Targeting bacterial pathogens with next-generation glycoconjugate vaccines: understanding the parameters governing immune responses to carbohydrate antigens

Sorieul, C.E.J.

Chapter 3

Approach to a Classic Glycoconjugate Vaccine for Evaluating the Immunogenicity of *Streptococcus suis* Rhamnose-Rich Polysaccharide

Charlotte Sorieul*, Yao Shi*, Lindert Benedictus, Nina M. van Sorge, Jeroen Codée and Sander van Kasteren

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* Equal contribution

Introduction

Streptococcus suis is an encapsulated Gram-positive pathogenic bacterium affecting pigs worldwide and is responsible for considerable economic losses in the swine industry.[1, 2] *S. suis* is also an emerging zoonotic agent, primarily in Asia,[2] and in both pigs and humans, *S. suis* can cause septicemia, endocarditis, arthritis, and most notably meningitis, often leading to irreversible sequelae such as hearing loss in humans.[3] A 2005 outbreak in China highlighted the deadly potential of this pathogen in human populations, with the majority of fatal cases attributed to streptococcal toxic shock syndrome.[4]

The widespread and extensive use of antibiotics in pig farms has significantly increased *S. suis* resistance to several antibiotics, making it crucial to develop a new strategy to combat this pathogen.[5] While vaccination remains one of the preferred approaches, progress is hindered by the substantial genetic and phenotypic variability of *S. suis* and there is currently no commercially available vaccine that provides broad protection. Among the identified antigens proposed as potential vaccine candidates, there is a significant interest in the polysaccharides surrounding the bacteria.[2, 5, 6] Capsular polysaccharides (CPS) compose the outermost layer of *S. suis* and CPS-based vaccines have been shown to provide protection to pigs.[7] However, CPS are serotype-specific structures and there are more than 29 serotypes of *S. suis*[8], which complicates vaccine development. Apart from CPS, the cell wall of Gram-positive bacteria like streptococci can be decorated with various other polysaccharides, including wall teichoic acids and rhamnose-rich polysaccharides (RPS), the latter of which can constitute up to 50% of the cell wall mass. As their name implies, RPS are distinguished by a high content of L-rhamnose, a 6-deoxyhexose found in bacteria but not in humans. These RPS are

complex glycopolymers, consisting of a polyrhamnose backbone substituted with variable oligo- or polysaccharide side chains.[9, 10]

As a key component of the bacterial cell wall, RPS has significant therapeutic potential. The molecular machinery producing RPS could offer attractive targets for developing new antimicrobials to combat antibiotic resistance[11], and RPS themselves hold promise as broad-spectrum vaccine candidates, due to their highly conserved nature across some streptococci strains.[12, 13] Notably, *Streptococcus pyogenes* (group A Streptococcus, GAS) harbors a well-studied RPS known as group A carbohydrate (GAC), which has been extensively studied by companies such as GSK[14] and Vaxcyte[15]. Funding from nonprofit organizations like CARB-X has earned Phase 1 clinical trials after validation in animal models.[16] Preclinical research is ongoing and innovative constructs of GAC-based vaccines[17] further support its potential against GAS.

In *S. suis*, genes involved in RPS biosynthesis have recently been identified and are considered vital for the bacterium. *S. suis* RPS thus emerged as a promising broad-spectrum vaccine candidate due to the conservation of glycan motifs across pathogenic *S. suis* strains[18], though its antigenic and protective potential requires evaluation. This Chapter describes the generation of a glycoconjugate RPS vaccine and its initial evaluation in piglets.

A major limitation of carbohydrate antigens for vaccine development is their inherent low immunogenicity. To overcome this, polysaccharides are usually conjugated to so-called protein carriers, generating glycoconjugate vaccines that can elicit T cell help, leading to a more robust and long-lasting immune response.[19-22] Cross-reactive material 197 (CRM_{197}) is a toxoid mutant of diphtheria toxin which has been used extensively and successfully as carrier in many licensed glycoconjugate vaccines, providing a long track record of safety and efficacy.[23, 24] CRM_{197} was thus selected as carrier to generate a model *S. suis* RPS-vaccine.

Polysaccharide conjugation is typically achieved in a random manner, which can disrupt glycan epitopes.[25] To properly assess isolated RPS, it is crucial to reduce the impact of conjugation on potentially important epitopes and ensure that the polysaccharide remains as exposed as possible. It was therefore decided to conjugate RPS at its reducing end, allowing the glycans to be oriented radially around the protein.[19, 26, 27] Carbohydrate antigens, whether isolated from bacteria or synthetically produced, are usually obtained through a painstaking, labor-intensive process, often making them the most valuable component of glycoconjugate vaccines. The approach taken in this Chapter thus focused on minimizing RPS material losses.

Reductive amination, a common method for polysaccharide conjugation, involves converting aldehydes to amines. This is typically done after sodium periodate oxidation of vicinal hydroxyl groups on the polysaccharide to introduce aldehyde groups[28, 29], and it is a process used in several licensed vaccines[30]. However, the reducing sugar of a polysaccharide can also be used for a reductive amination, after ring opening of the hemiacetal.[31] After reaction with an amine, the resulting Schiff base can be reduced for a stable functionalization[32], which is usually done with reductants such as sodium cyanoborohydride ($NaBH_3CN$).

Conjugation at the reducing end via reductive amination can be achieved in a single step directly onto the protein (**Figure 1a**).[14, 33-35] In this case, underivatized protein and carbohydrate are mixed in the presence $NaBH_3CN$, allowing the amines of the lysine residues to react with the terminal sugar aldehyde. Although this single-step method is convenient, it is slow, results in low conjugation degrees and requires large excesses of carbohydrate. This method can produce substantial amounts of unconjugated carbohydrate and protein[14], leading to material losses. In addition, it has been suggested that the lack of spacing between the carbohydrate and protein resulting from this method may reduce immunogenicity.[35]

An alternative approach involves activating the polysaccharide at its reducing end with ammonium salts[28, 36, 37] or other amine-containing linkers, like propargylamine[38, 39] (**Figure 1b**), to introduce functional groups such as an amine or azide for subsequent conjugation to the protein. Adipic acid dihydrazide (ADH)[35, 39-42] is another option for derivatizing polysaccharides at the reducing end (**Figure 1c**), which has the advantage of reacting more efficiently with aldehydes than primary amines[43].

A classic follow-up step after functionalization involves reacting the sugar with adipic acid bis(N-hydroxysuccinimide) ester (also called SIDEA) at the amino[33, 36, 37, 44-46] or hydrazido[45, 47] group(s) at the polysaccharide's terminal end[33, 36, 37, 42, 46] (or on its oxidized diols[45]), to introduce NHS esters which can then react with the lysine residues on the protein. SIDEA is a popular choice for its efficiency and the immunosilence of the resulting linker[46], but NHS esters are highly susceptible to hydrolysis[48]. This requires careful storage of derivatized sugars and their resuspension in organic solvents, which complicates water-based reactions with proteins, as organic solvents may compromise the integrity of the carrier. Additionally, hydrolysis of the NHS ester competes with the primary amine reaction and pH control is a critical factor for optimal conjugation.[49, 50]

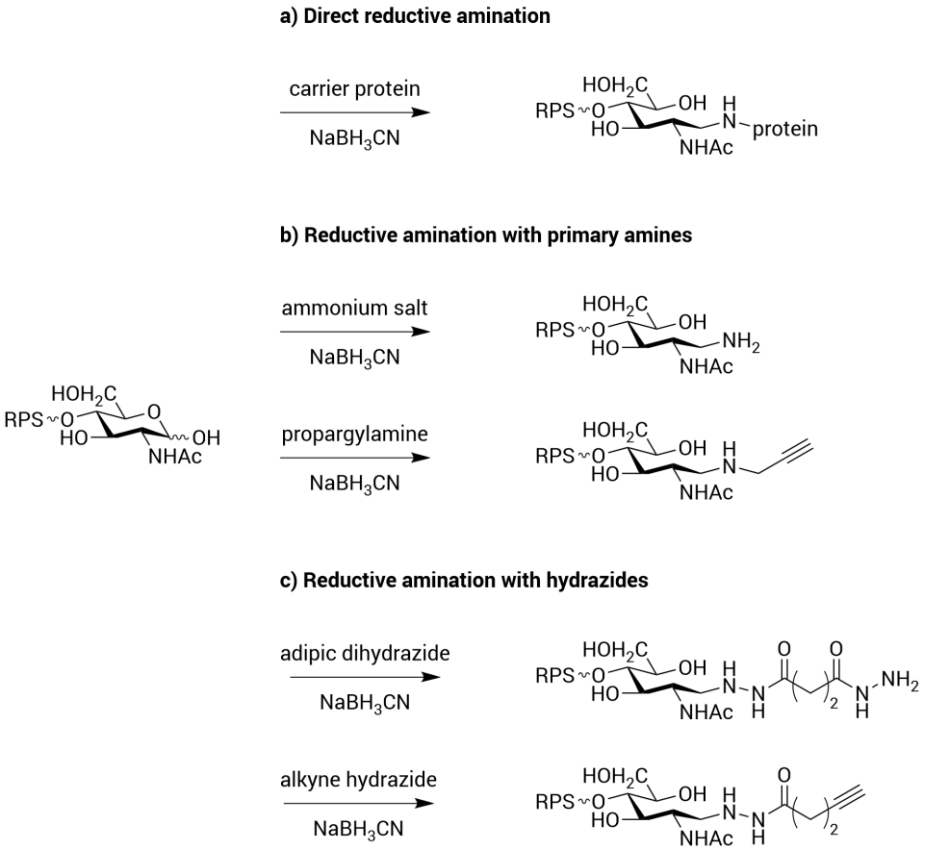

Figure 1. Conjugation strategies based on the reductive amination of the *S. suis* RPS polysaccharide N-acetylglucosamine (GlcNAc) reducing end, as released upon mild acid hydrolysis[18, 52]. **a)** Direct conjugation to a carrier protein through reductive amination is possible and has been used for glycoconjugation to the carrier's lysine residues sidechain. **b)** Functionalization of the polysaccharide's reducing end can be achieved with primary amines, such as those provided by ammonium salts or propargylamine. **c)** Functionalization with hydrazides have a distinct advantage over amines due to their enhanced reactivity with aldehydes, such as the one hidden at the polysaccharide's reducing end.

With homobifunctional linkers such as ADH and SIDEA, there is also the risk of crosslinking polysaccharides. A common approach to minimize crosslinking is to use a large excess of linker. However, a study by Bystricky *et al*. showed that an excess of

ADH only slightly reduced crosslinking in carboxymethylated polysaccharides[51], and this may also apply to SIDEA. In addition, each step requires purification, which inevitably contributes to material losses.

Click chemistry[53, 54] has only been recently applied to the synthesis of glycoconjugate vaccines, and its popularity for this purpose has increased significantly over the past decade.[15, 39, 40, 55-60] A well-known click reaction, the Huisgen 1,3-dipolar cycloaddition, involves a bioorthogonal reaction between an azide and an alkyne to form a triazole. This reaction has been used in glycoconjugate synthesis through two main variants: the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) and the strain-promoted azide-alkyne cycloaddition (SPAAC). Both variants offer similar advantages, including a high efficiency and specificity, as well as mild reaction conditions, although there are some concerns about the potential immunogenicity of the resulting linkage[56, 57] and the toxicity of residual copper in CuAAC.[61] Considering the potential greater impact of the large cyclooctene linkers resulting from SPAAC conjugation and the minimal copper amounts expected after purification for CuAAC, this Chapter explores the latter method for the conjugation of RPS to the CRM_{197} carrier protein.

Click chemistry has so far mostly been employed in the context of site-selective modification of the carrier protein[15, 55, 59] and the work from Stefanetti *et al*.[39, 40] is the only example that uses click chemistry of a cyclooctyne group introduced through reductive amination at the carbohydrate's reducing end for conjugation to a classic carrier. This Chapter proposes a streamlined approach using a single RPS derivatization step followed by efficient click chemistry for a smooth glycoconjugation process to generate a RPS-CRM_{197} vaccine modality evaluated in piglets.

Results and Discussion

RPS from *S. suis* was isolated from two pathogenic strains: S10, a virulent sequence type 1 strain originally isolated from healthy pig tonsils, and 861160, a sequence type 20 strain isolated from a human meningitis patient. These strains were selected to represent RPS of disease-associated *S. suis* lineages based on a bioinformatics analysis containing 1,719 *S. suis* genomes.[18] RPS from the S10 strain is composed of rhamnose, galactose and N-acetylglucosamine (GlcNAc), whereas 861160 RPS contains additional glucose, though the exact structure has yet to be determined. Although these two RPS variants in the pathogenic lineage of *S. suis* are different, the RPS resulting from the *srpL* gene deletion (Δ*srpL*), coding for the SrpL glycosyltransferase, is conserved. As a

result, Δ*srpL* RPS isolated from strains S10 and 861160 is highly similar, making it a promising candidate for developing a broad-spectrum vaccine against *S. suis*.

The nature of the terminal sugar of the natural RPS depends on the release method[52], and here, mild acid hydrolysis (0.02 N HCl) after chemical N-acetylation of the S10 and 861160 strains resulted in a GlcNAc terminal sugar.[18, 52] The linkage at the C-4 (**Figure 2a**) was confirmed by GC-MS analysis.[18]

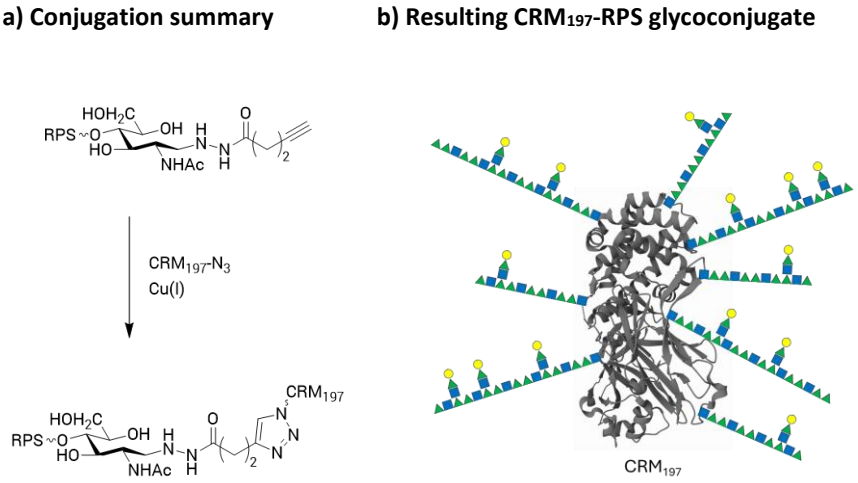

Figure 2. **a)** Resulting linkage after Cu(I)-catalyzed click chemistry glycoconjugation, where alkyne-hydrazide-derivatized RPS is conjugated to azide-derivatized CRM_{197}. **b)** Schematic representation of the generated CRM_{197}-RPS glycoconjugate, with radially conjugated 861160 Δ*srpL* RPS molecules attached to the carrier protein (CRM_{197}, PDB 7RRW).

Purified 861160 Δ*srpL* RPS was used to prepare the glycoconjugates using two different linkers for derivatization: propargylamine and alkyne-hydrazide. Propargylamine was initially chosen for its more established use in reductive amination at the reducing end and was compared with alkyne-hydrazide to evaluate the effectiveness of hydrazides against primary amines in this reaction. 861160 Δ*srpL* RPS was first quantified using a modified anthrone assay[52, 62], which measures the colorimetric change resulting from the reaction between furfural derivatives (formed

by the hydrolysis and dehydration of carbohydrates) and anthrone in sulfuric acid, producing a blue-green complex.[63] RPS was then reacted under acidic conditions with 500 molar equivalents of propargylamine or 300 molar equivalents of alkyne-hydrazide, respectively, in the presence of $NaBH_3CN$. The concentration of RPS was maintained high (at 10 mg/mL) to drive the reaction to completion. Both reactions were carried out with gentle shaking overnight, although shorter incubation times with ADH have been reported to be effective[39]. The resulting solutions were purified by ultracentrifugation in water and lyophilized overnight.

The derivatized 861160 Δ*srpL* RPS was subsequently reacted with conjugation-ready EcoCRM-azide (EcoCRM-N_3) from Fina Biosolutions, carrying 15 azide groups on average (**Figure 2a**). For the CuAAC conjugation reaction, EcoCRM-N_3 was solubilized and buffer-exchanged into phosphate-buffered saline (PBS) to remove EDTA from the lyophilized protein. The RPS-alkyne was resuspended in water at an estimated concentration of 100 mg/mL. The conjugation was conducted at a 1:1 molar ratio of azide-to-alkyne, in the presence of $CuSO_4$. The reaction volume was kept low to maintain high concentrations of EcoCRM-N_3 and RPS (approximately 10 and 30 mg/mL, respectively). The reaction proceeded at room temperature (RT), with samples taken periodically and stopped by adding Laemmli sample buffer to follow the reaction by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (**Figures 2b** and **3**).

For reaction analysis, 2 µg of conjugate sample were loaded onto a 7.5% acrylamide gel. The low acrylamide content was chosen to stretch the conjugate smear, for better visualization of the bands. Fortunately, the isolated RPS was relatively homogeneous, allowing clear distinction of the resulting conjugates, with each band corresponding to the addition of one RPS molecule. Up to seven additions were visible (notably in lane 0 h, under the alkyne-hydrazide category) before the saccharide loading became too high and the smear too dense to be separated on the 7.5% gel. Distinct differences were observed between propargylamine- and alkyne-hydrazide-derivatized RPS, with the latter showing a high degree of conjugation and no free protein remaining after 1 hour, while the former produced conjugates averaging 1-2 RPS molecules.

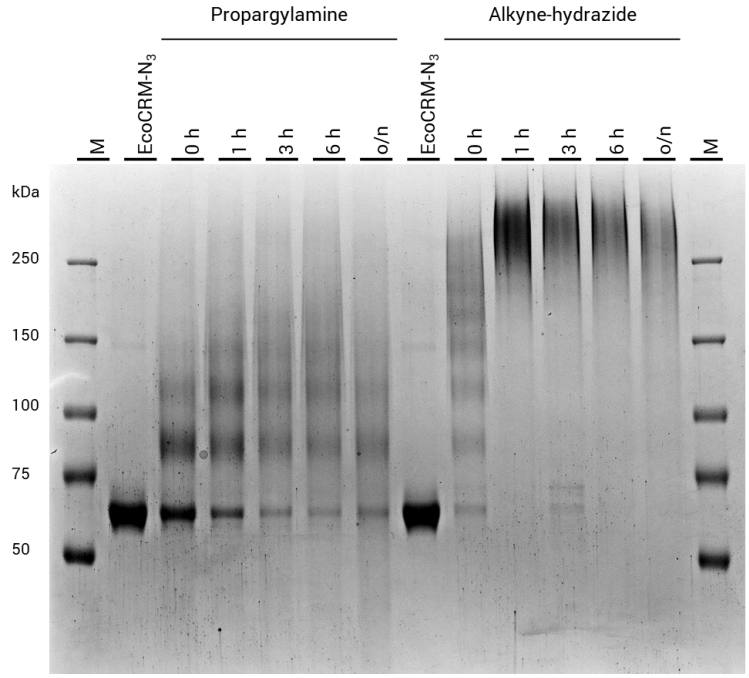

Figure 3. SDS-PAGE analysis of conjugation trials using 861160 Δ*srpL* RPS derivatized with propargylamine (left panel) or alkyne-hydrazide (right panel); analysis over time of the conjugation reaction (in hours), with the '0 h' sample being taken right after mixing and quenched with Laemmli buffer, corresponding to around 5 minutes of reaction; M stands for the molecular weight marker, and o/n indicates overnight incubation.

Interestingly, for both derivatization methods, the protein bands began to fade after 1 hour of reaction time, with smearing toward the bottom of the gel, suggesting potential protein degradation. This degradation likely resulted from the extended incubation in the reaction medium with $CuSO_4$, which could have compromised the protein structure[64]. As no improvement in loading was observed beyond 1 hour, this reaction time was chosen for the subsequent conjugations. However, given the substantial protein loading observed at '0 h' (corresponding to approximately 5 minutes of reaction time), a shorter incubation, e.g. 30 min, could achieve similar results.

The reductive amination with alkyne-hydrazide appeared to be very effective, but this made it difficult to visualize the degree of conjugation by SDS-PAGE, raising concerns about the structural integrity of EcoCRM. Although fully preserving the structural integrity of the protein carrier is generally not essential for glycoconjugates, as immune responses can be obtained despite heavy modifications, excessive instability can have a negative impact.[65] Protein aggregation or over-conjugation can affect T cell epitopes, potentially disrupting antigen processing and/or presentation. The saccharide loading level is a key parameter for the success of a glycoconjugate vaccine, with shorter chains typically requiring a higher degree of conjugation than longer ones to elicit an optimal immune response.[66-68] Based on the roughly estimated 15 kDa size of the 861160 RPS, it was decided to conjugate just enough polysaccharide to eliminate any free, unconjugated EcoCRM-N_3, to simplify the purification step.

To determine optimal reaction conditions, various azide-to-alkyne molar ratios, ranging from 1:0.4 to 1:0.7 (corresponding to a maximum of 6 to 10.5 RPS chains per protein, respectively), were explored (**Figure 4**), but no significant differences were observed. However, the lower conjugation level provided some insight into the conjugation efficiency: with an estimated 3-4 RPS per protein at a 1:0.4 ratio and 4-5 RPS per protein at a 1:0.6 ratio, the overall conjugation efficiency appeared to be between 50% and 60%.

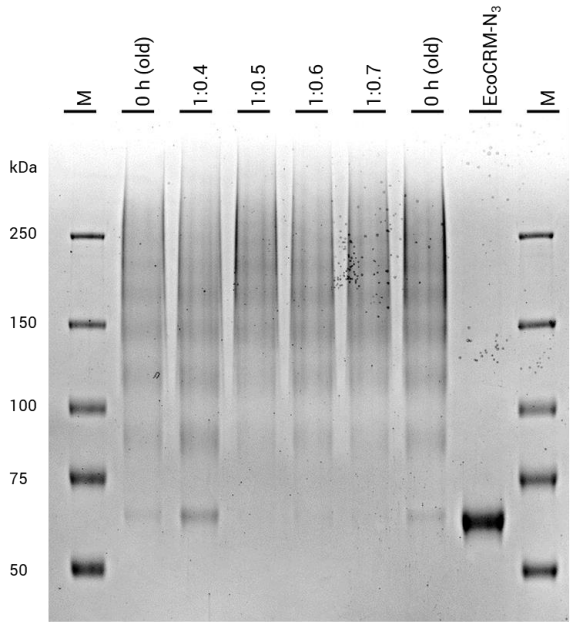

Figure 4. SDS-PAGE analysis comparing different azide-to-alkyne molar ratios. A sample from the previous 1:1 trial (0 h for alkyne-hydrazide, see **Figure 3**) was included twice to function as a reference ladder for visualizing the degree of conjugation.

Next, a large batch of conjugates was produced using newly derivatized RPS (**Figure 5**). A test run was conducted to determine the azide-to-alkyne ratio for the newly derivatized RPS (**Figure 5a**). The new test batch appeared to have a slightly lower saccharide loading, as seen in the 1:1 azide-to-alkyne ratio lane compared to the initial conjugation reaction at 1 hour (**Figure 3**) for the same ratio, perhaps due to a less efficient reductive amination step. Once again, no major differences between the various ratios were observed, and the 1:0.7 azide-to-alkyne ratio was selected for the large EcoCRM-RPS conjugate batch prepared for immunization (**Figure 5b**). This time, the saccharide loading appeared to be somewhat higher, which may be due to the larger volumes used, which allowed for greater pipetting precision. The conjugate was purified using ultracentrifugation with an Amicon 30 kDa filter, which permitted separation from the smaller RPS molecules.

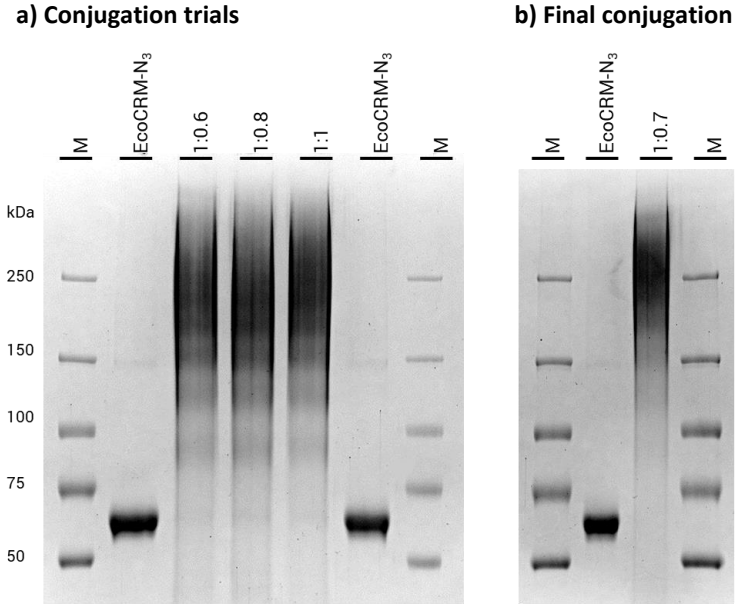

Figure 5. SDS-PAGE analysis of EcoCRM-RPS glycoconjugates using newly alkyne-hydrazide-derivatized RPS for the production of a large vaccine batch. **a)** Evaluation of different azide-to-alkyne molar ratios to determine the optimal conjugation degree. **b)** Large-scale production of EcoCRM-RPS conjugate at a 1:0.7 azide-to-alkyne ratio, corresponding to 10.5 RPS chains per EcoCRM-N_3 molecule.

Determination of the conjugation degree by MALDI-TOF was attempted, but it was unsuccessful, likely due to the high RPS content preventing proper ionization of the conjugate. The saccharide content of the purified conjugates was therefore assessed using the colorimetric anthrone assay, which estimated a loading of 2.3 RPS per protein. This result contradicted the estimation from the gel (**Figure 5b**), which suggested a much higher loading. The discrepancy in the anthrone assay results could be due to an unexpectedly smaller RPS Mw than anticipated.

The immunogenicity of the 861160 Δ*srpL* RPS-conjugate vaccine was assessed in 3-week-old piglets, which were immunized on days 0 and 14 by intramuscular injection with 2 mL of the EcoCRM-RPS glycoconjugate vaccine (corresponding to 109 µg of carrier protein and approximately 200 µg RPS) formulated with the X-Solve adjuvant, based on DL-α-tocopheryl acetate and light liquid paraffin[69, 70]. Blood samples were collected on days 0 (pre-immunization), 14 and 28 to evaluate anti-RPS antibody levels

by flow cytometry (FACS) (**Figure 6a**). To this end, streptavidin-coated beads were loaded with biotinylated 861160 Δ*srpL* RPS, incubated with the raised sera then tagged with a fluorescently-labelled anti-pig IgG (Fc) antibody for detection. No difference from the control group was observed after the first immunization on day 14, but some piglets showed a response after the second one. By day 28, a difference from the control could be observed, with the sera from the immunized piglets binding better to the beads, although significant variation in IgG levels was observed.

Next, streptavidin-coated beads were functionalized with six different RPS variants (WT, Δ*srpL* and Δ*srpP* from both *S. suis* strains, S10 and 861160), which were biotinylated for conjugation to the beads, to evaluate cross-reactivity of the antibodies raised with the 861160 Δ*srpL* RPS-EcoCRM conjugate. Interestingly, antibodies were capable of recognizing both the WT and Δ*srpL* mutants of *S. suis* strains S10 and 861160 with comparable IgG levels (**Figure 6b**), confirming that the conserved glycan motif from Δ*srpL* RPS is sufficient to elicit an immune response and be cross-reactive. The glycosyltransferase SrpP is predicted to be a N-acetylglucosamine-phosphate-undecaprenol (GlcNAc-P-Und) synthase and is essential for the sidechain biosynthesis. GC-MS-based glycosyl linkage analysis showed that RPS isolated from the *srpP* deletion mutant (Δ*srpP*) has a linear structure without sidechain.[18] Surprisingly, there was no significant difference in recognition of the RPS backbone (lacking the primary side chain of RPS) between the vaccinated and control groups, which may indicate potential pre-existing immunity. It may be that the rhamnose-rich backbone shares structural features with cell wall polysaccharides from other bacteria or certain commensal *S. suis* strains, and is thus recognized by pre-existing circulating antibodies. The presence of the primary sidechain on the RPS apparently blocks the recognition of the backbone by these antibodies, as the binding to the WT and Δ*srpL* RPS is significantly diminished.

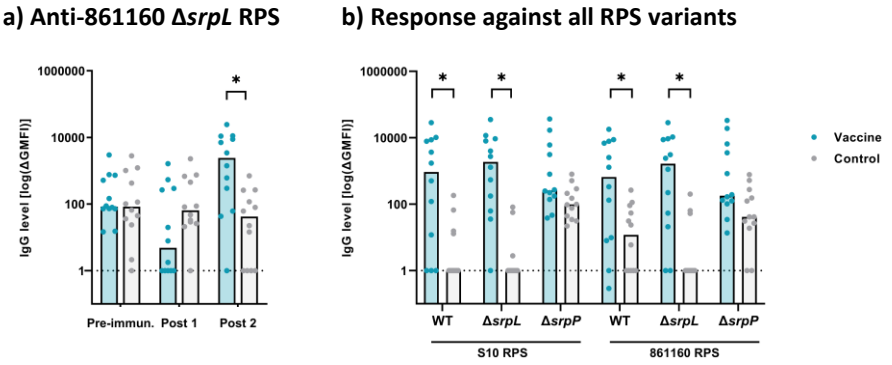

Figure 6. Immunogenicity evaluation of the EcoCRM-RPS conjugate. **a)** Antibody (IgG) levels against 861160 Δ*srpL* RPS post first (day 14) and post second (day 28) immunization. **b)** Recognition of different RPS variants from both S10 and 861160 *S. suis* strains with post second immunization sera. The difference observed of the binding of 861160 Δ*srpL* RPS-coated beads between the two figures could be due to the batch-to-batch variability of the sugar used for bead coating; the bars represent the median values from 12 piglets sera; non-responders (negative values) were set to 0 (indicated by the dotted line); statistical analysis was performed using the Mann-Whitney test, followed by Holm-Šídák's correction for multiple comparisons; * indicates $p \leq 0.05$ and non-significant pairwise comparisons are not shown. Figure adapted from the doctoral thesis of Yao Shi.[18]

Overall, the immune responses across piglets were highly heterogeneous. This indicates that further optimization of the conjugate could be beneficial, e.g. through investigating optimal saccharide-to-protein ratio or using a more targeted protein derivatization to better preserve the structural integrity of the carrier. The immune response could also be improved by exploring alternative adjuvants, administering a third vaccination or extending the time between immunizations. Lastly, the variability of the immune response may also be linked to the use of young 21-day-old piglets in the study, as their immune system is not yet fully developed.[71-73] Another strategy could thus involve immunizing sows and transferring the resulting antibodies to the piglets through colostrum afterwards.

Conclusion

A glycoconjugate vaccine was developed using commercially available EcoCRM and isolated *S. suis* RPS, using a modified version of a classic conjugation chemistry targeting the reducing end of the carbohydrate through reductive amination with an alkyne-functionalized hydrazide. The approach demonstrated rapid and efficient conjugation, with improved efficiency and likely reduced material loss compared to similar methods reported in the literature. The chemistry seems to have preserved the carbohydrate epitopes effectively, as the resulting glycoconjugate successfully elicited an immune response against Δ*srpL* RPS as well as several of its variants, including the WT.

To improve the glycoconjugate vaccine, the conjugation protocol could be further optimized, and the separate transformations (hydrazide formation[74] and click reaction[75]) validated in a more quantitative manner, which because of time constraints has not been done in this Chapter. For the determination of saccharide loading, size-exclusion chromatography with multi-angle static light scattering (SEC-MALS) is an attractive method.[25] As a complementary means to analyze the IgG levels in the control and raised sera, enzyme-linked immunosorbent assays (ELISA) could be used. Presumably, the RPS is too short to bind effectively to the 96-well plate and therefore biotinylated RPS can be immobilized on streptavidin-coated ELISA plates. Alternatively, other glycoconjugates based on a different carrier protein, e.g. bovine serum albumin (BSA) can be used as coating antigens.

Lastly, an important consideration is the immunogenicity of the linker used in the conjugate. The linkage between the carrier and carbohydrate comprises a hydrazido and a triazole moiety. It has been reported that triazoles can have immunomodulatory properties.[76, 77] Also, the hydrazido moiety in the linker can have some impact on the overall response, as there have been reports on their immunogenicity.[35] Evaluating the generated sera for reactivity against the linker, or separate parts of it, could provide valuable information and guide further optimization of the conjugation process.

Acknowledgements

This work was carried out in collaboration with Yao Shi as part of his doctoral research.

Material and Methods

Preparation of EcoCRM-RPS glycoconjugates

The EcoCRM-RPS glycoconjugates were prepared with 861160 Δ*srpL* RPS. RPS was extracted from the bacterial cell wall through mild acid hydrolysis following chemical N-acetylation and purified by size exclusion chromatography on a BioGel P150 column (Bio-Rad) equilibrated in sodium acetate-sodium chloride (NaOAc-NaCl) buffer, as described in [18]. The rhamnose concentration was measured using a modified anthrone assay.[62] The purified RPS was derivatized at its reducing end with alkyne-hydrazide (Lumiprobe) through reductive amination, using a molar ratio of RPS: hydrazide at 1:300:1000. This reaction was performed in 100 mM NaOAc (pH 4.5) at RT overnight with gentle shaking. The alkyne-derivatized RPS was subsequently purified by ultracentrifugation, through 25 cycles of washing with water (each cycle approximately a twofold dilution), using centrifugal filter units (Amicon Ultra Centrifugal Filter, 3 kDa MWCO; Millipore) as per the manufacturer's recommendations (40° fixed-angle rotor, 14,000 x g, RT).

The alkyne-derivatized RPS was then conjugated to CRM_{197}-azide (CRM_{197}-N_3) through copper-catalyzed azide-alkyne cycloaddition (CuAAC). Conjugation-ready CRM_{197}-N_3 was ordered from Fina Biosolutions, pre-derivatized with approximately 15 azide groups for reaction with alkyne groups. The click reaction was carried out with final concentrations of copper sulfate ($CuSO_4$) at 1 mM, sodium ascorbate at 10 mM, tris(benzyltriazolylmethyl)amine (THPTA) at 1 mM, and aminoguanidine at 10 mM in 30 mM HEPES buffer (pH 8.0). Different CRM_{197}-N_3:RPS-alkyne ratios were tested to determine the optimal conditions, analyzed by SDS-PAGE. A final molar ratio of azide:alkyne of 1:0.7 (CRM_{197}:RPS = 1:10.5) was selected for glycoconjugate synthesis. The reaction proceeded at RT for 1 hour, after which a sample was collected and quenched with Laemmli Sample Buffer containing β-mercaptoethanol (Bio-Rad) and analyzed by SDS-PAGE. The conjugates were purified by ultracentrifugation, with 25 cycles of washing in PBS, using Amicon Ultra Centrifugal Filters (30 kDa MWCO; Millipore) with 25 cycles of PBS washing (swinging bucket rotor, 4,000 x g, RT). The final products were sterile-filtered through 0.22 µm filters (Millex polyethersulfone syringe filter; Millipore), with the filters washed twice with PBS to maximize recovery.

Characterization of EcoCRM-RPS glycoconjugates

Protein and glycan concentrations were measured using the BCA Protein Assay Kit (Pierce, Thermo Scientific) as per the manufacturer's recommendations and the modified anthrone assay, respectively. Rhamnose (Rha) content was estimated using a minor modification of the anthrone procedure: 0.08 mL of the sample was mixed with 0.32 mL of anthrone reagent (0.2% in concentrated H_2SO_4), heated at 100°C for 10 min, cooled to RT and absorbance was read at 580 nm. Rha concentrations were calculated using an L-Rha standard curve.

Pig immunization and serum collection

Animal experiment and serum collection were conducted in MSD Animal Health and described in the doctoral thesis of Yao Shi. Briefly, CRM_{197}-RPS conjugates (molar ratio of approximately 2.3 RPS per CRM_{197}, as determined by anthrone assay) and placebo were adjuvanted with X-Solve (an oil-in-water emulsion) in a 1:1 volume ratio. Twenty-four 3-week-old piglets (Duroc and Yorkshire, both sexes) were divided into two groups (vaccine and control). The piglets, born and housed at the same farm, were evenly distributed across groups. Each piglet was intramuscularly immunized twice (days 0 and 14) with 2 mL of the vaccine formulation (109 µg glycoconjugate, protein-based). Blood samples were collected on days 0, 14 and 28 to assess serum antibody levels. Total serum IgG against RPS variants was measured by flow cytometry.[18]

Antigen-loaded streptavidin beads antibody binding assay

Purified RPS was biotinylated at its reducing end using biotin-amine (AxisPharm) and $NaBH_3CN$ (Sigma-Aldrich) at a molar ratio of 1:600:2500 in 80 mM NaOAc buffer (pH 5.5), with dimethyl sulfoxide (DMSO) added to improve linker solubility. The reaction proceeded overnight at RT in the dark. Biotinylated RPS was purified using Amicon Ultra Centrifugal Filters (3 kDa MWCO; Millipore).[18]

Streptavidin beads (Invitrogen) were coated with biotinylated RPS by incubating 5 × 10^7 beads (in 20 µL PBS) with 60 µL 0.2 mM RPS at RT for 15 minutes with shaking. The coated beads were washed thrice with PBS and resuspended in PBS containing 0.1% BSA and 0.05% Tween-20 (PBS-BSA-T20). Coating was validated via a lectin-binding assay and analyzed by flow cytometry (BD FACSCanto II; BD Biosciences).[18]

For antibody binding, 1×10^5 RPS-coated or uncoated beads in 12.5 µL PBS-BSA-T20 were mixed with an equal volume of pig serum (1:100 dilution) in a 96-well U-bottom plate and incubated at 4°C for 20 minutes in the dark with shaking. After a wash with PBS-BSA-T20 using a magnetic plate holder, beads were incubated with 25 µL of 10 µg/mL goat anti-pig IgG (Fc):FITC (Bio-Rad) for 20 minutes at 4 °C in the dark. Beads were washed and resuspended in 100 µL PBS-BSA-T20, and fluorescence was analyzed by flow cytometry.[18]

Acquired data was processed using FlowJo (v10.10.0). Background staining was measured using uncoated beads with primary serum and secondary antibodies, and antibody levels were reported as delta geometric mean fluorescence intensity (ΔGMFI), calculated by subtracting background GMFI from the tested samples' GMFI.[18]

References

1. Neila-Ibáñez, C., Casal, J., Hennig-Pauka, I., et al., Stochastic Assessment of the Economic Impact of Streptococcus suis-Associated Disease in German, Dutch and Spanish Swine Farms. *Front Vet Sci*, 8: p. 676002 (**2021**).
2. Haas, B. and Grenier, D., Understanding the virulence of Streptococcus suis: A veterinary, medical, and economic challenge. *Médecine et Maladies Infectieuses*, 48(3): p. 159-166 (**2018**).
3. Hughes, J.M., Wilson, M.E., Wertheim, H.F.L., et al., Streptococcus suis: An Emerging Human Pathogen. *Clinical Infectious Diseases*, 48(5): p. 617-625 (**2009**).
4. Yu, H., Jing, H., Chen, Z., et al., Human Streptococcus suis Outbreak, Sichuan, China. *Emerging Infectious Disease journal*, 12(6): p. 914 (**2006**).
5. Uruén, C., García, C., Fraile, L., et al., How Streptococcus suis escapes antibiotic treatments. *Veterinary Research*, 53(1): p. 91 (**2022**).
6. Segura, M., Streptococcus suis vaccines: candidate antigens and progress. *Expert Review of Vaccines*, 14(12): p. 1587-1608 (**2015**).
7. Goyette-Desjardins, G., Calzas, C., Shiao, T.C., et al., Protection against Streptococcus suis Serotype 2 Infection Using a Capsular Polysaccharide Glycoconjugate Vaccine. *Infect Immun*, 84(7): p. 2059-2075 (**2016**).
8. Goyette-Desjardins, G., Auger, J.P., Xu, J., et al., Streptococcus suis, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. *Emerg Microbes Infect*, 3(6): p. e45 (**2014**).
9. Guérin, H., Kulakauskas, S., and Chapot-Chartier, M.P., Structural variations and roles of rhamnose-rich cell wall polysaccharides in Gram-positive bacteria. *Journal of Biological Chemistry*, 298(10): p. 102488 (**2022**).
10. Mistou, M.Y., Sutcliffe, I.C., and van Sorge, N.M., Bacterial glycobiology: rhamnose-containing cell wall polysaccharides in Gram-positive bacteria. *FEMS Microbiol Rev*, 40(4): p. 464-79 (**2016**).
11. Zorzoli, A., Meyer, B.H., Adair, E., et al., Group A, B, C, and G Streptococcus Lancefield antigen biosynthesis is initiated by a conserved α-d-GlcNAc-β-1,4-l-rhamnosyltransferase. *Journal of Biological Chemistry*, 294(42): p. 15237-15256 (**2019**).
12. van Sorge, N.M., Cole, J.N., Kuipers, K., et al., The classical lancefield antigen of group a Streptococcus is a virulence determinant with implications for vaccine design. *Cell Host & Microbe*, 15(6): p. 729-740 (**2014**).
13. Walkinshaw, D.R., Wright, M.E.E., Mullin, A.E., et al., The Streptococcus pyogenes vaccine landscape. *NPJ Vaccines*, 8(1): p. 16 (**2023**).
14. Di Benedetto, R., Mancini, F., Carducci, M., et al., Rational Design of a Glycoconjugate Vaccine against Group A Streptococcus. *International Journal of Molecular Sciences*, 21(22) (**2020**).
15. Gao, N.J., Uchiyama, S., Pill, L., et al., Site-Specific Conjugation of Cell Wall Polyrhamnose to Protein SpyAD Envisioning a Safe Universal Group A Streptococcal Vaccine. *Infectious Microbes & Diseases*, 3(2): p. 87-100 (**2021**).
16. Ajay Castro, S. and Dorfmueller, H.C., Update on the development of Group A Streptococcus vaccines. *npj Vaccines*, 8(1): p. 135 (**2023**).
17. Ajay Castro, S., Thomson, S., Shaw, H.A., et al., Rhamnose Polysaccharide-Decorated Outer Membrane Vesicles as a Vaccine Candidate Targeting Group A Streptococcus

from Streptococcus pyogenes and Streptococcus dysgalactiae subsp. equisimilis. *bioRxiv*: p. 2024.08.23.609347 (**2024**).

18. Shi, Y., *Sweet Immunization: Identifying conserved glycan motifs to develop vaccines against diverse bacterial pathogens*. 2024, Amsterdam Medical Center.
19. Sorieul, C., Dolce, M., Romano, M.R., et al., Glycoconjugate vaccines against antimicrobial resistant pathogens. *Expert Review of Vaccines*, 22(1): p. 1055-1078 (**2023**).
20. Rappuoli, R., Glycoconjugate vaccines: Principles and mechanisms. *Science Translational Medicine*, 10(456): p. 1-6 (**2018**).
21. Khatun, F., Toth, I., and Stephenson, R.J., Immunology of carbohydrate-based vaccines. *Adv Drug Deliv Rev*, 165-166: p. 117-126 (**2020**).
22. Stefanetti, G., Borriello, F., Richichi, B., et al., Immunobiology of Carbohydrates: Implications for Novel Vaccine and Adjuvant Design Against Infectious Diseases. *Front Cell Infect Microbiol*, 11 (**2022**).
23. Micoli, F., Adamo, R., and Costantino, P., Protein Carriers for Glycoconjugate Vaccines: History, Selection Criteria, Characterization and New Trends. *Molecules*, 23(6) (**2018**).
24. van der Put, R.M.F., Metz, B., and Pieters, R.J., Carriers and Antigens: New Developments in Glycoconjugate Vaccines. *Vaccines*, 11(2): p. 219 (**2023**).
25. Biemans, R., Micoli, F., and Romano, M.R., *8 - Glycoconjugate vaccines, production and characterization*, in *Recent Trends in Carbohydrate Chemistry*, A.P. Rauter, et al., Editors. 2020, Elsevier. p. 285-313.
26. Micoli, F., Costantino, P., and Adamo, R., Potential targets for next generation antimicrobial glycoconjugate vaccines. *FEMS Microbiol Rev*, 42(3): p. 388-423 (**2018**).
27. Romano, M.R., Berti, F., and Rappuoli, R., Classical- and bioconjugate vaccines: comparison of the structural properties and immunological response. *Current Opinion in Immunology*, 78: p. 102235 (**2022**).
28. Berti, F. and Adamo, R., Antimicrobial glycoconjugate vaccines: an overview of classic and modern approaches for protein modification. *Chemical Society Reviews*, 47(24): p. 9015-9025 (**2018**).
29. Feemster, K., Buchwald, U.K., Banniettis, N., et al., Immunogenicity of Current and Next-Generation Pneumococcal Conjugate Vaccines in Children: Current Challenges and Upcoming Opportunities. *Open Forum Infectious Diseases*, 11(5) (**2024**).
30. Del Bino, L., Østerlid, K.E., Wu, D.-Y., et al., Synthetic Glycans to Improve Current Glycoconjugate Vaccines and Fight Antimicrobial Resistance. *Chemical Reviews*, 122(20): p. 15672-15716 (**2022**).
31. Hermanson, G.T., *Chapter 15 - Immobilization of Ligands on Chromatography Supports*, in *Bioconjugate Techniques (Third Edition)*, G.T. Hermanson, Editor. 2013, Academic Press: Boston. p. 589-740.
32. Lees, A., Sen, G., and LopezAcosta, A., Versatile and efficient synthesis of protein–polysaccharide conjugate vaccines using aminooxy reagents and oxime chemistry. *Vaccine*, 24(6): p. 716-729 (**2006**).
33. Kabanova, A., Margarit, I., Berti, F., et al., Evaluation of a Group A Streptococcus synthetic oligosaccharide as vaccine candidate. *Vaccine*, 29(1): p. 104-114 (**2010**).
34. Pitirollo, O., Di Benedetto, R., Henriques, P., et al., Elucidating the role of N-acetylglucosamine in Group A Carbohydrate for the development of an effective glycoconjugate vaccine against Group A Streptococcus. *Carbohydrate Polymers*, 311: p. 120736 (**2023**).

35. St. Michael, F., Cairns, C.M., Filion, A.L., et al., Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: conjugates based on core oligosaccharides. *Glycoconjugate Journal*, 31(1): p. 25-39 (**2014**).
36. Tontini, M., Romano, M.R., Proietti, D., et al., Preclinical studies on new proteins as carrier for glycoconjugate vaccines. *Vaccine*, 34(35): p. 4235-4242 (**2016**).
37. Costantino, P., Viti, S., Podda, A., et al., Development and phase 1 clinical testing of a conjugate vaccine against meningococcus A and C. *Vaccine*, 10(10): p. 691-8 (**1992**).
38. Upadhyay, K.K., Meins, J.F.L., Misra, A., et al., Biomimetic Doxorubicin Loaded Polymersomes from Hyaluronan-block-Poly(γ-benzyl glutamate) Copolymers. *Biomacromolecules*, 10(10): p. 2802-2808 (**2009**).
39. Stefanetti, G., Allan, M., Usera, A., and Micoli, F., Click chemistry compared to thiol chemistry for the synthesis of site-selective glycoconjugate vaccines using CRM(197) as carrier protein. *Glycoconjugate Journal*, 37(5): p. 611-622 (**2020**).
40. Stefanetti, G., Saul, A., MacLennan, C.A., and Micoli, F., Click Chemistry Applied to the Synthesis of Salmonella Typhimurium O-Antigen Glycoconjugate Vaccine on Solid Phase with Sugar Recycling. *Bioconjugate Chemistry*, 26(12): p. 2507-13 (**2015**).
41. Cheng, S., Wantuch, P.L., Kizer, M.E., et al., Glycoconjugate synthesis using chemoselective ligation. *Organic & Biomolecular Chemistry*, 17(10): p. 2646-2650 (**2019**).
42. Micoli, F., Rondini, S., Gavini, M., et al., O:2-CRM(197) conjugates against Salmonella Paratyphi A. *PloS One*, 7(11): p. e47039 (**2012**).
43. Solberg, A., Mo, I.V., Omtvedt, L.A.-A., et al., Click chemistry for block polysaccharides with dihydrazide and dioxyamine linkers - A review. *Carbohydrate Polymers*, 278: p. 118840 (**2022**).
44. Micoli, F., Alfini, R., Di Benedetto, R., et al., GMMA Is a Versatile Platform to Design Effective Multivalent Combination Vaccines. *Vaccines (Basel)*, 8(3) (**2020**).
45. Micoli, F., Romano, M.R., Tontini, M., et al., Development of a glycoconjugate vaccine to prevent meningitis in Africa caused by meningococcal serogroup X. *Proceedings of the National Academy of Sciences of the United States of America*, 110(47): p. 19077-82 (**2013**).
46. Liao, G., Zhou, Z., Liao, J., et al., 6-O-Branched Oligo-β-glucan-Based Antifungal Glycoconjugate Vaccines. *ACS Infect Dis*, 2(2): p. 123-31 (**2016**).
47. De Benedetto, G., Salvini, L., Gotta, S., et al., Investigation on Sugar–Protein Connectivity in Salmonella O-Antigen Glycoconjugate Vaccines. *Bioconjugate Chemistry*, 29(5): p. 1736-1747 (**2018**).
48. Klykov, O. and Weller, M.G., Quantification of N-hydroxysuccinimide and N-hydroxysulfosuccinimide by hydrophilic interaction chromatography (HILIC). *Analytical Methods*, 7(15): p. 6443-6448 (**2015**).
49. Magsumov, T., Ziying, L., and Sedov, I., Comparative study of the protein denaturing ability of different organic cosolvents. *International Journal of Biological Macromolecules*, 160: p. 880-888 (**2020**).
50. Nojima, Y., Iguchi, K., Suzuki, Y., and Sato, A., The pH-dependent formation of PEGylated bovine lactoferrin by branched polyethylene glycol (PEG)-N-hydroxysuccinimide (NHS) active esters. *Biol Pharm Bull*, 32(3): p. 523-6 (**2009**).
51. Bystricky, S., Machová, E., Malovíková, A., and Kogan, G., Determination of the cross-linking effect of adipic acid dihydrazide on glycoconjugate preparation. *Glycoconjugate journal*, 16: p. 691-5 (**1999**).

52. Rush, J.S., Parajuli, P., Ruda, A., et al., PplD is a de-N-acetylase of the cell wall linkage unit of streptococcal rhamnopolysaccharides. *Nature Communications*, 13(1): p. 590 (**2022**).
53. Kolb, H.C., Finn, M.G., and Sharpless, K.B., Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew Chem Int Ed Engl*, 40(11): p. 2004-2021 (**2001**).
54. Tornøe, C.W., Christensen, C., and Meldal, M., Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *The Journal of Organic Chemistry*, 67(9): p. 3057-3064 (**2002**).
55. Hu, Q.-Y., Allan, M., Adamo, R., et al., Synthesis of a well-defined glycoconjugate vaccine by a tyrosine-selective conjugation strategy. *Chemical Science*, 4(10): p. 3827-3832 (**2013**).
56. Nilo, A., Allan, M., Brogioni, B., et al., Tyrosine-directed conjugation of large glycans to proteins via copper-free click chemistry. *Bioconjugate Chemistry*, 25(12): p. 2105-11 (**2014**).
57. Adamo, R., Hu, Q.-Y., Torosantucci, A., et al., Deciphering the structure–immunogenicity relationship of anti-Candida glycoconjugate vaccines. *Chemical Science*, 5(11): p. 4302-4311 (**2014**).
58. Stefanetti, G., Hu, Q.Y., Usera, A., et al., Sugar-Protein Connectivity Impacts on the Immunogenicity of Site-Selective Salmonella O-Antigen Glycoconjugate Vaccines. *Angew Chem Int Ed Engl*, 54(45): p. 13198-203 (**2015**).
59. Kapoor, N., Uchiyama, S., Pill, L., et al., Non-Native Amino Acid Click Chemistry-Based Technology for Site-Specific Polysaccharide Conjugation to a Bacterial Protein Serving as Both Carrier and Vaccine Antigen. *ACS Omega*, 7(28): p. 24111-24120 (**2022**).
60. Zong, G., Toonstra, C., Yang, Q., et al., Chemoenzymatic Synthesis and Antibody Binding of HIV-1 V1/V2 Glycopeptide-Bacteriophage Qβ Conjugates as a Vaccine Candidate. *International Journal of Molecular Sciences*, 22(22) (**2021**).
61. Moses, J.E. and Moorhouse, A.D., The growing applications of click chemistry. *Chemical Society Reviews*, 36(8): p. 1249-1262 (**2007**).
62. Zamakhaeva, S., Chaton, C.T., Rush, J.S., et al., Modification of cell wall polysaccharide guides cell division in Streptococcus mutans. *Nature Chemical Biology*, 17(8): p. 878-887 (**2021**).
63. Roe, J.H., The determination of sugar in blood and spinal fluid with anthrone reagent. *Journal of Biological Chemistry*, 212(1): p. 335-43 (**1955**).
64. Zuily, L., Lahrach, N., Fassler, R., et al., Copper Induces Protein Aggregation, a Toxic Process Compensated by Molecular Chaperones. *mBio*, 13(2): p. e0325121 (**2022**).
65. Carboni, F., Kitowski, A., Sorieul, C., et al., Retaining the structural integrity of disulfide bonds in diphtheria toxoid carrier protein is crucial for the effectiveness of glycoconjugate vaccine candidates. *Chemical Science*, 13(8): p. 2440-2449 (**2022**).
66. Stefanetti, G., MacLennan, C.A., and Micoli, F., Impact and Control of Sugar Size in Glycoconjugate Vaccines. *Molecules*, 27(19) (**2022**).
67. Micoli, F., Stefanetti, G., and MacLennan, C.A., Exploring the variables influencing the immune response of traditional and innovative glycoconjugate vaccines. *Front Mol Biosci*, 10 (**2023**).
68. Anish, C., Beurret, M., and Poolman, J., Combined effects of glycan chain length and linkage type on the immunogenicity of glycoconjugate vaccines. *npj Vaccines*, 6(1): p. 150 (**2021**).

69. Mogler, M.A., Kitikoon, P., Puttamreddy, S., et al., *Swine influenza a virus vaccine*. 2018, Intervet Inc.
70. Jacobs, A.A.C., Grommen, A.W.F., Badbanchi, S., et al., Serotype independent protection induced by a vaccine based on the IgM protease of Streptococcus suis and proposal for a new immunity-based classification system. *Porcine Health Management*, 10(1): p. 41 (**2024**).
71. Hammerberg, C., Schurig, G.G., and Ochs, D.L., Immunodeficiency in young pigs. *American Journal of Veterinary Research*, 50(6): p. 868-74 (**1989**).
72. Bianchi, A.T., Scholten, J.W., Moonen Leusen, B.H., and Boersma, W.J., Development of the natural response of immunoglobulin secreting cells in the pig as a function of organ, age and housing. *Dev Comp Immunol*, 23(6): p. 511-20 (**1999**).
73. Poonsuk, K. and Zimmerman, J., Historical and contemporary aspects of maternal immunity in swine. *Animal Health Research Reviews*, 19(1): p. 31-45 (**2018**).
74. Svobodova, Z., Jankovicova, B., Plichta, Z., et al., Evaluation of Colorimetric BCA-Based Quantification of Hydrazide Groups on Magnetic Particles. *Journal of Spectroscopy*, 2018(1): p. 5492893 (**2018**).
75. Presolski, S.I., Hong, V.P., and Finn, M.G., Copper-Catalyzed Azide-Alkyne Click Chemistry for Bioconjugation. *Current Protocols in Chemical Biology*, 3(4): p. 153-162 (**2011**).
76. Staśkiewicz, A., Ledwoń, P., Rovero, P., et al., Triazole-Modified Peptidomimetics: An Opportunity for Drug Discovery and Development. *Frontiers in Chemistry*, 9 (**2021**).
77. Bhunia, D., Pallavi, P.M.C., Bonam, S.R., et al., Design, Synthesis, and Evaluation of Novel 1,2,3-Triazole-Tethered Glycolipids as Vaccine Adjuvants. *Archiv der Pharmazie*, 348(10): p. 689-703 (**2015**).

Supplementary information

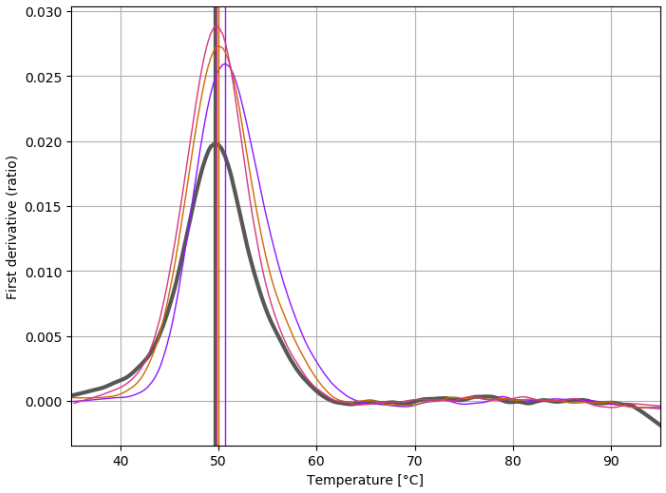

Figure S1. NanoDSF analysis of the thermal stability of CRM_{197} in DMSO. CRM_{197} was incubated with 5% (in orange), 10% (in pink) and 15% (in bold grey) DMSO for 1 h against a reference in HEPES pH 8.0 (in purple).