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Articles

Sequential Linkage of Carbohydrate Antigens to Mimic Capsular Polysaccharides: Toward Semisynthetic Glycoconjugate Vaccine Candidates against *Streptococcus pneumoniae* Serotype 14

Bruna M. S. Seco, Fei-Fei Xu, Andrea Grafmüller, Naresh Kottari, Claney L. Pereira, and Peter H. Seeberger*

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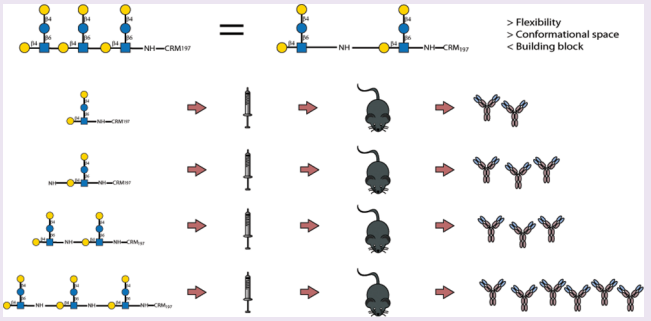
ABSTRACT: Vaccines based on isolated polysaccharides successfully protect humans from bacterial pathogens such as *Streptococcus pneumoniae*. Because polysaccharide production and isolation can be technically challenging, glycoconjugates containing synthetic antigens are an attractive alternative. Typically, the shortest possible oligosaccharide antigen is preferable as syntheses of longer structures are more difficult and time-consuming. Combining several protective epitopes or polysaccharide repeating units as blocks by bonds other than glycosidic linkages would greatly reduce the synthetic effort if the immunological response to the polysaccharide could be retained. To explore this concept, we bridged the well-understood and immunologically potent RU of *S. pneumoniae* serotype 14 (ST14) with an aliphatic spacer and conjugated it to the carrier protein CRM197. Mice immunized with the spacer-bridged glycan conjugates produced high levels of specific antibodies after just one or two vaccine doses, while the tetrasaccharide repeating unit alone required three doses. The antibodies recognized specifically ST14 CPS, while no significant antibody levels were raised against the spacer or unrelated CPS. Synthetic vaccines generated antibodies with opsonic activity. Mimicking polysaccharides by coupling repeating unit antigens via an aliphatic spacer may prove useful also for the development of other glycoconjugate vaccine candidates, thereby reducing the synthetic complexity while enhancing a faster immune response.



Bacterial capsular polysaccharides (CPS) comprised of repeating units have been identified as major virulence factors of bacterial pathogens.[1] Polysaccharide and glycoconjugate vaccines based on isolated CPS induce a protective immune response in people and prevent millions of deaths every year caused by pathogenic bacteria such as *Streptococcus pneumoniae*.[2] Almost all currently marketed vaccines rely on CPS isolated from bacterial culture. The isolation and purification of CPS from pathogens in sufficient quantities[3,4] can be challenging as other cellular polysaccharides are frequently found in CPS preparations even though their implications on the human immune system are not known. Certain CPS degrade during isolation or formulation, thus rendering the vaccine ineffective.[5−8] Semisynthetic glycoconjugate vaccines,[9,10] containing a synthetic oligosaccharide antigen resembling the CPS coupled to a carrier protein, have emerged as an attractive option with great potential for understanding glycan immunology and rationally designing efficacious bacterial vaccines.

A key consideration during synthetic vaccine design is antigen length. While CPS are many hundreds or thousands of monosaccharides in length, a majority of CPS repeating units vary fom two to six monosaccharides. Usually, just one or two synthetic repeating units are suffient to induce a protective immune response.[11] To produce specific antibodies against the CPS, B cells need to be activated and differentiated after binding to the glycoconjugate. The process relies primarily on B cell receptors (BCR) to sense the foreign antigen triggering a molecular cascade turning naïve B cells into mature antigen-specific B cells.[12] Numerous studies have shown that multivalent antigens are more efficient than monovalent antigens in promoting an antibody response, mainly because it induces BCR clustering leading to greater signal activation and consequently B cell differentiation.[13−16] Thus, earlier and stronger immune response improvement is crucial mainly when targeting newborns and infants.

Given the challenges associated with the synthesis of oligosaccharide antigens of increasing length, identifying

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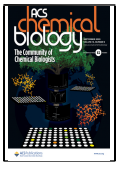

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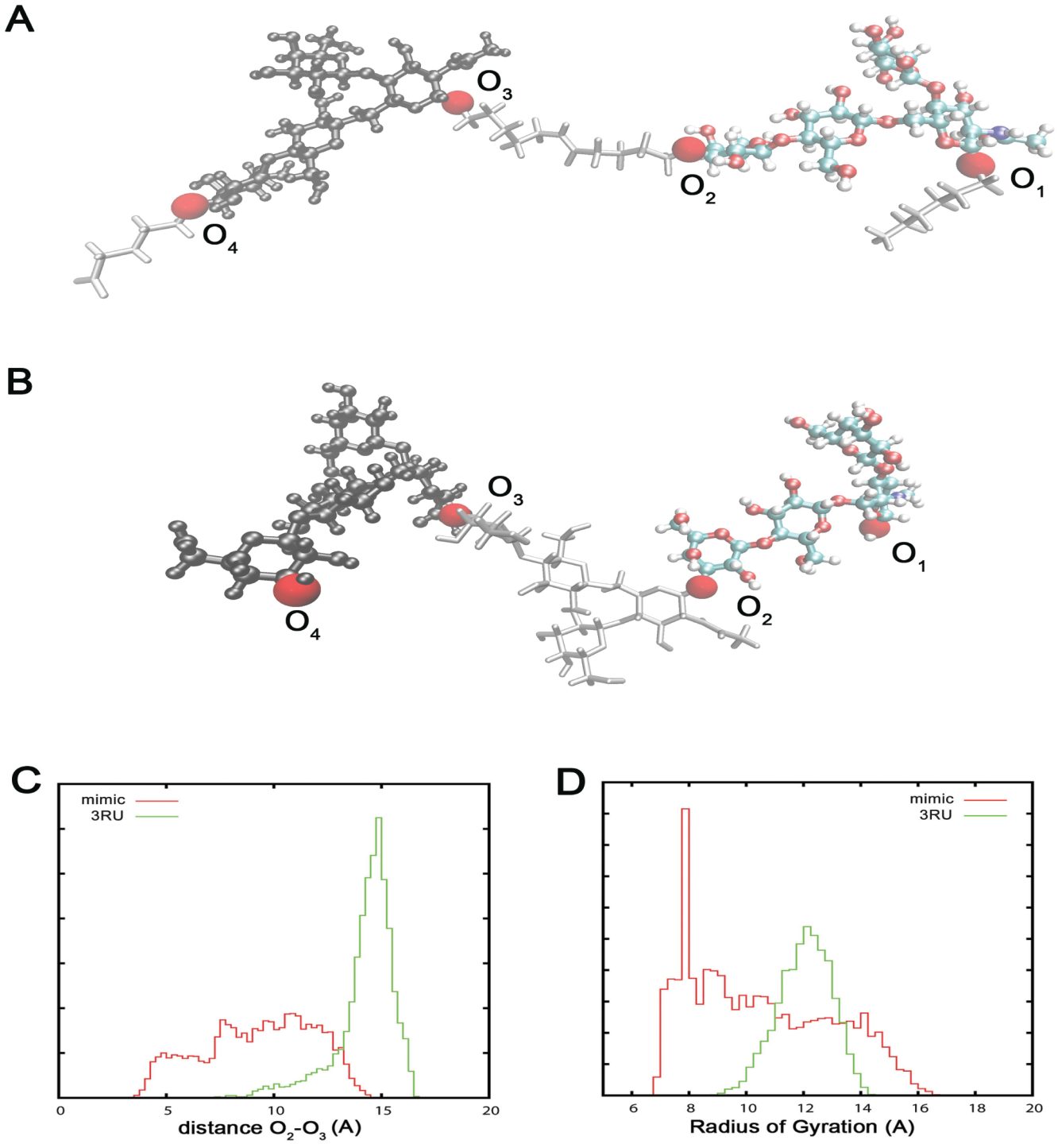

Figure 1. Molecular dynamcs simulations of the ST14 polysaccharide and ST14 polysaccharide mimic. (A and B) Simulation snapshots of the divalent ST14 mimic and a three-repeat unit ST14 polysaccharide, respectively. Comparison of the distance distributions from 1μs of simulation data for the distance between O2 and O3 corresponding to the effective linker lenght (C) and the radius of gyration (D). A complete set of the distance distributions between highlighted oxigen atoms (O1-O4) are shown in the Supporting Information.

minimal oligosaccharide epitopes is important. It remains to be seen whether an oligosaccharide chimera consisting of oligosaccharide repeating units connected via simple spacers is sufficient to induce a strong immune response associated with the specific response to polysaccharides. To address this question at the conceptual level, we focused on the capsular polysaccharide of *S. pneumoniae* serotype 14 (ST14). *S. pneumoniae* are Gram-positive bacteria that cause severe invasive pneumococcal diseases (IPDs) such as pneumonia, septicemia, meningitis, and otitis media.[17−20] ST14 is the most common of the more than 95 serotypes in the human population[21] and accounts for ≤29% of IPDs in children worldwide.[22] The ST14 CPS consists of tetrasaccharide [β-D-Gal*p*-(1→4)-][→6)β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→] repeating unit[23] that is the minimal structure required to induce ST14-specific antibodies.[24−26]

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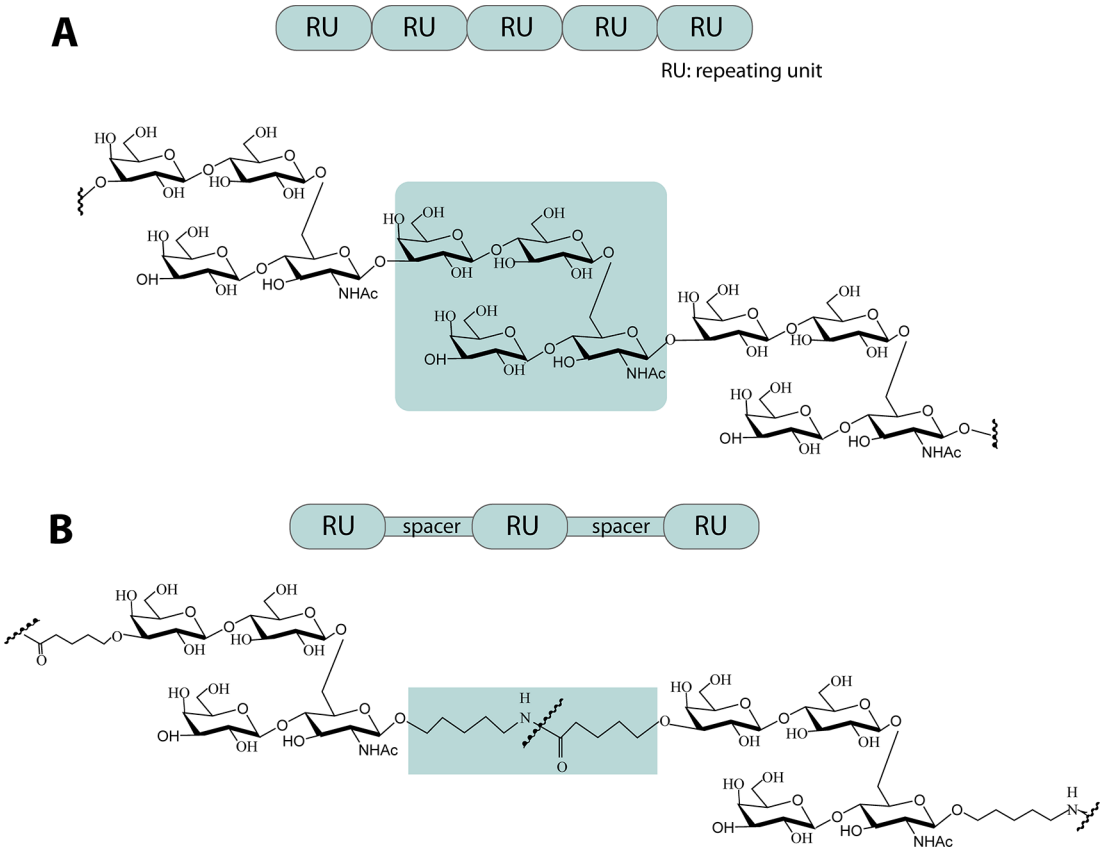

Figure 2. (A) Structure of the ST14 capsular polysaccharide. (B) Spacer-bridged ST14 oligosaccharide mimic of the capsular polysaccharide.

To design a polysaccharide mimic consisting of two ST14 tetrasaccharide repeating units (RU) connected by a linker, molecular dynamics (MD) simulations[27,28] were employed to sample the conformational space of an oligosaccharide consisting of three ST14 RUs (Figure 1B). On the basis of that model, a linker was designed such that the resulting chimeric structure where the middle RU is replaced by the spacer corresponds approximately to that of the native glycan (Figure 1A). To assess the structural similarity between the synthetic ST14 mimic and the native glycan, the conformational ensembles of the two molecules were compared using all-atom MD simulations.

On the basis of this design, just one tetrasaccharide instead of a dodecasaccharide has to be synthesized and is conjugated to another unit (Figure 2). The spacer-bridged oligosaccharide derivatives were designed for conjugation to carrier protein CRM197 and *in vivo* immunological assessment. With proper spacing, the repeating units will interact with B cell receptors and result in a robust immune response.

RESULTS AND DISCUSSION

Hybrid Design by Molecular Modeling. The ST14 dodecasaccharide consisting of three ST14 tetrasaccharide RUs was modeled with all-atom MD using the $GLYCAM_{OSMO,14}$ force field.[27,28] In designing a linker/spacer to bridge the middle tetrasaccharide RU, we adopted a two-stage process. A flexible and immunologically silent linker was envisioned that would carry minimal functional groups but could be installed in one simple synthetic manipulation. Amide bond formation connecting two alkyl linkers was selected for an initial quick assessment using MacroModel 8.0[29] that identified the union of a pentenyl amine and a butanoic acid as a potential solution for the linker challenge at hand. The polysaccharide mimic was simulated with MD, using parameters from the Amber SB99 force field[30,31] and partial charges derived using the R.E.D. tools scripts[32] for the linker residue. On the basis of these simulations, the linker segment introduced in the ST14 mimic is much more flexible than the central tetrasaccharide it replaces, allowing the molecule to sample a greater conformational space, as reflected in the wider probability distributions for the distances shown in panels C and D of Figure 1 (for a more detailed description, see the Supporting Information). The synthetic molecule reaches both more compact and more extended conformations than the comparatively rigid polysaccharide, and a significant overlap between the distributions for the two molecules remains. In addition, the conformational states within one tetrasaccharide repeat unit remain identical between the two molecules. Thus, the two saccharides in the ST14 mimic frequently access conformations that are very similar to those in the polysaccharide.

Synthetic Strategy. The synthesis of ST14 repeating unit oligosaccharides (**1**−**3**) with linkers at the reducing and nonreducing ends was based on key ST14 repeating unit tetrasaccharide **4** (Scheme 1). Tetrasaccharide antigen **4** containing two linkers was assembled by the linear combination of linker **5**, as well as building blocks **6**,[33] **7**, **8**,[34] and **9**. Tetrasaccharide **10** that lacks the linker at the nonreducing end was prepared the same way using building block **11**[35] instead of **9**.

Oligosaccharide Assembly. Glucosamine building block **6**, glucosyl **8**, and galactosyl **11** were synthesized by following the reported procedures.[33−35] The synthesis of both galactosyl

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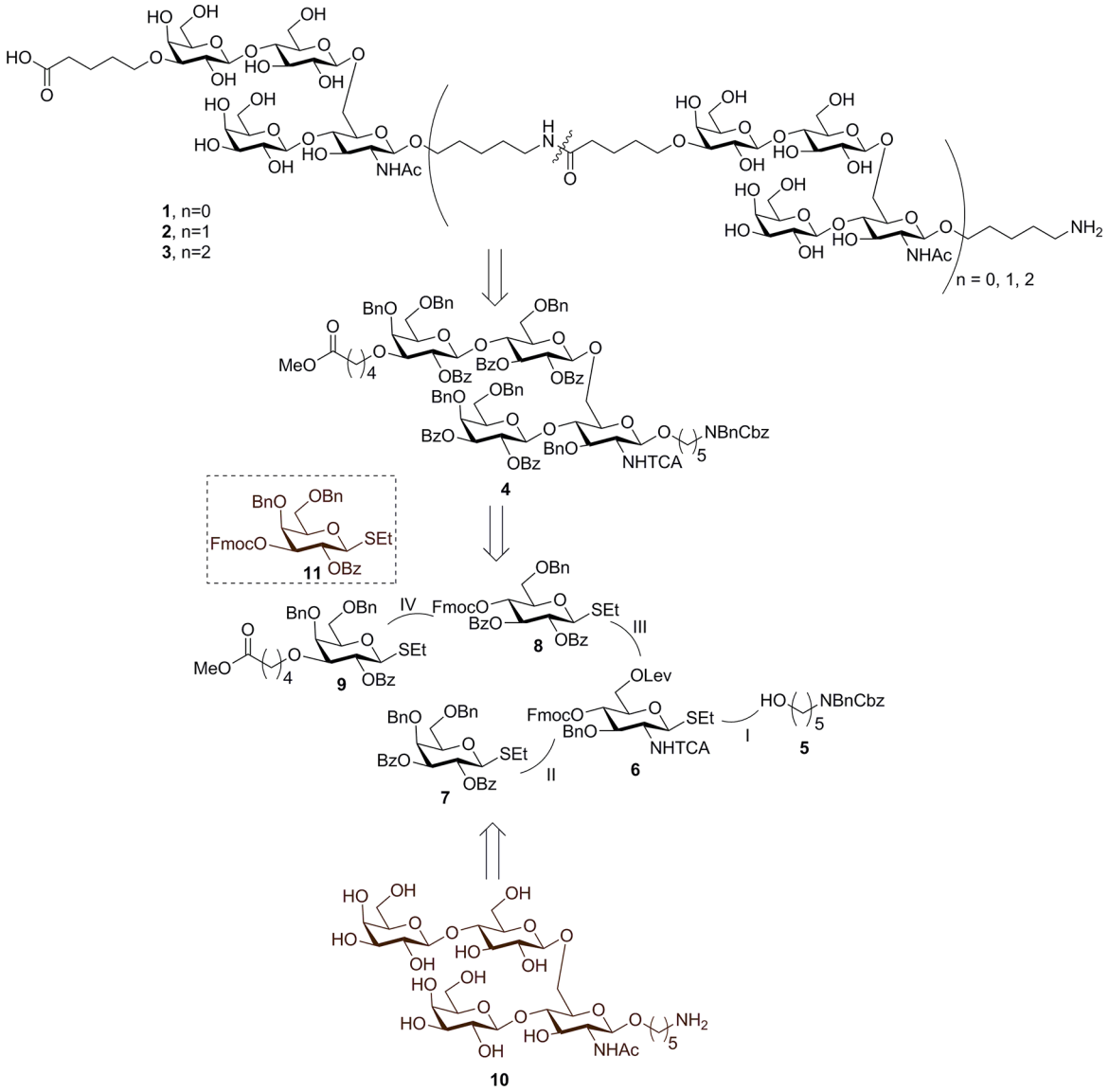
Scheme 1. Retrosynthetic Analysis of Capsular Oligosaccharide Derivatives 1−3 and 10



building blocks **7** and **9** commenced with ethyl 4,6-*O*-benzylidene-1-thio-*β*-D-galactopyranoside (see Scheme S1).

With four building blocks in hand, the stage was set to access tetrasaccharide **4**. The protected amino linker was introduced at the reducing end by glycosylation of **5** and thioglycoside **6** promoted by *N*-iodosuccinimide (NIS) in the presence of triflic acid (TfOH), affording **12**. Removal of the temporary fluorenylmethyloxycarbonyl (Fmoc) protecting group gave compound **13**, which was further reacted with galactose **7**, thus yielding the fully protected disaccharide **14**. Cleavage of levulinoyl (Lev) ester with hydrazine hydrate yielded **15** as an acceptor that was glycosylated with building block **8** to furnish trisaccharide **16**. Cleavage of the Fmoc group followed by coupling with thioglycoside **9** produced tetrasaccharide **4** (Scheme 2A).

Tetrasaccharide **18** was prepared by glycosylation of trisaccharide **17** and galactose building block **11** activated with NIS/TfOH. Removal of Fmoc, benzoyl, and benzyl protecting groups yielded fully deprotected tetrasaccharide **10** (Scheme 2B).

The methyl ester at the nonreducing end of **4** was cleaved with a sodium hydroxide solution followed by addition of excessive sodium methoxide to remove all benzoyl esters, yielding compound **21**. Subsequent hydrogenolysis catalyzed by palladium on carbon produced tetrasaccharide **1** (Scheme 3A).

Hydrogenation of tetrasaccharide **4** with palladium on carbon yielded compound **22** that contains a free amino group at the reducing end. Amide bond formation to couple **21** and **22** proceeded well with benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP) and *N*,*N*-diisopropylethylamine (DIPEA) as 80% spacer-bridged divalent ST14 antigen **23** was produced (Scheme 3B). 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate[36] (HATU) also successfully catalyzed the coupling but produced some unidentified byproducts. Removal of all of the ester and ether protecting groups of **23** using the same procedure that was used for compound **4** yielded deprotected divalent ST14 **2**, containing 2 RU. Spacer-bridged trivalent ST14 **3** was

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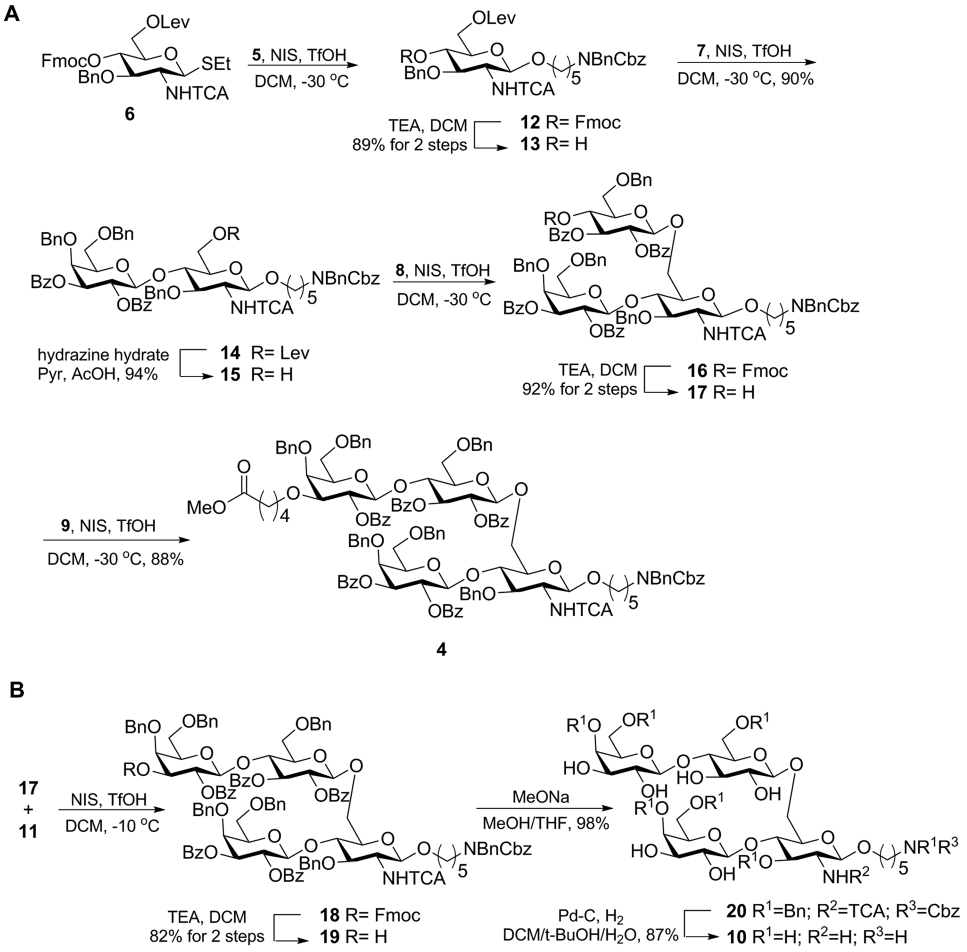
Scheme 2. Synthesis of Tetrasaccharides 4 and 10



prepared by PyBOP-mediated coupling of **22** and **24** to afford **25** followed by global deprotection.

A spacer dummy conjugate BSA-**35** was synthesized as a control to detect antibodies against the spacer (Scheme 4). The synthesis of **35** commenced with phenyl 2-azido-2-deoxy-4,6-*O*-benzylidene-1-seleno-α-D-galactopyranoside **27**, where the 5-methoxy-5-oxopentyl group was installed at position C3 to give **28** that was treated with NIS in the presence of water followed by 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride and cesium carbonate to afford imidate **29**. Glycosylation of linker **5** with **29** produced **30** before the resident azide was converted into an acetamino group with thioacetic acid in pyridine. Methyl ester **31** was hydrolyzed to yield **32** with a free carboxyl group, while hydrogenation resulted in **33** containing an amino group. Coupling of **32** and **33** with PyBOP furnished **34** that was fully deprotected to give **35**. The conjugation of **35** and BSA was achieved using bifunctional *p*-nitrophenyladipate (Scheme 4B) and characterized via MALDI-TOF (see Figure S3).

Human Anti-ST14 CPS Antibodies Bind Synthetic Glycans. Synthetic glycans **1**−**3** and **10** and native CPS14 were covalently immobilized on glass slides (Figure 3A). A spacer dummy conjugate BSA-**35** was used as a control to detect antibodies against the spacer. After incubation with a human reference serum of patients vaccinated with a CPS-based vaccine, the bound antibodies were detected using fluorescently labeled secondary anti-human antibodies. The human serum contained antibodies that bound all synthetic glycan derivatives **1**−**3**. Spacer **35** was not significantly bound by antibodies present in human sera (Figure 3B).

Preparation and Characterization of Glycoconjugates. The synthetic ST14-related glycans **1**−**3** and **10** were conjugated to carrier protein CRM197 using the homobifunctional reagent, adipate 4-nitro phenyl diester,[37] under mild conditions (Figure 4A). Coupling of 0.1 μmol of oligossaccharide to 1 mg of CRM197 resulted in glycoconjugates containing 10 (**1**), 11 (**2**), 7 (**3**), and 10 (**10**) glycans per molecule of CRM197. The loading was calculated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (see Figure S2).

Evaluation of the Immunogenicity of Glycoconjugates. Mice were immunized with the semisynthetic glycoconjugates to determine the immunogenicity of the synthetic ST14 glycans using aluminum hydroxide that is approved for human use as an adjuvant.[38] Immunizations with the vaccine formulation started on day 0, followed by a boost on days 14 and 28 (Figure 4B). Mice immunized with synthetic glycans produced specific antibodies against ST14 CPS. Interestingly, the antibody levels of mice immunized with ST14 glycans **3** were significantly higher than those with tetrasaccharide **10** already on day 14 ($p < 0.05$), while the levels of constructs **1** and **2** were significantly higher ($p < 0.001$) after the first boost (day 21) when compared to those with the synthetic tetrasaccharide **10** (Figure 4C). Overall, the

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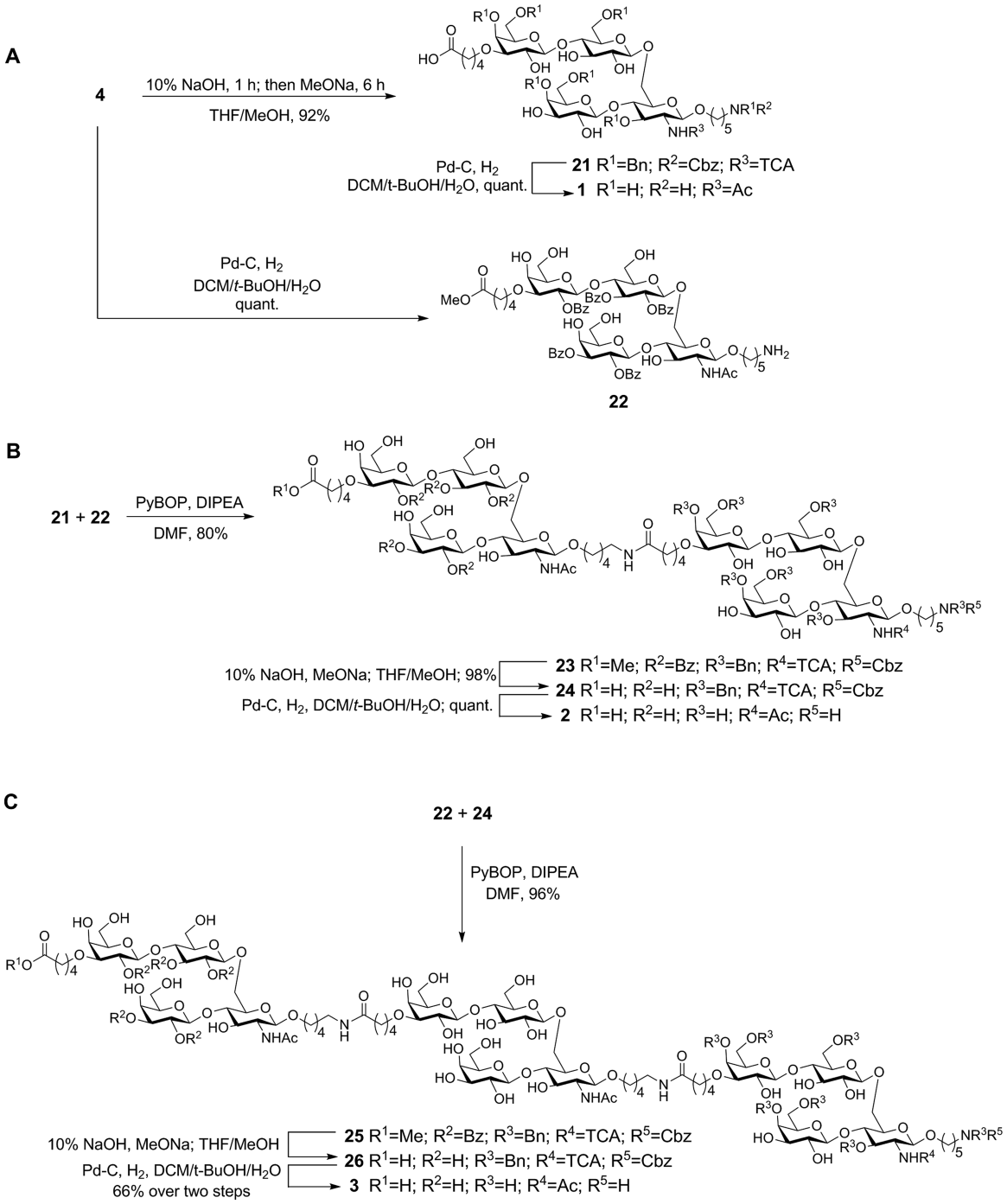
Scheme 3. Synthesis of Oligosaccharide Derivatives 1−3



trivalent derivative CRM197-**3** group showed an antibody titer higher than that of the tetrasaccharide CRM197-**10** group ($p < 0.001$) on days 14 and 35, supporting the concept that the increase in antigen length may lead to better BCR activation, eliciting better antibody production.[13]

Antibodies Raised against Synthetic Glycans Recognize the Specific Epitope of ST14 CPS. To test the specifity of binding of the antibody to ST14 CPS, mice sera from each group were incubated with the native capsular polysaccharide of serotype 2 and 14 and synthetic glycans on a microarray. Antibodies from individual mouse sera from each group recognized the synthetic structures of **1**−**3** and **10** and ST14 CPS, while no significant cross-reactivity was detected against ST2 CPS or the spacer. Antibodies that were produced following vaccinations with synthetic glycans were found to be specific to ST14 CPS (Figure 5B).

Antibodies Raised against Synthetic Glycans Show Opsonophagocytic Killling Activity *In Vitro*. Protective immunity against *S. pneumoniae* is mainly antibody-mediated.[39] To prove the functional activity of antibodies raised against synthetic glycans, we performed an opsophagocytic killing assay (OPKA) *in vitro*. Pooled sera of mice immunized with CRM197-**10**, CRM197-**1**, CRM197-**2**, CRM197-**3**, or PBS with aluminum hydroxide after three doses (Figure 6A) were incubated with HL-60 cells, baby rabbit complement, and ST14 bacteria. The human anti-pneumococcal reference serum

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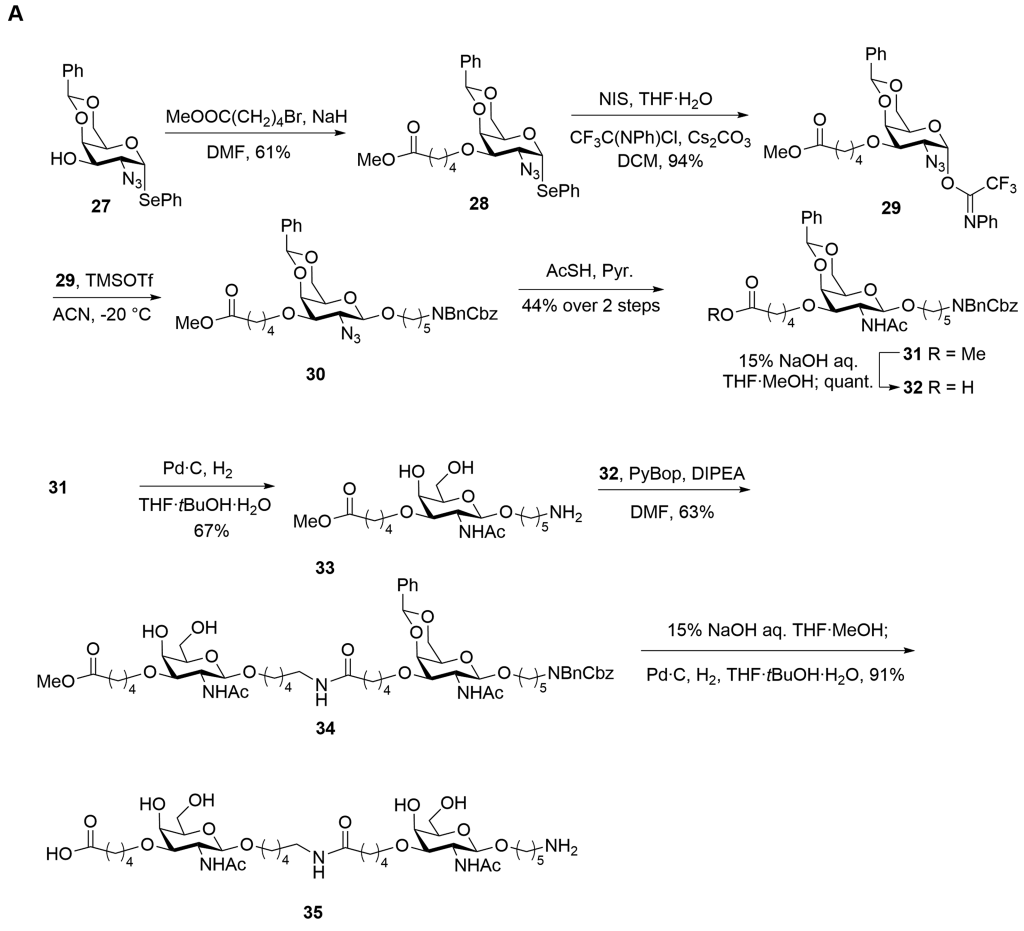
Scheme 4. (A) Synthesis of spacer 35 and (B) Conjugation of 35 to BSA



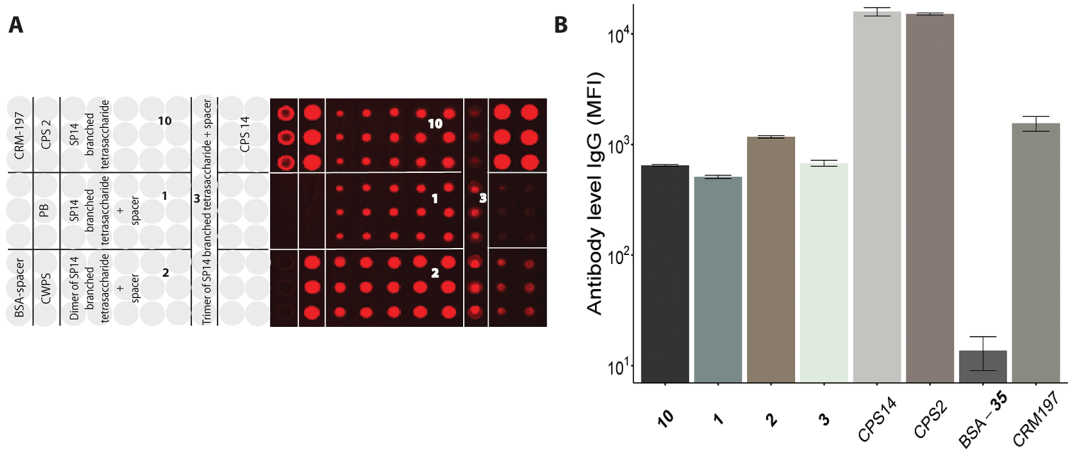


Figure 3. Native and synthetic oligosaccharides immobilized on a glycan microarray were incubated with the human reference serum of patients immunized with *S. pneumoniae* CPS. (A) Microarray in which glycans **1**−**3** and **10** were immobilized were bound by human IgG antibodies. (B) IgG antibody binding to synthetic glycans. No significant binding was detected against the spacer (BSA-**35**). A serum dilution of 1:100 was used in the analysis. Abbreviations: MFI, mean fluorescence intensity (mean ± standard deviation); PB, printing buffer; CPS, capsular polysaccharide; CWPS, cell wall polysaccharide; BSA, bovine serum albumin.

(007sp) was used as a standard reference.[40] The antibodies raised against synthetic antigens showed very similar antibacterial activity of 007sp, while sera from the PBS group showed no antibacterial activity (Figure 6B). A four-parameter

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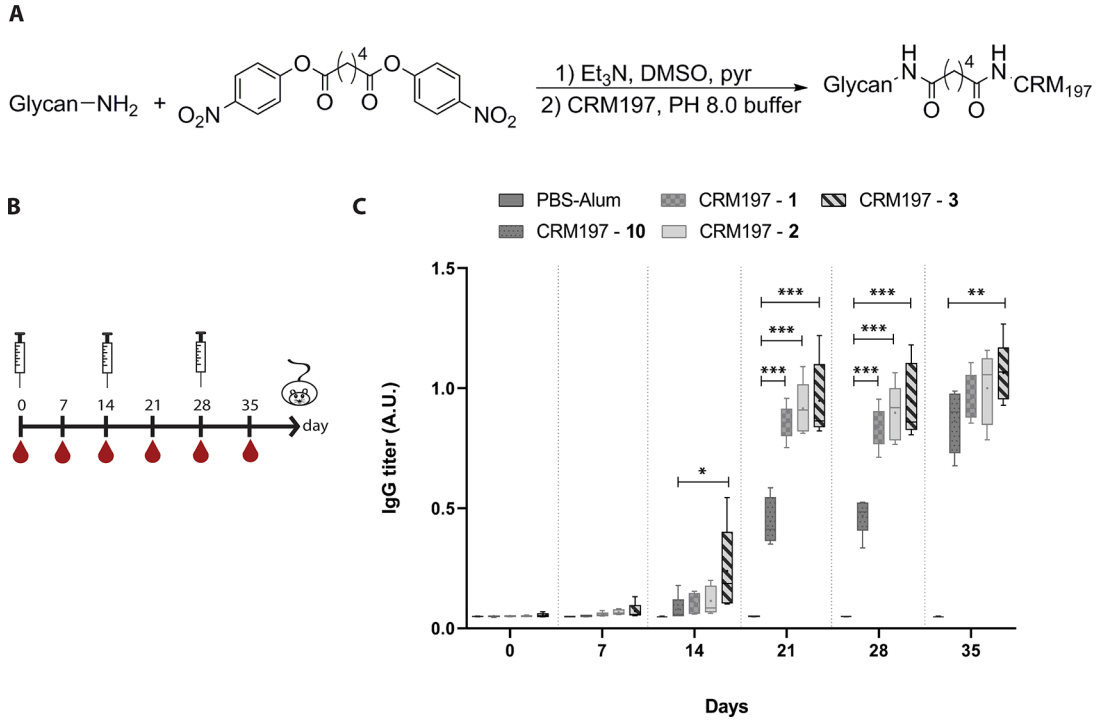

Figure 4. Antibody levels of mice immunized with glycoconjugates. (A) Preparation of conjugates. (B) Immunization schedule. Mice were immunized on day 0 followed by two boosts on days 14 and 28. Blood was collected at each time point. (C) IgG antibody levels of mice immunized with synthetic glycans were measured using ELISA plates coated with CPS of *S. pneumoniae* serotype 14. Glycoconjugate CRM197-**3** induced significantly higher antibody levels on day 14 and glycoconjugates CRM197-**1** and CRM197-**2** after the first boost (day 21) when compared to those of tetrasaccharide glycoconjugate CRM197-**10**, which increased markedly only after the second boost. Overall, CRM197-**3** showed significantly higher antibody titer when compared to that of tetrasaccharide CRM197-**10**. The 1:100 serum dilution was used in the analysis. Abbreviations: A.U., absorbance units; PBS, phosphate-buffered saline. ***$p < 0.001$. **$p < 0.01$. *$p < 0.05$ (mean ± standard deviation).

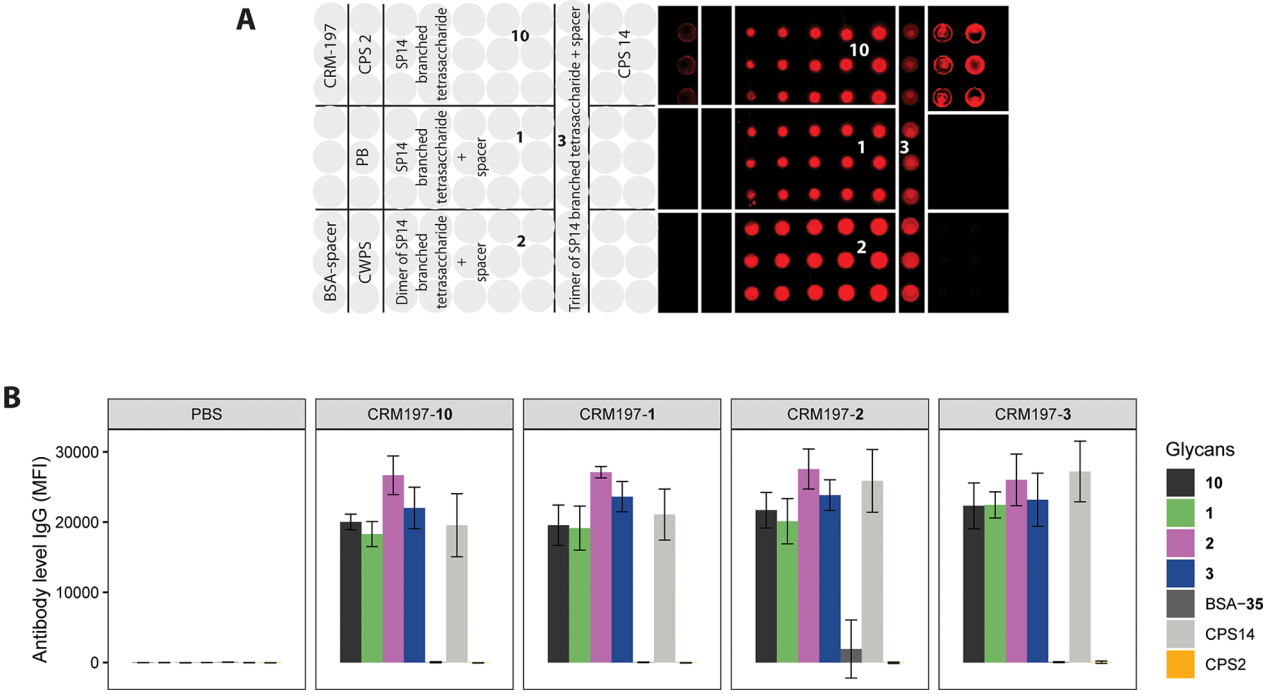

Figure 5. Glycan microarray incubated with sera of mice immunized with synthetic glycans conjugated to CRM197 and formulated with aluminum hydroxide. (A) Mice produced specific antibodies against native capsular polysaccharide CPS 14, synthetic glycan **10**, and oligosaccharide derivatives **1**−**3**. No significant binding to CPS 2 or spacer BSA-**35** was detected. (B) The divalent and trivalent derivative glycans showed the highest level of binding throughout the groups. A serum dilution of 1:100 was used in the analysis. Abbreviations: MFI, mean fluorescence intensity; PBS, phosphate-buffered saline.

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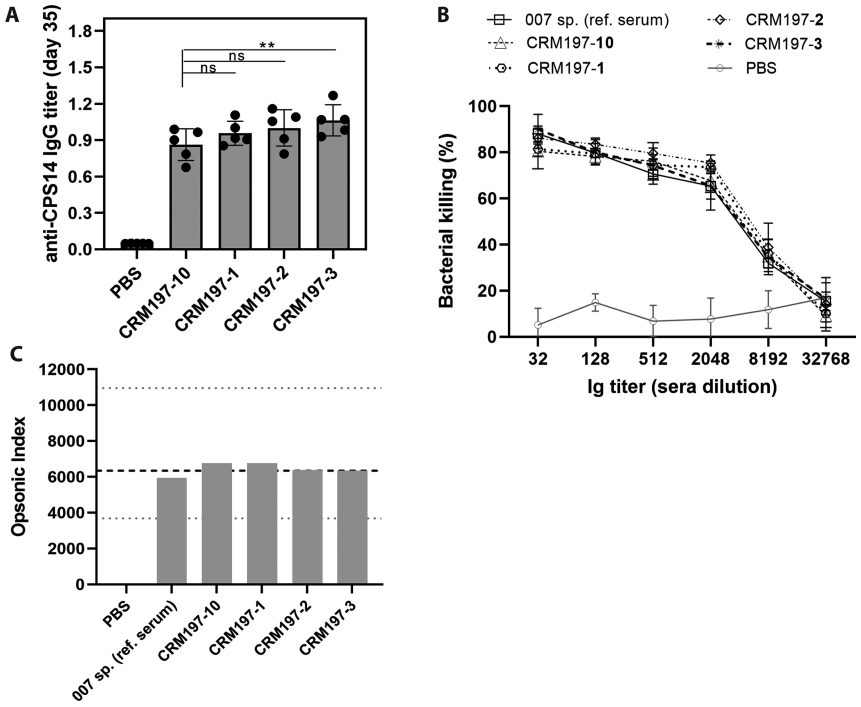

Figure 6. Opsonophagocytic killing activity *in vitro* of CRM197-**10**, CRM197-**1**, CRM197-**2**, and CRM197-**3** vaccine-generated antibodies. (A) Anti-CPS14 IgG titer of mice vaccinated with semisynthetic CRM197-**10**, CRM197-**1**, CRM197-**2**, and CRM197-**3** as well as with PBS adjuvanted after three vaccine doses (day 35) used in the assay. Data were analized by analysis of variance (mean ± standard deviation of five immunized mice). (B) Opsonophagocytic activity of polled sera of mice vaccinated with semisynthetic CRM197-**10**, CRM197-**1**, CRM197-**2**, CRM197-**3**, and PBS against ST14. Sera 007sp was used as the reference standard. Two independent experiments in duplicate were used in the analysis (mean ± standard deviation). Semisynthetic vaccines showed a pattern similar to that of the 007sp reference serum. (C) The opsonic index (OI), where 50% of the bacteria are killed, was based on a four-parameter logistic model of a generated cruve with four points from two independent assays. The 007sp reference serum was used as a standard, and the black dashed line represents the mean OI with a 95% confidence interval (CI) marked as dottted gray lines.[40] The OI values of semisynthetic vaccines are similar and in the range of the 95% CI of reference values. The sequential linkage of synthetic ST14 RU does not affect the opsonic killing acitivity against ST14. $^{**}p < 0.01$.

logistic model was applied to establish the serum dilution point at which 50% of the bacteria are killed (opsonic index). The results are in the range of the accepted confidence interval of reference serum,[40] and the opsonic indices of 007sp serum, CRM197-**10**, CRM197-**1**, CRM197-**2**, and CRM197-**3** were in a very similar range (Figure 6C). These results are expected because the tetrasacchairde RU alone has already been proven to elicit antibodies with opsonic activity.[24] The combination of two or three RU with a spacer should not change the antibody activity because the tetrasaccharide RU remains the same. Thus, the sequential linkage of ST14 RU does not affect the opsonic activity of generated antibodies against ST14.

SIGNIFICANCE

Mono-, di-, and trivalent ST14 derivatives **1**−**3**, respectively, were rapidly synthesized by bridging RUs with an aliphatic 10-carbon spacer. The ST14 oligosaccharide derivatives were conjugated with carrier protein CRM197 to form semisynthetic neo-glycoconjugates. These neoglycoconjugates were formulated with aluminum hydroxide and immunologically evaluated in mice. Both glycan array and ELISA analyses of the immune sera demonstrated that the aliphatic spacer decreased neither antigenicity nor immunogenicity. Interestingly, the ST14-specific antibody responses against derivatives **1**−**3** were significantly higher after only one or two immunizations when compared to that of the branched tetrasaccharide RU only. Most importantly, the sequential linkage of ST14 repeating units does not impair the opsonic killing activity of generated antibodies.

The strategy of spacing synthetic repeating unit glycan antigens with linkers of the appropriate length to mimic capsular polysaccharides is promising for the design of semisynthetic glycoconjugate vaccine candidates and results in a faster, specific antibody response. Higher antibody levels holding opsonic activity in the earlier immunization phase are important for constraining pneumococcus infection at an early stage.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00360.

Experimental procedures for molecular dynamics simulations, synthesis of glycan building blocks, vaccine characterization, biological assays, animal experiments, and NMR spectra (PDF)

AUTHOR INFORMATION

Corresponding Author

Peter H. Seeberger − *Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, 14476 Potsdam, Germany; Department of Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany;*

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orcid.org/0000-0003-3394-8466;
Email: peter.seeberger@mpikg.mpg.de

Authors

Bruna M. S. Seco − *Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, 14476 Potsdam, Germany; Department of Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany*

Fei-Fei Xu − *Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, 14476 Potsdam, Germany; Department of Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany*

Andrea Grafmüller − *Department of Theory and Bio-Systems, Max Planck Institute of Colloids and Interfaces, 14476 Potsdam, Germany;* orcid.org/0000-0002-1671-3158

Naresh Kottari − *Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, 14476 Potsdam, Germany*

Claney L. Pereira − *Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, 14476 Potsdam, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acschembio.0c00360

Author Contributions

B.M.S.S. and F.-F.X. contributed equally to this work. F.-F.X., C.L.P., and P.H.S. planned the study. F.-F.X., B.M.S.S., and A.G. conducted the experiments and analyzed the data. B.M.S.S., F.-F.X., C.L.P., and P.H.S. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Finland, M., and Dowling, H. F. (1935) Cutaneous Reactions and Antibody Response to Intracutaneous Injections of Pneumococcus Polysaccharides. *J. Immunol. 29*, 285−299.

(2) Astronomo, R. D., and Burton, D. R. (2010) Carbohydrate vaccines: developing sweet solutions to sticky situations? *Nat. Rev. Drug Discovery 9*, 308−324.

(3) Tree, J. A., Richardson, C., Fooks, A. R., Clegg, J. C., and Looby, D. (2001) Comparison of large-scale mammalian cell culture systems with egg culture for the production of influenza virus A vaccine strains. *Vaccine 19*, 3444−3450.

(4) Gonçalves, V. M., Zangirolami, T. C., Giordano, R. L. C., Raw, I., Tanizaki, M. M., and Giordano, R. C. (2002) Optimization of medium and cultivation conditions for capsular polysaccharide production by *Streptococcus pneumoniae* serotype 23F. *Appl. Microbiol. Biotechnol. 59*, 713−717.

(5) Pujar, N. S., Huang, N. F., Daniels, C. L., Dieter, L., Gayton, M. G., and Lee, A. L. (2004) Base hydrolysis of phosphodiester bonds in pneumococcal polysaccharides. *Biopolymers 75*, 71−84.

(6) Sturgess, A. W., Rush, K., Charbonneau, R. J., Lee, J. I., West, D. J., Sitrin, R. D., and Hennessey, J. P. (1999) *Haemophilus influenzae* type b conjugate vaccine stability: catalytic depolymerization of PRP in the presence of aluminum hydroxide. *Vaccine 17*, 1169−1178.

(7) Wessels, M. R., Paoletti, L. C., Guttormsen, H.-K., Michon, F., D'Ambra, A. J., and Kasper, D. L. (1998) Structural Properties of Group B Streptococcal Type III Polysaccharide Conjugate Vaccines That Influence Immunogenicity and Efficacy. *Infect. Immun. 66*, 2186−2192.

(8) Schumann, B., Anish, C., Pereira, C. L., and Seeberger, P. H. (2013) CHAPTER 3 Carbohydrate Vaccines. In *Biotherapeutics: Recent Developments using Chemical and Molecular Biology*, pp 68−104, The Royal Society of Chemistry.

(9) Pozsgay, V. (2008) Recent Developments in Synthetic Oligosaccharide-Based Bacterial Vaccines. *Curr. Top. Med. Chem. 8*, 126−140.

(10) Verez-Bencomo, V., Fernández-Santana, V., Hardy, E., Toledo, M. E., Rodríguez, M. C., Heynngnezz, L., Rodriguez, A., Baly, A., Herrera, L., Izquierdo, M., Villar, A., Valdés, Y., Cosme, K., Deler, M. L., Montane, M., Garcia, E., Ramos, A., Aguilar, A., Medina, E., Toraño, G., Sosa, I., Hernandez, I., Martínez, R., Muzachio, A., Carmenates, A., Costa, L., Cardoso, F., Campa, C., Diaz, M., and Roy, R. (2004) A Synthetic Conjugate Polysaccharide Vaccine Against *Haemophilus influenzae* Type b. *Science 305*, 522−525.

(11) Schumann, B., Reppe, K., Kaplonek, P., Wahlbrink, A., Anish, C., Witzenrath, M., Pereira, C. L., and Seeberger, P. H. (2018) Development of an Efficacious, Semisynthetic Glycoconjugate Vaccine Candidate against *Streptococcus pneumoniae* Serotype 1. *ACS Cent. Sci. 4*, 357−361.

(12) Davis, M. M. (2004) The evolutionary and structural 'logic' of antigen receptor diversity. *Semin. Immunol. 16*, 239−243.

(13) Puffer, E. B., Pontrello, J. K., Hollenbeck, J. J., Kink, J. A., and Kiessling, L. L. (2007) Activating B Cell Signaling with Defined Multivalent Ligands. *ACS Chem. Biol. 2*, 252−262.

(14) Yang, J., and Reth, M. (2016) Receptor Dissociation and B-Cell Activation. In *B Cell Receptor Signaling* (Kurosaki, T., and Wienands, J., Eds.) pp 27−43, Springer International Publishing, Cham, Switzerland.

(15) Volkmann, C., Brings, N., Becker, M., Hobeika, E., Yang, J., and Reth, M. (2016) Molecular requirements of the B-cell antigen receptor for sensing monovalent antigens. *EMBO J. 35*, 2371−2381.

(16) Bennett, N. R., Zwick, D. B., Courtney, A. H., and Kiessling, L. L. (2015) Multivalent Antigens for Promoting B and T Cell Activation. *ACS Chem. Biol. 10*, 1817−1824.

(17) AlonsoDeVelasco, E., Verheul, A. F., Verhoef, J., and Snippe, H. (1995) *Streptococcus pneumoniae:* virulence factors, pathogenesis, and vaccines. *Microbiol. Rev. 59*, 591−603.

(18) Inostroza, J., Vinet, A. M., Retamal, G., Lorca, P., Ossa, G., Facklam, R. R., and Sorensen, R. U. (2001) Influence of Patient Age on *Streptococcus pneumoniae* Serotypes Causing Invasive Disease. *Clin. Diagn. Lab. Immunol. 8*, 556−559.

(19) Hausdorff, W. P., Bryant, J., Paradiso, P. R., and Siber, G. R. (2000) Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin. Infect. Dis. 30*, 100−121.

(20) Boulnois, G. J. (1992) Pneumococcal proteins and the pathogenesis of disease caused by Streptococcus pneumoniae. *J. Gen. Microbiol. 138*, 249−259.

(21) Bentley, S. D., Aanensen, D. M., Mavroidi, A., Saunders, D., Rabbinowitsch, E., Collins, M., Donohoe, K., Harris, D., Murphy, L., Quail, M. A., Samuel, G., Skovsted, I. C., Kaltoft, M. S., Barrell, B., Reeves, P. R., Parkhill, J., and Spratt, B. G. (2006) Genetic Analysis of the Capsular Biosynthetic Locus from All 90 Pneumococcal Serotypes. *PLoS Genet. 2*, e31.

(22) Johnson, H. L., Deloria-Knoll, M., Levine, O. S., Stoszek, S. K., Freimanis Hance, L., Reithinger, R., Muenz, L. R., and O'Brien, K. L. (2010) Systematic Evaluation of Serotypes Causing Invasive Pneumococcal Disease among Children Under Five: The Pneumococcal Global Serotype Project. *PLoS Med. 7*, e1000348.

(23) Lindberg, B., Lonngren, J., and Powell, D. A. (1977) Structural studies on the specific type-14 pneumococcal polysaccharide. *Carbohydr. Res. 58*, 177−186.
(24) Laferriere, C. A., Sood, R. K., de Muys, J.-M., Michon, F., and Jennings, H. J. (1998) *Streptococcus pneumoniae* Type 14 Polysaccharide-Conjugate Vaccines: Length Stabilization of Opsonophagocytic Conformational Polysaccharide Epitopes. *Infect. Immun. 66*, 2441−2446.
(25) Verheul, A. F., Versteeg, A. A., De Reuver, M. J., Jansze, M., and Snippe, H. (1989) Modulation of the immune response to pneumococcal type 14 capsular polysaccharide-protein conjugates by the adjuvant Quil A depends on the properties of the conjugates. *Infect. Immun. 57*, 1078−1083.
(26) Verheul, A. M., Versteeg, A. A., Westerdaal, N. A. C., Van Dam, G. J., Jansze, M., and Snippe, H. (1990) Measurement of the humoral immune response against *Streptococcus pneumoniae* type 14-derived antigens by an ELISA and ELISPOT assay based on biotin-avidin technology. *J. Immunol. Methods 126*, 79−87.
(27) Kirschner, K. N., Yongye, A. B., Tschampel, S. M., González-Outeiriño, J., Daniels, C. R., Foley, B. L., and Woods, R. J. (2008) GLYCAM06: A generalizable biomolecular force field. Carbohydrates. *J. Comput. Chem. 29*, 622−655.
(28) Sauter, J., and Grafmüller, A. (2016) Predicting the Chemical Potential and Osmotic Pressure of Polysaccharide Solutions by Molecular Simulations. *J. Chem. Theory Comput. 12*, 4375−4384.
(29) Mohamadi, F., Richards, N. G. J., Guida, W. C., Liskamp, R., Lipton, M., Caufield, C., Chang, G., Hendrickson, T., and Still, W. C. (1990) Macromodel—an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. *J. Comput. Chem. 11*, 440−467.
(30) Hornak, V., Abel, R., Okur, A., Strockbine, B., Roitberg, A., and Simmerling, C. (2006) Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins: Struct., Funct., Genet. 65*, 712−725.
(31) Wang, J., Cieplak, P., and Kollman, P. A. (2000) How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? *J. Comput. Chem. 21*, 1049−1074.
(32) Dupradeau, F.-Y., Pigache, A., Zaffran, T., Savineau, C., Lelong, R., Grivel, N., Lelong, D., Rosanski, W., and Cieplak, P. (2010) The R.E.D. tools: advances in RESP and ESP charge derivation and force field library building. *Phys. Chem. Chem. Phys. 12*, 7821−7839.
(33) Hahm, H. S., Broecker, F., Kawasaki, F., Mietzsch, M., Heilbronn, R., Fukuda, M., and Seeberger, P. H. (2017) Automated Glycan Assembly of Oligo-N-Acetyllactosamine and Keratan Sulfate Probes to Study Virus-Glycan Interactions. *Chem. 2*, 114−124.
(34) Nokami, T., Tsuyama, H., Shibuya, A., Nakatsutsumi, T., and Yoshida, J.-i. (2008) Oligosaccharide Synthesis Based on a One-pot Electrochemical Glycosylation−Fmoc Deprotection Sequence. *Chem. Lett. 37*, 942−943.
(35) Hahm, H. S., Liang, C.-F., Lai, C.-H., Fair, R. J., Schuhmacher, F., and Seeberger, P. H. (2016) Automated Glycan Assembly of Complex Oligosaccharides Related to Blood Group Determinants. *J. Org. Chem. 81*, 5866−5877.
(36) Salta, J., Dernedde, J., and Reissig, H.-U. (2015) Synthesis of multivalent carbohydrate mimetics with aminopolyol end groups and their evaluation as L-selectin inhibitors. *Beilstein J. Org. Chem. 11*, 638−646.
(37) Wu, X., Ling, C.-C., and Bundle, D. R. (2004) A New Homobifunctional p-Nitro Phenyl Ester Coupling Reagent for the Preparation of Neoglycoproteins. *Org. Lett. 6*, 4407−4410.
(38) Clements, C. J., and Griffiths, E. (2002) The global impact of vaccines containing aluminium adjuvants. *Vaccine 20* (Suppl. 3), S24−S33.
(39) Brandileone, M. C., Zanella, R. C., Almeida, S. C. G., Cassiolato, A. P., Lemos, A. P. S., Salgado, M. M., Higa, F. T., Minamisava, R., and Andrade, A. L. (2019) Long-term effect of 10-valent pneumococcal conjugate vaccine on nasopharyngeal carriage of Streptococcus pneumoniae in children in Brazil. *Vaccine 37*, 5357−5363.
(40) Burton, R. L., Antonello, J., Cooper, D., Goldblatt, D., Kim, K. H., Plikaytis, B. D., Roalfe, L., Wauters, D., Williams, F., Xie, G. L., Nahm, M. H., and Akkoyunlu, M. (2017) Assignment of Opsonic Values to Pneumococcal Reference Serum 007sp for Use in Opsonophagocytic Assays for 13 Serotypes. *Clin. Vaccine Immunol. 24*, e00457-16.

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