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TITLE:

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

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SUMMARY:

Genetic code expansion is applied for the introduction of an unnatural amino acid bearing a biorthogonal functional group on a carrier protein at a defined site. The biorthogonal function is further used for the site-selective coupling of a carbohydrate antigen to provide a homogeneous glycoconjugate vaccine.

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. Glycococonjugate 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.

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 b[1]. Glycococonjugate vaccines are made of purified bacterial polysaccharides that form the capsules of bacteria or synthetic oligosaccharides that mimic these surface-expressed polysaccharides[2], 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 antigens[3]. 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 ratio[3]. 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 connectivity[4]. 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 modifications[11] or to graft biophysical probes[12] or drugs[13] but which are ideal handles for further conjugation with carbohydrate antigens. Successful examples have been claimed by Biotech[14] using cell-free protein synthesis[15] but preparation of glycoconjugate vaccines according to this strategy still waits for becoming popularized.

Application of this 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 glycococonjugates. 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)

1.1 Synthesis of N^{α}-Boc-propargyl-lysine[18]

1.1.1 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.

1.1.2 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.

1.1.3 Warm the reaction mixture to room temperature and continue stirring for 10 h.

1.1.4 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.

1.1.5 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.

1.1.6 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 CH_2Cl_2-methanol (9:1) as eluent.

1.1.7 Dry the combined organic layers over $MgSO_4$, filter off the solid phase and concentrate the filtrate under reduced pressure on a rotary evaporator.

1.1.8 Dissolve a sample of the crude oily N^{α}-Boc-propargyl-lysine in deuterated chloroform ($CDCl_3$) and control its identity by ^{1}H NMR.

CAUTION: Extraction may result in a buildup of pressure. Release any pressure buildup frequently.

1.2 Synthesis of the unnatural amino acid propargyl-L-lysine (PrK)

1.2.1 Introduce N^{α}-Boc-propargyl-lysine in a round bottom flask equipped with a septum.

1.2.2 Add 4 mL of anhydrous dichloromethane (CH_2Cl_2) to the flask under argon to dissolve the N^{α}-Boc-propargyl-lysine.

1.2.3 Add 4 mL of trifluoroacetic acid (TFA) dropwise using a syringe while stirring.

1.2.4 Stir the reaction mixture for 1 h at RT. Monitor the reaction by TLC using CH_2Cl_2-methanol (9:1) as eluent.

1.2.5 Concentrate the reaction mixture under reduced pressure.

1.2.6 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.

1.2.7 Filter the PrK in the form of a white solid on a fritted-glass.

1.2.8 Dissolve an aliquot of the PrK in D_2O. Then carry out NMR analyses to control its identity and purity.

1.2.9 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

2.1 Plasmid preparation

2.1.1 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 *BamHI* and *XhoI* restriction sites of the pET24d plasmid. This will introduce a His_6 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.

2.1.2 Construct a second expression plasmid (pEVOL-MmPylRS) containing two copies of the gene coding for the pyrrolysyl-tRNA synthetase from *Methanosarcina mazei* (MmPylRS) and the gene coding for the corresponding $tRNA^{Pyr}$ as previously described[19]. Use this specially designed plasmid vector, pEVOL, for efficient incorporation of UAAs.

NOTE: The detailed plasmids information is described in **Supplemental Materials**.

2.2 Co-transformation of plasmids into the expression strain

2.2.1 Thaw a 100 µL aliquot of chemically competent *Escherichia coli* BL21(DE3) on ice for 5 min.

2.2.2 Add 1 µL of each plasmid (50-100 ng of each) into the cells and incubate for 30 min on ice.

2.2.3 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.

2.2.4 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.

2.3 Expression of proteins modified with PrK

2.3.1 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.

2.3.2 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.

2.3.3 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.

2.4 Analyze crude cell extracts from the 5 mL culture samples by SDS-PAGE and western Blot analysis

2.4.1 Resuspend 5 mL cell pellets into 250 µL of lysis buffer (50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 8, 5 mM imidazole, 0.2 mM PMSF) and transfer it into a 1.5 mL microtube.

2.4.2 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.

2.4.3 Centrifuge samples at 17,000 x *g* for 10 min to eliminate cell debris.

2.4.4 Take 10 µL of the supernatant and add 5 µL of water and 5 µL 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.

2.5 Protein purification by gravity flow-bench affinity chromatography using Nickel-NTA beads

2.5.1 Resuspend the cell pellets (from the 495 mL culture) into 20 mL of lysis buffer (50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 8, 5 mM imidazole, 0.2 mM PMSF).

2.5.2 Add 5 µL of DNase I (1 mg/mL) and 500 µL of lysozyme (50 mg/mL) into the suspension and allow lysis by incubating the suspension at 37 °C during 30 min.

2.5.3 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 µm filter.

2.5.4 Add Ni-NTA resin to the suspension (500 µL for 500 mL of cell culture) and mix gently at 4 °C for 1 h.

2.5.5 Pour the suspension into a polypropylene column and collect the unbound fraction.

2.5.6 Wash the resin with 10 mL of washing buffer containing 50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, 10 mM imidazole. Wash the resin a second time with 5 mL of washing buffer (50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, 20 mM imidazole). Collect the wash fractions.

2.5.7 Elute the his-tagged protein with 1 mL of elution buffer (50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, 300 mM imidazole). Repeat this step 4 times and collect all the elution fractions.

2.5.8 Analyze the crude lysate as well as the 7 purification fractions by SDS-PAGE on a 12% acrylamide gel.

2.5.9 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 $cm^{-1} \cdot M^{-1}$ and a molecular weight of 34.14 kDa for mPsaA.

3. **Removal of the histidine tag by TEV protease digestion**

3.1 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.

3.2 Add 100 µL of TEV protease (add 1 µL containing 10 units of TEV protease for 20 µg of protein to digest).

3.3 Add 50 µL of 0.1 M dithiothreitol (DTT).

3.4 Complete with TEV buffer (50 mM Tris HCl, 0.5 mM EDTA, pH 8) up to 5 mL.

3.5 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.

3.6 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 Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, 5 mM imidazole).

3.7 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.

3.8 Pour the suspension into a polypropylene column. Collect the unbound fraction and wash the column with 5 mL of washing buffer (50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, 10 mM imidazole)

NOTE: The protein of interest should be recovered in the unbound and washing fractions.

3.9 Elute the TEV protease and the undigested protein by adding 5 mL of elution buffer (50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, 300 mM imidazole) on the column. Check the fractions for protein contents at 280 nm and by SDS-PAGE analysis.

3.10 Check the efficiency of the digestion by loading digested samples on an SDS PAGE with the undigested protein as a control.

3.11 Dialyze the digested protein against 1 L of click buffer (50 mM Na_2HPO_4/NaH_2PO_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 $cm^{-1}{\cdot}M^{-1}$ 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.

4.1 Take 432.5 µL of PrK-mutated protein at a concentration of 57.8 µM into a 2 mL microtube.

NOTE: A minimum concentration of 2 µM 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.

4.2 Add 10 µL of 5 mM 6-hexachloro-fluorescein-azide and then add a premix of 2.5 µL of $CuSO_4$ solution at 20 mM and 7.5 µL of Tris(benzyltriazolylmethyl)amine (THPTA) at 50 mM (stock solutions concentrations).

4.2.1 Add 25 µL of aqueous 100 mM aminoguanidine hydrochloride.

4.2.2 Add 25 µL of 20 mg/mL an extemporaneously prepared aqueous solution of sodium ascorbate.

4.2.3 Close the tube, mix by inverting several times and incubate at room temperature for 2 h.

4.2.4 Stop the reaction by adding 50 µL of 0.5 M EDTA.

4.2.5 Take 15 µL of the reaction mixture and put it a microtube, add 5 µL of loading buffer (bromophenol blue, SDS, β-mercaptoethanol), heat the mixture at 100 °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-N_3) by click chemistry

5.1 Coupling

5.1.1 Take 432.5 µL of PrK-mutated protein at 57.8 µM into a 2 mL microtube.

5.1.2 Add 10 µL of 5 mM Pn14TS-N_3[21] in water then add a premix of 2.5 µL of $CuSO_4$ solution at 20 mM and 7.5 µL of THPTA at 50 mM.

NOTE: Synthesis of Pn14TS-N_3, 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.

5.1.3 Add 25 µL of 100 mM aminoguanidine hydrochloride.

5.1.4 Add 25 µL of 20 mg/mL extemporaneously prepared aqueous solution of sodium ascorbate.

5.1.5 Close the tube, mix by inverting several times and incubate at RT during 2 h.

5.1.6 Stop the reaction by adding 50 µL of 0.5 M EDTA.

5.1.7 Take 15 µL of samples and analyze by SDS-PAGE.

5.2 Gel filtration purification of the glycoconjugate

5.2.1. 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.

5.2.2. Collect the fractions containing the glycoconjugate.

NOTE: For prolonged storage, dialyze the glycoconjugate against 1 L of H_2O twice for 2 h and then overnight at 4 °C by using dialysis membrane (cut-off Mw 6000-8000 Da), then freeze-dry and store the glycoconjugate at -80 °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**). Pneumoccocal surface adhesion 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[23,21] 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 (EMBL)[19] containing orthogonal tools necessary to incorporate the propargyl-lysine, into competent *E. coli* BL21(DE3) strain. Positive co-transformed clones were selected using 25 µg/mL kanamycin and 30 µg/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 sidechain 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 and 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 4A**). The histidine tag was removed upon proteolytic cleavage using TEV protease (**Figure 4B**). 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→4)-β-D-Glcp-(1→6)-[β-D-Galp-(1→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 fluoroprobe (**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-N_3 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 reassignment[25]. 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].

Figure 2: Propargyl-lysine synthesis. (**A**) Steps of propargyl-lysine synthesis. Insert: Monitoring of the deprotection of Boc-L-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: CH_2Cl_2/MeOH (9:1), left lane: Boc-L-Lys(prop-2-ynyloxycarbonyl)-OH (R_f 0.90), right lane: crude propargyl-lysine, (R_f

0.38). 400 MHz ^{1}H (**B**) and ^{13}C NMR spectra (**C**) of propargyl-lysine recorded in D_2O.

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.

Figure 4: Affinity purification using Ni-NTA beads 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; Lane 6-9: Elution fractions with 300 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 33 105 Da and (bottom) mPsaA K32PrK, theoretical 33 184 Da, found 33 192 Da. The found masses are within the expected margin error.

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 Å[26]).

Figure 6: Histidine–tag digestion and conjugation of mPsaA with (A) fluorescein-N_3 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-$mPsaA^{K32PrK}$ theoretical MW 34 091 Da, found 34 088 Da.

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 protein[29], 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 it 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 response[8]. Since increasing the hapten/protein ratio is often correlated with enhanced anti-hapten humoral response when using short haptens[30], 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.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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616 be turned into effective vaccines by multivalent presentation. *The Journal of Infectious*
617 *Diseases*. (2019).
618

Figure 1

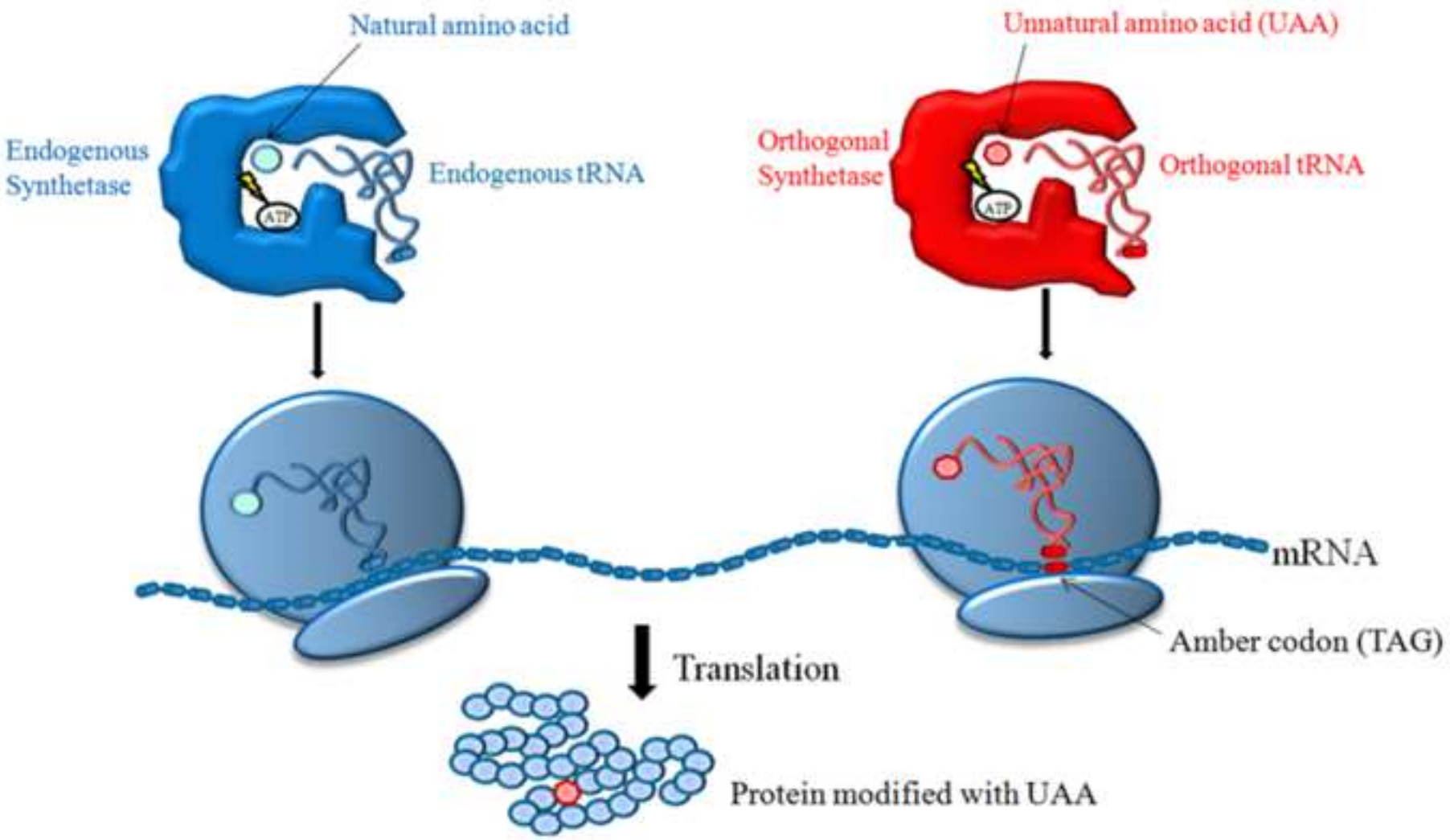


Figure 2

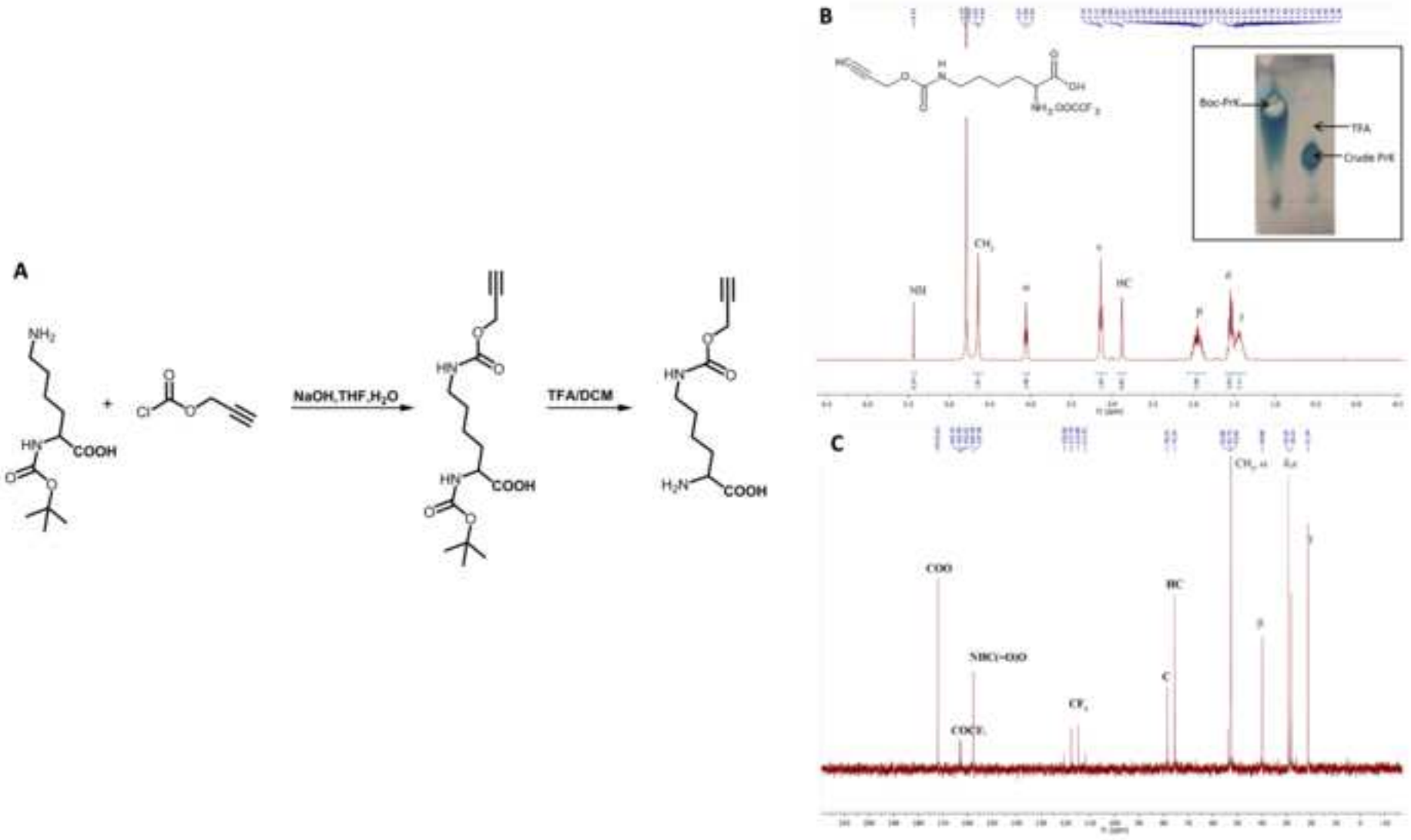


Figure 3

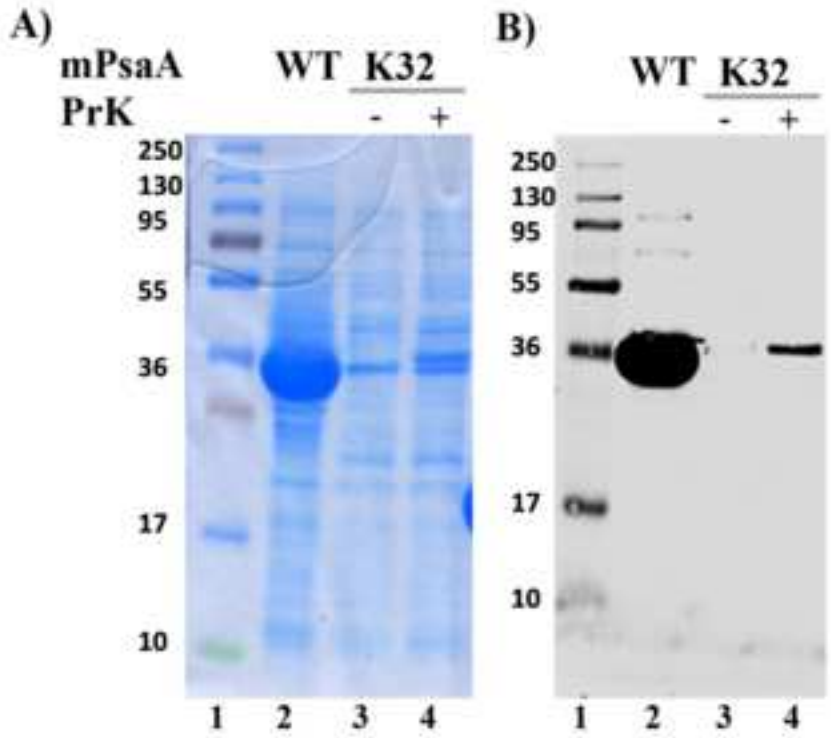


Figure 4

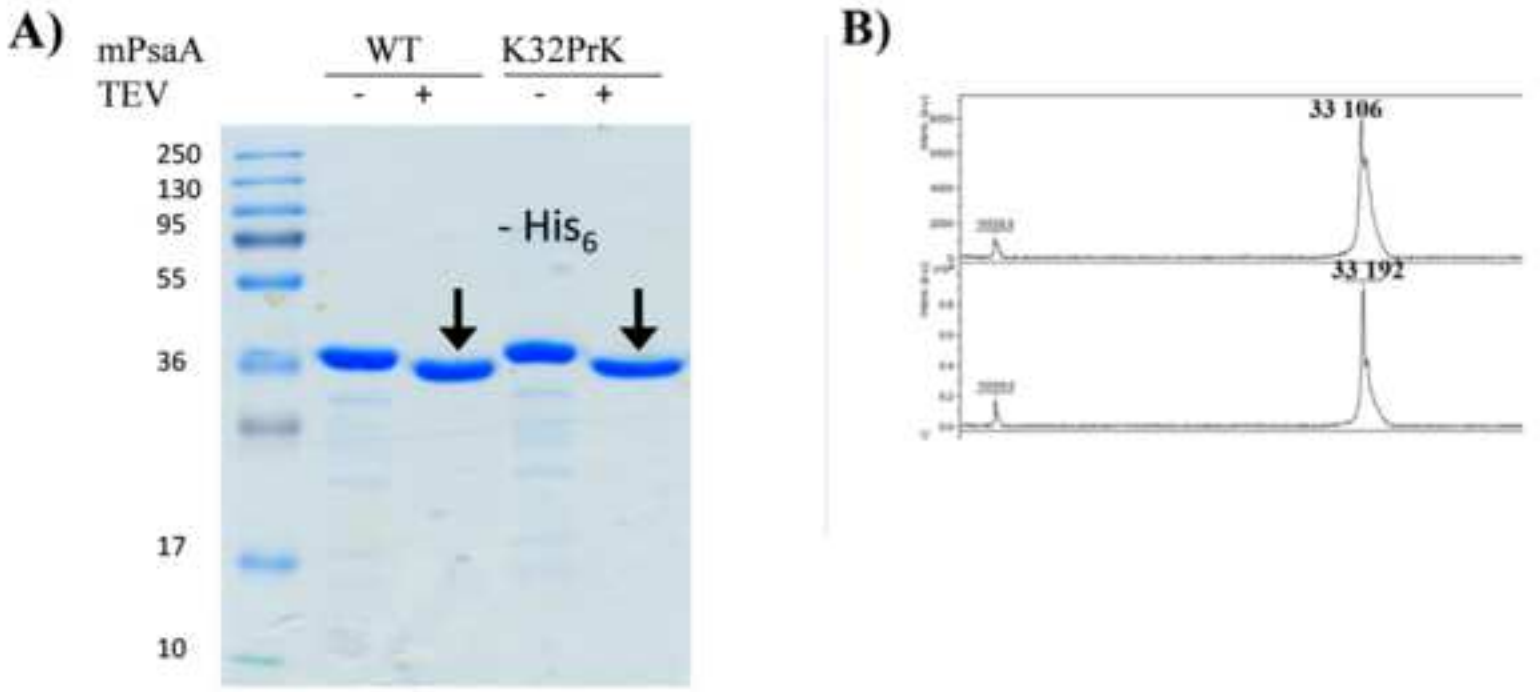


Figure 5

Click here to access/download;Figure;Figure 5.tif

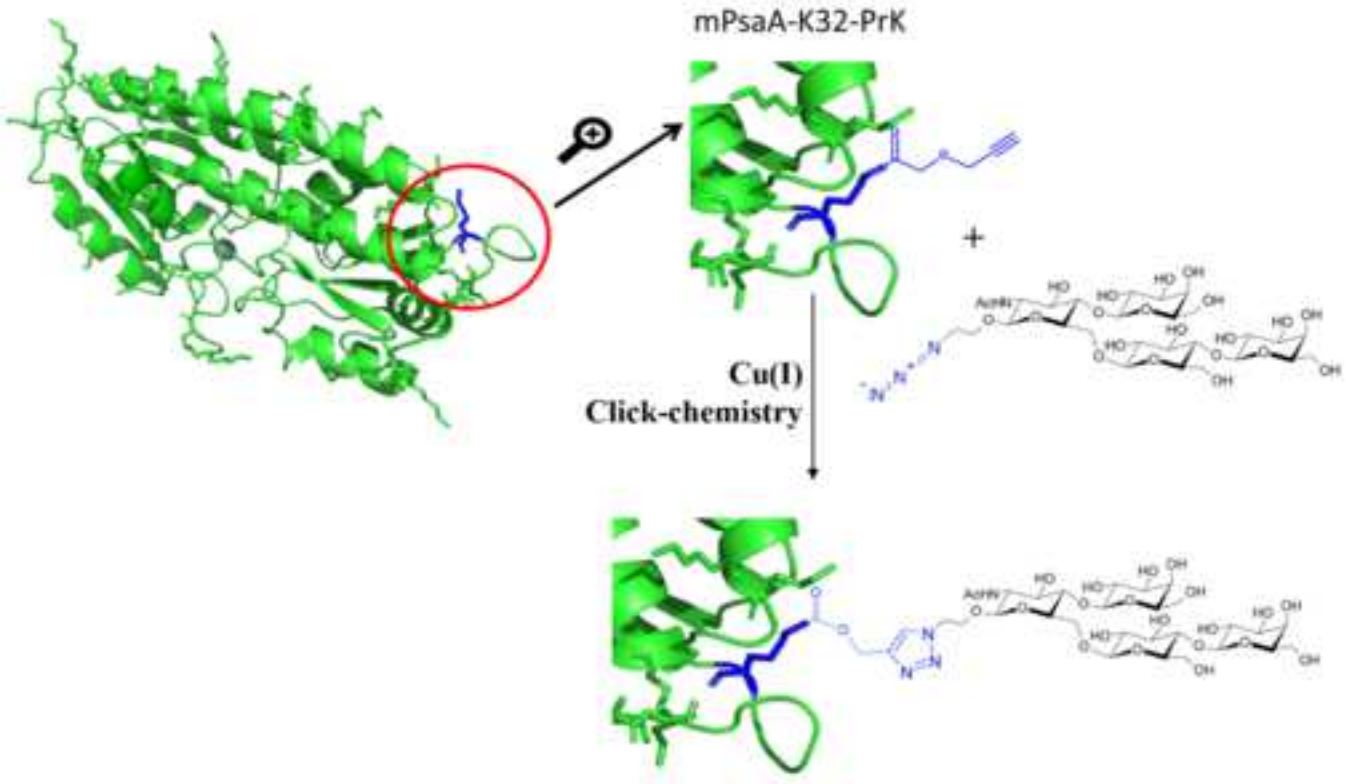
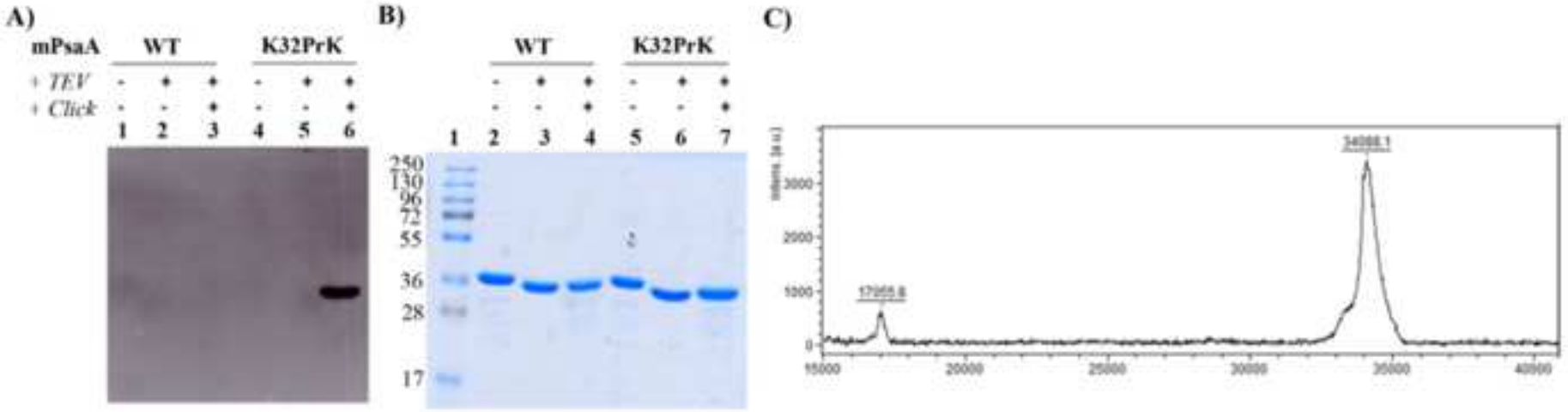


Figure 6

Click here to access/download;Figure;Figure 6.tif



Name of Material/ Equipment	Company	Catalog Number
AIM (autoinductif medium)	Formedium	AIMLB0210
Boc-Lys-OH	Alfa-Aesar Merck	H63859
BL21(DE3)	Novagen	69450
Dialysis membrane		
DNAseI		
Filter 0.45µm		
L-arabinose		
lysozyme		
	Machery	
Ni-NTA resin	Nagel	Protino
Pall centrifugal device		
pET24d-mPsaAK32TAG-ENLYFQ-HHHHHH	this study	
pET24d-mPsaA-WT	this study	
	gift fromEdward Lemke EMBL	
pEVOL plasmid	(ref 19)	
Propargyl chloroformate	Sigma-Aldrich	460923
Sonicator	Thermo Fisher	FB120-220

Comments/Description

Solid powder
Solid powder

E. coli str. B, F^{-} *ompT gal dcm lon* $hsdS_B(r_B^{-}m_B^{-})$ λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) $[malB^{+}]_{K\text{-}12}(\lambda^{S})$

Ni-NTA beads in suspension into 20% ethanol

same as pET24d-mPsaA-WT but with a K32TAG mutation in the mPsaA gene
pET24d plasmide with the Wt mPsaA gene cloned between the *BamHI* and *XhoI* restriction sites with a TEV protease sequence followed

plasmide with p15A origin, two copies of MmPylRS (one under GlnS promoter and one under pAra promoter), one copy of the $tRNA^{CUA}$ ι

Liquid

by a His_6 tag at the C-terminal end of mPsaA gene and carrying the Kanamycine resistance gene

ınder the ProK promoter, the chloramphenicol resistance gene

Dear Dr Bajaj,
Please find our answers to the editor and reviewer comments:

Editorial and production comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
done
2. Please reword the title to make it concise.
We don't really see what you expect as our title is shorter than most titles of other JOVE videos in the same field; Ex:Residue-specific Incorporation of Noncanonical Amino Acids into Model Proteins Using an *Escherichia coli* Cell-free Transcription-translation System.
Ex2:Optical Control of a Neuronal Protein Using a Genetically Encoded Unnatural Amino Acid in Neurons
Also we think that shortening the title will make it loose meaning.
It has, however, been slightly modified in response to one of Reviewer's 3 critique
3. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Sutrovax, HiLoad 15/600 Superdex™, GE Healthcare etc.
Done
4. Please expand all abbreviation during the first-time use.
done
5. Please include the goal of the manuscript as well in the introduction section.
Added at the end of the introduction: "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"
6. Please include a single line space between each step, substep and note in the protocol section.
done
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.
done
8. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
done
9. Please ensure you answer the "how" question, i.e., how is the step performed?
done
10. Step 2.1: Please briefly describe how the plasmid preparation is done or include citations for both
details were added for the pET24d-mPsaAK32TAG-ENLYFQ-HHHHHH plasmid and a note already indicates "The detailed plasmids information is described in Supplementary MATERIALS." There is no reference available for this plasmid but a reference was already included in the text for pEVOL.
11. To maintain consistency, please use the same terminology throughout. The title suggests combined unnatural amino acid incorporation and click chemistry. However, in the entire manuscript especially protocol/results these terms are not used. Please bring out clarity.
done
12. Please ensure that the protocol fits the 10-page limit including all the headings and spacings.
done

Video:

1. Please remove the university logo on the upper right side from the video.
As the video was made by the video service of University of Nantes on its sole funding it is part of the university charter to display its logo on the video.
2. Please use gloves for performing all the steps involving bacterial culture.
In France the health and safety rules do not include wearing gloves for manipulation of level 1 microorganism. That is why we didn't sear them during the shooting of the bacterial culture steps.
3. Please include the title card both in the beginning and in the end.
done
4. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text.
5. Please ensure that the subsections' titles are the same in both the video and the text.
Done, except for part IV which is a unique section in the video when it corresponds to two sections in the text, because the procedure is really similar for paragraphs 4 and 5 in the text and it would be redundant to show it twice on the video.
6. Please include all figures both in the text and the video.
done
7. Please ensure all the result figures are shown in the video as well.
done

Production comment:
• Please include a single space between Glycoconjugate and Vaccine in the title cards.
done
• Consider leaving the title cards up a little longer- they are hard to read without pausing the video.
The video service could not grant us enough time on our video to redo the whole sound mixing
Check the center alignment on the titles as well- they may be a little offset improperly.
We have checked the alignment of the titles and they are already centered.
• There is interlacing (line "combing") in the protocol footage, editing in a progressive frame mode or deinterlacing footage beforehand will reduce/eliminate these video artifacts.
done
• I'm not sure if the presenter is speaking English around 0:26. It sounds like she switches in mid-sentence. Spoken discussion and VO should be in English unless an exception has been specifically made with JoVE.
We confirm that the presenter is speaking English at 0:26. She is saying "codon suppression method" which is quite difficult to pronounce for French speakers.
• Please remove the Organization's watermark in the upper right of the frame.
same as editor's #1 comment on video

Reviewer #1 :
Major Concerns:
1. Please explain whether the K32Prk mutation affects the stability of proteins.
Added In the result part "The stability of the mPsaA^{K32PrK} thus obtained was assessed by circular dichroism which showed that the structure of the protein was not affected by the mutation of the Lysine 32 into a propargyl-lysine."

2. If the protein is coupled with azido-fluorescein, is there any result that confirms the conjugation? for example, analyse the conjugation efficacy by a fluorescence spectroscopy or scanner.

The conjugation of the protein with the azido-fluorescein is already presented in figure 6A. We have added precisions in the text of the results: “mPsaA WT were then conjugated to the fluoroprobe (**Figure 6A**)”

Minor Concerns:
Please use some figures with high resolution

Reviewer #2:

MAJOR/MINOR CONCERNS

(1) GENERAL. The authors emphasise the generation of "homogeneous" products as one of the major advantages of their protocol. This is based on the general concept and the bioorthogonality of the two reaction partners (propargyl lysine and azide). However, they have not actually experimentally demonstrated the "homogeneity" of their product. This should be made clearer in the manuscript.
The orthogonality of the click chemistry has been demonstrated a long time ago and researchers who use it usually do not perform supplementary experiment to prove its specificity. We have however verified that 1) our mutation was at the correct position by gene sequencing, 2) the protein was modified with the PrK UAA and then the tetrasaccharide was conjugated by mass spectrometry.

(2) MANUSCRIPT

- P7: "Pn14TS-N3" - The abbreviation of the tetrasaccharide needs to be explained, and its source and/or a reference for its synthesis included.
The abbreviation is further explained page 8 by addition of “a tretrasaccharide mimicking the *Streptococcus pneumoniae* serotype 14 capsular polysaccharide”.
The reference 21 was already included in the text to specify the source of the Pn14TS-N3

- P7: "Any carbohydrate antigen containing an azide function can be used." - While this is true in theory, in practice, it is almost certainly not. Cu-catalysed cycloaddition reactions can be notoriously capricious, depending on the exact structure of the substrate. Please rephrase this sentence and/or add a note of caution.
Added:” Theoretically, any carbohydrate antigen containing an azide function can be used”

(3) VIDEO:

- The introduction would be easier to follow if it was illustrated with relevant schematics (e.g. Fig 1 from the manuscript), and not only show the researcher.
added figure 1 at the beginning of the video

- Synthesis: include the names of compounds that are used in the commentary, on the slides (0:56 ff)
- Molecular biology/protein biochemistry: Show the names and quantities of key reagents e.g., in speech bubbles (e.g., at 4:56 min: E coli BL-21 DE3, 100uL)
We thought about adding names and quantities of the reagents used when we prepared the video in the first place but we decided not to because given the number of different reagents used we thought it will fill the image and it might be annoying for the audience to see bubbles appear and disappear all the time. We thought it was the role of the written protocol to give information on the reagent and quantities and that the video is here to show how the solutions obtained after each step should look like and how to perform the gestures. When possible we have tried to write legibly on the tubes and glassware the names of the reagents.

I am also wondering if it might be useful to briefly explain some fundamental operations, e.g., the need

for sterilisation of equipment for the molecular biology/protein expression part, the measurement of protein concentration by UV, or the correct use of a rotary evaporator. Because of the cross-disciplinary nature of the experiment (see comments above), the video will be particularly valuable to scientists who have expertise in e.g., chemistry, but may not be familiar with even basic techniques in molecular biology/protein biochemistry. Alternatively, a slide with relevant links to relevant resources could be included at the end.
There is no need to sterilize equipment for the protein purification.
We think the operations described here are really basic and information can be found easily elsewhere. Also the video will be much longer if we have to explain how to use each instrument and it won't add value to the video as these instruments are not particularly unusual.

Reviewer #3:

Major Concerns:
1. The introduction should be ended with aim of the project/study mentioning the candidate used as representative.
Done as answered to editorial comment #5
2. Results section has a bit of discussion also which may be carefully avoided as the results and discussion sections are written separately in the manuscript.
removed from results section and explained in the discussion section:" However, no conclusion can be drawn since the PrK incorporation site is close to the N-terminus and any truncated form produced is probably degraded by the bacteria »
3. Protocol and Results section do not mention tests like polysaccharide(oligosaccharide) to protein ratio, conjugation yields in terms of protein and oligosaccharide used in beginning versus that received in the purified glycoconjugate. These two are important parameters in characterizing glyconjugates
Added in the text page10: "The conjugation by click chemistry being quantitative the majority of the mPsaA^{K32PrK} was conjugated with the Pn14TS-N_3 as illustrated by the mass spectrometry results (**Figure 6C**)."

4. The discussion section need further elaboration on following points: Next steps e.g. immunogenicity studies; discussion about impact of very high protein to oligosaccharide ratio in the conjugates so obtained in comparison to conventionally obtained conjugates; the conjugation yields in comparison to conventional conjugates and hence cost implications; finally discuss scope to optimize the Protein to Polysaccharide ratio by incorporation of more than one UAAs. The later one seems to be touched upon in abstract but missing in the discussion section.
added at the end of discussion section: 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 unvaluable tools to evaluate the impact of the hapten/protein carrier connectivity on the immune response.[8] Since increasing the hapten/protein ratio is often correlated with enhanced anti-hapten humoral reponse when using short haptens,[30] 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.

Minor Concerns:
1. References need to have same format which is quite mixed in the current format
done
2. The word 'vaccine' may be removed from the title which generally is indicative of the formulated glycoconjugate

The title was reformulated to : “Homogeneous Glycoconjugate Produced by Combined Unnatural Amino Acid Incorporation and Click-Chemistry for vaccine purpose »

3. Not sure of the reason but the title and abstract have some typo differences in very first page and second page of the PDF file
Done, correction of homogenous to homogeneous if this is what the reviewer meant
4. Line 40-41: drug regulatory agencies done; Replace "classical bioconjugation strategies' with 'classical conjugation strategies' done
5. Line 46: ..and efficient in a broad age group including young infants. done
6. Line 47: ..provide the optimal defense.. done
7. Line 53: Apart from a... done
8. Line 56: ..linker, if used... done
9. Line 221-222: share range of the temperature and time which may be required done
10: Line 245: Recheck if the title is really aligned (accessibility and functionality) with the text in section 4, if not, please align
The title is aligned with the content of the section 4 as the point of this procedure is to conjugate by click chemistry a fluoroprobe carrying an azide on the protein mutated with PrK and visualize the conjugate by fluorescence which shows that the PrK was accessible and functional to be conjugated
11. Line 254-255: Mention if 20 and 50 mM are working or final concentrations
Done, it is now specified in the text that it is initial concentrations
12: Line 313-314: It would be useful to provide the amino acid sequence of PsaA highlighing the K32 where modification is done, this could be clubbed in figure 5we don’t see which information necessary to understand the results this new figure would add
13: Fig 5: Cu+ or CuI, Please check
We confirm it is Cu^{I}
14. Fig 6(B): Worth mentioning the differences in apparent variation in mol. wt. of bands in lane 2 vs3,4 and 5 vs 6,7
Added at the end of the results section: “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.”

General comment:
Share the appropriate composition of various buffers unless it is very commonly known in scientific fraternity
Phosphate buffers, click chemistry buffer and TEV buffer are already detailed in the text, we don’t see which other buffer needs to be more detailed.
Use full form of uncommon abbreviations, when used first in abstract and main manuscript
done

Dear Dr Nguyen,

Please find the modifications made regarding the video as an answer to editorial comments:

Changes to be made by the Author(s) regarding the video:

1. If feasible, please convert this editing project settings to Progressive Scan and try to reduce/remove the "combing" effect seen throughout. We are publishing on a website, which does not benefit from interlaced footage.

The previous version of the video was already set to Progressive scan as is the version now resubmitted. We have checked with the service who did the video and they have already changed for a format compatible with a website.

2. The narration sounds good except for the background noise. Please attempt to run noise reduction on the narration audio track.

Background noise has been removed

3. What is the speaker saying at 00:25-00:28? This is not clear. Please re-record this for clarity.
The speaker is saying: “codon suppression method”. As it is not possible for us to record again the speaker we have added a title to the figure (i.e. “codon suppression method”) which appears in the video as she is saying these words. We hope that it will help understand what she is saying.

4. The music in the protocol can be removed.

The music is removed