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Review

# Site-selective reactions for the synthesis of glycoconjugates in polysaccharide vaccine development

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ABSTRACT

Carbohydrates are often found linked to lipids or proteins, to form glycoconjugates such as glycolipids, glycoproteins and proteoglycans. These glycoconjugates play important biological roles, involving cell-cell recognition, inflammation, immune response, tumor metastasis and in viral/bacterial/parasitic infections. However, it is difficult to obtain these naturally occurring glycoconjugates as they are often heterogeneous compositions, which causes batch-to-batch variability of structure and activity often leading an incomplete understanding of their mechanism of action. Thus, the efficient preparation of synthetic glycoconjugates, possessing well-defined compositions and reproducible biological properties is highly desirable. In the present review, we summarize the advances in site-selective approaches for glycoconjugate synthesis and, in particular, the preparation of polysaccharide vaccines.

## 1. Introduction

Carbohydrates are often found linked to lipids or proteins, to form glycoconjugates such as glycolipids, glycoproteins and proteoglycans. These glycoconjugates play important biological roles, involving cell-cell recognition, inflammation, immune response, tumor metastasis and in viral/bacterial/parasitic infections (Colombo, Pitirollo, & Lay, 2018; Davis, 1999; Varki et al., 2015-2017). Among these are glycoconjugate vaccines, composed of oligosaccharide or polysaccharide antigens covalently linked to carrier proteins, which represent an effiective means to reduce morbidity and death casued by infectious diseases (Cloutier, Muru, Ravicoularamin, & Gauthier, 2018; Dagan, Poolman, & Siegrist, 2010; Kay, Cuccui, & Wren, 2019; Micoli et al., 2019; Rappuoli, 2018). However, it is difficult to obtain these naturally occurring glycoconjugates as they are often heterogeneous compositions, which causes batch-to-batch variability of structure and activity often leading an incomplete understanding of their mechanism of action (Berti & Adamo, 2018). An understanding of the mechanisms underlying the immune response to glycoconjugates is crucial in the production of highly protective knowledge-based vaccines. For example, capsular polysaccharides (CPS) of bacterial pathogens have long been recognized as major targets for eliciting carbohydrate specific antibody responses to confer protection from these pathogens. These polysaccharides are typically conjugated to immunogenic protein carriers to enhance the immunogenicity, stimulating B-cell maturation to memory cells and so evoke a long-lasting T-cell memory response (Avci & Kasper, 2010; Avery & Goebel, 1931). Recently, the cellular and molecular mechanisms for adaptive immune activation mediated by glycoconjugate vaccines were elucidated by the Kasper lab (Avci, Li, Tsuji, & Kasper, 2011; Sun, Stefanetti, Berti, & Kasper, 2019) and the Avci Lab (Middleton, Sun, Paschall, & Avci, 2017). Kasper and co-workers discovered a mechanism that uptake of a glycoconjugate vaccine by antigen presenting cells (APC) resulted in the presentation of a carbohydrate epitope by the MHC class II, thus stimulating carbohydrate specific $CD4^+$ T cells. Subsequently, Avci and coworkers (Middleton et al., 2017) provided further evidence for the functional roles of Pn3P-specific $CD4^+$ T cells utilizing mouse immunization schemes that induce Pn3P-specific IgG responses in a carbohydrate-specific T cell–dependent manner. This mechanistic knowledge guides the vaccine community towards designing knowledge-based, structurally defined,

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new-generation vaccines with reproducible biological properties.

Generally, polysaccharides can be easily modified at multiple sites and coupled randomly to proteins in a clustered form. However, these random modifications may alter the natural epitopes of polysaccharide antigens and such conjugates consequently may not elicit specific antibodies to native antigens (Berti & Adamo, 2013; Colombo et al., 2018). In addition, random modifications and couplings are difficult to reproduce from batch-to-batch in vaccine preparation (Colombo et al., 2018). Since there has been a recent rise in the emergence of new multi-drug resistant bacterial strains (Berti & Adamo, 2018; Micoli, Costantino, & Adamo, 2018), developing novel and efficient methods to obtain new defined carbohydrate antigens of higher purity is at the forefront of the vaccine development over the past twenty years, especially the evolution of site selective glycoconjugation, which should contribute to a better understanding of the mode of action of vaccines (Adamo, 2017; Villadsen, Martos-Maldonado, Jensen, & Thygesen, 2017). In the current review, we focus on advances in site-selective approaches for glycoconjugate synthesis and, in particular, the preparation of vaccines from unprotected carbohydrates reported since 2001.

## 2. Approaches for the synthesis of glycoconjugates

Glycoconjugation generally relies on the modification of carboxyl, hydroxyl, phenoxyl, hemiacetyl, mercapto/disulfide and amino/imino functional groups (Kuberan & Linhardt, 2000). The direct conjugation between carbohydrate and protein, peptide or lipid generally results in low yields and complex product mixtures, particularly when the reactants involve proteins and glycans having multiple reactive sites or with reactive sites with high levels of steric hindrance. Thus, the basic strategy for the preparation of homogeneous glycoconjugates often requires the installation of an appropriate linker (Fig. 1) between protein and saccharide, reducing the steric hindrance and controlling the conjugation in a highly regio- and stereoselective manner (Adamo, 2017; Basu, Shrivastav, & Kariya, 2003). This review summarizes the most common conjugation approaches used including reductive amination, click chemistry, Michael addition chemistry, cycloaddition chemistry and chemoenzymatic synthesis, for easy transformation of native, unprotected carbohydrates into useful glycoconjugates. A brief introduction of a microreactor, a very promising technology for chemical and biochemical processes, is presented, which we believe could be widely applied in the preparation of glycoconjugates in the future, promoting the transfer of glycoconjugation from laboratory- scale synthesis to industrial production.

### 2.1. Reductive amination

Reductive amination, where a mixture of an aldehyde or ketone and amine is treated with a reductant in a one-pot reaction, is one of the most straightforward methods for glycoconjugates synthesis (Dalpathado, Jiang, Kater, & Desaire, 2005). Almost all of the carbohydrates have a “reducing end”, a terminus with an aldehyde or a ketone functionality, facilitating site-selective conjugation (Villadsen et al., 2017). Conjugation chemistry involving reductive amination typically utilizes sodium cyanoborohydride to selectively reduce intermediate imine adducts, known as Schiff bases. However, due to the poor efficiency this direct coupling method has generally only been applied into carbohydrate derivatization to enhance detection sensitivity in structural analysis (Zhang et al., 2018). Thus, the use of reductive amination to introduce a glycosylamine or a bifunctional spacer arm containing an amino group, offers a preparative approach to functionalize the reducing end of carbohydrate chain prior to further conjugation with proteins (Kuberan & Linhardt, 2000) (Fig. 2A).

Selective conjugation at the non-reducing end of an oligosaccharide or polysaccharide by reductive amination can be also achieved under certain circumstances, if there is an *N*-Acetylneuraminic acid (NeuAc) residue at the non-reducing end of the carbohydrate chain. Adamo et al. (Nilo et al., 2015) reported a controlled periodate oxidation of PSV, a complex anionic polysaccharide, that targeted 20 % of the available diol system of NeuAc glycerol chain, followed by reductive amination to introduce a free amine group, which could then be further conjugated with proteins (Fig. 2B).

In summary, due to its mild conditions and simple operations, reductive amination is widely used in the synthesis of glycoconjugate. However, the toxic residual cyanide from reducing agent sodium cyanoborohydride limits the application of the approach and in many cases it is inefficient and difficult to industrialize.

### 2.2. Click chemistry

Click chemistry is widely used to synthesize glycoconjugate clusters as carbohydrate-mediated multi-valent interactions play important roles in numerous biological processes, such as bacterial and viral infections, cell-growth and recognition, and cancer metastasis (Chabre & Roy, 2013; Elchinger et al., 2011). This strategy was also well established in site-selective conjugation. Novel substrates and linkers (Fig. 1, compound **1**, **2**, **5** and **7**) have been developed based on click chemistry (Adamo, 2017). The two most common types of click reactions, Huisgen 1, 3-dipolar cycloaddition and thiol-click chemistry are reviewed in Sections 2.2.1 and 2.2.2.

#### 2.2.1. Huisgen 1, 3-dipolar cycloaddition

Huisgen 1, 3-dipolar cycloaddition is a Cu (I)-catalyzed chemoselective coupling between organic azides and terminal alkynes, and represents the most popular click reaction to date owing to its convenient, quick, and quantitative reaction properties and orthogonality

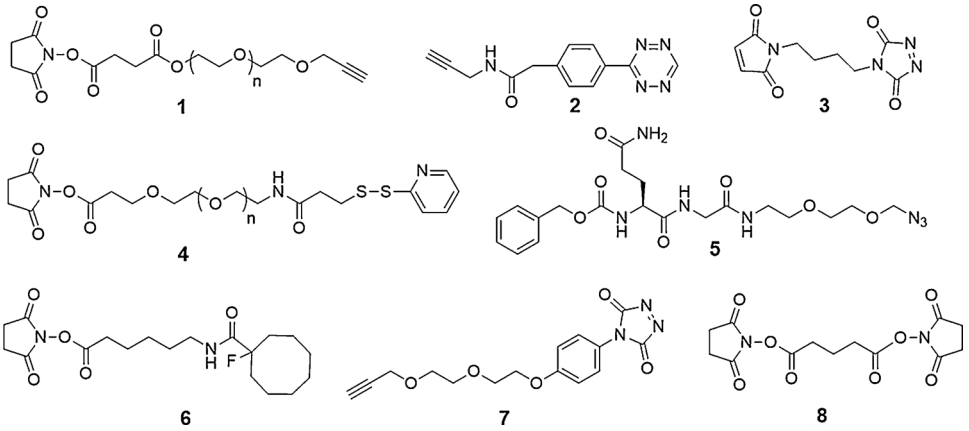


**Fig. 1.** Common linkers for glycoconjugate synthesis.

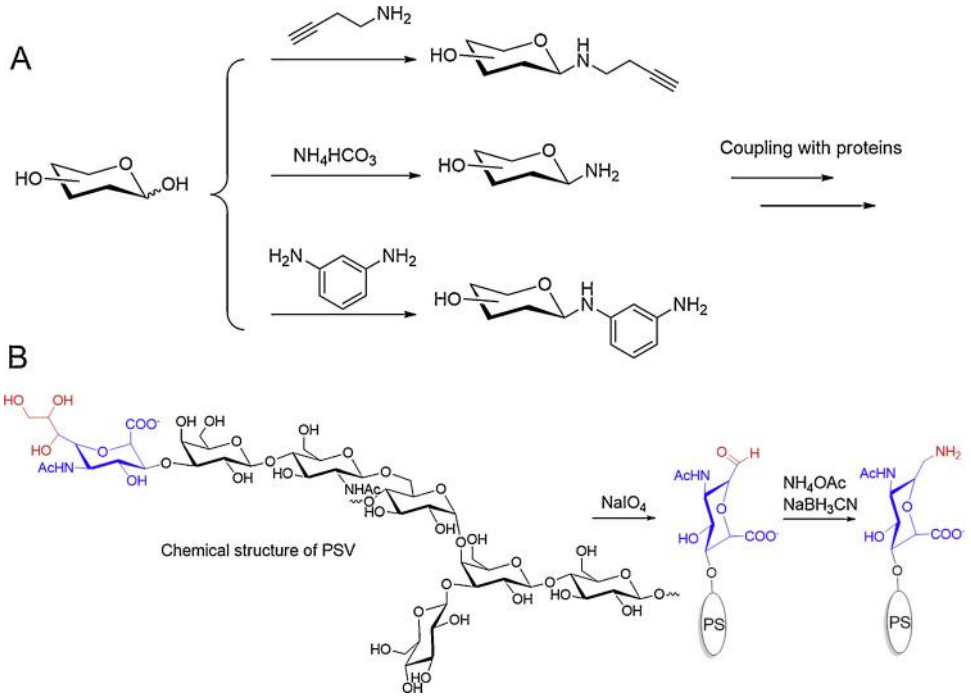


**Fig. 2.** (A) Reductive amination at the reducing end of carbohydrate chain. Glycosylamine or a bifunctional spacer arm is utilized to improve the conjugation efficiency. (B) Reductive amination at the non-reducing end. The black moiety of the PSV structure is designated with the symbol "PS".

(Rostovtsev, Green, Fokin, & Sharpless, 2002). A diverse collection of saccharides with anomeric positions functionalized with azido or alkyne groups have been developed for click chemistry (Salmon, Williams, Maresca, Supuran, & Poulsen, 2011). Click chemistry can also be performed in solid phase (Stefanetti, Saul, MacLennan, & Micoli, 2015) allowing the possibility of recovering unreacted sugar and simplifying the purification process, thus, reducing the cost of glycoconjugate vaccine production. This strategy was also applicable in the preparation of glycoconjugate functionalized fullerene (Pereira, Santos, Luduvico, Alves, & de Freitas, 2010) or magnetic nanoparticles (Raval et al., 2015). Tzeng et al. (Raval et al., 2015) reported the synthesis of polyethylene oxide stabilized magnetic nanoparticles (PEO-MNPs) that were bio-functionalized with sialyl lactose derivative, Neu5Ac(α2-3)Gal(β1-4)Glcβ-sp (Fig. 3A). A nitrated 3,4-dihydroxy-L-phenylanaline (nitro-DOPA) was coupled with an alkyne containing linker **1** (Figs. 1 and 3A) followed by ligation exchange with nanoparticle and click chemistry with the azido modified sialyl lactose derivative. The resulting glycoconjugate functionalized nanoparticles were demonstrated as potent anti-adhesion agents for reducing enterotoxigenic *E. coli* infection.

However, the use of Cu(I) as catalyst in Huisgen 1, 3-dipolar cycloaddition presents a certain risk as excessive copper can cause dizziness, nausea, and even toxicity to the liver and kidney. High-energy cyclic alkynes could be used to avoid the use of Cu(I), while the regioselectivity is sacrificed. Moreover, the alkyne and azide groups typically have to be installed at the end of the alkyl chain to ensure the progress of click chemistry, which increases the difficulty of chemical synthesis.

#### 2.2.2. Thiol-click chemistry

Thiols are prone to react either by radical or catalyzed processes under very mild conditions with a multitude of substrates, and are becoming popular reactants in the synthesis of glycoconjugates (Hoyle, Lowe, & Bowman, 2010; McSweeney, Dénès, & Scanlan, 2016). Glycosyl thiols are useful building blocks for the thiol-click chemistry, enabling certain *S*-linked glycoconjugates synthesis, which may have enhanced chemical stability and resistance to enzymatic hydrolysis (De Leon, Levine, Craven, & Pratt, 2017). Davis et al. (Bernardes, Gamblin, & Davis, 2006; Floyd, Vijayakrishnan, Koeppe, & Davis, 2009) developed a direct and general manner for the preparation of glycosyl thiols from unprotected sugars using Lawesson's reagent. Instead of chemical modification to functionalize peptides prior to their conjugation, a "tag-modify" approach was developed, introducing a non-natural amino acid (L-homoallylglycine) that introduced a double bond into a target protein through the expression of the appropriate gene sequence in *E. coli*. Subsequently, the thiol-ene ligation reaction with thiosugar in a radical photoinitiated system was employed to furnish the *S*-linked glycoprotein conjugates (Fig. 3B). Mild reaction conditions and a breadth of applicable substrates are advantages of thiol-click chemistry. However, the reaction orthogonality is not as good as other kinds of click reactions due to the high activity of thiol group. In addition, an unpleasant thiol odor is prevalent in thiol-click reactions, especially for low molecular weight reactants.

### 2.3. Michael reactions

Michael reactions have been used widely in organic synthesis for their efficient C–C bond-forming ability. The Michael reaction is not confined to the addition of carbanions, rather, a wide variety of nucleophiles (Michael donors) having hetero-atoms, such as N, O, S, P, can add to an electron deficient C=C bond in a molecule (Michael acceptor) in bioconjugation synthesis known as aza-Michael addition (Rulev, 2011). Thiol-type reactions (Chen, Zhou, Peng, & Yoon, 2010; Dénès, Pichowicz, Povie, & Renaud, 2014), involving the addition of thiols or thiolate anions to electron deficient C=C bonds, are particularly useful in bioconjugation synthesis due to the robust formation of thioether bonds with fast reaction kinetics, high regioselectivity, and an often quantitative yield across a broad range of substrates (Nair et al., 2014). Chen et al. (Wang, Shi, Wu, & Chen, 2014) reported an efficient fluorescent labeling on the non-reducing end of low molecular weight heparin (LMWH) through an aza-Michael addition (Fig. 4A). This conjugation was initiated from a specific enzyme hydrolysis of glycosaminoglycans to produce oligosaccharides containing a $\Delta^{4,5}$-unsaturated carboxylic acid at the non-reducing terminus. The resulting

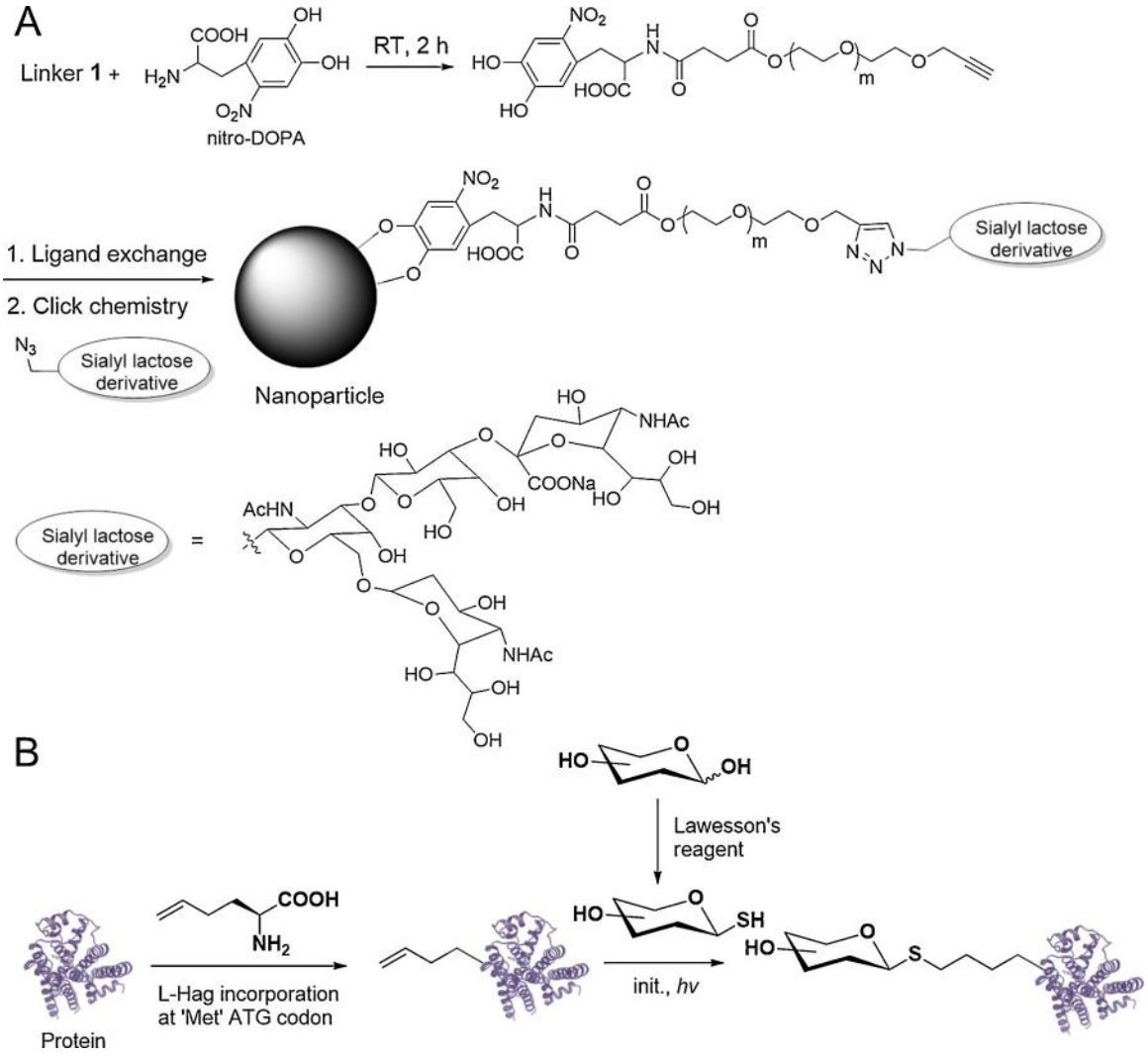


**Fig. 3.** (A) Synthesis of heterobifunctional polyethylene oxide coated nanoparticles bio-functionalized with sialic-acid derivative via Huisgen 1, 3-dipolar cycloaddition. (B) Synthesis of *S*-linked glycoproteins by thiol-ene ligation.

carboxylic acid of oligosaccharide was subsequently converted to a carboxylic benzyl ester, which is a more reactive acceptor for aza-Michael addition with thiol-containing fluorescent molecule. This method could be potentially used for the preparation of unnatural glycosaminoglycan-peptide conjugates with the glycan non-reducing end selectivity attached to a thiol-containing peptide. However, since the substrates must contain α, β-unsaturated system, this inevitably limits the application of this method. Also stereoselective control in Michael reactions can be challenging.

### 2.4. Amidation

Amide forming chemistry using DCC (dicyclohexylcarbodiimide), water soluble EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), or sulfo-NHS (*N*-hydroxysulfosuccinimide) activation of carboxylic groups for coupling with amino functionality in protein or carbohydrate has been widely used to form glycopeptides. Among these, the reactions of oxyamine and hydrazides become powerful methods as the fact that oxime and hydrazine formation occur in aqueous medium with a high degree of chemoselectivity with unprotected biomolecules, such as peptides, proteins, carbohydrates or oligonucleotides (Ostergaard, Christensen, Hjuler, Jensen, & Thygesen, 2018). For example, adipic acid dihydrazide (ADH, Fig. 4B), widely used as a homobifunctional linkers in bioconjugate chemistry, could be specifically reacted with the reducing end of the carbohydrate by reductive amination. The remaining acyl hydrazide could be further modified with protein or lipid derivatives by amidation to obtain glycoconjugates (Leitner et al., 2014). Adipic acid bis (*N*-hydroxysuccinimide) ester (SIDEA, Fig. 4B) is another important bifunctional glutaryl linker popularized in the conjugation reactions due to its conjugation efficiency. Moreover, this linker would not elicit antibodies against itself or affect the immunological properties of resultant glycoconjugate (Liao et al., 2016). The combination of SIDEA and ADH also guarantees a higher conjugation efficiency than the use of SIDEA alone (De Benedetto, Salvini, Gotta, Cescutti, & Micoli, 2018) (Fig. 4B). However, substrates must contain carboxyl and amine groups to ensure the progress of amidation and it is usually difficult to control the site placement if there are multiple carboxyl or amine groups in the substrates.

### 2.5. Diels-Alder reaction

The Diels–Alder (DA) reaction between a diene and a dienophile is an important tool in glycoconjugate synthesis and variations in reaction conditions can allow for selective product formation with high levels of regio- and stereo-control (Pozsgay, Vieira, & Yergey, 2002; Sylvain, Sylvestre, & José, 2016). However, multiple chemical steps are required to prepare the DA reaction precursor of either glycan or protein increasing the cost of glycoconjugate synthesis. Instead of chemical

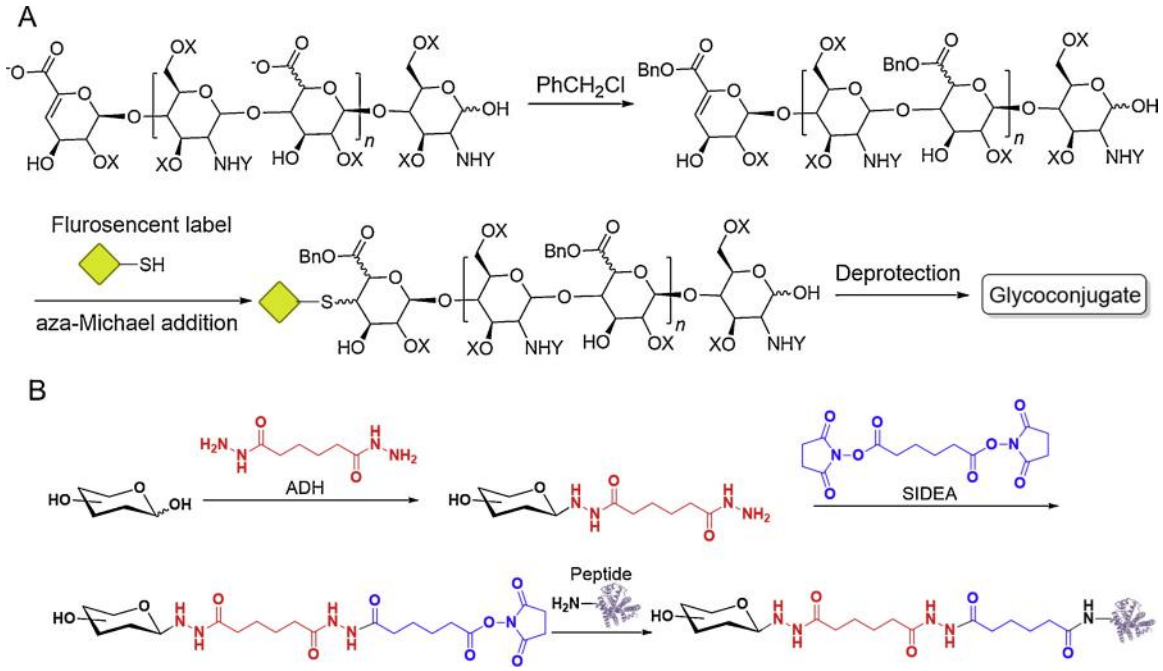


**Fig. 4.** (A) Synthetic route to the fluorescent labeling of low molecular weight heparin at the non-reducing end. X = $SO_3Na$ or H; Y = $COCH_3$, $SO_3Na$ or H. (B) Glycoconjugate synthesis through sequential insertion of ADH and SIDEA as linkers.

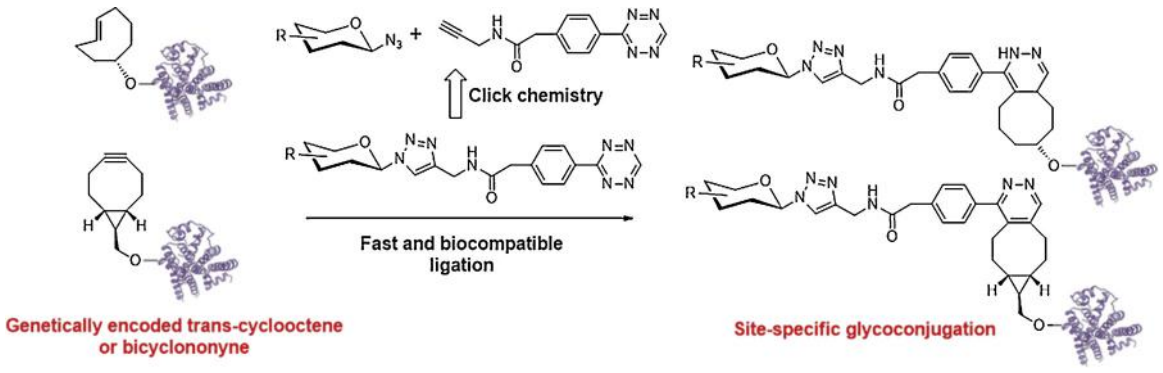


**Fig. 5.** Glycoconjugate synthesis using Diels-Alder reaction (Machida et al., 2015).

approach, Winssinger et al. (Machida, Lang, Xue, Chin, & Winssinger, 2015) achieved the rapid preparation of protein that was modified with a trans-cyclooctene or bicyclononyne derivatized lysine using a generic strategy (Fig. 5). Functionalized protein was chemoselectively conjugated by DA reaction with tetrazine functionalized glycans, prepared by click chemistry between glycosyl azide and an alkyne-tetrazine, to achieve the site-specific glycoconjugation. Since performing a selective glycoconjugation in complex systems would require a bioorthogonal conjugation without toxic reagents (such as copper), this chemicals-free method utilizing the inverse cycloaddition of a tetrazine with strained alkenes and alkynes would be an ideal for live cell experiments. Although this method solves the cumbersome problem of multi-step chemical synthesis, it depends on complex genetic engineering techniques to introduce non-natural amino acids. The resulting unnatural amino acids may have potential risk to cause immune responses in the body. In addition, this method is not as fast as click chemistry and may be difficult to commercialize.

### 2.6. Enzymatic approach

In contrast to conventional chemical synthesis, which usually requires tedious protection/de-protection manipulations to achieve regio-selectivity and stereo-selectivity, enzymatic glycosylation can provide perfect control of the anomeric configuration and high regioselectivity without the need for protecting groups (Schmaltz, Hanson, & Wong, 2011; Yamamoto, 2013). Wang et al. (Li, Zeng, Hauser, Song, & Wang, 2005; Wang & Huang, 2009) explored synthetic sugar oxazolines as donor substrates for ENGase-catalyzed *N*-glycopeptide synthesis (Fig. 6A). A heterogeneous *N*-glycoprotein was deglycosylated by enzyme to form a homogeneous GlcNAc-glycoprotein as acceptor. Oligosaccharide oxazoline corresponding to the *N*-glycan core Man3GlcNAc subsequently served as a donor for a regio-specific and stereo-specific transglycosylation to form corresponding *N*-glycopeptides. Azido modified oligosaccharide ligand could be accepted by ENGases and, thus, achieved bioorthogonal labeling. This strategy was based on the assumption that the ENGase-catalyzed reaction proceeds through a mechanism of the substrate-assisted catalysis involving an oxazolinium ion intermediate.

Enzymatic approaches have also been demonstrated to be applicable in the synthesis of glycoconjugate polymers. Miura et al. (Miura, Ikeda, & Kobayashi, 2003; Miura, Ikeda, Wada, Sato, & Kobayashi, 2003) reported the regioselective esterification of maltitol and lactitol with divinyl sebacate that was catalyzed by lipase to form 6-vinyl esters. The conversion and chemoselective transesterification were

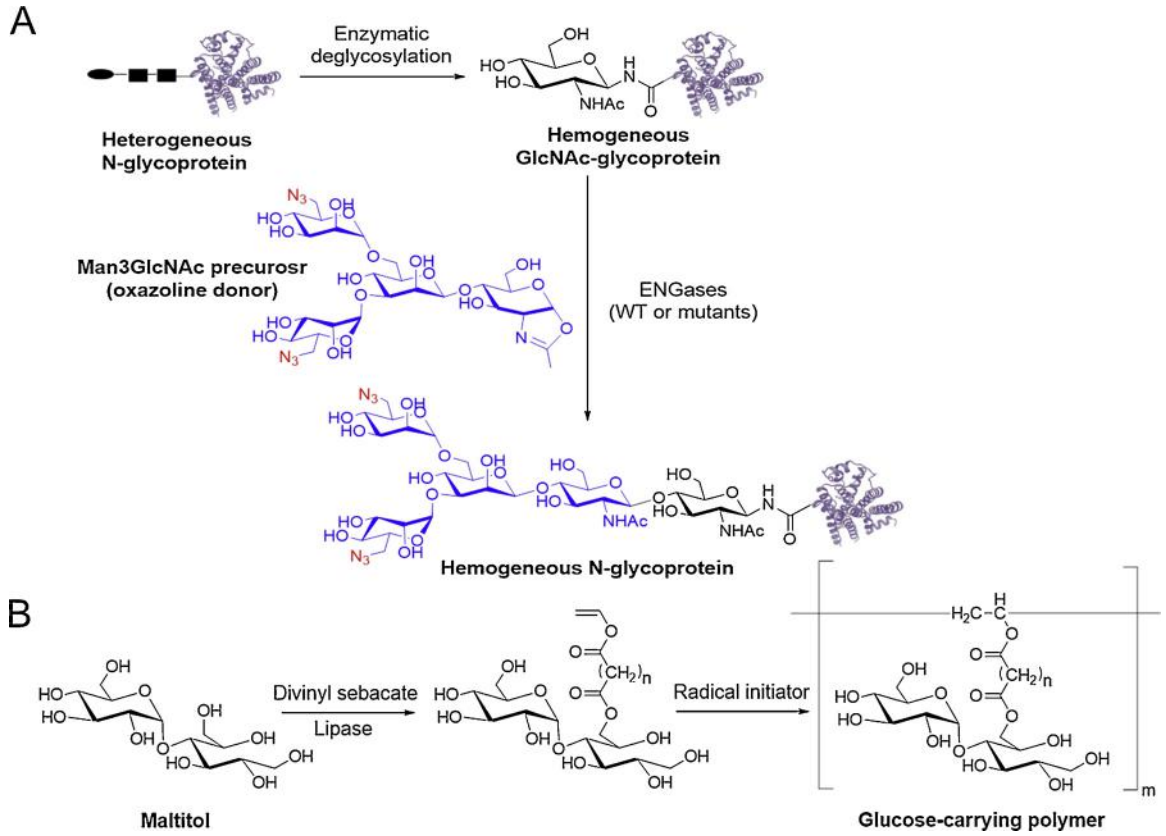


**Fig. 6.** (A) ENGase-catalyzed transglycosylation for *N*-glycoprotein synthesis (Wang & Huang, 2009). (B) Synthesis of glycoconjugate polymers through enzymatic esterification of sugar alcohols and subsequent radical polymerization.

dependent on lipases. The resulting vinyl ester was subjected to radial polymerization to obtain the corresponding glycoconjugate polymers (Fig. 6B). This approach should be useful for developing various glycoconjugate polymers with high biological activities due to the multivalent glyco-cluster effect and biodegradability due to their poly (vinyl alcohol) backbone and ester linkage. However, a major disadvantage of enzymatic approach is the low reaction yields of enzyme-catalyzed glycosylation, and the glycosidic bonds of product can be easily hydrolyzed by enzyme as well. Although major breakthroughs on glycosyltransferase development have been made, the poor availability of enzymatic substrates and the potential inactivation of the enzyme largely limited the application of this approach.

### 2.7. Cysteine-type reaction

Sulfhydryl of thiol-containing amino acids (e.g., cysteine) is the prime attachment site for conjugation, due to the nucleophilic character of thiol especially at physiological pH. The most popular thiol-reactive conjugation handle is maleimide (Henkel, Röckendorf, & Frey, 2016), as it specifically and efficiently couples with *S*-alkyl. Trapping the glycosyl amine with iodoacetic acid followed by nucleophilic substitution between iodoacetyl and thiol is another common cysteine-type reaction to access glycoconjugate (Kuberan & Linhardt, 2000; Murase, Tsuji, & Kajihara, 2009) (Fig. 7A). The orthogonal condensation reactions between 1, 2-aminothiols and aldehydes are very useful since 1, 2-aminothiols are naturally present in proteins as *N*-terminal cysteine (Fig. 7B). This reaction offered a catalyst-free alternative to click chemistry and the resulting thiazolidine product was reported highly stable at both acidic and neutral pH (Bermejo-Velasco, Nawale, Oommen, Hilborn, & Varghese, 2018). The efficiency of such a reaction has already been demonstrated in coupling between peptides and oligonucleotides, aliphatic aldehyde or biotin (Wade, Domagalam, Rothacker, Catimel, & Nice, 2001), and we believe it will be popularized in the glycoconjugate synthesis as well in the near future.

Linhardt et al. (Cheng et al., 2019) developed a chemoselective ligation method for glycopeptides synthesis using adipic dihydrazide (ADH) as a linker followed by selective ligation with cysteine residue of peptides (Fig. 7C). $PhNH_2$/acetate buffer was found to be the optimal coupling condition to form carbohydrate-ADH and was even able to achieve the efficient preparation of the complex glycosaminoglycans-ADH conjugates in high yields. The remaining acyl hydrazide was oxidatively converted to an acyl azide and then captured as a thioester and transesterified with the cysteine residue of a peptide to obtain a thioester-linked glycoconjugate. When this cysteine is at the *N*-terminus of the peptide chain, the thioester rapidly rearranges to form a stable amide linkage between the carbohydrate and peptide. This method is applicable to access site-selective conjugation for both carbohydrate and peptides (Fang et al., 2011), however, a cysteine residue is indispensable for the substrate peptides, limiting the application of this approach.

### 2.8. Other chemistries

The high reactivity of 4-phenyl-1,2,4-triazoline-3,5-dione towards the phenol functionality in water is particularly attractive in the site-selective conjugation. Hu et al. (Hu et al., 2013; Stefanetti, Hu et al., 2015) developed a combination technology with tyrosine-selective alkynylation and a click chemistry mediated glycoconjugation sequence, which can access a well-defined glycan, protein carrier connectivity (Fig. 8A). The phenol residue of the tyrosine from peptide was selectively attached to the linker **7** (Figs. 1 and 8A) through a triazolidinone-ene reaction. The alkyne group of the linker in the other end was subsequently conjugated with azido tagged carbohydrate to form the glycoconjugate. All these reactions were conducted under mild neutral

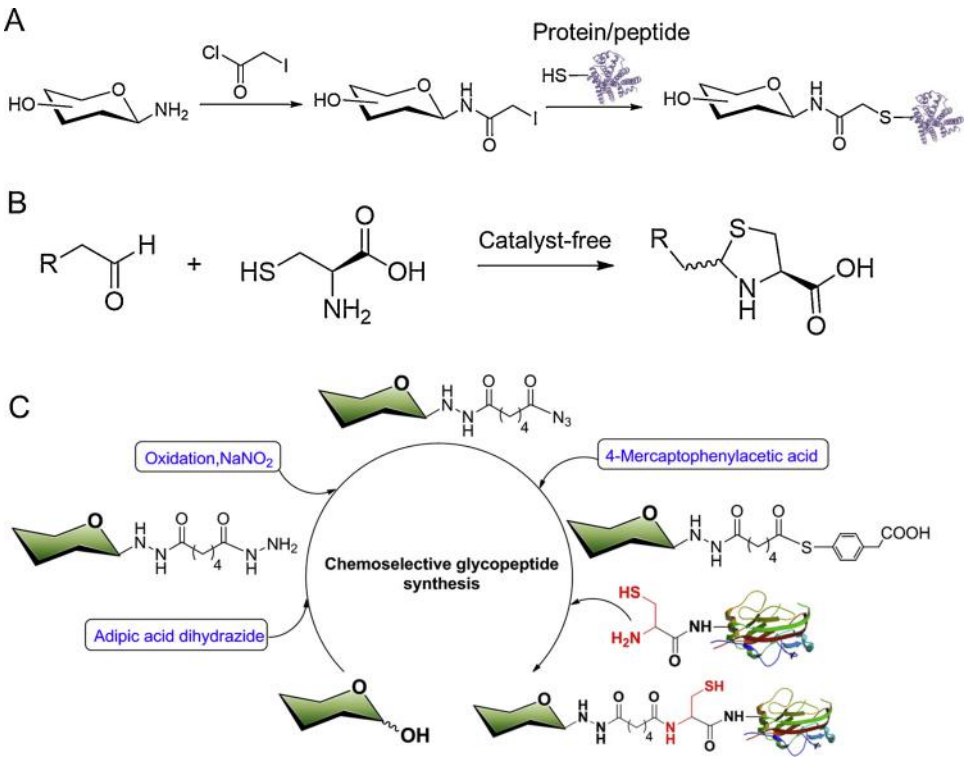


**Fig. 7.** (A) Nucleophilic substitution between iodoacetyl and thiol to form glycoconjugate. (B) Scheme of the condensation between aldehyde and L-cysteine through thiazolidine formation. (C) Glycopeptide preparation. Peptides where cysteine is not at N-terminus could also be synthesized but instead result in a thioester linkage (Cheng et al., 2019). Copyright 2019 The Royal Society of Chemistry.

conditions.

Squarate mediated coupling is regarding a unique bifunctional linker both of whose ethyoxyl groups are reactive with amines but under different pH conditions. For example, when treated with ethylenediamine, only one of the ethyoxyl groups is replaceable by amine in neutral buffer (pH 7) and the second ethyoxyl group of squarate is active towards amines in basic solution (pH 9), thus, enabling the conjugation of oligosaccharide–squarate to proteins (Fig. 8B) (Wang, Chang, Guttormsen, Rosas, & Kasper, 2003; Wang, Asnani, & Auzanneau, 2010).

### 2.9. Microreactors

Microreactors, devices with narrow channels that are constructed from stable and inert materials, represents a very promising technology for chemical and biochemical processes and, thus, for glycoconjuagte synthesis (Geyer & Seeberger, 2007; Ratner et al., 2005). These microreactors have a high surface area/volume ratio in microreactor, allowing rapid heat exchange and mass transfer. This approach can greatly increase the reaction yields and simplify the product separation while greatly lowering energy consumption. Although promising, to date there have been few examples of glycoconjugate synthesis using the microfluidic reactor devices. Seeberger et al. (Kikkeri, Laurino, Odedra, & Seeberger, 2010) pioneered a single-phase microfluidic

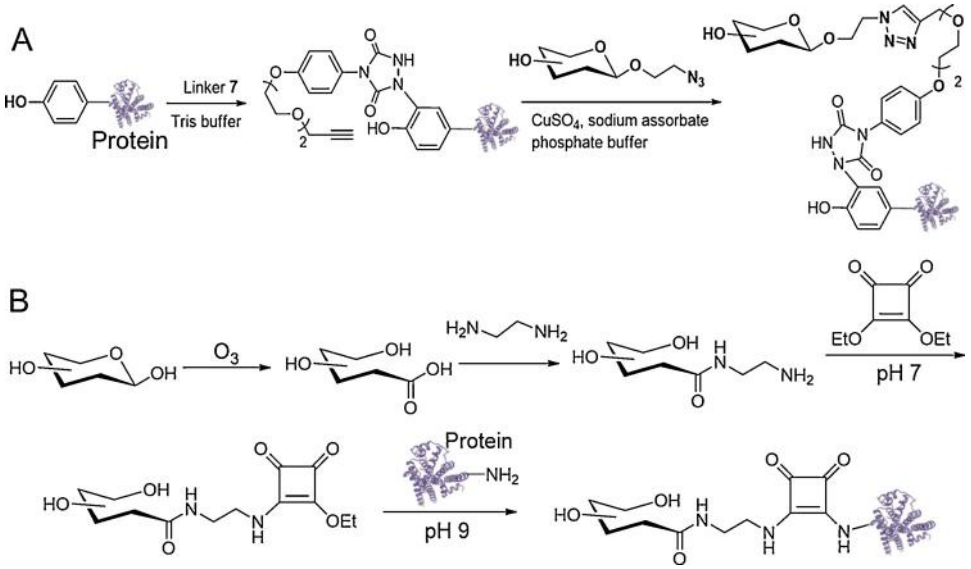


**Fig. 8.** (A) Tyrosine-selective alkynylation and a click chemistry mediated glycoconjugation sequence. (B) Squarate mediated coupling in the glycoconjugate preparation.

system for the synthesis of CdSe and CdTe nanoparticles (quantum dots, QDs), which were further conjugated with carbohydrate that was pre-modified with thiol groups. The continuous-flow microreactor enables precise temperature control as well as efficient mixing, allowing for the preparation of QDs with narrow size distribution as well as controlling the QDs size by simply varying the reaction time in the flow reactor. In addition, glycoconjugate synthesis using immobilized enzymes in a microreactor have also been investigated (Haneda, Oishi, Kimura, & Inazu, 2018). Microreactors offer promise in promoting the transfer of a laboratory-scale synthesis to industrial production, while proof-of-concept reactions have been demonstrated, more complex synthesis of glycoproteins or glycolipids still need to be demonstrated. Moreover, currently microreactors can only be applied to the reactions that have been studied thoroughly, and the application to understand reactions is difficult. Meanwhile, the microfluidic channels are easily clogged if the reaction system is highly adherent or easily precipitated.

## 3. Conclusions and perspectives

There is a thick layer of oligosaccharides and polysaccharides covering the cell surfaces of pathogens, mediating their receptor binding to host cells for initial adhesion and organism invasion. Thus, these exposed saccharides are attractive targets as antigens for vaccine design. Furthermore, polysaccharide immunogenicity can be strongly enhanced by conjugation to an immunogenic carrier protein, providing T cell-dependent glycoconjugate antigens able to stimulate B cell maturation to memory cells and induce immunoglobulin class switching from IgM to polysaccharide-specific IgG. Thus, glycoconjugation represents a key tool in carbohydrate vaccine preparation. Conjugation reaction chemistry can strongly impact the efficiency of conjugation, saccharide to protein ratio, and structure and size of the resulting conjugate, all of which has consequences in the immunogenicity of a carbohydrate vaccine. Moreover, the immunogenic activity of fully homogeneous conjugates still requires sophisticated biological studies, as the immune response towards carbohydrate-protein conjugates depends on multiple factors including the size of the carbohydrate epitope, the carrier protein, the nature and number of covalent bonds and the nature of the linker arm. Methods simplifying and accelerating the preparation of glycoconjugates have matured over the past decade. The chemical and enzymatic approaches presented in this review provide multiple options for researches to design specific glycoconjugate vaccines, however, sometimes it may be necessary to combine multiple approaches to successfully facilitate glycoconjugate synthesis. We also expect chemoenzymatic approaches, automated solid-phase synthesis and microreactor technology to make carbohydrate production considerably more attractive for industrialization.

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