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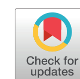
Translating basic research into novel vaccine

Precise protein conjugation technology for the construction of homogenous glycovaccines

Annabel Kitowski[1], Francisco Corzana[2], Gonçalo J.L. Bernardes[1,3,*]

[1]Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal
[2]Departamento de Química, Universidad de La Rioja, Centro de Investigación en Síntesis Química, E-26006 Logroño, Spain
[3]Department of Chemistry, University of Cambridge, Lensfield Road, CB2 1EW Cambridge, UK



The introduction of vaccines for the treatment and prevention of bacterial or viral diseases in the early 19th century marked a crucial turning point in medical history. Since then, extensive immunization campaigns have eradicated smallpox and drastically reduced the number of diphtheria, tetanus, pertussis and measles cases worldwide. Although a broad selection of vaccines is available, there remains a need to develop additional vaccine candidates against a range of dangerous infectious diseases, preferably based on precise syntheses that lead to homogenous formulations. Different strategies for the construction of this type of vaccine candidates are being pursued. Glycoconjugate vaccines are successful in the fight against bacterial and viral infectious diseases. However, their exact mechanism of action remains largely unknown and the large-scale production of chemically defined constructs is challenging. In particular, the conjugation of the carbohydrate antigen to the protein carrier has proved to be crucial for the properties of these vaccines. This review highlights some of the latest findings and developments in the construction of glycoconjugate vaccines by means of site-specific chemical reactions.

Introduction

Vaccines are one of the most important tools in our fight against various infectious diseases. As resistance against many antibiotics increases and new viruses appear, the development of new vaccine candidates becomes ever more important. Glycoconjugate vaccines represent one of the most successful strategies to elicit a strong and long-lasting immune response, especially in infants. In general, the conjugation of a polysaccharide antigen to an immunogenic protein carrier generates a T-cell dependent immune response, which leads to the formation of enduring memory B-cells [1,2]. By following this approach, glycoconjugate vaccines have been licensed for a number of infectious diseases, such as *Haemophilus influenzae* type b, *Samonella* Typhi, *Neisseria meningitidis* and *Streptococcus pneumoniae* [3,4].

In recent years, efforts have been made to understand the exact mechanism by which glycoconjugates are processed by the immune system, and to develop more defined vaccine constructs. So far, glycoconjugate vaccines have been obtained largely through random conjugation reactions of polysaccharide antigens to immunogenic protein carriers [3,5]. These reactions, like reductive aminations, active esters or carbodiimide-mediated couplings, commonly take place on functionalities of the carbohydrate or through the installation of a linker moiety between the sugar and a different

*Corresponding author:: Gonçalo J.L. Bernardes (gb453@cam.ac.uk), (gbernardes@medicina.ulisboa.pt)

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amino acid, typically lysine, aspartic or glutamic residues, on the carrier protein (Fig. 1, I–III) [6]. Currently, there are six carrier proteins being used for licensed vaccines, namely tetanus toxoid (TT), diphtheria toxoid, CRM_{197}, recombinant exotoxin A of *P.aeruginosa*, Protein D from *H. influenzae* and the outer membrane protein complex of meningococcus B. Despite the efficiency of the resulting constructs, the current approach comes with drawbacks like batch-to-batch variations and rather undefined products. To overcome these problems, new methods have been developed for the chemical synthesis of desired glycan antigens and for their site-selective conjugation on the protein carrier. The goal of a defined site-selective conjugation on a carrier can be addressed in different ways and we highlight some of them here, together with recent examples of chemically-defined glycoconjugate constructs.

Examples for selective glycoconjugate vaccines

Fully synthetic conjugate – Quimi-hib

The biggest success in synthetic glycoconjugate vaccine design so far is arguably the fully synthetic glycoconjugate vaccine Quimi-Hib against *Haemophilus influenza* type b in 2004 [7]. The authors identified four key points that facilitated the successful production of this defined glycoconjugate vaccine on a large scale: a synthetic route for a precursor disaccharide with just one chromatographic purification step, a high-yielding polycondensation reaction to obtain the necessary oligosaccharide structure, efficient deprotection of the oligosaccharide, and selective conjugation of the glycan-antigen to the TT carrier. By following this strategy, Verez-Bencomo *et al.* were able to produce a glycoconjugate vaccine candidate in bulk and translate findings from a prototype vaccine with human serum albumin as carrier protein into a commercial product. The coupling strategy of the synthetic antigen, in average eight repeating units of polyribosylribitol phosphate, relies on thiolation of lysine ε-amino groups of tetanus toxoid, which results in a 1:2.6 ratio by weight of antigen-to-tetanus toxoid [7]. Although the successful synthesis of the complex polyribosylribitol phosphate antigen was a clear milestone in the development of synthetic glycoconjugate vaccine candidates, the conjugation reaction to the protein carrier still has potential for improvement in the future. As will be discussed later, frequently abundant lysine residues might not be the most ideal targeting point for conjugation strategies, which promote formation of non-uniform conjugation products.

Quimi-Hib remains the only licenced fully synthetic glycoconjugate vaccine, but more recent examples for glycoconjugate vaccine candidates with entirely synthetic polysaccharide antigens are now in early stages of clinical development. A synthetic glycoconjugate vaccine against Shigella Flexneri 2a, SF2a-TT15 is currently under investigation in two phase 2 clinical trials [8,9]. A synthetic pentadecasaccharide, which is part of the *O*-specific polysaccharide domain of the bacterial lipopolysaccharide, was conjugated to ε-amino groups of lysine residues of tetanus toxoid [10,11]. Extensive optimisation of the conjugation strategy enabled improved control over the antigen:carrier protein ratio for which 17 ± 5:1 was optimal in terms of immunogenicity. The ongoing clinical trials SF2a-TT15 use a conjugate with an average molar ratio of 15:1 (pentadecapolysaccharide/tetanus toxoid). Additionally, a phase I trial is being conducted on the safety of AV0328, a glycoconjugate vaccine comprising synthetic fragments of bacterial poly-*N*-acetylglucosamine that are conjugated to lysine residues of tetanus toxoid [12]. The current candidate AV0328 consists of a pentamer (5GlcNH2:TT) and has been shown to elicit protective antibodies against *Rhodococcus equi* in horse foals [13].

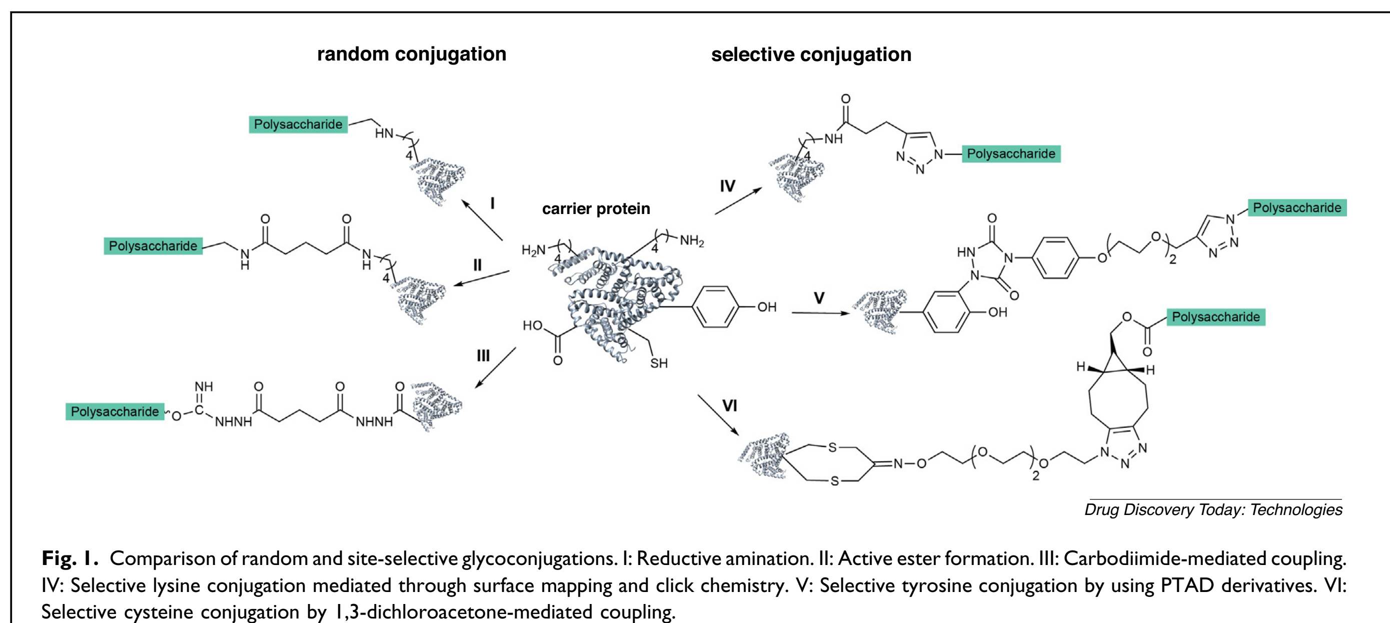

Fig. 1. Comparison of random and site-selective glycoconjugations. I: Reductive amination. II: Active ester formation. III: Carbodiimide-mediated coupling. IV: Selective lysine conjugation mediated through surface mapping and click chemistry. V: Selective tyrosine conjugation by using PTAD derivatives. VI: Selective cysteine conjugation by 1,3-dichloroacetone-mediated coupling.

The discussed approaches for the construction of synthetic glycoconjugate vaccines focus on the chemical synthesis of the carbohydrate antigen moiety, which allows better control relative to the use of antigen fragments. The conjugation strategies of these licenced or advanced vaccine candidates, however, still relies on random conjugation towards reactive amino acid residues of the carrier protein. To be able to construct a chemically defined glycoconjugate vaccine, efforts should be placed on conjugation reactions that enable conjugation at a specific site(s) on the carrier protein. To do so, different strategies are being developed to enable these highly selective conjugation steps and some of them will be discussed in the following sections.

Site-selective protein modification – non-canonical amino acids

There are a number of different routes available when it comes to the development of new site-selective modification strategies. The options include modifying proteinogenic amino acids, as in the selective glycoconjugates examples above, or introducing non-canonical amino acids. The use of non-canonical amino acids for modification reactions brings the advantage of new functional groups and usually minimizes unwanted side reactions [14]. A range of technologies introduce non-canonical amino acids (ncAAs) into proteins and some of them will be outlined below.

One of the first methods to be developed uses auxotrophic strains of *Escherichia coli*. These strains cannot synthesize certain amino acids so rely on the uptake of these from the growth medium [15]. Here, an ncAA analogue with the desired functional group can replace the absent amino acid in the growth medium and will be used by the bacterium for protein biosynthesis. Its incorporation can be supported, for example, by overexpression of the relevant tRNA transferase. A drawback of this method is that not only the amino acid in one particular position of the protein is replaced, but any amino acid of the same kind, which does not fulfil the criteria for a site-selective modification.

Another strategy, which was developed recently, is genetic code expansion [16]. By using an aminoacyl-tRNA synthetase and a suitable tRNA, it is possible to insert a non-canonical amino acid at a particular site into a growing peptide chain. Tyrosyl-tRNA synthetases, from *Methanococcus jannaschii* and *E. coli*, and *E. coli* leucyl-tRNA-synthetase and pyrrolysyl-tRNA-synthetase, from *Methanosarcinae*, can incorporate ncAAs into proteins [14]. This approach allows the introduction of various chemical reporter groups in a defined sequence position, which enables the construction of homogeneously and site selectively modified protein constructs. For example, the introduction of *trans*-cyclooctene or bicyclononyne reporter groups in sfGFP, allowed the site-selective conjugation of tetrazine-modified glycan moieties [17] through inverse electron-demand Diels–Alder reaction [18]. Similarly, high-mannose N-type glycans featuring an azido group were site-selective installed into a bicyclononyne-tagged antibody fragment through strain-promoted alkyne–azide cycloaddition [19]. Because the general concept of genetic code expansion relies on defined pairs of suitable tRNA-synthetase/tRNAs and an expression system like *E. coli*, the translation of this strategy towards large-scale productions for clinical settings remains challenging. A potential solution towards this goal was the development of a cell-free, *E. coli* based system, presented in 2011. The so-called open cell-free synthesis allows large-scale production of fully functional proteins, which can be modified to contain ncAAs suitable for site-selective glycoconjugation [20]. This system is currently used by Vaxcyte for the production of their lead product VAX-24, a 24-valent pneumococcal conjugate vaccine that is currently in the preclinical phase [21].

This new technology is clearly an important milestone on the road to fully synthetic and defined glycoconjugate vaccines. However, strategies that rely on targeting and selective modification of endogenous, native amino acids would accelerate testing of new vaccines. In the following section, we highlight some of the recent developments for the selective modification of proteinogenic amino acids and translation of these techniques into defined vaccine candidates.

Site-selective protein conjugates – proteinogenic amino acids

Selective lysine conjugates

Lysine represents the most abundant amino acid residue in carrier proteins like CRM_{197} or TT and presents a nucleophilic ε-amine sidechain. However, the high reactivity makes site-selective reactions on this sidechain particularly challenging and difficult to control. Nevertheless, methods have been developed to discriminate different lysine residues based on their reactivity and to use this differentiation for selective conjugations. Crotti *et al.* were able to map 37 of 39 lysine residues of carrier protein CRM_{197} in different labeling reactions, which led to the identification of more surface-exposed residues. With a two-step conjugation method based on a copper(I)-catalyzed azide-alkyne cycloaddition reaction, they could then selectively conjugate glycans to these lysine residues and prepare defined glycoconjugate candidates (Fig. 1, IV) [22].

Another selective glycoconjugate construct based on lysine modification was obtained through enzyme catalysis. Microbial transglutaminase was used to catalyze the selective modification of Lysine37/39 in CRM_{197} with a ZQG linker molecule at a controlled pH. The azide-containing linker enabled the final coupling reaction to *Salmonella* O-antigen to afford a model glycoconjugate vaccine candidate [23].

In addition to these defined conjugate constructs, several other methods for selective lysine modifications have been developed, but these have not yet been translated into

vaccine candidates. For example, successful site-selective modification on a single lysine residue was achieved with sulfonyl acrylate reagents (Fig. 2a) [24]. In this context, computational calculations were performed to determine the most reactive lysine residue. This approach allowed the selective modification of one lysine over others and present cysteine residues. A different strategy for a site-selective modification on a single lysine residue was developed by Zhang *et al*. By using a peptide-guided fluorodinitrobenzene or isothiocyanate reactions, they were able to site-selectively modify a single lysine residue on an SH3 domain and ubiquitin-like proteins like SUMO or mammalian LC3 proteins (Fig. 2a) [25]. A few other methods for site-selective modifications on lysine residues have been developed, but because of the high abundance and reactivity of this amino acid, it is difficult to develop selective reactions for a broad spectrum of different proteins. In addition, because of the many lysines present on a protein, it is difficult to ensure batch-to-batch reproducibility.

Selective tyrosine conjugates

Unlike lysine and cysteine, tyrosine is an aromatic amino acid and its phenolic side chain typically provides reactivities that are orthogonal to cysteine or lysine residues. Also, the hydrophobic character of the phenol group often causes this amino acid to be partially buried within the protein surface, which leaves the polar hydroxyl group exposed to solvent [26].

A selective conjugation in two steps was developed in 2013, when Hu *et al*. used an alkyne functionalized derivative of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) to modify tyrosine residues of CRM_{197} (Fig. 1, V) [27]. For a defined glycoconjugate vaccine candidate, a synthetic hexasaccharide representing an immunogenic glycan antigen of *Candidiasis*, was coupled to a tyrosine-modified CRM_{197} and tested against a vaccine candidate achieved through random lysine conjugation in immunization studies. Here, a linker moiety was introduced on the protein carrier in an ene-type reaction, whereas the glycan antigen was coupled to the linker through an additional copper-mediated azide-alkyne [3+2] cycloaddition reaction. In follow-up studies a copper-free conjugation strategy was developed. This reaction relied on glycan antigens modified with cyclooctyne handles and a carrier protein that contains an azido-linker selectively introduced on tyrosine residues [28]. A defined glycoconjugate vaccine candidate was constructed by using the established carrier protein CRM_{197} and polysaccharide antigen from Group B *Streptococcus*. In further experiments, tyrosine residues of GBS pilus proteins GBS80 and GBS67 were successfully modified and selective glycoconjugate candidates were obtained by using the described strain-promoted azide-alkyne [3+2] cycloaddition reactions. Subsequently, in studies on the use of GBS80 as carrier protein in glycoconjugate vaccines, Nilo *et al.* showed that these constructs can elicit potent antibodies and demonstrated the protective effect of these vaccine candidates against GBS infection in new-born mice [29]. Equally, good results were obtained for the second explored GBS pilus protein GBS67, which was used in comparative studies using different tyrosine and lysine specific conjugation strategies [30].

Additional tyrosine-targeting chemical reactions are available and complete the range of technologies available. For instance, a selective reaction on tyrosine was achieved through the application of a three-component Mannich-type coupling reaction under mild conditions (Fig. 2c) [31]. Multiple proteins were successfully modified in a selective manner by using cyclic diazodicarboxamide compounds, which were able to react specifically with phenol side chains of tyrosine in an ene-like reaction (Fig. 2c) [27,32]. *In situ* generation of reactive cyclic diazodicarboxyamide enabled a tyrosine-selective reaction, in which luminol derivatives were applied in the presence of stoichiometric amounts of H_2O_2. The bio-orthogonal character of this reaction was proven by a successful dual-modification procedure together with N-hydroxysuccinimide-ester chemistry on lysine [33].

Besides selective strategies for lysine or tyrosine residues, the modification of cysteine side-chains is probably one of the most promising strategies to achieve site-selective conjugation products [34].

Selective cysteine conjugates

Cysteine is less abundant in native protein sequences but their sulfydril side-chain is very nucleophilic, so they represent another possibility for the construction of site-selective protein conjugates. Classic approaches like maleimide or haloalkyl substitutions have been used for some time, however efforts have been made to improve the reaction kinetics and selectivity to obtain defined products [35–37]. Commonly, the modification of cysteine residues relies on the reduction of interchain disulfide bonds, which results in free thiol functionalities for further modification. Disulfide bonds are involved in protein stability and structural integrity, so selective modifications in this position through rebridging reagents can maintain these functions.

In the case of carrier protein CRM_{197}, two disulfide bonds are present in the protein and although the disulfide bond between C186 and C201 is more surface exposed, the one that connects C461 and C471 was found to be more shielded inside the protein. This differentiation was used for a partial reduction that enabled selective modification of C186 and C201 by using 1,3-dichloroacetone [23]. The introduced ketone handle was further used to install an azido functionality through oxime formation. In a final step, *O*-antigen from *Salmonella typhimurium* was conjugated in a cysteine-selective manner to the protein carrier (Fig. 1, VI). Comparative studies with lysine-conjugates or random conjugated controls showed significantly higher antibodies against the

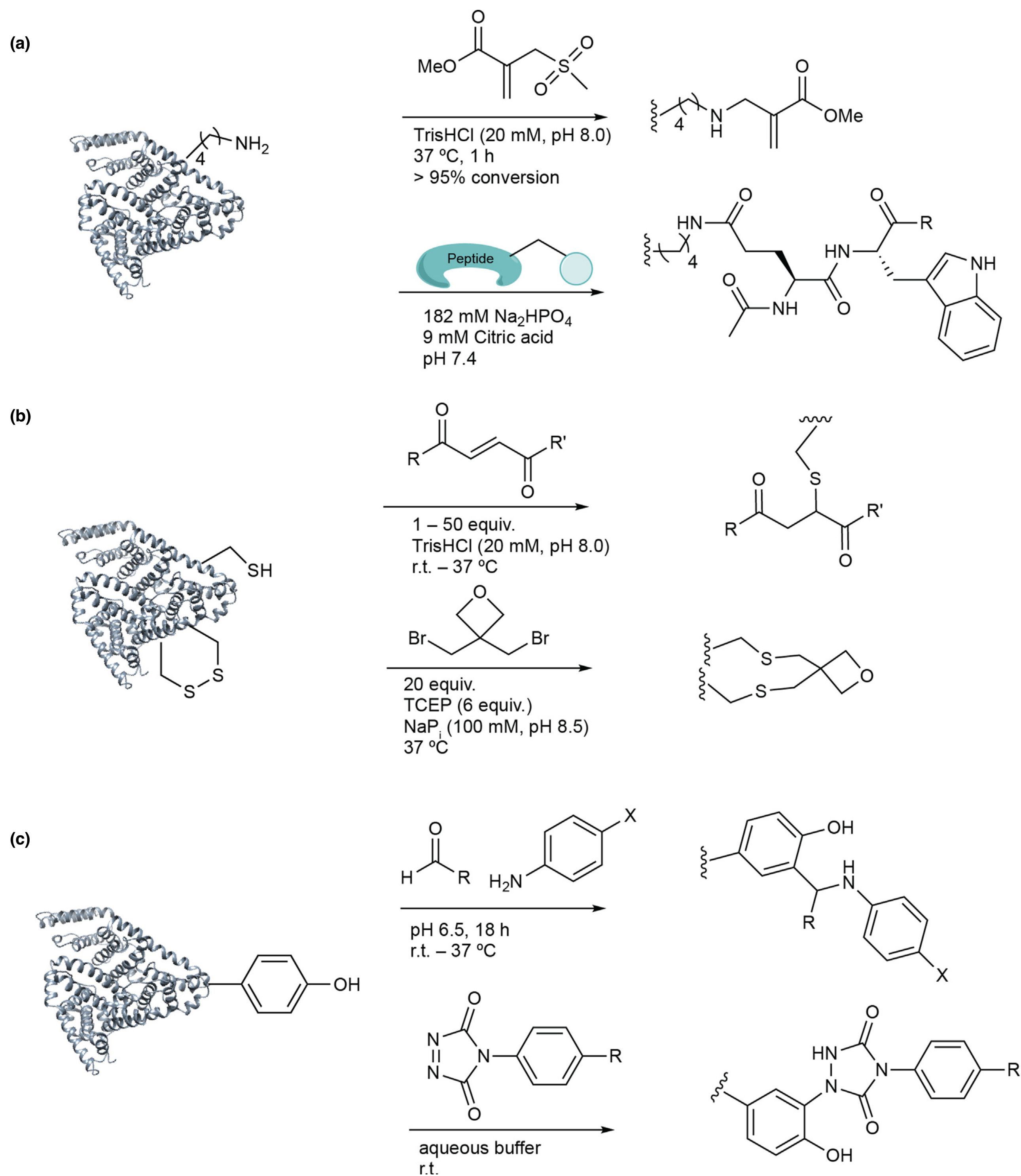

Fig. 2. Chemical site-selective protein glycosylation. **(a)** Site-selective lysine modifications. **(b)** Site-selective cysteine modifications. **(c)** Site-selective tyrosine modifications.

conjugated bacterial antigen, which demonstrates the importance of the conjugation site on the immunization outcome [23].

When using 3,3-bis(bromomethyl)oxetane for the rebridging reaction of C186 and C201 after selective reduction, the resulting modified CRM_{197} elicited a significantly higher amount of protein specific antibodies in mice (Fig. 2b) [38]. This promising result highlights the importance of site-selective modifications in carrier proteins and can be further used for the construction of glycoconjugate constructs. Successful

selective rebridging reactions were also reported with commercial allyl sulfone compounds and divinylpyrimidine [38–40]. A critical point when modifying interchain disulfide bonds remains the influence of the introduced functionalities on protein structure and function. When looking into cysteine modifications without bridging functionalities, several strategies are available. An attractive alternative to classical maleimide chemistry was developed by Bernardim *et al.* and describes the use of carbonylacrylic reagents for site-selective cysteine modification (Fig. 2b) [41]. Here only a few equivalents of reagent are required, which together with fast kinetics and mild reaction conditions show the advantages of this method and make it a promising tool for the construction of conjugate constructs.

The cases described herein for site-selective glycoconjugate constructs emphasize the success that has been made in recent years; however the limited number of examples indicates the need for more techniques for site-selective introduction of modifications in potential carrier proteins.

An antigen either at ncAA or an endogenous residue has advantages and disadvantages. The introduction of ncAA enables precise, selective conjugation, but the production of carrier proteins-tagged with a particular ncAA is elusive. The introduction of multiple ncAAs is complicated and prevents the construction of multivalent constructs. Nevertheless, recent developments into the large-scale production of proteins-tagged with ncAA promise to facilitate the construction of vaccines based on chemistries targeting unnatural side-chains. Site-selective conjugation of endogenous amino acids relies on native protein sequences, which is an advantage for the production of these carriers. However, modifying a protein at multiple defined sites when targeting endogenous amino acids is a difficult task.

A possible scenario for future developments on glycoconjugate vaccines is the combination of more than one approach, for example, by using site-selective chemical reactions, genetic encoding of ncAAs and new technologies for production of tagged- carrier proteins.

Effects of protein modification on function and immunogenicity of glycoconjugate vaccines [42]

The conjugation of carbohydrate antigens towards immunogenic protein carriers is a key point to enable the generation of polysaccharide specific IgG antibodies and the development of long-lasting T-cells [2,43]. Although the exact mechanism of glycoconjugate vaccines in terms of immune recognition is little understood, different factors are known to influence the immunogenicity of these constructs. Length and density of the carbohydrate antigen, together with the conjugation method to the carrier protein are important for the immunological properties of the constructs. The latter was subject of several studies, which aimed to compare glycoconjugates received from random conjugation experiments with site-selective constructs [30,44,45]. A study that demonstrated the impact of the conjugation site on the immunogenicity of the vaccine candidate was conducted by using different constructs out of CRM_{197} as carrier protein and an *O*-antigen of *Salmonella typhimurium* [23]. Site-selective glycoconjugates were synthesized by targeting either different lysine, tyrosine or cysteine residues and a randomly conjugated construct was obtained by targeting aspartic or glutamic acid. Analysis of the immune response *in vivo* revealed that conjugation at two specific cysteine residues resulted in significantly higher amounts of polysaccharide specific antibodies than a comparable conjugate to lysine residue. In a similar study with the same carrier protein and a synthetic β-glucan hexasaccharide, site-selective glycoconjugates through defined tyrosine residues elicited a higher titer of anti-glycan IgG antibodies than a site-selective conjugate on lysine residues [44].

The effect of the site of modification and multivalency on the immunogenicity of glycoconjugate vaccine candidates remains to be fully understood. However, the use and comparison of different conjugation strategies using established synthetic antigens could provide further insights into a future platform for the construction of chemically-defined glycoconjugate vaccines. Since many strategies require the use of spacers that are potentially immunogenic, the construction of well-defined conjugates could also facilitate the impact of the spacer towards immunogenicity and potential side-effects.

Concluding remarks

These exemplary studies demonstrate the importance of site-selective conjugation for the development of efficient vaccine candidates. Ongoing research to introduce defined modifications in potential carrier proteins may provide useful tools on the road to a fully synthetic and defined conjugate vaccine. As bacterial resistance to current antibiotic treatments increases and new viruses emerge, vaccines will be crucial in our fight against them.

Conflict of interest

The authors declare that they have no conflict of interest.

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