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Video Article

Homogeneous Glycoconjugate Produced by Combined Unnatural Amino Acid Incorporation and Click-Chemistry for Vaccine Purposes

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Abstract

Genetic code expansion is a powerful tool to introduce unnatural amino acids (UAAs) into proteins to modify their characteristics, to study or create new protein functions or to have access to protein conjugates. Stop codon suppression, in particular amber codon suppression, has emerged as the most popular method to genetically introduce UAAs at defined positions. This methodology is herein applied to the preparation of a carrier protein containing an UAA harboring a bioorthogonal functional group. This reactive handle can next be used to specifically and efficiently graft a synthetic oligosaccharide hapten to provide a homogeneous glycoconjugate vaccine. The protocol is limited to the synthesis of glycoconjugates in a 1:1 carbohydrate hapten/carrier protein ratio but amenable to numerous pairs of biorthogonal functional groups. Glycoconjugate vaccine homogeneity is an important criterion to ensure complete physico-chemical characterization, thereby, satisfying more and more demanding drug regulatory agency recommendations, a criterion which is unmet by classical conjugation strategies. Moreover, this protocol makes it possible to finely tune the structure of the actual conjugate vaccine, giving rise to tools to address structure-immunogenicity relationships.

Video Link

The video component of this article can be found at <https://www.jove.com/video/60821/>

Introduction

Glycoconjugate vaccines are essential elements of the vaccine arsenal available for the prophylactic treatment of infectious diseases. They are safe, well-tolerated and efficient in a broad age group including young infants. They provide the optimal defense against infections caused by capsulated bacteria like meningococcus, pneumococcus or *Haemophilus influenzae* type b¹. Glycococonjugate vaccines are made of purified bacterial polysaccharides that form the capsules of bacteria or synthetic oligosaccharides that mimic these surface-expressed polysaccharides², which are covalently linked to a carrier protein. The presence of a carrier protein is essential to promote protective humoral immune responses directed against the antigenic determinant expressed by the carbohydrate antigens³. Apart from a careful selection and production of the carbohydrate antigen, the features known to exert an influence on the efficacy of a glycoconjugate vaccine are: the nature of the carrier protein, the conjugation chemistry (including the nature and the length of the linker if used), or the saccharide/protein ratio³. Obviously, the positions at which the saccharide is conjugated to the protein as well as the number of connectivity points are relevant for immunogenicity. To date, these two parameters have hardly been studied because the preparation of the glycoconjugates remains largely empirical. Their synthesis usually relies on the use of amine or carboxylic acid functions of, respectively, lysine or aspartic/glutamic acid side-chain residues present on the carrier protein sequence. This leads not to a single but to a heterogeneous mixture of glycoconjugates.

Playing on the reactivity, accessibility or distribution of the amino acid residues in the protein gives rise to more defined glycoconjugates that are more reliable to document the effect of saccharide/protein connectivity⁴. A step forward towards this goal can be achieved by applying protein glycan coupling technology, a recombinant process that allows the production of controlled glycoconjugate vaccines in cell factories^{5,6}. However, the glycosylation exclusively takes place at an asparagine residue within D/EXNYS/T sequons (whereby X is any out of the 20 natural amino acids), not naturally present on the carrier proteins.

Site selective mutagenesis and in particular incorporation of cysteines to exploit their highly and selective reactivity appears as an alternative^{7,8}. Production of carrier proteins incorporating UAAs in their sequence can offer even more flexibility for homogeneous glycoconjugate vaccine preparation. More than 100 UAAs have been developed and further incorporated into various proteins^{9,10}. Many of them contain bioorthogonal functions usually used to carry out post translational modifications¹¹ or to graft biophysical probes¹² or drugs¹³ but which are ideal handles for further conjugation with carbohydrate antigens. Successful examples have been claimed by Biotech¹⁴ using cell-free protein synthesis¹⁵ but preparation of glycoconjugate vaccines according to this strategy still waits for becoming popularized.

Application of the in vivo strategy for the production of mutated carrier protein needs a modified translational machinery that includes a specific codon, a tRNA recognizing the codon and an aminoacyl-tRNA synthetase (aaRS) which specifically catalyzes the transfer of the UAA on the

tRNA (Figure 1)¹⁶. The pyrrolysine amber stop codon suppression is one of the most widely used methods to incorporate UAA, in particular the propargyl-lysine (PrK)¹⁷. The latter can in turn react with azido-functionalized carbohydrate haptens to provide fully defined, homogeneous glycoconjugates. In the present manuscript we describe how to synthesize the propargyl-L-lysine, an UAA carrying an alkyne handle, how to incorporate it into a target protein during its translation in a bacteria and finally how to perform conjugation between the modified protein and a hapten carrying an azide function using click chemistry.

Protocol

1. Synthesis of the UAA: propargyl-lysine (PrK)

- Synthesis of *N*^ε-Boc-propargyl-lysine¹⁸
 - Dissolve 500 mg of Boc-L-Lys-OH (2.03 mmol) in a mixture of aqueous 1 M NaOH (5 mL) and THF (5 mL) in a flask and fit the flask with a silicon septum.
 - Cool the flask in an ice bath and then add 158 μL of propargyl chloroformate (1.62 mmol) dropwise (over a 2-3 min period) using a microsyringe while stirring.
 - Warm the reaction mixture to room temperature and continue stirring for 10 h.
 - Cool down solutions of 50 mL of diethyl ether, 50 mL of aqueous 1 M hydrochloric acid and 60 mL of ethyl acetate in an ice bath.
 - Cool the crude reaction mixture in an ice bath and pour the mixture into a separation funnel. Extract the mixture with 50 mL of diethyl ether. Discard the organic layer.
 - Cautiously add aqueous 1 M hydrochloric acid to the aqueous phase in the separation funnel. Then extract the aqueous layer twice using 30 mL of ethyl acetate. Verify the presence of *N*-Boc-propargyl-lysine in the organic phase by TLC using CH₂Cl₂-methanol (9:1) as eluent.
 - Dry the combined organic layers over MgSO₄, filter off the solid phase and concentrate the filtrate under reduced pressure on a rotary evaporator.
 - Dissolve a sample of the crude oily *N*^ε-Boc-propargyl-lysine in deuterated chloroform (CDCl₃) and control its identity by ¹H NMR. CAUTION: Extraction may result in a buildup of pressure. Release any pressure buildup frequently.
- Synthesis of the unnatural amino acid propargyl-L-lysine (PrK)
 - Introduce *N*^ε-Boc-propargyl-lysine in a round bottom flask equipped with a septum.
 - Add 4 mL of anhydrous dichloromethane (CH₂Cl₂) to the flask under argon to dissolve the *N*^ε-Boc-propargyl-lysine.
 - Add 4 mL of trifluoroacetic acid (TFA) dropwise using a syringe while stirring.
 - Stir the reaction mixture for 1 h at RT. Monitor the reaction by TLC using CH₂Cl₂-methanol (9:1) as eluent.
 - Concentrate the reaction mixture under reduced pressure.
 - Add diethyl ether to the crude residue and incubate it at 4 °C for 1 h to precipitate the PrK. When working on higher scale, if the PrK is not completely precipitated, triturate to precipitate it and extend the incubation time if needed.
 - Filter the PrK in the form of a white solid on a fritted-glass.
 - Dissolve an aliquot of the PrK in D₂O. Then carry out NMR analyses to control its identity and purity.
 - For further use, dissolve the unnatural amino acid PrK in distilled water at a final concentration of 100 mM and store at -20 °C as 1 mL aliquots.

2. Production of the recombinant protein modified by PrK

- Plasmid preparation
 - Construct an expression plasmid (pET24d-mPsaAK32TAG-ENLYFQ-HHHHHH) that contains the target mature Pneumococcal surface adhesin A (mPsaA) gene (pET24d-mPsaA-WT) followed by a Tobacco Etch Virus (TEV) protease sequence by cloning the insert between the *Bam*HI and *Xho*I restriction sites of the pET24d plasmid. This will introduce a His₆ tag at the C-terminus of the protein. Replace the codon of lysine-32 with the amber codon (TAG), using conventional site-directed mutagenesis technique.
 - Construct a second expression plasmid (pEVOL-MmPyIRS) containing two copies of the gene coding for the pyrrolysyl-tRNA synthetase from *Methanosarcina mazei* (MmPyIRS) and the gene coding for the corresponding tRNA^{pyr} as previously described¹⁹. Use this specially designed plasmid vector, pEVOL, for efficient incorporation of UAAs.

NOTE: The detailed plasmids information is described in **Supplemental File 1**.
- Co-transformation of plasmids into the expression strain
 - Thaw a 100 μL aliquot of chemically competent *Escherichia coli* BL21(DE3) on ice for 5 min.
 - Add 1 μL of each plasmid (50-100 ng of each) into the cells and incubate for 30 min on ice.
 - Transfer the 1.5 mL microtube with the thawed competent cells in an incubator at 42 °C for 45 s and then move it back to ice for 2 min.
 - Add 900 μL of LB medium and incubate under shaking for 1 h at 37 °C to allow antibiotic expression. Then plate the bacteria onto LB agar with 25 μg/mL of kanamycin and 30 μg/mL of chloramphenicol. Allow bacteria growth overnight at 37 °C.
- Expression of proteins modified with PrK
 - Inoculate a single co-transformed colony in 5 mL of LB medium with antibiotics (25 μg/mL of kanamycin and 30 μg/mL of chloramphenicol). Incubate overnight at 37 °C with shaking.
 - Dilute the primary culture (5 mL) into 500 mL of auto-induction medium containing antibiotics, 0.02% of L-arabinose and 1 mM of the unnatural amino acid PrK and incubate it at 37 °C for 24 h with shaking. Include a negative control by performing the culture without PrK in parallel and a positive control by performing the culture of a clone containing the wt protein.

3. Aliquot 5 mL out of the 500 mL culture medium and centrifuge for 10 min at 5,000 x g. Discard the supernatant and freeze the pellet at -20 °C. Harvest cells from the remaining 495 mL by centrifugation for 10 min at 5,000 x g. Discard the supernatant and freeze the pellet at -20 °C.
4. Analyze crude cell extracts from the 5 mL culture samples by SDS-PAGE and western Blot analysis
 1. Resuspend 5 mL cell pellets into 250 μ L of lysis buffer (50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 150 mM NaCl, pH 8, 5 mM imidazole, 0.2 mM PMSF) and transfer it into a 1.5 mL microtube.
 2. Lyse cells by freezing the tubes in liquid nitrogen, thawing it in a 42 °C bath and vortexing at high speed for 30 s. Repeat this step 3 times.
 3. Centrifuge samples at 17,000 x g for 10 min to eliminate cell debris.
 4. Take 10 μ L of the supernatant and add 5 μ L of water and 5 μ L of loading buffer (bromophenol blue, SDS, β -mercaptoethanol). Heat the samples for 5 min at 100 °C and carry out SDS-PAGE and western Blot analyses.
5. Protein purification by gravity flow-bench affinity chromatography using Nickel-NTA beads
 1. Resuspend the cell pellets (from the 495 mL culture) into 20 mL of lysis buffer (50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 150 mM NaCl, pH 8, 5 mM imidazole, 0.2 mM PMSF).
 2. Add 5 μ L of DNase I (1 mg/mL) and 500 μ L of lysozyme (50 mg/mL) into the suspension and allow lysis by incubating the suspension at 37 °C during 30 min.
 3. Sonicate the cells during 5 min (cycles of 5 s-5 s, amplitude 50%) and then remove the cell debris by centrifugation at 20,000 x g for 30 min followed by filtration on 0.45 μ m filter.
 4. Add Ni-NTA resin to the suspension (500 μ L for 500 mL of cell culture) and mix gently at 4 °C for 1 h.
 5. Pour the suspension into a polypropylene column and collect the unbound fraction.
 6. Wash the resin with 10 mL of washing buffer containing 50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 150 mM NaCl, 10 mM imidazole. Wash the resin a second time with 5 mL of washing buffer (50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 150 mM NaCl, 20 mM imidazole). Collect the wash fractions.
 7. Elute the his-tagged protein with 1 mL of elution buffer (50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 150 mM NaCl, 300 mM imidazole). Repeat this step 4 times and collect all the elution fractions.
 8. Analyze the crude lysate as well as the 7 purification fractions by SDS-PAGE on a 12% acrylamide gel.
 9. Combine the fractions containing pure His-tagged protein and dialyze it against 1 L of TEV protease buffer (50 mM Tris-HCl, 0.5 mM EDTA, pH 8) overnight by using a dialysis membrane (cut-off MW 6000-8000 Da). Measure the concentration of the protein at 280 nm with a molar extinction coefficient of 37 360 $\text{cm}^{-1}\cdot\text{M}^{-1}$ and a molecular weight of 34.14 kDa for mPsaA.

3. Removal of the histidine tag by TEV protease digestion

1. Collect the protein sample into a 50 mL tube and add TEV buffer (50 mM Tris HCl, 0.5 mM EDTA, pH 8) up to 1 mL at a concentration of 2 mg/mL.
NOTE: The concentration may vary according to previous results. Protein concentrations that we have tested are in a typical 2-3 mg/mL range.
2. Add 100 μ L of TEV protease (add 1 μ L containing 10 units of TEV protease for 20 μ g of protein to digest).
3. Add 50 μ L of 0.1 M dithiothreitol (DTT).
4. Complete with TEV buffer (50 mM Tris HCl, 0.5 mM EDTA, pH 8) up to 5 mL.
5. Incubate overnight at 4 °C with slow shaking.
NOTE: If digestion is not complete, add more TEV protease, incubate for longer time or at higher temperature up to 30 °C.
6. Dialyze the digested protein to remove EDTA at 4 °C overnight by using a dialysis membrane (cut-off 6000-8000 Da) against phosphate buffer (50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 150 mM NaCl, 5 mM imidazole).
7. To eliminate the TEV protease and the undigested protein, incubate the mix with Ni-NTA beads and mix gently for 1 h at 4 °C.
8. Pour the suspension into a polypropylene column. Collect the unbound fraction and wash the column with 5 mL of washing buffer (50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 150 mM NaCl, 10 mM imidazole)
NOTE: The protein of interest should be recovered in the unbound and washing fractions.
9. Elute the TEV protease and the undigested protein by adding 5 mL of elution buffer (50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 150 mM NaCl, 300 mM imidazole) on the column. Check the fractions for protein contents at 280 nm and by SDS-PAGE analysis.
10. Check the efficiency of the digestion by loading digested samples on an SDS PAGE with the undigested protein as a control.
11. Dialyze the digested protein against 1 L of click buffer (50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH8) at 4 °C overnight with a dialysis membrane (cut-off 6000-8000 Da) to remove imidazole as well as to exchange the buffer, and measure the concentration of the protein at 280 nm with molar extinction coefficient and molecular weight of mPsaA (37 360 $\text{cm}^{-1}\cdot\text{M}^{-1}$ and MW 34.14 kDa).

4. Assessment of the unnatural amino acid propargyl-lysine accessibility and functionality for click chemistry

NOTE: Conjugate the mPsaA with 6-hexachloro-fluorescein-azide using the protocol described by Presolski et al.²⁰ for click chemistry.

1. Take 432.5 μ L of PrK-mutated protein at a concentration of 57.8 μ M into a 2 mL microtube.
NOTE: A minimum concentration of 2 μ M of alkyne is acceptable. If the protein concentration is lower, concentrate it with a centrifugal concentrator or favor the balance of the reaction by increasing azide/alkyne molar ratio.
2. Add 10 μ L of 5 mM 6-hexachloro-fluorescein-azide and then add a premix of 2.5 μ L of CuSO_4 solution at 20 mM and 7.5 μ L of Tris(benzyltriazolylmethyl)amine (THPTA) at 50 mM (stock solutions concentrations).
 1. Add 25 μ L of aqueous 100 mM aminoguanidine hydrochloride.
 2. Add 25 μ L of 20 mg/mL an extemporaneously prepared aqueous solution of sodium ascorbate.
 3. Close the tube, mix by inverting several times and incubate at room temperature for 2 h.

- Stop the reaction by adding 50 μ L of 0.5 M EDTA.
- Take 15 μ L of the reaction mixture and put it in a microtube, add 5 μ L of loading buffer (bromophenol blue, SDS, β -mercaptoethanol), heat the mixture at 100 $^{\circ}$ C for 5 min, and then load it into a 12% acrylamide gel. After migration, visualize the fluorescent conjugate on the gel under UV light at 312 nm.

5. Conjugation of mPsaA with an azido-functionalized carbohydrate antigen (Pn14TS-N₃) by click chemistry

- Coupling
 - Take 432.5 μ L of PrK-mutated protein at 57.8 μ M into a 2 mL microtube.
 - Add 10 μ L of 5 mM Pn14TS-N₃²¹ in water then add a premix of 2.5 μ L of CuSO₄ solution at 20 mM and 7.5 μ L of THPTA at 50 mM.
NOTE: Synthesis of Pn14TS-N₃, a tetrasaccharide mimicking the *Streptococcus pneumoniae* serotype 14 capsular polysaccharide, has been described in reference 21. Theoretically, any carbohydrate antigen containing an azide function can be used.
 - Add 25 μ L of 100 mM aminoguanidine hydrochloride.
 - Add 25 μ L of 20 mg/mL extemporaneously prepared aqueous solution of sodium ascorbate.
 - Close the tube, mix by inverting several times and incubate at RT during 2 h.
 - Stop the reaction by adding 50 μ L of 0.5 M EDTA.
 - Take 15 μ L of samples and analyze by SDS-PAGE.
- Gel filtration purification of the glycoconjugate
 - Purify the glycoconjugate by applying it to a steric exclusion agarose column (15 x 600 bed dimensions, 3,000-70,000 fractionation range), equilibrated with 100 mM PBS buffer, pH 7.3 at a 0.8 mL/min flow with detection at 280 nm.
 - Collect the fractions containing the glycoconjugate.
NOTE: For prolonged storage, dialyze the glycoconjugate against 1 L of H₂O twice for 2 h and then overnight at 4 $^{\circ}$ C by using dialysis membrane (cut-off Mw 6000-8000 Da), then freeze-dry and store the glycoconjugate at -80 $^{\circ}$ C.

Representative Results

In this project, a homogeneous glycoconjugate vaccine was prepared using the amber stop codon suppression strategy to introduce an UAA at a defined site (**Figure 1**). Pneumococcal surface adhesin A was selected as the carrier protein moiety. This protein is highly conserved and expressed by all strains of *Streptococcus pneumoniae*²². It is highly immunogenic and previously used as a carrier in pneumococcal vaccine formulations^{21,23}. As a proof-of-concept, the UAA propargyl-lysine efficiently charged by the wild type pyrrolysyl-tRNA synthetase (PylRS)/tRNA pair of the archaea *Methanosarcina mazei* was investigated. The propargyl-lysine is commercially available but can be advantageously prepared from Boc-L-lysine in only two synthetic steps (**Figure 2**). An amber codon was generated at a desired position in a pET24d plasmid containing the mPsaA gene. This plasmid was co-transformed with a pEVOL plasmid (a kind gift from Edward Lemke (EMBL¹⁹) containing orthogonal tools necessary to incorporate the propargyl-lysine, into competent *E. coli* BL21(DE3) strain. Positive co-transformed clones were selected using 25 μ g/mL kanamycin and 30 μ g/mL chloramphenicol. The plasmid pEVOL contains originally not one but two copies of the gene coding for MmPylRS to incorporate the propargyl-lysine residue: the first copy is under the control of a constitutive promoter while the expression of the other one is inducible in the presence of arabinose. However, we have noticed no dramatic decrease of propargyl-lysine incorporation if the MmPylRS gene under the control of constitutive promoter is suppressed.

The propargyl-lysine was introduced at position 32 in replacement of a lysine near the N-terminus of the PsaA. Any residue with a surface-exposed side-chain can virtually be exchanged in view of carrying out further conjugation. The mutated protein was produced in its mature form (mPsaA^{K32PrK}) with inclusion of a cleavable 6-histidine tag sequence at its C-terminus. The efficiency of the mPsaA^{K32PrK} production was checked by SDS-PAGE and Western Blot analysis using an anti-Histidine tag antibody, when growth was performed in the presence or the absence of the UAA propargyl-lysine and in comparison with the production of the wild type mPsaA (**Figure 3**). Visualization revealed a protein band at an expected molecular weight (Lanes 4, **Figure 3A & 3B**). The presence of a full-length protein strongly indicates the successful incorporation of the PrK into mPsaA. The intensity is, however, lower than that observed for wild type mPsaA (Lanes 2, **Figure 3A & 3B**). Leakage (i.e., production of the full-length protein without incorporation of the UAA) and premature release of the protein by the Release Factor RF1 during translation are two main drawbacks frequently encountered during this process. On one hand, no band at the expected molecular weight is visualized in the absence of propargyl-lysine meaning that no leakage occurred (Lanes 3, **Figure 3A & 3B**) and indirectly confirmed that the band observed on Lanes 4 corresponds to mPsaA^{K32PrK}. On the other hand, no band can be seen at low molecular weight that could correspond to the truncated form of mPsaA (Lane 4 on **Figure 3A**). The mPsaA^{K32PrK} was then purified by affinity chromatography, with a typical yield of 8 mg/L (in comparison to 12-20 mg/L for the wild type protein) and the incorporation of the propargyl-lysine residue was finally confirmed by mass spectrometry (**Figure 4**). The histidine tag was removed upon proteolytic cleavage using TEV protease (**Figure 4**). The stability of the mPsaA^{K32PrK} thus obtained was assessed by circular dichroism, which showed that the structure of the protein was not disturbed by the mutation of the Lysine 32 into a propargyl-lysine (data not shown).

Having the mPsaA^{K32PrK}, the reactivity of the alkyne for click chemistry was assessed using an azido-functionalized fluorescein and further used to conjugate a synthetic oligosaccharide antigen β -2-azidoethyl d-Galp-(1 \rightarrow 4)- β -d-Glcp-(1 \rightarrow 6)-[β -d-Galp-(1 \rightarrow 4)]- β -d-GlcpNAc (Pn14TS) (Figure 5). This tetrasaccharide is related to the *S. pneumoniae* type 14 capsular polysaccharide and has previously been conjugated to mPsaA using different conjugation chemistries^{8,21,24}. Experiments here were done in comparison with wild type mPsaA as a control. The histidine tag was first removed upon proteolytic cleavage using the TEV protease. The digested mPsaA^{K32PrK} and mPsaA WT were then conjugated to the fluorophore (Figure 6A) or Pn14TS (Figure 6B). The reaction was assessed by SDS-PAGE. The small increase in the molecular weight of the sample between lane 6 and 7 (Figure 6B) indicates a successful conjugation with the tetrasaccharide Pn14TS. Finally, the glycoconjugate was purified by gel filtration and its identity confirmed by mass spectrometry (Figure 6C). The conjugation by click chemistry being quantitative the majority of the mPsaA^{K32PrK} was conjugated with the Pn14TS-N₃ as illustrated by the mass spectrometry results (Figure 6C).

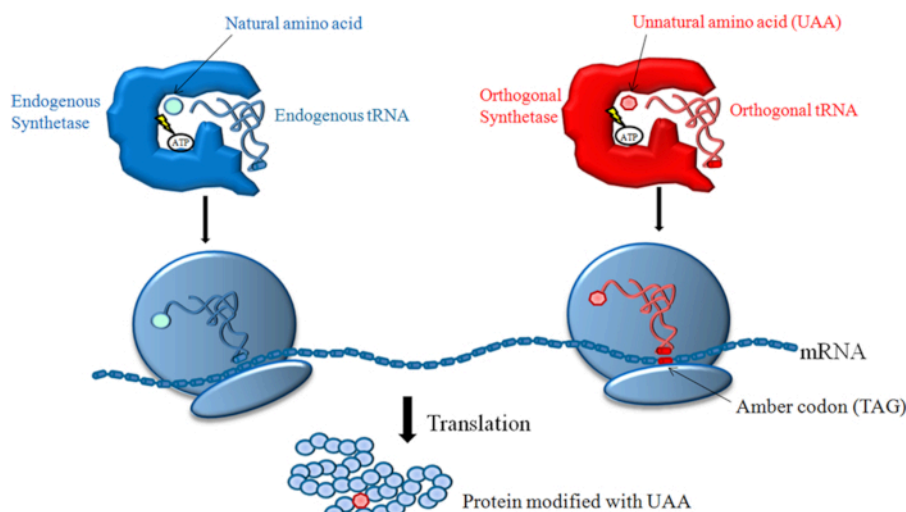


Figure 1: Incorporation of propargyl-lysine (PrK) into mPsaA during translation using an orthogonal pyrrolysyl-tRNA synthetase/tRNA pair and TAG codon reassignment²⁵. During translation, endogenous synthetases catalyze the link between amino acids and corresponding tRNAs. Then, loaded tRNAs are used by the ribosomal machinery to generate the neo-synthesized polypeptide. According to the amber stop codon suppression strategy, an orthogonal aminoacyl-tRNA synthetase (aaRS) (herein a pyrrolysyl-tRNA synthetase from *M. mazei*), loads an UAA (herein PrK) on its cognate tRNA which designed anticodon can read the amber stop codon (TAG) on the mRNA. This specific recognition directs the incorporation of the UAA into the specific site on the target protein. Figure reproduced from Wang et al.²⁵. [Please click here to view a larger version of this figure.](#)

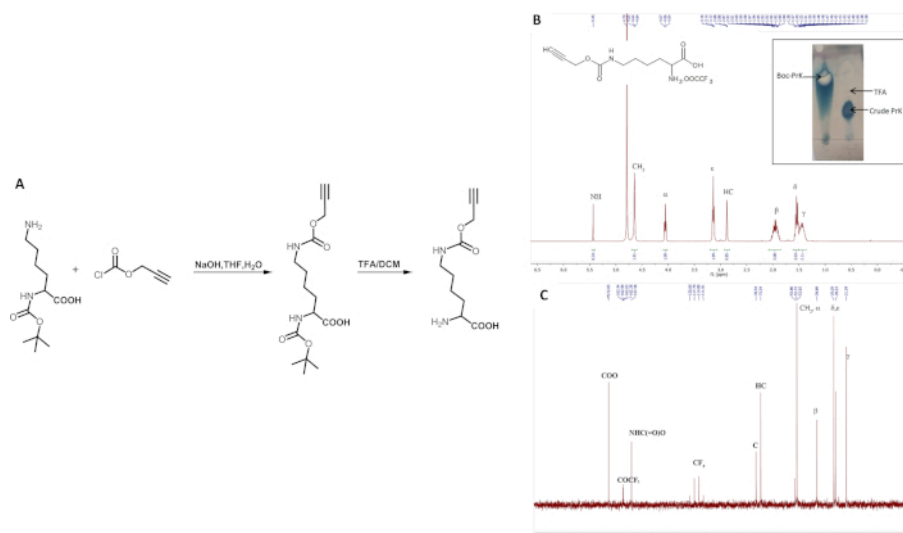


Figure 2: Propargyl-lysine synthesis. (A) Steps of propargyl-lysine synthesis. Insert: Monitoring of the deprotection of Boc-Lys(prop-2-ynyloxycarbonyl)-OH intermediate: thin-layer chromatography on 0.25 mm silica gel plates with fluorescent indicator (GF254) and visualised by charring with vanillin in sulfuric acid/ethanol (1.5:95 v/v); eluent: CH₂Cl₂/MeOH (9:1), left lane: Boc-Lys(prop-2-ynyloxycarbonyl)-OH (*R_f* 0.90), right lane: crude propargyl-lysine, (*R_f* 0.38). 400 MHz ¹H (B) and ¹³C NMR spectra (C) of propargyl-lysine recorded in D₂O. [Please click here to view a larger version of this figure.](#)

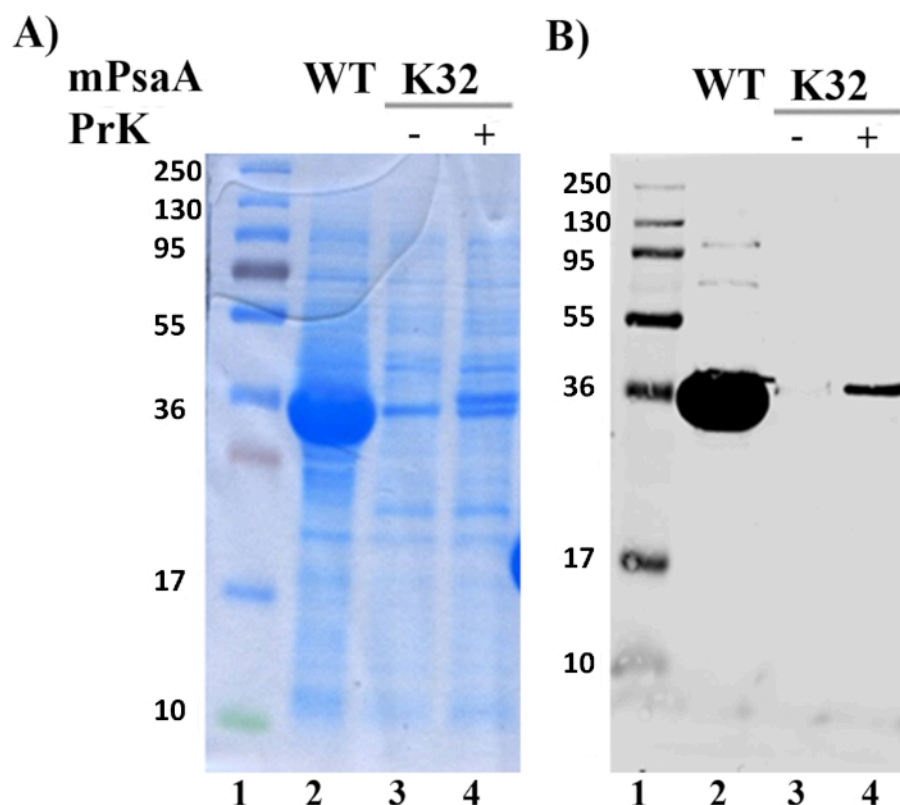


Figure 3: Analysis of crude cell samples. (A) SDS-PAGE analysis and (B) Western blot analysis on crude cell samples. Lane 1: unstained protein marker; Lane 2: crude cell extract of wild type mPsaA; Lane 3: crude cell extract of mPsaA^{K32TAG} grown in the absence of PrK; Lane 4: crude cell extraction of mPsaA^{K32TAG} grown in the presence of PrK. Conditions: 12% acrylamide gel, running at 100 V, 2 h. SDS-PAGE stained by Coomassie blue; Western Blot revealed using anti-histidine tag antibody and secondary antibody coupled with AlexaFluor680. [Please click here to view a larger version of this figure.](#)

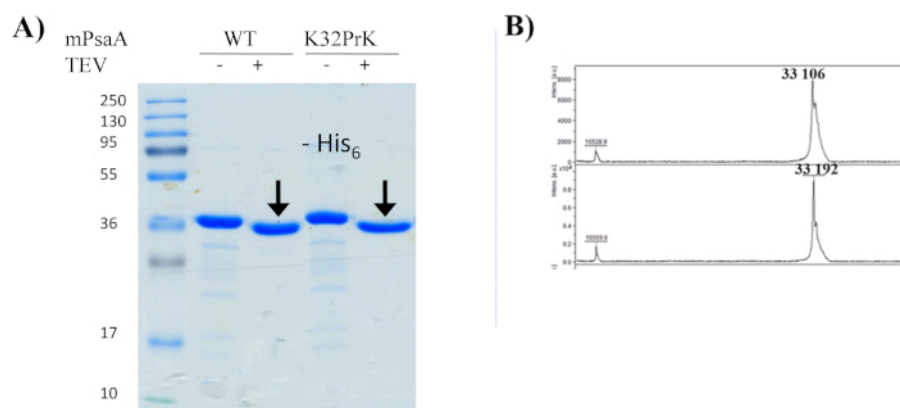


Figure 4: Histidine tag removal and mass spectrometry analysis. (A) SDS-PAGE analysis. Lane 1: unstained protein marker; Lane 2: crude cell extract; Lane 3: unbound fraction; Lane 4: wash fraction with 10 mM imidazole; Lane 5: wash fraction with 20 mM imidazole; Conditions: 12% acrylamide gel running at 100 V for 2 h, and stained by Coomassie blue; (B) MALDI-TOF-MS spectra of (top) mPsaA WT, theoretical MW 33 103 Da, found 33 106 Da and (bottom) mPsaA K32PrK, theoretical 33 184 Da, found 33 192 Da. The found masses are within the expected margin error. [Please click here to view a larger version of this figure.](#)

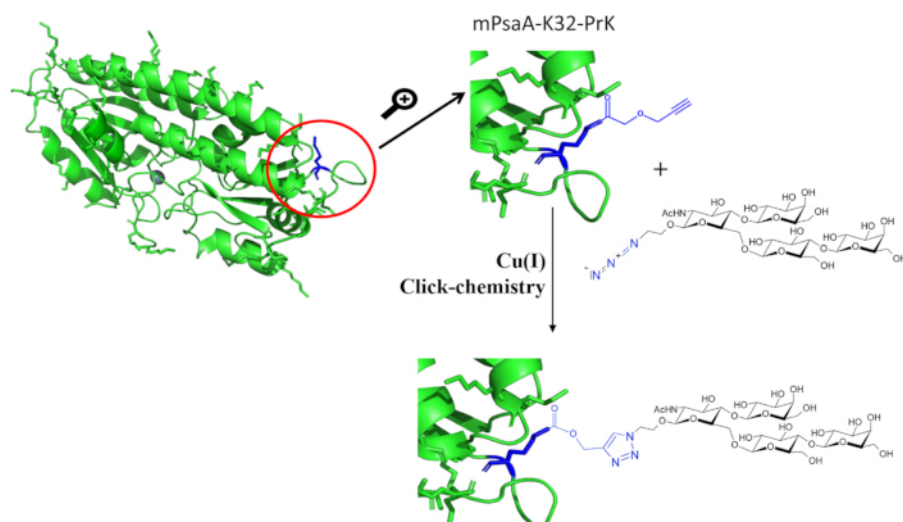


Figure 5: Schematic representation of the conjugation strategy by click chemistry. A single tetrasaccharide bearing an azide is specifically coupled to its complementary biorthogonal alkyne group on mPsaA K32PrK (mPsaA representation based on the 1PSZ PDB file, with a resolution of 2.0 Å²⁶). [Please click here to view a larger version of this figure.](#)

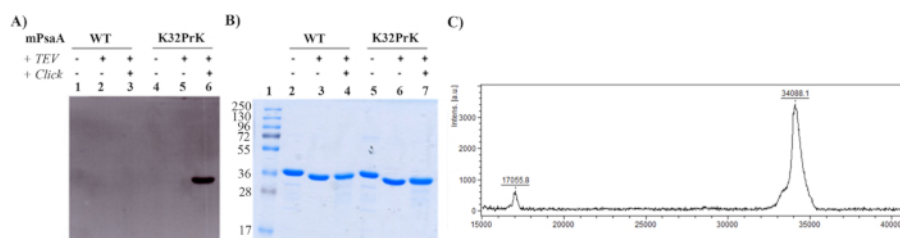


Figure 6: Histidine-tag digestion and conjugation of mPsaA with (A) fluorescein-N₃ and with (B) Pn14TS. (A) SDS-PAGE Lanes 1-3: WT mPsaA; Lane 4-6: mPsaA^{K32PrK}; (B) Lane 1: unstained protein marker; Lane 2-4: WT mPsaA; Lane 5-7: mPsaA^{K32PrK}. 2 µg protein sample/lane, 12% acrylamide, 100 V, 2 h; (C) MALDI-TOF-MS spectra of the Pn14TS-mPsaA^{K32PrK} conjugate. Theoretical MW 34 091 Da, found 34 088 Da. [Please click here to view a larger version of this figure.](#)

Supplemental File 1. [Please click here to view this file \(Right click to download\).](#)

Discussion

Site-directed mutagenesis is a straightforward strategy to incorporate specific amino acids at a defined position of a protein which remains barely used with the aim of preparing glycoconjugate vaccines^{7,8,14}. Classical mutagenesis based on the 20 natural amino acids approach is highly efficient since no modification of the translation machinery is required. Cysteine mutations are usually targeted to further explore the unique thiol reactivity either directly or in two steps (e.g., after its modification into a dehydroalanine intermediate, a strategy called post-translational mutagenesis)^{27,28}. Genetic code expansion is perhaps even more attractive and flexible since it allows the direct incorporation of a wide range of UAAs with diverse functionalities^{9,10}. While several UAAs can be incorporated simultaneously within a protein²⁹, the number of mutations is usually more limited. We herein applied the related amber stop codon strategy to introduce a single propargyl-lysine in a carrier protein. The incorporation can take place at any position provided the sidechain of the initial amino acid was surface-exposed, a criterion easily determined from X-ray crystallographic structures or *in silico* modeling. Moreover, it is not limited to propargyl-lysine but can be extended to any UAA functionalized with a biorthogonal function which will later serve as an anchor to graft the incoming carbohydrate antigen and for which an orthogonal aaRS/tRNA pair exists.

One of the drawbacks of the strategy is the possible production of truncated protein, resulting from the release of the peptidyl sidechain when reading the amber stop codon, as a side-product. Even if we did not observe any truncated form here (probably degraded by the bacteria because of its very small size), a histidine tag has been added at C-terminus of the protein to facilitate the purification of the expected full-length mutated protein from impurities and noticeably from the truncated protein (which by essence does not express the histidine tag sequence). This can become essential if the UAA incorporation is carried out near the protein C-terminus since purification cannot be attempted using alternative chromatography techniques like gel filtration.

For most applications removal of the histidine tag is not mandatory. However, it may be useful regarding the design of glycoconjugate vaccine as part of the immune system may be diverted against the tag sequence. For this proof-of-concept, we inserted an amino acid length sequence specifically cleaved by the TEV protease which leaves five extra amino acids on the carrier protein after digestion.

The conjugation step between the alkyne of the propargyl-lysine and a representative synthetic oligosaccharide Pn14TS related to a pneumococcal capsule and bearing a complementary azide was carried out according to a click chemistry protocol reported by Presolski et al.²⁰ If necessary, completion of the reaction can easily be reached by increasing the reaction time or by modifying the ratio between the alkyne,

azide and copper reactants and reagents. Copper salts are eliminated by treatment with excess EDTA followed by a short purification by steric exclusion chromatography.

The glycoconjugate obtained with the technique described in the present work can then be used to immunize mice. Having such fully-defined and easily modulated glycoconjugate in hands provides invaluable tools to evaluate the impact of the hapten/protein carrier connectivity on the immune response⁸. Since increasing the hapten/protein ratio is often correlated with enhanced anti-hapten humoral response when using short haptens³⁰, one might be interested in testing conjugates with multiple haptens. The incorporation of multiple UAAs however needs some adjustments of the protocol as the incorporation of an UAA in the protein tends to decrease the yield of protein production due to the RF1 activity.

In definitive, this method is a powerful tool to gain access to homogeneous glycoconjugate vaccines facilitating their physico-chemical characterization and further carbohydrate antigen/carrier connectivity-immunogenicity relationship studies.

Disclosures

The authors have nothing to disclose.

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