

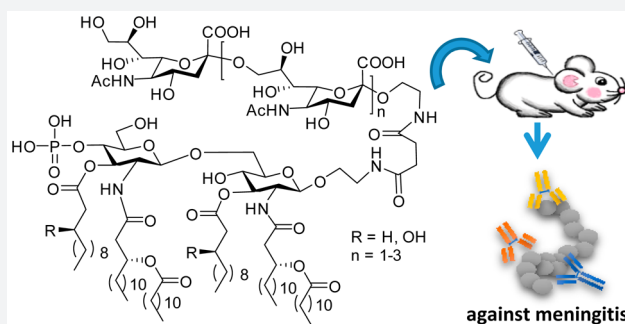
Fully Synthetic Self-Adjuvanting α -2,9-Oligosialic Acid Based Conjugate Vaccines against Group C Meningitis

Guochao Liao,[†] Zhifang Zhou,[†] Sharad Suryawanshi, Mohabul A. Mondal, and Zhongwu Guo^{*}

Department of Chemistry, Wayne State University, 5101 Cass Avenue, Detroit, Michigan 48202, United States

Supporting Information

ABSTRACT: α -2,9-Polysialic acid is an important capsular polysaccharide expressed by serotype C *Neisseria meningitidis*. Its protein conjugates are current vaccines against group C meningitis. To address some concerns about traditional protein conjugate vaccines, a new type of fully synthetic vaccines composed of oligosialic acids and glycolipids was explored. In this regard, α -2,9-linked di-, tri-, tetra-, and pentasialic acids were prepared and conjugated with monophosphoryl lipid A (MPLA). Immunological studies of the conjugates in C57BL/6J mouse revealed that they alone elicited robust immune responses comparable to that induced by corresponding protein conjugates plus adjuvant, suggesting the self-adjuvanting properties of MPLA conjugates. The elicited antibodies were mainly IgG2b and IgG2c, suggesting T cell dependent immunities. The antisera had strong and specific binding to α -2,9-oligosialic acids and to group C meningococcal polysaccharide and cell, indicating the ability of antibodies to selectively target the bacteria. The antisera also mediated strong bactericidal activities. Structure–activity relationship analysis of the MPLA conjugates also revealed that the immunogenicity of oligosialic acids decreased with elongated sugar chain, but all tested MPLA conjugates elicited robust immune responses. It is concluded that tri- and tetrasialic acid–MPLA conjugates are worthy of further investigation as the first fully synthetic and self-adjuvanting vaccines against group C meningitis.



INTRODUCTION

With the rapid growth in drug resistance, bacterial infections have again become a major threat to human health.¹ For infectious disease control, vaccination is considered an effective strategy.^{1–8} When it comes to antibacterial vaccine development, the abundant, exposed, and conserved capsular polysaccharides (CPSs) on the bacterial cell surface are valuable antigens.^{9,10} However, carbohydrates are usually weakly immunogenic and T cell independent,^{11,12} thus they have to be covalently linked to an immunologically active carrier to become T cell dependent.^{13–15} In recent decades, antibacterial vaccines composed of polysaccharides and proteins have witnessed great success, and their clinic use has kept many infectious diseases under control.^{16,17}

Despite the great success of polysaccharide–protein conjugate vaccines, they have inherent problems. First, the polysaccharides utilized to create vaccines are derived from bacteria and are thus heterogeneous and easily contaminated.^{15,17} Moreover, in order to promote the conjugation with protein, CPSs have to be modified, making their composition more complex. To address the issue, structurally defined repeating units of CPSs have been explored for antibacterial vaccine development.^{18,19} An example of this approach's success is the oligosaccharide-based *Haemophilus influenzae* type b vaccine now in clinical use.²⁰ Second, carbohydrate–protein conjugation usually affords complex

mixtures that are difficult to control and duplicate. Third, carrier proteins can elicit strong B cell responses that may suppress the desired immune responses to carbohydrate antigens.^{21,22} A promising strategy to overcome these problems is to employ fully synthetic vaccines,^{1,16,23–28} which not only are homogeneous, structurally defined, and easy-to-control but also are free of bacterial contamination.

To facilitate fully synthetic glycoconjugate vaccine development, we have recently explored a new carrier, i.e., monophosphoryl lipid A (MPLA):^{29,30} a lipid A derivative. Like lipid A, MPLA is a strong immunostimulant that can interact with toll-like receptor 4 (TLR-4) to promote antigen presentation, T-helper cell activation, and T cell mediated immune response.^{31–35} In contrast to lipid A, however, MPLA is essentially nontoxic and thus has been approved as a human vaccine adjuvant.^{33,36} We have studied several MPLA-based anticancer vaccines that were proved to be self-adjuvanting and to elicit robust T cell dependent immunity.^{29,30,37,38} Based on these findings, we envisioned the potential application of MPLA to create fully synthetic antibacterial vaccines. To probe this hypothesis, a CPS of serotype C *N. meningitidis*^{39,40} was utilized as the target antigen to develop anti-group C meningitis vaccines. In addition, this research also provides in-depth

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analysis of the structure–activity relationships of oligosaccharide antigens.

RESULTS AND DISCUSSION

Group C *N. meningitidis* is one of the two bacterial strains mainly responsible for meningitis epidemics in developed countries.^{7,41} α -2,9-Polysialic acid is its characteristic CSP,^{39,40} which has been used to develop successful vaccines against group C meningitis^{42–44} and is still a hot target for new vaccine design.^{45–47} Accordingly, we prepared a series of α -2,9-oligosialic acids, including di-, tri-, tetra-, and pentasialic acids, conjugated them with MPLAs—the 4'-*O*-phosphoryl form of *N. meningitidis* lipid A (1–4, Figure 1) and its analogue without

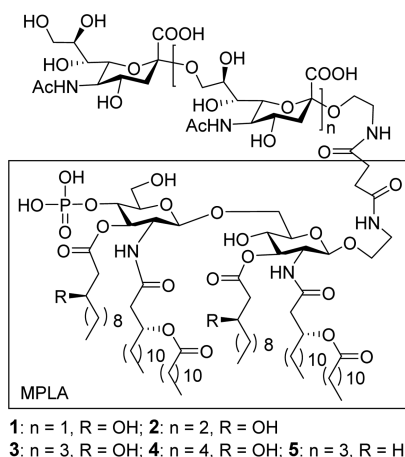


Figure 1. Structures of designed MPLA–oligosialic acid conjugates 1–5.

the hydroxyl groups on the lipid side chains (5)—and studied the resultant conjugates in mice. These oligosialic acids were also linked to keyhole limpet hemocyanin (KLH) and human serum albumin (HSA) to obtain conjugates that were utilized as the positive control and as capture reagents for enzyme-linked immunosorbent assay (ELISA) of oligosialic acid specific antibodies, respectively.

Synthesis of Glycoconjugates 1–5. As outlined in Scheme 1, MPLA derivatives 6 and 7 with a free carboxylic group³⁸ and oligosialic acids 10–13 carrying a free amino group at the reducing end⁴⁸ were prepared according to reported procedures. Then, 6 and 7 were converted into active esters 8 and 9, which were coupled with 10–13 to afford 14–18. Finally, 14–18 were subjected to catalytic hydrogenolysis under an H_2 atmosphere to remove all of the benzyl groups to yield 1–5. On the other hand, the KLH and HSA conjugates of oligosialic acids were prepared by coupling 10–13 with KLH and HSA, respectively, via the bifunctional glutaryl (Supporting Information), as described before.⁴⁸

Immunologic Evaluation of Glycoconjugates 1–5. Immunologic studies of 1–5 were carried out with female C57BL/6J mice using liposomes of 1–5 made with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol in a 10:65:50 molar ratio.^{37,38} Liposomes are expected to improve the water solubility of 1–5 and their presentation to immune cells.^{49,50} The immunization protocol was to subcutaneously (sc) inject each conjugate (9 μg of sugar) to a group of five mice, with or without an external adjuvant, on days 1, 14, 21, and 28, respectively. The mice were bled on day 0 before initial immunization and on day 27 and day 38 after

boost immunizations. Blood samples were used to prepare antisera for ELISA detection of oligosialic acid specific antibodies with oligosialic acid–HSA conjugates as capture reagents. Antibody titer was calculated by linear regression analysis of adjusted optical density (OD) values, i.e., after deduction of the OD values of day 0 mouse sera, against serum dilution numbers in logarithmic scale and was defined as the dilution number yielding an adjusted OD value of 0.20.

Influence of External Adjuvant on the Immune Response to 1. Our previous studies showed the self-adjuvanting properties of MPLA conjugates.^{37,38} However, all clinical vaccines are currently used with adjuvants, so we sought to probe the influence of an external adjuvant on the immunologic properties of MPLA-based antibacterial vaccines. We examined 1 alone or 1 with complete Freund's adjuvant (CFA), alum, or TiterMax Gold adjuvant. Antisera were prepared from blood samples collected 10 days after the last boost immunization and analyzed by ELISA with disialic acid–HSA as the capture reagent. As depicted in Figure 2, all four groups of mice exhibited similar immunologic responses, with the production of high total and IgG2b antibody titers and moderate IgG2c, IgM, and IgG3 antibody titers. Thus, the external adjuvants had no or little impact on the immunologic responses to 1. According to the literature,^{11,51,52} IgG antibody production is associated with T cell dependent immunity. Therefore, 1 was proved to be self-adjuvanting and elicit robust, antigen-specific T cell dependent immune responses in mice.

Impact of Vaccine Dose on the Immune Response to 1. To study the dose–immunity correlation, three groups of mice were immunized with liposomes of 1 containing 1, 9, and 18 μg of disialic acid per injection, respectively. The titers of total and isotypes of antibodies detected by ELISA (Figure 3) showed that the vaccine dose had a small impact on the immune response and mice in the 9 μg dose group had the highest titers of all tested antibody isotypes. However, high total and IgG2b antibody and moderate IgG2c, IgM, and IgG3 antibody levels were observed with all dose groups, and the dose did not have an obvious impact on antibody isotypes. It seemed that a low dose of 1, e.g., 1 μg of sialic acid per injection, was sufficient to elicit a robust immune response. Thus, although higher doses may help enhance the immune response, exceedingly high doses are not necessarily beneficial.

Comparing the Immunologic Properties of MPLA and KLH Conjugates. Mice were immunized with liposomes of 1 alone (9 μg of sialic acid/injection) or with a KLH–disialic acid–TiterMax Gold Adjuvant emulsion (6 μg of sialic acid/injection).^{53,54} ELISA results of their antisera revealed that both conjugates elicited strong and a similar pattern of antibody response, mainly IgG2b and IgG2c and some IgG1 and IgG3 (Figure 4). Moreover, compared to KLH conjugate, 1 elicited a slightly lower IgG1 antibody titer but a higher IgG3 antibody titer. The results were consistent with the observation^{37,38,48} that glycoproteins often elicit IgG1 antibody response whereas glycolipids elicit carbohydrate-specific IgG3 antibody response. Most significantly, both 1 and KLH–disialic acid elicited high titers of IgG2b and IgG2c antibodies, indicating T cell dependent immunity.^{11,27,51,52} Thus, these studies further verified that 1 alone could elicit robust immune responses comparable to that elicited by the corresponding KLH conjugate plus adjuvants.

Structure–Immunogenicity Relationships of Oligosialic Acid Antigens. Mouse immunization and ELISA were carried out by aforementioned protocols. As shown in Figure 5, all of

Scheme 1. Synthesis of the Target MPLA–Oligosialic Acid Conjugates 1–5

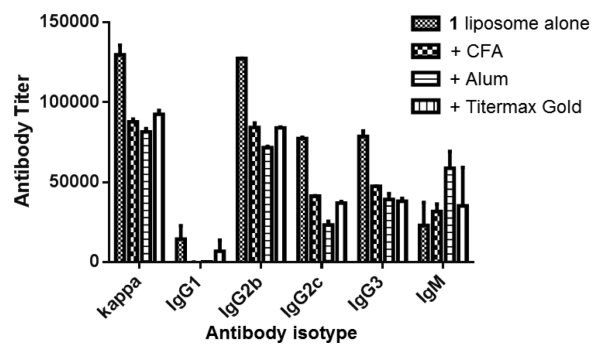
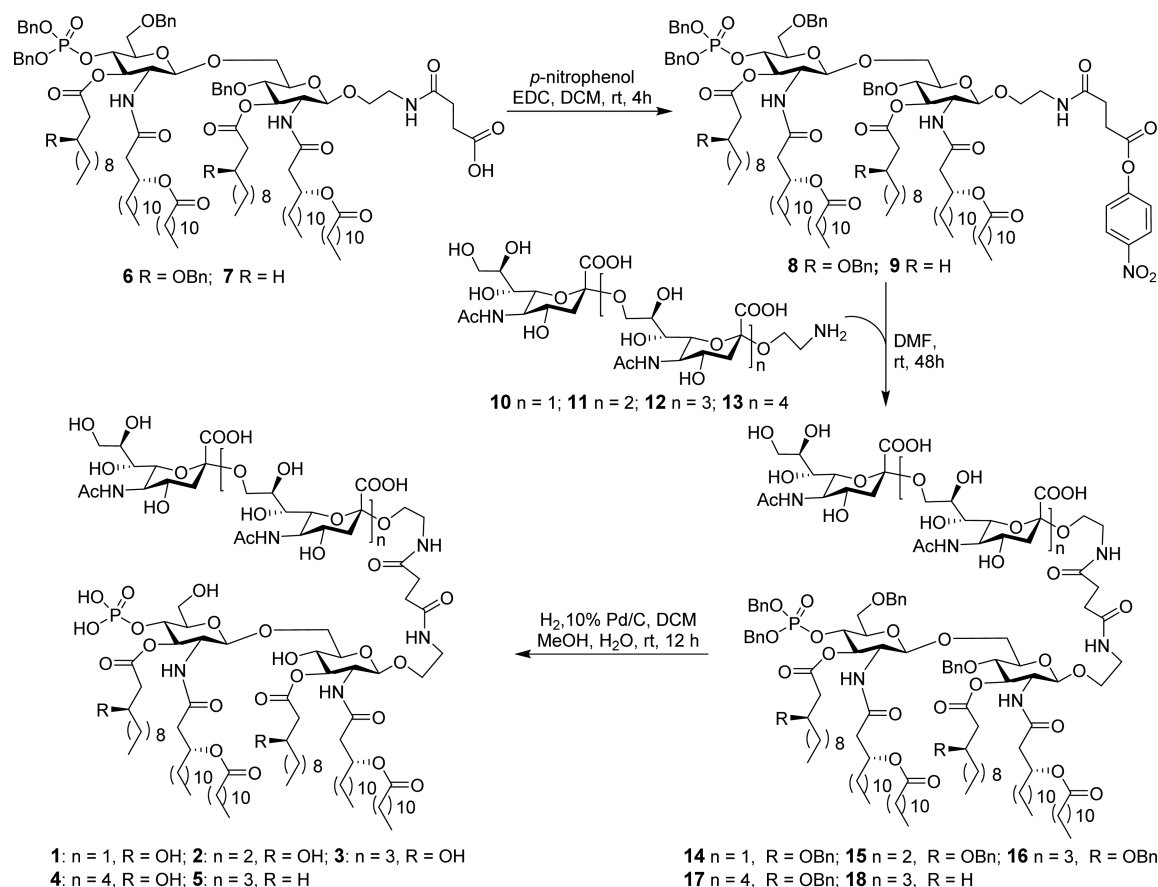


Figure 2. ELISA results of disialic acid specific total (anti-kappa), IgG1, IgG2b, IgG2c, IgG3, and IgM antibodies elicited by liposomes of **1** alone or in combination with an adjuvant, including CFA, alum, and TiterMax Gold adjuvant. The error bar represents the standard error of three parallel experiments.

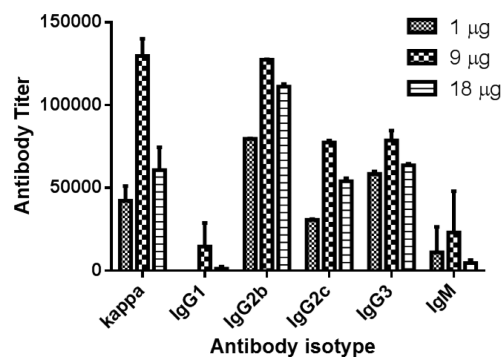


Figure 3. ELISA results of the titers of various disialic acid specific antibodies in the day 38 pooled antisera of **1** injected in doses of 1, 9, and 18 μg of carbohydrate antigen/mouse. The error bar represents the standard error of three parallel experiments.

the conjugates **1–5** elicited strong immune responses, revealed by high total antibody titers, and IgG2b was the major antibody subclass, indicating T cell dependent immunity.^{11,27,51,52} Moreover, antibody titers for **1–4** decreased progressively with sugar chain elongation, suggesting that shorter oligosialic acids were overall better immunogens than longer ones. Nevertheless, the IgG2b antibody titer for **4** was still high. In addition, **5** and **3**, which have the same oligosialic acid but different MPLA, gave different total antibody titers (Figures S5, S6), indicating the impact of MPLA structure on the immunogenicity of these conjugates. However, the IgG2b

antibody titers for **5** and **3** were only slightly different (<2-fold), thus **5** could still elicit strong T cell dependent immunity.

Cross-Reactivity of Each Antiserum with All Other Oligosialic Acids. Cross-reactions between the antisera of **1–4** and all synthetic oligosialic acids were assessed by ELISA. As shown in Figure 6A, antiserum **1** had strong reactions with disialic acid, but its reactivity with other oligosialic acids decreased significantly: di- > tri- > tetra- > pentasaccharide. Thus, a portion of antibodies elicited by **1** was probably specific to disialic acid, not reactive with longer oligosialic acids. A potential explanation was that the conformation of disialic acid in **1** was affected by the carrier molecule to result in antibodies that could only recognize specific conformers. Antiserum **2** had

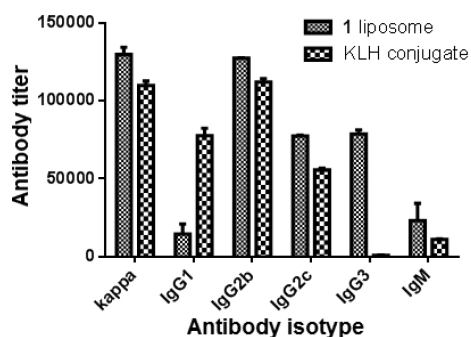


Figure 4. ELISA results of the titers of various disialic acid specific antibodies in the pooled antisera of disialic acid–MPLA conjugate 1 and disialic acid–KLH conjugate. Error bar represents the standard error of three parallel experiments.

a similar decreasing binding trend but to a lesser intensity. In contrast, antisera 3 and 4 had essentially the same reactivity with all oligosialic acids. Moreover, we discovered that the antisera did not react with other sialic acid containing antigens,

such as GM3, sTn, and α -2,8-polysialic acid.⁴⁸ Thus, the majority of antibodies elicited by 2–4 should recognize a common and specific antigenic epitope, that is, α -2,9-linked sialic acids.

Reactivity of the Antisera with α -2,9-Polysialic Acid, the Natural CPS of Group C *N. meningitidis*. As revealed by the ELISA results (Figure 6B), all of the antisera 1–4 had strong reactions with the natural CPS of group C *N. meningitidis*. Antisera 2–4 had similar reactivity and their antibody titers were comparable to that of the reaction with corresponding oligosialic acids, but antiserum 1 exhibited decreased reactivity with the CPS. Nonetheless, the reaction of 1 was still strong, indicating that a significant portion of the antibodies elicited by 1 could recognize and bind to the CPS. Through a competitive ELISA experiment we have also demonstrated that the bacterial CPS could inhibit antibody binding to oligosialic acids (Supporting Information), further verifying the specific binding of elicited antibodies to the natural antigen.

Binding between Antisera and *N. meningitidis* Cell.

These assays were carried out using a Bio-Dot microfiltration apparatus equipped with a PVDF membrane. Prefixed *N.*

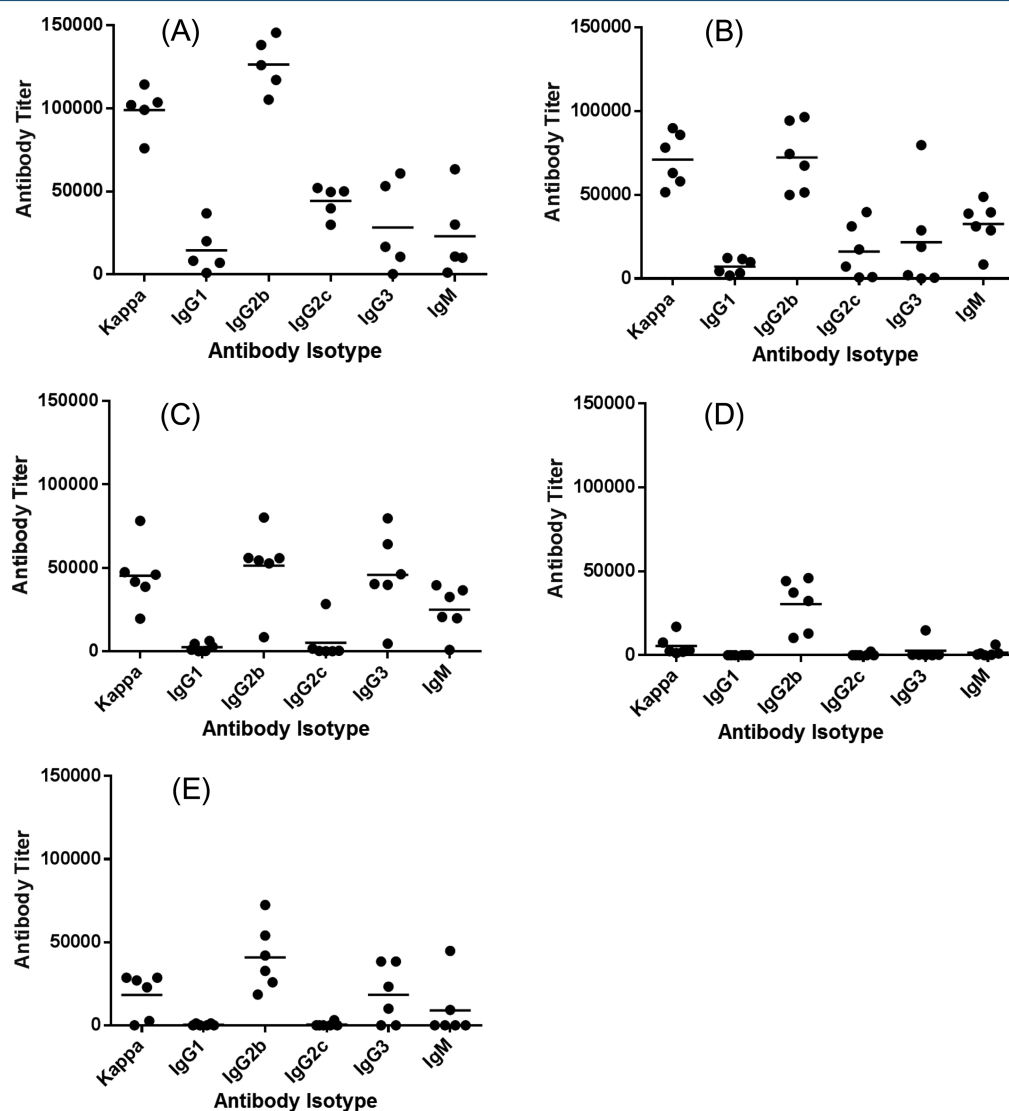


Figure 5. ELISA results of the day 38 antisera of 1 (A), 2 (B), 3 (C), 4 (D), and 5 (E). Each dot represents the antibody titer of an individual mouse, and each black bar represents the average titer for each group.

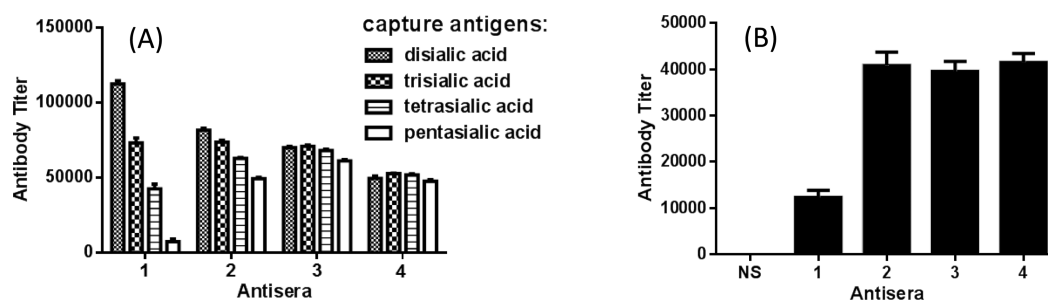


Figure 6. ELISA results of the cross-reactions of normal mouse serum (NS) and pooled antisera of 1–4 with (A) di-, tri-, tetra-, and pentasialic acids and (B) the CPS of group C *N. meningitidis*. The error bar represents the standard error of three parallel experiments.

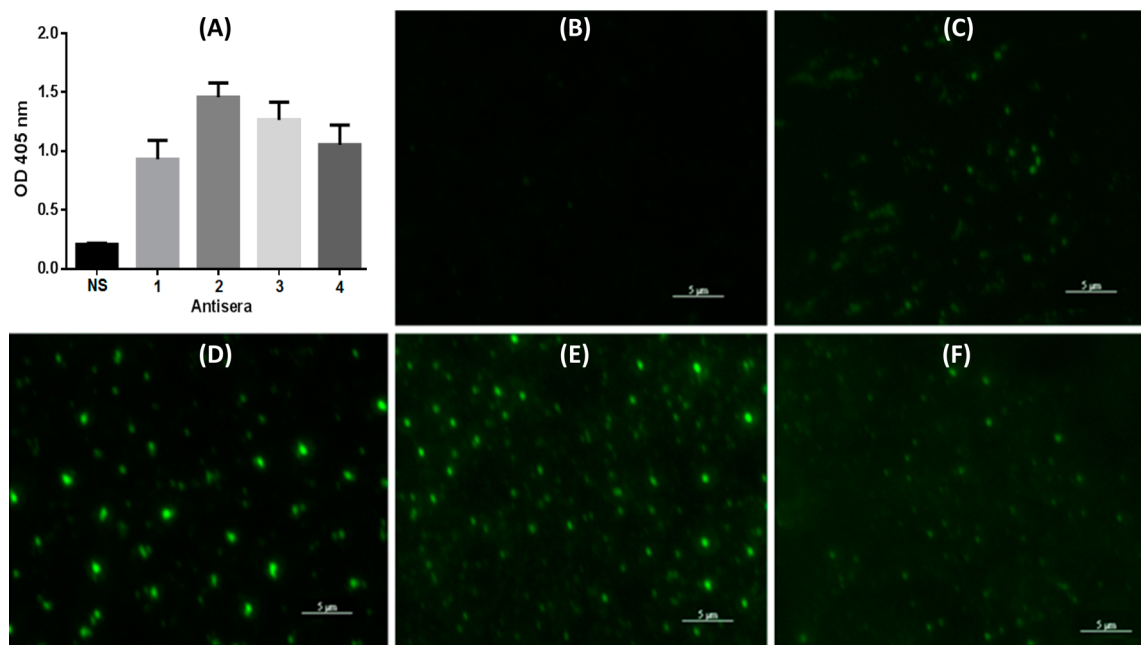


Figure 7. (A) Results of the binding between *N. meningitidis* cell and pooled day 38 antisera of 1–4, with normal mouse sera (NS) as negative controls. All of the mouse sera were 1:100 diluted. Error bars show the standard errors of three parallel experiments. The differences between NS and all antisera of 1–4 were statistically significant ($P < 0.05$). (B–F) Fluorescent images of *N. meningitidis* cells stained with NS (B) or with the antisera of 1 (C), 2 (D), 3 (E), and 4 (F). The white reference bar is 5 μm.

meningitidis cells were incubated with antisera 1–4 and then an alkaline phosphatase (AP) conjugated antibody, and finally examined at 405 nm wavelength. The results (Figure 7A) revealed that antibodies in the antisera could recognize and bind to the bacterial cell, as shown by the fluorescent images of cells treated with antisera and FITC-labeled anti-kappa antibody (Figures 7B–7F). Interestingly, antiserum 1, which exhibited the highest total antibody titer, had significantly weaker binding as compared to antisera 2–4. Thus, at least a portion of antibodies elicited by 1 did not bind to α -2,9-polysialic acid on the bacterial cell, which is consistent with conclusions of the cross-reactivity assays (Figure 6). Overall, the binding of antisera 2–4 to bacterial cells mirrored the ELISA results (Figure 5). Despite the decreased binding at high dilution numbers, this effect was still significant at 1:800 (Supporting Information). Moreover, similar to our previous discovery,⁴⁸ antisera 1–4 did not have obvious binding to cancer cells expressing other sialoglycans than α -2,9-polysialic acids (Supporting Information). These results have verified that antibodies elicited by 1–4 could recognize and target specifically group C *N. meningitidis* cell. Collectively these

data suggest that 1–4 and especially 2–4 can be functional vaccines.

Bactericidal Activity of Antisera 1–4 to *N. meningitidis* Cell. In this study, bacteria were incubated with serially diluted normal mouse serum or the antisera of 1–4, respectively, in the presence of rabbit complements. The number of surviving bacteria were then counted after culture on agar plates. As depicted in Figure 8, all of the antisera mediated strong, serum concentration dependent toxicity to the bacterial cell. At a 1:2 dilution >98% of the bacterial cells were killed by the antisera, while the dilution number for 50% killing was ca. 1:8 for all antisera. These results provided strong evidence for the synthetic vaccines to elicit protective immunities against group C meningitis. It was interesting to note that all of the antisera exhibited similar bactericidal activity despite the lower CPS-binding capability of antiserum 1 compared to other antisera, suggesting that antibacterial vaccines are protective so long as they provoke the proper and robust antibody responses.^{14,42}

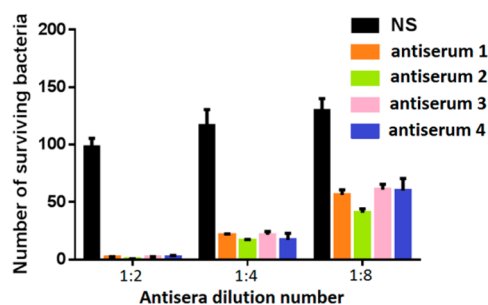


Figure 8. Results of bactericidal assays using normal mouse serum (NS) and pooled antisera of 1–4. The error bar represents the standard error of three parallel experiments.

CONCLUSION

Two series of MPLA conjugates of α -2,9-linked oligosialic acids were prepared and evaluated as fully synthetic vaccines. These structurally defined conjugates enabled in-depth immunological studies and the use of structure–activity relationship analysis to help identify proper α -2,9-oligosialic acid antigens and MPLA conjugates for anti-group C meningitis vaccine development.

Immunological studies on α -2,9-oligosialic acid-MPLA conjugates 1–4 showed that they alone elicited high total and IgG antibody titers, verifying their self-adjuncting property. Conjugates 1–4 were used as liposomes prepared with phosphatidylcholine (PC) and cholesterol to improve their water solubility and to create homogeneous formulations. Although liposomes could improve the immunogenicity of conjugate vaccines^{49,50} and negatively charged liposomes consisting of PC, cholesterol, and diacetylphosphate were found to have adjuvant activity,⁵⁵ liposomes consisting of PC and cholesterol did not have adjuvant activity.⁵⁵ Thus, the adjuvant activity of 1–4 was due to MPLA. In addition, Boons and co-workers^{56,57} showed that glycoconjugates made of immunosilent lipopeptides, instead of TLR agonists, did not elicit robust IgG antibody responses, and we found that conjugates made of protected MPLA were immunologically inactive.³⁷ The MPLA structure was also demonstrated to have a significant impact on the immunogenicity of these conjugates. For example, the immune response to 3 was stronger than that to 5 carrying the same oligosialic acid but a different MPLA. Our detailed analysis of cytokine and chemokine releases induced by similar MPLA conjugates⁵⁸ provided further support that the self-adjuncting activity of 1–4 was due to the TLR agonist property of MPLA.

We have shown that the vaccine dose used for immunization had only a small impact on the immune response. Among the tested doses for 1 (1, 9, and 18 μ g/mouse), the 9 μ g group had the highest titers of total and various isotypes of antibodies. Nonetheless, mice in all the dose groups exhibited a robust and similar pattern of immune responses.

Structure–activity relationship studies on 1–4 revealed the difference of oligosialic acids in their immunogenicity, which decreased with sugar chain elongation from di- to pentasialic acids. Thus, 1 induced the highest titers of antigen-specific total and IgG2b antibodies. However, some of the antibodies elicited by 1 bound to disialic acid but not to other oligosialic acids or bacterial CPS. On the other hand, antibodies induced by 2–4 could recognize and react with all oligosialic acids and the CPS and antiserum 2 exhibited the highest binding to bacterial cells. Despite the fact that the titers of antibodies induced by 1–4 decreased as the sugar chain was elongated and that some of

the antibodies elicited by 1 did not bind to the CPS, 1 and 4 elicited robust immune responses and sufficient antibodies that could effectively recognize and bind to the bacterial cell.

More importantly, 1 and corresponding KLH conjugate elicited a strong and similar pattern of immune responses, namely, both mainly inducing IgG2b, IgG2c, and some IgG1 and IgG3 antibodies. Similar antibody responses were also observed with other MPLA conjugates. Robust IgG antibody response indicates T cell dependent immunity, meaning desirable antibody affinity maturation, better antibody-mediated cell and complement-dependent cytotoxicity, and long-term immunologic memory.^{11,27,51,52} IgG antibody subclasses are defined according to their Fc regions and differ in their ability to activate the immune system, and their activity hierarchy is IgG2a \sim IgG2b > IgG1 \gg IgG3.⁵⁹ C57BL/6J mouse does not have IgG2a gene but expresses the allelic IgG2c antibody.^{60,61} In addition, among various IgG antibody subclasses, IgG2b is the most potent for effector response activation and antimicrobial immunity.^{11,27,51,52} The induction of high levels of IgG antibody, especially IgG2b and IgG2c, by 1–4 suggested their great potential as protective vaccines against group C meningitis,^{62,63} which was eventually verified by the strong bactericidal activities observed with their antisera.

In conclusion, we have demonstrated in this work that oligosialic acid–MPLA conjugates 1–5 were self-adjuncting, which alone elicited strong T cell dependent immunity comparable to that induced by corresponding KLH conjugates. Especially, 2 and 3 elicited robust immune responses and high titers of antibodies that mediated effective killing of group C *N. meningitidis* cell. This conclusion agrees well with that of a study on oligosialic acid–KLH conjugates.⁴⁸ However, as aforementioned, fully synthetic vaccines with defined structures have a number of advantages over conventional carbohydrate–protein conjugates. Thus, 2 and 3 were identified as promising anti-group C meningitis vaccines worthy of further investigation.

EXPERIMENTAL SECTION

Vaccine Formulation. Liposomes of 1–5 were prepared by a previously reported protocol.^{37,38} After a conjugate (0.42 μ mol), DSPC (2.15 mg, 2.7 μ mol), and cholesterol (0.81 mg, 2.1 μ mol) in 10:65:50 molar ratio were dissolved in a mixture of CH₂Cl₂, MeOH, and H₂O (3:3:1, v/v, 2 mL), solvents were removed under reduced pressure via rotary evaporation, which created a thin lipid film on the vial wall. This film was hydrated in HEPES buffer (20 mM, pH 7.5, 3.0 mL) containing 150 mM of NaCl in a water bath at 60 °C. The mixture was shaken on a vortex mixer and then sonicated for 20 min to generate liposomes used for immunization. The size of these liposomes was determined by dynamic light scattering (DLS) measurement, and the average diameter was about 1500 nm with a polydispersity index (PDI) of around 0.60.

CFA, alum, and TiterMax Gold Adjuvant emulsions of vaccines were obtained by thoroughly mixing the liposomes of 1 (0.75 mL) or KLH–disialic acid with an adjuvant (0.75 mL), according to the manufacturer’s instruction.⁵³

Immunization of Mouse. Each group of five mice was inoculated via subcutaneous (sc) injection of a liposome of 1–5 (0.1 mL) on day 1 for initial immunization. In the CFA, alum, and TiterMax groups, mice were inoculated via intramuscular (im) injection of an emulsion of the adjuvant and a specific conjugate (0.1 mL). After initial immunization, mice were boosted 3 times on days 14, 21, and 28 by injection of the same conjugate (0.1 mL). Antisera were prepared from blood

samples collected via the mouse leg veins prior to initial immunization on day 0 and after immunization on days 28 and 38. Antisera were stored at $-80\text{ }^{\circ}\text{C}$ before immunologic assays.

ELISA.³⁸ ELISA plates were coated with a specific oligosaccharic acid–HSA conjugate ($100\text{ }\mu\text{L}$, $2\text{ }\mu\text{g}$ of sialic acid/mL) dissolved in the coating buffer (0.1 M bicarbonate, pH 9.6) at $37\text{ }^{\circ}\text{C}$ for 1 h. After washing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST), the plates were treated with a blocking buffer [10% bovine serum albumin (BSA) in PBST] at rt for 1 h. Then, a pooled or an individual mouse antiserum ($100\text{ }\mu\text{L}$) with serial half-log dilutions from 1:300 to 1:656100 in PBS was added, which was followed by incubation at $37\text{ }^{\circ}\text{C}$ for 2 h. The plates were washed with PBST and incubated at rt for 1 h with 1:1000 diluted solution ($100\text{ }\mu\text{L}$ /well) of AP-coupled goat anti-mouse kappa, IgM, IgG1, IgG2b, IgG2c, and IgG3 antibodies, respectively. The plates were washed with PBST and then treated with a *p*-nitrophenylphosphate (PNPP) buffer ($100\text{ }\mu\text{L}$, 1.67 mg/mL) at rt for 30 min. Finally, the plates were examined at 405 nm wavelength. The OD values after deducting background OD of the day 0 sera were plotted against dilution numbers, and the best-fit equation was obtained for each set of data and used to calculate the antibody titer, defined as the dilution number giving an adjusted OD value of 0.20.

Antiserum Binding to *N. meningitidis* Cell. A modified protocol for ELISA using a Bio-Dot microfiltration apparatus⁴⁸ was used to assess antiserum binding to the bacterial cell. Briefly, the PVDF membrane was pretreated in the blocking buffer (1% BSA in PBST) and then set on the apparatus. A suspension of killed *N. meningitidis* (ATCC 31275) cells in PBS ($50\text{ }\mu\text{L}$, OD 0.2 at 600 nm) was added. After PBS buffer was removed through filtration, the bacterial cells remaining in the wells were incubated with the same blocking buffer ($200\text{ }\mu\text{L}$) at rt for 1 h to mask any nonspecific binding sites on the bacterial cell, and the blocking buffer was removed through filtration under vacuum. The plate was washed with PBST ($350\text{ }\mu\text{L}$) 3 times, followed by addition of $100\text{ }\mu\text{L}$ of normal mouse serum or pooled antisera (1:100 dilution in PBS) of 1–4. The plate was incubated at $37\text{ }^{\circ}\text{C}$ for 2 h and washed 6 times with PBST ($350\text{ }\mu\text{L}$). To each well was added 1:1000 diluted AP-conjugated goat anti-mouse kappa antibody solution ($100\text{ }\mu\text{L}$ /well), and the plate was incubated at rt for 1 h. Finally, the plate was washed with PBST 6 times and developed with a PNPP solution (1.67 mg/mL in buffer, $200\text{ }\mu\text{L}$) at rt for 30 min. An aliquot of the solution ($100\text{ }\mu\text{L}$) in each well was transferred into a clear, round-bottom 96-well plate for colorimetric readout at 405 nm wavelength. The binding between antibodies and the cell was reflected by the observed OD value.

For cell imaging, a suspension of killed *N. meningitidis* cells was smeared on IF microscope slides. The slides were dried, washed with PBST, and then treated with 3% FBS blocking buffer at $37\text{ }^{\circ}\text{C}$ for 1 h. After washing, the slides were incubated with normal mouse serum or a 1:10 diluted antiserum at $37\text{ }^{\circ}\text{C}$ for 2 h. The slides were washed with PBST and treated with FITC-labeled goat anti-mouse kappa antibody at rt for 30 min. The slides were washed and mounted with the fluoromount aqueous mounting medium. The cell images were obtained with a Zeiss ApoTome Imaging System using a $100\times/1.30$ oil objective lens.

Bactericidal Assays of the Antisera. The protocol was similar to that reported in the literature.¹⁴ A $-80\text{ }^{\circ}\text{C}$ stock culture of serogroup C *N. meningitidis* (ATCC 31275) was streaked and incubated in #953 broth at $37\text{ }^{\circ}\text{C}$ for 6 h.

Thereafter, the bacterial cells were suspended in #953 broth and adjusted to a concentration of 10^5 cfu/mL. The bacterial suspension ($20\text{ }\mu\text{L}$, 2000 cfu) was mixed and incubated with 2-fold serially diluted (1:2 to 1:16) normal mouse serum or pooled day 38 antiserum of 1–4 ($10\text{ }\mu\text{L}$) at $37\text{ }^{\circ}\text{C}$ for 15 min. In the meantime, a mixture of #953 broth ($10\text{ }\mu\text{L}$) and bacterial suspension ($20\text{ }\mu\text{L}$) was set as the complement-only control. After incubation, $50\text{ }\mu\text{L}$ of rabbit complement (1:1 diluted in #953 broth) was added, followed by incubation at $37\text{ }^{\circ}\text{C}$ for 1 h. Finally, $6\text{ }\mu\text{L}$ of the mixture was spread on the #953 agar plates. After 36 h of culture, the number of surviving bacteria in each sample was counted.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acscentsci.5b00364](https://doi.org/10.1021/acscentsci.5b00364).

Instrumentation, general methods, synthetic procedures, biological assays, and NMR and MS spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: (+1)-313-577-2557. E-mail: zwguo@chem.wayne.edu.

Author Contributions

[†]G.L. and Z.Z. contributed equally to the present work.

Notes

The authors declare no competing financial interest.

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