INTRODUCTION

Acinetobacter baumannii is a Gram-negative bacterium, which can cause a wide range of hospital and community-acquired infections such as bacteremia, pneumonia, meningitis, urinary tract infections, and skin and soft tissue infections. Predominantly, it is an opportunistic pathogen that can cause severe hospital-acquired infections, especially among immunocompromised individuals. A. baumannii can also colonize in humans without causing infections or symptoms and commonly exists in the natural environment. Bacterial resistance to multidos is posing a global threat to the public health and severely affecting the effectiveness of public health management. A. baumannii has demonstrated the ability to acquire resistance to numerous classes of antibiotics via multiple resistance mechanisms. A. baumannii is thought to exhibit extensive resistance to most last-line antibiotics in recent years, yet it remains sensitive to most antibiotics discovered before the 1970s. Carbapenems have served as the last-resort antibiotics to treat A. baumannii infections for years. However, the increasing trend of carbapenem resistance in A. baumannii worldwide has limited their efficacy and promoted the use of polymyxins and tigecycline as the last-line drugs. However, the emergence of A. baumannii strains that are resistant to colistin and tigecycline has been reported, aggravating clinical problems caused by carbapenem-resistant (CR) A. baumannii. The treatment of A. baumannii infections has become difficult due to the emergence of multidrug-resistant strains; hence, development of new strategies for preventing and treating infections caused by this pathogen becomes necessary. Among the 12 “priority pathogens” requiring urgent antibacterial R&D published by the World Health Organization (WHO) in 2017, A. baumannii is on the top of this list as the top priority for immediate attentions. Development of new antibacterial drugs against multidrug-resistant (MDR) A. baumannii has been pursued by researchers worldwide. Apart from a search for novel antibiotics, vaccination or immunotherapy is an alternative strategy to protect people from bacterial infections and combat multidrug resistance. Over the past decades, a growing number of vaccine candidates against A. baumannii including whole bacteria, outer membrane vesicles or complexes, DNA-based vaccines, and purified or recombinant subunits have been proposed and studied. Bacterial surface carbohydrates have been proven effective as potential antigens for development of vaccines against infectious diseases. Glycoconjugate vaccines have been successfully developed and effectively used against Haemophilus influenzae type B, selected serotypes of Streptococcus pneumoniae, Neisseria meningitidis serogroups A, C, W, and Y, Salmonella typhi, and Campylobacter jejuni. The structure of carbohydrate-based antibacterial vaccines is commonly composed of carbohydrate antigens, linkers, and carrier proteins. The carbohydrate antigen can be the bacterial surface polysaccharide isolated from cultured bacteria, as in the case of Prevnar 13 (Pfizer, approved by the FDA in 2010), which contains cell capsule sugars of 13 serotypes of S. pneumoniae (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 23F, and 33F).
9V, 14, 18C, 19A, 19F, and 23F) conjugated to diphtheria CRM197 carrier protein. However, not all pathogens can be readily cultured, and bacterial polysaccharide extraction can be plagued with contaminations. Alternatively, synthetic carbohydrate antigens are structurally defined and free from cell-derived contaminants; an example is Quimi-Hib (CIGB, approved in 2004 in Cuba), which contains synthetic polyribosylribitol phosphate conjugated to tetanus toxoid carrier protein. Various synthetic carbohydrate-based vaccine candidates against different pathogens are being explored.

Pseudaminic acid (Pse), which belongs to the nonulosonic acid family, is widely distributed in numerous pathogenic bacteria as a component of repeating units used to construct cell surface-associated glycans, such as lipopolysaccharide (LPS) in P. aeruginosa, Shigella Boydii, and Vibrio vulnificus, capsular polysaccharide (CPS) in A. baumannii, pili in P. aeruginosa, and flagella in Aeromonas caviae, H. pylori, and Campylobacter jejuni. Although the evolutionary origin and exact function of Pse on the bacterial cell surface remain unclear, it is likely that Pse plays an important role in bacterial pathogenicity, as it is highly associated with virulence factors LPS, CPS, and flagella and is unique to Gram-negative bacteria and structurally reminiscent to mammalian sialic acids. On the other hand, CPS from pathogenic bacteria containing Pse has been regarded as a potential target for vaccine development. Recently, Wu et al. reported that the bacteriophage ΦAB6 tailspike protein was capable of specifically recognizing the exopolysaccharide (EPS) of A. baumannii strain S4149 and depolymerizing it to oligosaccharide Pse5NAc7NAc-(2→6)-Glcp-β-(1→3)-Galp-β-(1→3)-GdnAc-p-β-(1→3)-GalNAc-p-β-(1→3) as the major product. The resultant oligosaccharide was conjugated to the carrier protein and used as the vaccine, and the boosted sera from vaccinated rabbit recognized the EPS from A. baumannii strain S4149 but not EPS from strain SK44, which shared most sugar components with AB 54149 except for Pse. These studies demonstrated the critical role of Pse as the epitope for immune response and the potential therapeutic application of the Pse conjugate as antibacterial vaccines.

RESULTS AND DISCUSSION

Design and Synthesis of Pse-CRM197 conjugates.

Figure 1. Design and synthesis of Pse-CRM197 conjugates.

bacteriophage ΦAB6 tailspike protein was capable of specifically recognizing the exopolysaccharide (EPS) of A. baumannii strain S4149 and depolymerizing it to oligosaccharide Pse5NAc7NAc-(2→6)-Glcp-β-(1→3)-Galp-β-(1→3)-GdnAc-p-β-(1→3)-GalNAc-p-β-(1→3) as the major product. The resultant oligosaccharide was conjugated to the carrier protein and used as the vaccine, and the boosted sera from vaccinated rabbit recognized the EPS from A. baumannii strain S4149 but not EPS from strain SK44, which shared most sugar components with AB 54149 except for Pse. These studies demonstrated the critical role of Pse as the epitope for immune response and the potential therapeutic application of the Pse conjugate as antibacterial vaccines. Considering the challenges such as antigen heterogeneity and low batch-to-batch reproducibility encountered in isolating the CPS antigen from cultured bacteria and the structural limitation associated with the bacteriophage strategy due to the fact that naturally existing Pse molecules form diverse structures in pathogenic bacteria, we herein reported the first synthetic Pse-based vaccine against Pse-bearing bacterial pathogens. The constructed Pse-CRM197 conjugates were capable of stimulating high immune responses in a mouse model and conferred protection of the vaccinated mice against infections by Pse-bearing A. baumannii.
able to elicit an immune response against Pse-containing *A. baumannii* CPS and protect the vaccinated animal host from lethal infection. To test this hypothesis, we designed the α-pseudaminoside-based conjugate vaccine, in which pseudaminic acid bearing acetylated N5 and N7 sites linked to a tetraethylene glycol spacer via α-linkage was chosen as the epitope (Figure 1). The α-Pse5NAc7NAc unit has been identified as the component of K2 serotype CPS, which has been found in several antibiotic-resistant *A. baumannii* strains.33−35 The Pse species could be chemically synthesized by the *de novo* approach developed by our group,36 and the desired α-glycosidic linkage could be constructed using our additive controlled α-pseudamination method.37 As carbohydrates are T-cell-independent antigens that cannot generate strong, long-lasting, and memorable IgG antibodies, conjugation with an immunogenic carrier protein capable of activating helper T cells is necessary to enhance the antiglycan antibody titer. The nontoxic mutant of diphtheria toxin, CRM197, is currently widely used as the carrier protein for glycans to make them immunogenic. We designed an ortho-phthalaldehyde (OPA)−pseudaminic acid linker, where OPA has been demonstrated to be an efficient tool for bioconjugation via reaction with primary amines,38 to conjugate pseudaminic acid to the carrier protein.

To this end, the stereoselective glycosylation of the Pse thioglycoside donor 4 with Fmoc-protected PEG linker 5 gave α-pseudaminoside 6 in 80% yield (Figure 1c). After converting N5-azide and N7-benzyl carbamate to acetamides via hydrogenolysis and acetylation, the Fmoc group in 7 was removed, and the released free amine was coupled with acid 8 containing the o-phthalaldehyde methyl acetal moiety to give 9. The isopropyl ester and O-acettes were removed by treating 9 with lithium hydroxide, and the acetal was subsequently hydrolyzed by 10% aqueous acetic acid solution to give the Pse−OPA species 10. Finally, 10 was reacted with CRM197 carrier protein in phosphate buffered saline (PBS, pH 7.4) to generate Pse−CRM197 conjugates. Compared with the widely used thiol-maleimide conjugation, this single step OPA conjugation using purified Pse−OPA species could avoid the premodification of carrier protein and incorporation of bare linker species (in blocked form by cysteine treatment), which may induce the undesired immune response to the linker and suppress the desired response. To explore the difference in the immune response caused by different antigen loading, we synthesized the conjugates using 20, 30, and 50 equiv of the OPA−Pse
moiety to generate the Pse−CRM197 conjugate (sugar/protein ratio: 4.76), Pse−CRM197 (sugar/protein ratio: 8.27), and Pse−CRM197 (sugar/protein ratio: 14.34), respectively. In addition, to verify the anti-Pse antibody generated by the vaccination, we also synthesized Pse−bovine serum albumin (BSA) conjugate as the surrogate of a natural glycan in which a different alkyl linker and thiol-maleimide conjugation chemistry was used to diminish the unexpected recognition of the carrier protein and linker by the boosted antisera (for details, see Supporting Information).

**Antibody Responses to Pse−CRM197 Vaccines.** The immunogenicity of Pse−CRM197 conjugates was assessed by immunizing male C57BL/6J mice mixed with aluminum hydroxide as an adjuvant in a prime boost regimen (Figure 2a). The control group received CRM197 mixed with aluminum hydroxide in PBS. The Pse-specific antibody response in postimmune sera was characterized by ELISA using the Pse−bovine serum albumin (BSA) conjugate 11 as the surrogate of a natural glycan in which a different alkyl linker and thiol-maleimide conjugation chemistry was used to diminish the unexpected recognition of the carrier protein and linker by the boosted antisera (for details, see Supporting Information).

The antibody titers increased significantly on day 21, 1 week after receiving the second dose for all the three Pse−CRM197 vaccines (Figure 2c). The antibody response of Pse−CRM197 was found to be slightly lower when compared to that of Pse−CRM197 and Pse−CRM197 2, which might be due to the overcrowded sugar content. On day 35, 1 week after receiving the third dose, the antibody titers for all three Pse vaccines remained stable (Figure 2d). Furthermore, the antibody responses were sustainable for all the three Pse vaccines when measured within a 1-month period, which is 5 weeks after the last immunization (Figure 2e). In addition, the titers for Pse−CRM197 3 were comparable to levels of Pse−CRM197 1 and Pse−CRM197 2 finally. These data demonstrated that vaccination of the Pse vaccines elicited significant levels of IgG against Pse−BSA, whereas the control mice had no detectable antigen-specific IgG.

To characterize the immune responses elicited by immunization of the Pse−CRM197 vaccines, isotyping of Pse-specific antibodies in postimmune sera on day 35 was determined by ELISA (Figure 3). None of the test formulations induced the production of detectable amounts of IgA antibodies. For the three Pse−CRM197 vaccines, IgG1, IgG2b, and IgG3
contributed the bulk of the Pse-specific IgG titer, while a weak IgG2c response was observed, demonstrating that immunization with the Pse−CRM197 vaccines produced antibodies of three subtypes. In contrast, mice vaccinated with only CRM197 failed to elicit Pse-specific IgG. All the detected Pse-specific antibodies contained kappa light chains. These data indicate that vaccination of all the three Pse vaccines elicits humoral immune responses and produces sustainable and significant levels of Pse-specific antibodies of IgG type.

Flow Cytometry Analysis of Postimmune Sera toward A. baumannii Strain 00.191. The binding capacity of postimmune sera toward Pse-containing CPS was further determined by flow cytometry using A. baumannii strain 00.191, a multidrug-resistant strain recently isolated from a clinical sample. The serotype of strain Ab-00.191 was identified as K2 type via genomic sequencing, and the existence of Pse was verified by metabolic labeling study in our laboratory.39 Bacteria incubated with postimmune sera of animals treated with the three Pse vaccines exhibited similar profiles, whereas the CRM197 control resembled the negative control (Figure 4). The significant difference between the fluorescence strength of the test samples and the negative control illustrated that glycoconjugate-boosted sera can recognize bacteria bearing Pse on the surface.

Immunization with Pse−CRM197 Vaccines Protects Mice from A. baumannii Infection. To characterize the efficacy of the Pse vaccines, the vaccinated and control mice were challenged with A. baumannii strain Ab-00.191 using a clinical sample.
The LD$_{50}$ of strain Ab-00.191 was determined by infecting mice with different doses of bacteria. Infection of mice with $5.9 \times 10^6$, $1 \times 10^7$, and $5.9 \times 10^7$ CFU of strain Ab-00.191 led to 20, 50, and 100% mortality, respectively (Figure 5a). We next determined if the response produced by immunization with the Pse vaccines was sufficient to provide protection from infection with *A. baumannii*. Mice immunized as previously described, at weeks 0, 2 and 4, were challenged with *A. baumannii* strain Ab-00.191 2 weeks after the final immunization and monitored for survival over 7 days (*n* = 4 mice/group). The mice immunization and challenge experiments were repeated twice, and the data on Figure 5b,c were the combination of these two repeats. Vaccinated mice challenged with $2.0 \times 10^7$ CFU ($2 \times$ LD$_{50}$) of Ab-00.191 strain were completely protected from the challenge, whereas all control mice received CRM197 and negative control mice died within 36 h (Figure 5b). When challenged with $5.0 \times 10^7$ CFU ($5 \times$ LD$_{50}$) of strain Ab-00.191, 25% mortality was recorded for mice that received Pse–CRM197 1 and Pse–CRM197 3, 0% mortality for mice that received Pse–CRM197 2, and 100% mortality for control mice that received CRM-197 as well as the negative control mice (Figure 5c). These data indicate that all of the test Pse vaccines provide protection against infections caused by Pse-bearing *A. baumannii* strains.

Using the *A. baumannii* sepsis model, the effect of vaccination on postinfection tissue bacterial loads was determined by measuring the quantities of viable bacteria in blood and tissues of vaccinated and control mice (*n* = 4 mice/group) 12 h after infection with $5.0 \times 10^7$ CFU ($5 \times$ LD$_{50}$) of strain Ab-00.191. This experiment was repeated twice, and the data were the combination of these two repeats. Vaccination resulted in a reduction in tissue bacterial loads by $10^3$–$10^5$-fold when compared to the control mice for all tissues tested and $10^6$-fold for blood (Figure 6). Serum levels of proinflammatory cytokines IL-1$\beta$, IL-6, and TNF-$\alpha$ were also measured to determine if immunization with these Pse–CRM197 vaccines was able to protect infected mice from release of these cytokines during bacterial sepsis. It was found that the levels of all three cytokines were significantly lower in vaccinated mice when compared to the control mice, suggesting that the vaccinated mice did not experience the effect of proinflammatory cytokines, which were released during septic shock (Figure 7).

**CONCLUSION**

In summary, we have developed synthetic Pse-based glycoconjugates as antibacterial vaccines. A promising finding is that the monosaccharide Pse can serve as the effective antigen that elicits a specific antibody response and confers protection on mice against infection by Pse-producing *A. baumannii* strain Ab-00.191. *A. baumannii* is a troublesome pathogen that causes infections with limited therapy options. With ready access to the synthetic Pse moieties, development of Pse-based vaccines against *A. baumannii* infection becomes highly feasible. In this study, we further demonstrated that OPA chemistry is an effective tool for conjugating synthetic carbohydrates onto carrier proteins in glycoconjugate synthesis. Nevertheless, one limitation that we encounter in this study is that the CPS structures of *A. baumannii* exhibit high diversity and that there are *A. baumannii* serotypes that do not produce a Pse moiety. To address this issue, future research efforts can be oriented to the design and development of multivalent glycoconjugates with different types of bacterial carbohydrates. It is also expected that Pse-based vaccines will be also effective against other Pse-bearing Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Campylobacter jejuni*.
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Author Contributions

R.W., H.L., and T.W. performed the chemical synthesis, X.Y. performed the biological studies, R.W., X.Y., S.C., and X.L. analyzed the data. S.C. and X.L. initiated and supervised the project. S.C. and X.L. wrote the manuscript with input from other authors.

Notes

The authors declare no competing financial interest.

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DEDICATION

Dedicated to Professor Samuel Danishefsky for his 85th birthday.

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