana, V., Hardy, E., Toledo, M. E., Rodriguez, M. C., Heynngnezz,
9 L., Rodriguez, A., Baly, A., Herrera, L., Izquierdo, M., Villar, A., Valdes, Y., Cosme, K., Deler, M. L.,
10 Montane, M., Garcia, E., Ramos, A., Aguilar, A., Medina, E., Torano, G., Sosa, I., Hernandez, I., Martinez,
11 R., Muzachio, A., Carmenates, A., Costa, L., Cardoso, F., Campa, C., Diaz, M., and Roy, R. (2004) A
12 synthetic conjugate polysaccharide vaccine against Haemophilus influenzae type b, *Science* 305, 522-525.
- 13 43. Nikolaev, A. V., Chudek, J. A., and Ferguson, M. A. J. (1995) The chemical synthesis of Leishmania
14 donovani phosphoglycan via polycondensation of a glycobiosyl hydrogenphosphonate monomer, *Carbohydr.*
15 *Res.* 272 179-189.
- 16 44. Slattegard, R., Teodorovic, P., Kinf, H. H., Ravenscroft, N., Gammon, D. W., and Oscarson, S. (2005)
17 Synthesis of structures corresponding to the capsular polysaccharide of Neisseria meningitidis group A, *Org*
18 *Biomol Chem* 3, 3782-3787.
- 19 45. Litschko, C., Romano, M. R., Pinto, V., Claus, H., Vogel, U., Berti, F., Gerardy-Schahn, R., and Fiebig,
20 T. (2015) The capsule polymerase CslB of Neisseria meningitidis serogroup L catalyzes the synthesis of a
21 complex trimeric repeating unit comprising glycosidic and phosphodiester linkages, *J Biol. Chem.* 290,
22 24355-24366.
- 23 46. Willis, L. M., and Whitfield, C. (2013) Structure, biosynthesis, and function of bacterial capsular
24 polysaccharides synthesized by ABC transporter-dependent pathways, *Carbohydr. Res.* 378, 35-44.
- 25 47. Fiebig, T., Freiberger, F., Pinto, V., Romano, M. R., Black, A., Litschko, C., Bethe, A., Yashunsky, D.,
26 Adamo, R., Nikolaev, A., Berti, F., and Gerardy-Schahn, R. (2014) Molecular cloning and functional
27 characterization of components of the capsule biosynthesis complex of Neisseria meningitidis serogroup A:
28 toward in vitro vaccine production, *J. Biol. Chem.* 289, 19395-19407.
- 29 48. Safari, D., Dekker, H. A., Joosten, J. A., Michalik, D., de Souza, A. C., Adamo, R., Lahmann, M.,
30 Sundgren, A., Oscarson, S., Kamerling, J. P., and Snippe, H. (2008) Identification of the smallest structure
31 capable of evoking opsonophagocytic antibodies against Streptococcus pneumoniae type 14, *Infect. Immun.*
32 76, 4615-4623.
- 33 49. Broecker, F., Hanske, J., Martin, C. E., Baek, J. Y., Wahlbrink, A., Wojcik, F., Hartmann, L.,
34 Rademacher, C., Anish, C., and Seeberger, P. H. (2016) Multivalent display of minimal Clostridium difficile
35 glycan epitopes mimics antigenic properties of larger glycans, *Nat. Commun.* 7, 11224.
- 36 50. Micoli, F., Adamo, R., Proietti, D., Gavini, M., Romano, M. R., MacLennan, C. A., Costantino, P., and
37 Berti, F. (2013) Meningococcal X polysaccharide quantification by high-performance anion-exchange
38 chromatography using synthetic N-acetylglucosamine-4-phosphate as standard, *Anal Biochem* 442, 259-261.
- 39 51. Adamo, R., Micoli, F., Proietti, D., and Berti, F. (2014) Efficient synthesis of meningococcal X
40 polysaccharide repeating unit (N-Acetylglucosamine-4-phosphate) as analytical standard for polysaccharide
41 determination, *Synth. Commun.* 44, 1266-1273.
- 42
43

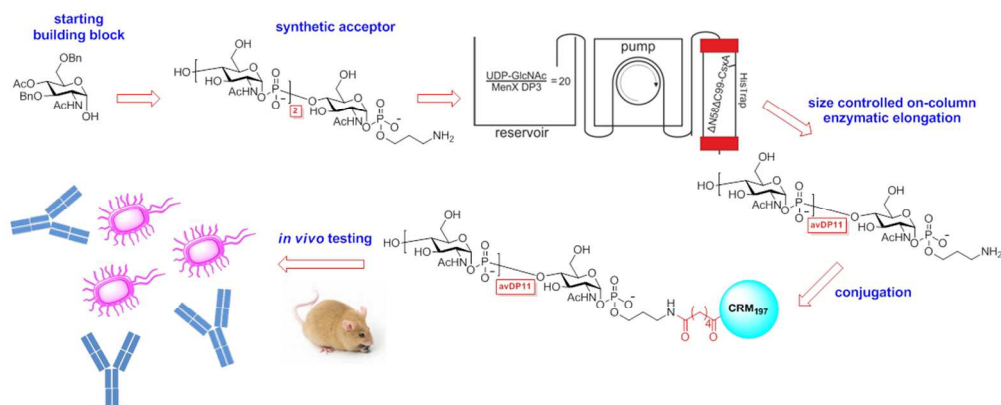
44 **Author contributions**

45
46 LL, RGS, TF, FB and RA conceived and designed the experiments; DO, MRR, TF, DP, MB, MT,
47 LS, LM, RDR performed the experiments; MRR, RGS, TF, FB and RA analyzed the results; DO,
48 TF and RA wrote the manuscript which was reviewed and approved by all the authors.
49

50 **Notes**

51
52
53
54 This work was done under a cooperative research and development agreement between Novartis
55 Vaccines and Hannover Medical School; in March 2015 the Novartis non-influenza Vaccines
56 business was acquired by the GSK group of companies which was involved in all stages of the
57
58
59

1
2
3 study conduct and analysis. DO is a student at the University of Rome and participates in a post
4 graduate studentship program at GSK. LM did a postgraduate internship at Novartis Vaccines. DO,
5 MRR, DP, MT, LS, RDR, FB and RA are employees of the GSK group of companies. TF, MB and
6 RGS are listed as inventors of a patent on the described topic.
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



305x121mm (96 x 96 DPI)