Addition of Laurydethylamine N-Oxide (LDAO) to a Copper-Free Click Chemistry Reaction Improves the Conjugation Efficiency of a Cell-Free Generated CRM197 Variant to Clinically Important Streptococcus pneumoniae Serotypes

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ABSTRACT: Strain-promoted azide−alkyne cycloaddition (SPAAC) reactions like click chemistry have the potential to be highly scalable, robust, and cost-effective methods for generating small- and large-molecule conjugates for a variety of applications. However, despite method improvements, the rates of copper-based click chemistry reactions continue to be much faster than the rates of copper-free click chemistry reactions, which makes broader deployment of click chemistry challenging from a safety and compatibility standpoint. In this study, we used a zwitterionic detergent, namely, lauryldimethylamine N-oxide (LDAO), in a copper-free click chemistry reaction to investigate its impact on the generation of conjugate vaccines (CVs). For this, we utilized an Xpress cell-free protein synthesis (CFPS) platform to generate a proprietary variant of CRM197 (eCRM) containing non-native amino acids (nnAA) with azide-containing side chains as a carrier protein for conjugation to several clinically relevant dibenzocyclooctyne (DBCO)-derivatized S. pneumoniae serotypes (types 3, 5, 18C, and 19A). For conjugation, we performed copper-free click chemistry in the presence and absence of LDAO. Our results show that the addition of LDAO significantly enhanced the reaction kinetics to generate larger conjugates, which were similarly immunogenic and equally stable to conjugates generated without LDAO. Most importantly, the addition of LDAO substantially improved the efficiency of the conjugation process. Thus, our results for the first time show that the addition of a zwitterionic surfactant to a copper-free click chemistry reaction can significantly accelerate the reaction kinetics along with improving the efficiency of the conjugation process.

INTRODUCTION

Strain-promoted azide−alkyne cycloaddition (SPAAC) reactions constitute an efficient, potentially scalable, and Good Manufacturing Practice (GMP)-compatible method for bio-orthogonal labeling and generation of antibody−drug conjugates or vaccines. Site-specific copper-free click chemistry eliminates use of toxic catalysts and can increase homogeneity and stability of antibody−drug conjugates in comparison to conjugates generated stochastically through utilization of endogenous lysine or cysteine residues. Meanwhile, both copper-catalyzed and copper-free SPAAC reactions are generally insensitive to solvent parameters and are mostly driven by a free energy gradient favoring generation of a covalent adduct. However, recent studies have shown that surfactants like sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and sodium decanoate can have a meaningful impact on the kinetics of both copper-catalyzed and copper-free click chemistry reactions. In a recent study, Schneider et al. showed that the addition of SDS and N-lauroylsarcosine

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enhanced the rate of a copper-catalyzed SPAAC reaction, resulting in higher yields of conjugated materials, while elsewhere, the presence of certain surfactants at concentrations above the critical micelle concentration (CMC) in click chemistry reactions was found to improve both conjugation efficiency and reaction kinetics. Recently, we also successfully utilized a zwitterionic detergent, lauryldimethylamine N-oxide (LDAO), to purify a protein vaccine antigen that showed high propensity for aggregation and precipitation by itself. Intriguingly, the addition of LDAO during the purification process dramatically improved the recovery yields of nonaggregated antigens. Since we are interested in utilizing these target antigens as carrier proteins for generating novel conjugate vaccines (CVs) using click chemistry, we wanted to further investigate the impact of LDAO on the copper-free SPAAC reaction.

In this study, we used SPAAC to conjugate dibenzocyclooctyne (DBCO)-derivatized several clinically important pneumococcal polysaccharide serotypes (types 3, 5, 18C, and 19A) to our proprietary in-house-generated catalytically inactive diphtheria toxin variant, namely, CRM197 (eCRM), in which specific amino acid residues are replaced with azide-containing non-native amino acids (nnAA). Interestingly, our results show that the addition of LDAO (at concentrations above CMC) significantly enhances the kinetics of conjugation of eCRM to each of the pneumococcal serotypes through stabilization of a low-energy state within the micellar architecture. Furthermore, conjugates generated in the presence of LDAO were larger in size, yet equally stable and as immunogenic as conjugates generated without the addition of LDAO to the reaction mixture. However, most importantly, our results demonstrate that even under nonoptimized conjugation conditions, the addition of LDAO alone to the SPAAC reaction was able to substantially increase the process efficiency, as assessed by estimation of % free azide in the purified conjugates, which may provide significant process improvement and conjugate recovery even at the commercial scale. Thus, to our knowledge, this is the first report showing that the addition of a zwitterionic detergent such as LDAO can significantly enhance the reaction kinetics and improve the efficiency of copper-free click chemistry reactions.

**RESULTS AND DISCUSSION**

Addition of LDAO Accelerates the Kinetics of the Copper-Free SPAAC Reaction through Stabilization of a Low-Energy Intermediate. To measure the effectiveness of the addition of LDAO on the kinetics of the copper-free SPAAC reaction in comparison to other surfactants (sodium deoxycholate (SDOC), PS80) or organic solvents (dimethyl sulfoxide (DMSO)), we performed the reaction of 3-azido-7-hydroxycoumarin (azidocoumarin) with DBCO-(PEG)$_4$-NH$_2$. Azidocoumarin has the advantage that it becomes substantially more fluorescent upon triazole formation post the SPAAC reaction. As shown in Figure 1A, the addition of LDAO strongly accelerates the conjugation reaction (orange curve), thereby promoting completion within an hour. In comparison, reactions performed in water alone or water supplemented with other surfactants or organic solvents were significantly slower.
slower. We calculated the reaction rate of LDAO, and we observed that LDAO accelerated the reaction by 4000 M$^{-1}$ s$^{-1}$, which is equivalent to 100-fold faster than rates calculated for the benzyl azide reaction with dibenzylcyclooctyne reagents accelerated by either SDS or by cationic detergents such as dodecyltrimethylammonium bromide (DTAB).21

Next, we performed SPAAC reactions in the presence of LDAO at concentrations above and below its CMC of 0.023%24 to investigate if the effect of LDAO was through improved ion-pairing or mediated by micellar catalysis (Figure 1B). To control the effects of the amount of catalyst on the reaction rate, we utilized sodium chloride to reduce the CMC to below 0.01%.28 While 0.01% LDAO was found to have no effect on reaction rate enhancement between eCRM and DBCO-activated polysaccharide (APS) type 9N in the absence of sodium chloride, the addition of sodium chloride produced a noticeable increase in the reaction rate (Figure 1B). This strongly suggests that the micellar structure is required for catalysis.

Because cycloaddition reactions between hydrophobic molecules have been shown to be accelerated (relative to the rate in a hydrophobic solvent) when colocalization is promoted within the cavity of an aqueous cyclodextrin,29 it is tempting to speculate that the hydrophobic interior of the micelle provides an environment that is conducive to catalysis. We favor the idea that the catalysis actually occurs on the surface of the micelle for several reasons. First, SPAAC reactions are actually slowed down in hydrophobic solvents.30 On the other hand, by the Gouy–Chapman theory, the surface of a micelle is made exceptionally hydrophilic due to the high concentration of salt. Second, substantial differences in catalysis have been noted between detergents of very similar chain length and tail composition.21 If it is the hydrophobic interior of the micelle accelerating the reaction, the rates should be similar. Lastly, it should be noted that while the micellar-based acceleration in the above-referenced study was shown to depend on having a hydrophobic moiety connected

Figure 2. Addition of LDAO facilitates the formation of larger conjugates. (A) Polysaccharide repeating unit (PSRU) for S. pneumoniae serotypes, types 3, 5, 18C, and 19A, shows vast structural diversity. (B) SEC-MALS analysis of the time course of the SPAAC reaction between eCRM and activated types 3, 5, 18C, and 19A PS (APS) estimates the generation of larger conjugates in the presence of LDAO.
to the azide, the azide group itself is a charged species and unlikely to enter the interior of the micelle to a great degree.

Next, we measured the effect of LDAO or DMSO addition on the spectral readout for azidocoumarin. As shown in Figure 1C, unlike DMSO, the addition of 0.1% LDAO causes a noticeable redshift in the absorbance with a substantial shoulder extending out to over 550 nm, which is consistent with the formation of a low-energy reactive state of the azide with the hydrophilic surface of the micelle. Furthermore, to see if the redshift we observed correlates with the ability of LDAO to catalyze SPAAC reactions, we compared the UV spectra of eCRM to those of para-azidomethyl-L-phenylalanine (pAMF) alone or another carrier protein with multiple mnAA sites (protein D) (Figure S1). As shown in Figure 1D, only the absorbance spectra for eCRM showed a significant redshift, consistent with the attainment of a favorable low-energy state for promoting faster kinetics for SPAAC. An alternate hypothesis for the redshift observed in our spectra is that localization within the micelle promotes a state which is similar to that of J-aggregates, which also have red-shifted bands. Typically, however, J-aggregate bands are very sharp and strong, absorbing over only a few nanometers in wavelength. Although our observed spectra are inconsistent with this, it is possible that a similar mechanism is at work.

**Figure 3.** Effect of LDAO on conjugation efficiency and stability. (A–D) SEC-MALS and SDS-PAGE analysis of large-scale conjugates generated with and without LDAO. Impact of LDAO on the (E) conjugation efficiency was estimated using a free azide measurement assay and (F) stability was determined by a % free polysaccharide estimation assay performed 0, 2, 4, and 9 weeks after storing the conjugates at 4 and 25 °C.
Mechanistically, if the favorable interaction of eCRM with the micellar surface occurs at a single site, then this would be expected to accelerate the initial intermolecular reaction rate between eCRM and APS, relative to the already rapid intramolecular reactions between subsequent pAMF residues and APS−DBCO. This would lead to a situation where eCRM molecules would interact at sites where they are incapable of fully reacting with a single APS and react instead with multiple APS chains, forming a spiderweb structure of eCRM/APS. The size of this structure would be considerably larger than a conjugate where each eCRM molecule would fully react with only one APS.

**Addition of LDAO Facilitates the Formation of Larger Conjugates with eCRM.** Several studies have shown that certain surfactants like SDS, CTAB, NLS, and sodium deoxycholate can increase the reaction kinetics of SPAAC[22] and also facilitate bioconjugation to efficiently generate antibody−drug conjugates.[23] Since the addition of LDAO already enhanced the kinetics of eCRM conjugation to APS type 9N (Figure 1B), we were interested in investigating its effect on the conjugation process more broadly. For this, we performed eCRM conjugation to several structurally diverse but clinically relevant *S. pneumoniae* serotypes (types 3, 5, 18C, and 19A) (Figure 2A), each of which has been implicated in the etiology of invasive pneumococcal disease.[5,26,32,33]

Conjugation reactions were performed at a small scale (1 mL), and size-exclusion chromatography-multidetector light scattering (SEC-MALS) analysis was performed for each reaction for samples harvested at the listed time points (Figure 2B). As shown, reactions with LDAO showed at least a 2-fold enhancement in the size of the conjugates at 0.5 h in comparison to conjugates generated without LDAO and continued to grow larger with time. Interestingly, conjugates generated using APS with more branched structures, like type 5 or 18C (Figure 2A), were substantially larger in size after 4 h in comparison to conjugates made with more linear APS, like types 3 and 19A. Surprisingly, a similar enhancement in size or the kinetics of the reaction was not observed when a different carrier protein (protein D, Figure S1) or a neutral surfactant (PS80) was added to the conjugation reaction (Figure S2). These results suggest that the addition of LDAO facilitates efficient formation of larger conjugates while utilizing and stabilizing carrier proteins like eCRM in a low-energy reactive state.

**Impact of LDAO on Conjugation Efficiency and Stability.** Using concentrations listed in Figure 2B, we scaled up conjugations of eCRM to APS (types 3, 5, 18C, and 19A) in the presence and absence of LDAO, followed by SEC-MALS and SDS-polyacrylamide gel electrophoresis (PAGE) analysis. As shown in Figure 3A–D, conjugates generated with LDAO were significantly larger in size when compared to conjugates generated without LDAO. Additionally, the SDS-PAGE analysis shows more efficient consumption of eCRM in the reactions with LDAO. Next, we utilized a free azide estimation assay to calculate the conjugation efficiency of the reactions. As shown in Figure 3E, conjugates generated with LDAO on average showed 8% lower free azide in comparison to conjugates made without LDAO, thereby confirming that the addition of LDAO facilitates utilization of more azide-containing nnAA sites on the carrier protein, hence improving the reaction efficiency. Finally, we hypothesized that increased utilization of azides would result in more densely crosslinked conjugates that might exhibit improved stability. Therefore, free APS analysis, measuring the portion of APS that is not covalently bound to the conjugate, was performed on the dialyzed conjugates to estimate the impact of LDAO on the product stability. Conjugates generated with type 19A were chosen for this analysis since native 19A polysaccharide has a high propensity to undergo autolytic degradation (data not shown). The study was performed by incubating the eCRM-19A conjugates made with and without LDAO at 4 °C or accelerated stability assessment at 25 °C. Samples were withdrawn at 0, 2, 4, and 9 weeks and % free APS analysis was performed. As shown in Figure 3F, estimation of % free APS shows no observable differences between the two groups, suggesting that the addition of LDAO does not differentially impact the stability of the conjugates generated using type 19A. However, performing similar analyses over longer time periods across multiple other clinically important serotypes may show a meaningful improvement in the overall stability of these conjugates.

**Assessment of In Vivo Immunogenicity of Conjugate Vaccines Generated with and without LDAO.** To assess in vivo immunogenicity, conjugate vaccines generated with and without LDAO were purified, and alum-adjuvanted formulations were administered intramuscularly to New Zealand white (NZW) rabbits using the dosing schedule presented in Figure 4A. A 0.11 μg dose of PS for each serotype was administered to...
In this study, for the first time, we show that the addition of a rabbit to match the allometrically scaled antigen dose in licensed pneumococcal vaccines. Enzyme-linked immunosorbent assay (ELISA) was performed using postimmunization bleeds while coating the underivatized target PS as the detection antigen. As shown in Figure 4B, conjugates generated with or without LDAO elicited similar immune responses post prime and boost vaccinations with no significant differences observed for any serotype. This shows that conjugations facilitated by LDAO lead to the generation of equally immunogenic CVs using clinically important serotypes of S. pneumoniae.

**CONCLUSIONS**

Several studies have shown beneficial effects of the addition of surfactants to improving the kinetics of SPAAC reactions. In this study, for the first time, we show that the addition of a zwitterionic detergent LDAO above its CMC greatly accelerates the kinetics of a copper-free click chemistry reaction to generate CV. Our results show that conjugates generated with and without LDAO are similarly stable and equally immunogenic in boosting antibody responses against the target serotypes. From a human safety point of view, amine-oxide surfactants have been shown to have a low toxicity and are used in a wide variety of commercial products. Most importantly, we show that conjugation reactions in the presence of LDAO are substantially more efficient at using a larger proportion of pAMF sites on the carrier protein, which may have a meaningful impact on the generation of these vaccines at a commercial scale.

**EXPERIMENTAL PROCEDURES**

**DBCO Derivatization of Pneumococcal Serotypes Using 1-Cyano-4-dimethylaminopyridinium Tetrafluoroborate (CDAP) Chemistry.** Serotypes 3, 18C, and 19A were activated via CDAP, while serotype 5 was activated via periodate, followed by reductive amination (as shown elsewhere). Briefly, CDAP (Sigma-Aldrich) is added to the PS solution in sodium borate buffer, followed shortly thereafter by DBCO-PEGamine (Sigma-Aldrich). The resulting activated polysaccharide (APS) is purified with tangential flow filtration (TFF) to remove small-molecule process-related impurities, filtered through a 0.2 μm-rated filter and stored frozen at −65 °C. The APS can then be reacted with eCRM to form conjugates.

**Generation of Conjugate Vaccine Using eCRM and S. pneumoniae PS (Types 3, 5, 18C, and 19A) Using SPAAC.** In a glass vial with a stirring bar, the following reagents were added in this order: water, potassium phosphate (Sigma-Aldrich) and serotypes 3, 18C, and 19A), DMSO (Sigma-Aldrich) for a final concentration of 15%, LDAO (Sigma Life Science) for a final concentration of 0.1%, APS-3 0.8 mg/mL, APS-5 0.3 mg/mL, APS-18C 1.5 mg/mL, and APS-19A 0.55 mg/mL, and lastly eCRM. Reactions were mixed for 4 h at room temperature and then quenched for 2 h with 4 equiv of sodium azide (Sigma-Aldrich) relative to the PSRU concentration. After quenching, reactions were dialyzed (100 kDa molecular weight cutoff (MWCO)) against 5 mM sodium succinate (Sigma-Aldrich, CAS #52378), pH 5.8, 150 mM NaCl buffer with at least two changes and then filtered through a 0.2 μm dual-layer filter (Thermo Fisher). The polysaccharide concentration was determined by the anthrone assay. Finally, the conjugates were characterized by SDS-PAGE and SEC-MALS to determine the molecular weight.

**Anthrone Assay for Estimating PS Concentration.** A stock of 2 mg/mL of the anthrone reagent (Sigma-Aldrich) was prepared in cold sulfuric acid (Sigma-Aldrich), while a 1 mM stock of polysaccharide repeating unit (PSRU) for each serotype was prepared in water. Thereafter, 100 μL/well of serially diluted PSRU stock or the 3× diluted unknown was plated (96-well plate) in triplicate, followed by the addition of 200 μL/well of the anthrone reagent stock. Using a multichannel pipet, all of the reactions were mixed to homogeneity, after which the plate was covered with a plastic cover, and the reactions were incubated at 95 °C for 10 min. Post incubation, the plate was carefully removed from the oven and placed at 4 °C for 10 min to facilitate cooling to ambient temperature. Finally, absorbance was measured at λ = 620 nm using a UV/vis plate reader. Using the slope and y-intercept of the least-squares fit, the concentration of the unknown sample was calculated using the average absorbance value measured at λ = 620 nm.

**ELISA Method for Measuring Antibody Responses.** Screening antigen: serotypes 3 and 19A diluted to 1 ug/mL, serotypes 5 and 18C diluted to 2.5 μg/mL in 1× phosphate-buffered saline (PBS). Each serotype (100 μL) was plated on NUNC ELISA plates (NUNC Maxisorp) and incubated overnight at 4 °C. The next day, the plates were washed with 0.5% PS20 in 1× PBS three times (Biotek EL406 or EL405 LS) and blocked with 3% bovine serum albumin (BSA) (Thermo Fisher) in 1× PBS for 1 h at room temperature. Plates were washed three times. Sera was diluted 1:10, then serially diluted by five, and 50 μL was added to the plate. The plates were then incubated for 1 h at 37 °C. After the incubation period, plates were washed six times, and 100 μL of 1:5000 diluted Donkey anti-Rabbit IgG (H + L) peroxidase-conjugated (Jackson ImmunoResearch) was added to the plates and incubated for another hour at room temperature. Plates were washed again six times, then 100 μL of 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) peroxidase substrate (1-component) (Seracare) was added, and plates were incubated for 30 min at room temperature. Finally, after 30 min, the plates were read at 415 and 570 nm (BioTek Power Wave HT plate reader). The antibody titer was calculated based on EC50.

**Multiangle Light Scattering (MALS) Analysis.** The SEC-MALS-UV-RI setup consists of an Agilent HPLC 1100 degasser, temperature-controlled autosampler (4 °C), column compartment (25 °C), and UV–vis diode array detector (Agilent) in line with a DAWN-HELEOS multilayer light scattering detector and Optilab T-Rex differential refractive interferometer (Wyatt Technology) coupled to three TOSOH columns in series: TSKgel Guard PWXL 6.0 mm ID × 4.0 cm long, 12 μm particle; TOSOH TSKgel 6000 PWXL 7.8 mm ID × 30 cm long, 13 μm particle; and TSKgel 3000 PWXL 7.8 mm ID × 30 cm long, 7 μm particle. A mobile phase consisting of 0.2 μm filtered 1× PBS (Sigma-Aldrich) + 5% acetonitrile (VWR) was used at a 0.5 mL/min flow rate, and 50 μg sample was injected for analysis. Agilent Open Lab software was used to control the HPLC, and Wyatt Astra 7 software was used for data collection and molecular weight analysis.

**Estimation of Free PS for Stability Assessment.** Briefly, 1% (w/v) sodium deoxycholate (DOC) (Thermo Scientific) solution at pH 6.8 ± 0.03 was prepared by weighing out 500 ± 0.1 mg of DOC and transferred to a 50 mL falcon tube. Milli-
Q water (50 mL) was added to the tube and mixed until all of the sodium deoxycholate was dissolved. The DOC was left at room temperature overnight to ensure that it had completely dissolved, and the pH was adjusted to 6.8. Once the DOC was made, we proceeded with the DOC precipitation. All conjugate samples, negative control (water), and reagents (1% DOC, pH 6.80 ± 0.03, and 1 M HCl) were brought to room temperature. To a 500 μL reaction containing a polysaccharide concentration of 0.250 mg/mL of each conjugate, 50 μL of 1% DOC, pH 6.80 ± 0.03, was added. After the addition of DOC, the reaction was mixed by pipetting for 10 cycles, and the samples were incubated on ice for 30 min, vortexed briefly after 15 min. After 30 min, 25 μL of 1 M HCl (Sigma-Aldrich) was added to all of the samples, making sure to mix very well and waited for 5 min for the precipitate to form. Then the supernatant was centrifuged once more at 10,000 rpm at 4 °C for 20 min. The supernatant was collected into a new tube again, and the pellet was discarded. The supernatant was centrifuged once more at 10,000 rpm at 4 °C for 10 min to ensure that all pellets were separated. The supernatant was collected into a new tube again, and the pellet was discarded. The samples were then analyzed for their “% free polysaccharide” (%FPS) content using the anthrone assay.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03481.

Effect of LDAO addition on conjugation of type 3 PS to protein D variant (Figure S1); effect of LDAO or PS80 addition on conjugation of type 5 PS to eCRM (Figure S2) (PDF)

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**Author Contributions**

1L.B. and L.P-P. are co-first authors. L.P-P., L.B., and N.K. designed the research. M.I. expressed and purified the protein, while O.M., C.B., P.A., M.S., and S.A. performed the polysaccharide derivatization. L.P-P., L.B., and E.C. performed the conjugation and ELISA assays. S.S. did the kinetics of SPAAC, and A.B. performed the SEC-MALS analysis. In vivo immunizations were performed at Covance Inc. L.P-P., L.B., E.C., and N.K. wrote the manuscript. All authors provided critical review and feedback and approved the final manuscript.

**Notes**

The authors declare no competing financial interest.

**ABBREVIATIONS**

SPAAC, strain-promoted azide–alkyne cycloaddition; LDAO, lauryldimethylamine N-oxide; CV, conjugate vaccines; CFPS, Xpress cell-free protein synthesis; eCRM, CRM197; DBCO, dibenzocyclooctyne; pAMF, para-azidomethyl-l-phenylalanine; CMC, critical micelle concentration; nnAA, non-native amino acids; PRSU, polysaccharide repeating unit; APS, activated polysaccharide; DMSO, dimethyl sulfoxide; PS80, polysorbate 80

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