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Engineering a new generation of carbohydrate-based vaccines

Kevin B. Weyant¹, Dominic C. Mills¹, and Matthew P. DeLisa^{1,2,3}

¹Robert Frederick Smith School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14853 USA

²Comparative Biomedical Sciences, Cornell University, Ithaca, NY 14853 USA

³Department of Biomedical Engineering, Cornell University, Ithaca, NY 14853 USA

Abstract

Recent advances in chemical synthesis, conjugation chemistry, engineered biosynthesis, and formulation design have spawned a new generation of vaccines that incorporate carbohydrate antigens. By providing better immunity against a variety of pathogens or malignant cells and lowering the cost of production, these developments overcome many of the limitations associated with conventional vaccines involving polysaccharides. Moreover, the resulting vaccine candidates are shedding light on how the immune system responds to carbohydrates and providing mechanistic insight that can help guide future vaccine design. Here, we review recent engineering efforts to develop and manufacture carbohydrate-based vaccines that are efficacious, durable, and cost-effective.

Introduction

The dense saccharide layer that coats many pathogens and malignant cells is an attractive target for subunit vaccination. Accordingly, carbohydrate-based vaccines first entered the market in the late 1970s and early 1980s with the release of a capsular polysaccharide (CPS) vaccine against *Haemophilus influenzae* type B (Hib) and a 14-valent CPS vaccine against *Streptococcus pneumoniae*. The vaccines were composed of a simple formulation of pathogen-derived polysaccharides that were sufficient to provide protective immunity in healthy adults. However, antibody responses were poor and non-protective in medically at-risk groups including children under two years old [1,2]. The poor response in these groups has been attributed to the T-cell independent nature of the immune response to carbohydrate antigens (discussed below) and, in the case of neonates and young children, the immature immune system that has not yet had the benefit of multiple exposures to cross-reactive natural flora. Since that time, carbohydrate vaccine development has almost exclusively focused on creating more advanced formulations that provide broader protection in these high-risk groups.

*Address correspondence to: Matthew P. DeLisa, Robert Frederick Smith School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14853. Tel: 607-254-8560; Fax: 607-255-9166; md255@cornell.edu.

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Pure carbohydrates, especially polysaccharides, typically activate antibody-producing B-cells by cross-linking the B-cell receptor (BCR), leading to the production of predominantly low-affinity and short-lived immunoglobulin M (IgM) antibodies (Fig. 1A) [3]. In contrast, protein antigens elicit additional activation signals, facilitating antibody class-switching to more high-affinity and long-lived IgGs in all age groups. These signals are provided by helper T-cells which recognize antigen-derived peptides presented on major histocompatibility complex (MHC) class II molecules [4]. Consequently, antibodies elicited from carbohydrates and proteins are generally classified as T-cell independent and T-cell dependent, respectively (exceptions, including T-cell dependent zwitterionic polysaccharides, are described elsewhere [5]). Interestingly, when the two biomolecules are covalently linked to form a conjugate, the protein component can elicit T-cell help to anti-carbohydrate B-cells, leading to the generation of anti-carbohydrate IgGs (Fig. 1B). The principle of glycoconjugate vaccines is now widely employed to improve immune responses against carbohydrate antigens and is critical for protection in the immature immune systems of young children.

Commercial glycoconjugate vaccines against the CPS of Hib, *S. pneumoniae*, and *Neisseria meningitidis* are now well established and have dramatically decreased disease rates in the countries where they have been introduced [6–8]. In light of this success, numerous additional glycoconjugate vaccines against bacterial pathogens, viral pathogens, or cancer have been developed with many of these in clinical trials [9]. However, while glycoconjugates have proven to be effective and safe, standard production methods for these compounds are complex, resulting in high-cost vaccines that cannot be administered wholesale in many developing countries [8]. Moreover, protection is inadequate against many disease targets, especially intracellular pathogens and cancer which require additional cell-mediated immune responses [10,11].

In this review, we provide an overview of the wide-ranging strategies that have been developed in recent years to engineer and manufacture carbohydrate-based vaccines. These methods have the potential to not only improve the efficacy, durability, and/or cost effectiveness of existing licensed glycoconjugate vaccines but may also provide new ways to prevent myriad other diseases for which licensed vaccines are not currently available.

Carbohydrate antigen production

Most commercial glycoconjugate vaccines are based on polysaccharide antigens isolated from pathogenic bacteria. The polysaccharide isolation step is costly, introduces safety concerns, and results in a heterologous mixture of glycans and contaminants that may have undesired effects on the immune response [12]. In addition, reliance on natural sources of polysaccharide antigens limits disease targets to those involving known carbohydrates that are well expressed and relatively easy to purify. Consequently, engineered approaches to carbohydrate production have become attractive alternatives.

Chemical synthesis of carbohydrate antigens has been studied for many years, and in 2004 its utility was demonstrated with the approval of a chemically synthesized oligosaccharide vaccine for Hib in Cuba [13]. Several other well-defined carbohydrate antigens have been chemically synthesized, including the repeat unit of PSII polysaccharide from *Clostridium*

difficile, the conserved core lipopolysaccharide (LPS) tetrasaccharide Hep2Kdo2 present on the surface of many bacterial pathogens, and the tumor-associated carbohydrate antigen (TACA) GM2 [14–16]. Chemical synthesis has also been used to further engineer oligosaccharides with enhanced stability or immunogenicity, including the CPS from *N. meningitidis* serogroups A and B [17,18]. Recent technologies that facilitate carbohydrate assembly or increase the number of synthesizable structures have broadened the scope of this approach [19]. One notable example is automated glycan assembly (AGA) [20]. This method eases the creation of glycan microarrays, which are used to uncover immunodominant glycan epitopes (glycotopes) that can subsequently be incorporated into glycoconjugate vaccines. This strategy was first demonstrated by Schumann *et al.* to design a vaccine that protects against *S. pneumoniae* serotype 8 [21]. Although prohibitive cost has generally limited AGA technology to microarray applications and prevented it from being used to synthesize sufficient quantities of carbohydrates for vaccination, further developments that streamline the process may increase its scalability [22].

Chemoenzymatic assembly is a scalable and reliable approach that exploits the inherent specificities of glycosyltransferase (GST) enzymes and can be used as an alternative to or in combination with chemical synthesis [12]. However, it requires identification of the natural pathways of glycan synthesis followed by recombinant expression and purification of functional enzymes, a process that can be challenging and time-consuming. Nonetheless, chemoenzymatic assembly has successfully been used to create CPS-based glycoconjugates for *N. meningitidis* serogroups A, C, and X [23–25].

In some instances, recombinantly expressed GSTs can be used to assemble non-native carbohydrate antigens within tractable host organisms such as *Escherichia coli*. For example, this method has been used for the *in vivo* assembly of CPS from several serotypes of *S. pneumoniae* [26]. Carbohydrate antigen production in *E. coli* has gained considerable attention more recently due to its powerful integration with bacterial protein-glycan coupling technology (PGCT) and glycosylated outer membrane vesicles (glycOMVs), which are described in more detail below.

Protein-based glycoconjugates

All currently licensed glycoconjugate vaccines contain carbohydrate antigens that are chemically conjugated to immunogenic carrier proteins such as tetanus toxoid (TT), diphtheria toxoid (DT) or a non-toxic mutant of DT called cross-reactive material 197 (CRM₁₉₇). The nature of the carrier protein can influence both the efficiency of T-cell help to carbohydrate antigen-specific B-cells and the risk of undesired interactions that may lead to immune suppression. For example, TT conjugates are typically more immunogenic than DT conjugates, but the former are more susceptible to carrier-induced epitopic suppression (CIES), a mechanism whereby pre-existing anti-carrier antibodies suppress the anti-carbohydrate immune response [27].

Prevalent functional groups on the carrier protein such as amino groups in lysine residues or carboxyl groups in acidic residues are typical carbohydrate attachment sites, which results in a heterologous conjugate population. However, growing evidence that attachment site and glycan density can significantly impact the immune response has led to the development of

conjugation chemistries that are more site-selective [28]. These include thiol-ene addition, which has been used for site-specific conjugation to a single-cysteine mutant of subtilisin, and tyrosine ligation, which has been used to conjugate an oligosaccharide to a controlled number of tyrosine residues in CRM₁₉₇ [29,30].

Functional groups on the carbohydrate antigen must be chemically activated to allow for attachment to the carrier protein. Such groups may be terminal, leading to the formation of a neoglycoconjugate, or repeated, leading to the formation of a lattice-type glycoconjugate (Fig. 2A and B) [31]. Immunogenicity of the latter is often superior. However, lattice-type glycoconjugates can only be formed using polysaccharide antigens, are difficult to characterize, and can display unnatural epitopes which are prone to eliciting non-protective antibodies [32].

Overall, chemical conjugation of carbohydrate antigens to protein carriers is often a laborious, complex, and expensive process that requires careful consideration of each variable to generate an effective vaccine. Detailed considerations regarding the protein carrier, attachment design, and linker chemistry are described in several recent reviews [28,31–33].

Protein glycosylation technology in non-pathogenic *E. coli*

The identification of the first general bacterial protein glycosylation system in *Campylobacter jejuni* followed by its successful transfer into *E. coli* [34] may open the door to a cheaper and more scalable method of creating protein-based glycoconjugates. The system relies on the oligosaccharyltransferase (OST) enzyme PglB from *C. jejuni* and the lipid anchor undecaprenyl pyrophosphate (Und-PP), which is present in the cytoplasmic membrane of *E. coli*. Und-PP naturally functions as an anchor for the construction of oligo- and polysaccharides including O-polysaccharide (O-PS) antigen in Gram-negative bacteria. Due to its glycan substrate promiscuity, heterologously expressed PglB can transfer Und-PP-linked glycans to target proteins that carry the consensus acceptor sequence D/E-X₁-N-X₂-S/T, where X₁ and X₂ are any amino acids but proline (Fig. 3A). Importantly, glycosylation by PglB is compatible with a wide variety of heterologous O-PS structures [35] as well as other non-native glycan structures that are produced from heterologous GST expression [36].

Glycoconjugate production in *E. coli*, or PGCT as it is sometimes called, first requires recombinant expression of all GSTs required for carbohydrate antigen synthesis. Second, an immunogenic carrier protein is genetically engineered to include one or more PglB acceptor sequences at defined locations, thus allowing greater control of glycosylation sites than most chemical conjugation methods. Finally, the glycosylation machinery and carrier protein are co-expressed in *E. coli* and the glycoprotein product is purified. Several preclinical studies have shown that effective glycoconjugate vaccines can be produced using PGCT. These include O-PS conjugates that protect against *Francisella tularensis* and *Burkholderia pseudomallei* as well as a CPS conjugate that protects against *Staphylococcus aureus* [37–39]. A few vaccines produced by this method have recently entered clinical trials, including O-PS conjugates for *Shigella dysenteriae* type I, *S. flexneri* 2a, and extraintestinal pathogenic *E. coli* (ExPEC) [40–42].

Lipid-based glycoconjugates

For many years, protein or peptide conjugation was considered a requirement to create a broadly effective carbohydrate-based vaccine. However, Chang *et al.* identified a mechanism of B-cell help that is lipid-dependent. Specifically, a subset of invariant natural killer T (iNKT) cells can recognize glycolipids on CD1d, a non-classical MHC-like protein expressed on B-cells, and provide the signals necessary for B-cells to produce high-affinity IgGs (Fig. 1C) [43]. For example, the glycolipid α -galactosylceramide (α -GalCer) and analogues can effectively stimulate iNKTs [44].

Proof that lipid conjugation can be used to enhance the immune response against poorly immunogenic carbohydrates was demonstrated with the creation of a synthetic vaccine with *S. pneumoniae* serotype 4 CPS conjugated to α -GalCer. The conjugate generated high-affinity IgGs and was protective in mice, whereas protection was poor with unconjugated CPS [45]. This work and similar studies [46,47] have opened the door to a potential new class of lipid-linked glycoconjugate vaccines (Fig. 2C) that may provide better, longer lasting protection than glycoproteins for some disease targets, given the different roles of helper T-cells and iNKTs in immunity [44,48]. Moreover, carbohydrate conjugation to iNKT epitopes like α -GalCer can conveniently be performed using similar techniques to those established for protein-based glycoconjugates [45].

Glycosylation of bacterial outer membrane vesicles (OMVs)

In *E. coli*, the native O-antigen ligase enzyme WaaL can conjugate a non-native carbohydrate antigen assembled on Und-PP to the core oligosaccharide of lipid A, forming a unique hybrid LPS structure that is transported to the outer membrane (Fig. 3B). Until recently, the utility of this mechanism in the context of carbohydrate-based vaccines was limited to either the production of glycolipids that required subsequent purification and chemical conjugation to an immunogenic carrier or the creation of whole-cell *E. coli* vaccines that surface display desired carbohydrate antigens. In addition, the conserved lipid A component of LPS is not known to bind CD1d or stimulate iNKTs [49]. However, recent studies describing the generation of bacterial outer membrane vesicle (OMVs) whose exteriors are decorated with designer glycotopes (Fig. 2D) have sparked interest in exploiting this simple glycolipid conjugation mechanism for vaccine engineering.

OMVs are 20–250 nm liposomes that are derived from the outer membrane of Gram-negative bacteria. They contain a variety of macromolecules including proteins, DNA, and glycolipids (*e.g.*, LPS) and can serve as nonliving immunogenic mimics of their parental bacteria that stimulate both innate and adaptive immunity and possess intrinsic adjuvant properties [50]. These characteristics are exemplified by OMVs isolated directly from *N. meningitidis*, which induce potent protective immune responses [51] and are now included in the formulation of Bexsero®, a commercial vaccine that protects against *N. meningitidis* serogroup B (MenB) [52]. Studies within the last decade have shown that strong and specific immune responses can be generated against protein antigens heterologously expressed on the exterior or in the lumen of OMVs derived from *E. coli* [53–58].

Several recent publications established that strong immune responses can also be generated against lipid A-bound carbohydrate antigens displayed on OMVs (glycOMVs). For example, glycOMVs displaying *F. tularensis* O-PS elicited significant titers of O-PS-specific serum IgG antibodies (as well as vaginal and bronchoalveolar IgA antibodies) and protected mice that were subsequently challenged with lethal doses of virulent *F. tularensis* [59]. Likewise, high IgG titers were generated in mice immunized with glycOMVs displaying the O-PS from uropathogenic *E. coli* serotype O25b or CPS from *S. pneumoniae* serotype 14 [60,61].

While these recent studies focused on bacterial polysaccharide antigens, OMVs can also be engineered to display TACAs. For example, Valentine *et al.* remodeled the exterior of OMVs with the disaccharide Thomsen-Friedenreich (T) antigen as well as polysialic acid (PolySia) [62], both of which represent potential targets for cancer immunotherapy. As self-antigens, both T antigen and PolySia are poorly immunogenic [63]. However, OMV-mediated delivery of these glycotopes to the immune system generated statistically significant IgG titers against both carbohydrates in mice.

Many factors likely contribute to the strong immune response generated against carbohydrate antigens on OMVs. For example, OMVs contain several microbe-associated molecular patterns (MAMPs) such as lipid A, DNA, and peptidoglycan which enhance inflammation. Furthermore, although the carbohydrate is not covalently linked to a T-cell epitope, proteins on the vesicles may provide T-cell help for carbohydrate-specific B-cells. Finally, like some synthetic liposome adjuvants, OMVs can induce greater cell-mediated immunity than subunit vaccines [64,65]. This latter characteristic may have been critical for the protection induced by glycOMV vaccine candidates against the intracellular pathogen *F. tularensis*.

Overall, glycOMVs represent a promising new approach for carbohydrate-based vaccine development that have the potential to be produced at low cost via large-scale bacterial fermentation. Nonetheless, more studies are needed to establish their clinical viability. For example, the impact from repeated dosing of glycOMVs has not been established; efficacy may decrease over time due to CIES [27]. In addition, the high toxicity of lipid A in humans needs to be addressed. While most lipid A is removed through detergent-extraction of LPS in clinically approved OMVs from *N. meningitidis*, treating glycOMVs in the same way would potentially destroy their conjugated glycotopes. Fortunately, genetic remodeling of the lipid A structure resulted in greatly reduced toxicity with retention of protective efficacy in mice [59]. Whether such genetically detoxified lipid A is safe for use in humans remains to be determined.

Noncovalent association between carbohydrates and T-cell epitopes

Covalent attachment between the antigen and a carrier with a T-cell epitope (*i.e.*, proteins, peptides, or lipids that bind CD1d) is not a strict requirement for carbohydrate-based vaccines. However, they do need to be physically associated to achieve robust immunity [45,66]. For example, a variety of formulations composed of carbohydrate antigens and T-cell epitopes bound to nanoparticles induce T-cell dependent carbohydrate antigen-specific IgGs. These include gold nanoparticles with helper T-cell epitopes [67], synthetic liposomes with helper T-cell epitopes (Fig. 2E) [68], and synthetic liposomes with iNKT epitopes (Fig.

2F) [46]. GlycOMVs (Fig. 2D) likely induce anti-carbohydrate IgGs via similar mechanisms, but the significance of the covalent linkage to lipid A warrants further study. In addition to nanoparticles, other novel formulations for co-delivery of carbohydrate antigens and T-cell epitopes have been developed. In one study by Zhang *et al.*, proteins were genetically fused to a biotin-binding domain and bound to biotinylated polysaccharide antigens, forming complexes analogous to lattice-type glycoconjugates (Fig. 2B) [69]. In addition, Thanawastien *et al.* created a vaccine composed of polysaccharide antigens trapped in a protein matrix (Fig. 2G) [70].

Overall, these formulations could simplify the creation of broadly effective carbohydrate vaccines compared to their conventional glycoconjugate counterparts. Moreover, unlike PGCT and glycOMVs, the formulations can incorporate carbohydrate antigens that are synthesized *in vitro*. This can be beneficial for some targets because *in vitro* synthesis allows for the creation of modified carbohydrate antigens that are more immunogenic, stable, and structurally well-defined. Nonetheless, additional studies comparing the efficacy and safety of these newer formulations with that of marketed glycoconjugate vaccines are clearly warranted.

Conclusion

Several novel technologies have arisen in the past few years that have the potential to overcome the shortcomings of prototypical glycoconjugate vaccines. Carbohydrate synthesis methods and new protein- or lipid-based conjugation strategies have been critical to the creation of more protective formulations. PGCT has emerged as a potential low-cost alternative for the biosynthesis of glycoconjugate vaccines in host organisms that are amenable to large-scale fermentation. Finally, glycOMVs and glyco-modified nanoparticles have developed as highly immunogenic alternatives that eliminate the need for labor-intensive conjugation to a known T-cell epitope. In the case of glycOMVs, the intrinsic adjuvant properties of OMVs [71] and the tunability of their lipid A moieties [72] holds promise for the generation of a self-adjuvanting, non-toxic delivery system for carbohydrate antigens.

The next generation of carbohydrate vaccines will likely involve a combination of these technologies. For example, a recent technology called cell-free glycoprotein synthesis (CFGpS) combines aspects of chemoenzymatic assembly and PGCT and may soon be used to manufacture glycoconjugate vaccines [73]. Moreover, the vaccine candidates developed with these technologies will inform our understanding of immune responses to carbohydrate antigens and in turn guide further vaccine optimization. As carbohydrate vaccine formulations continue to be improved, so too will their clinical potential beyond pathogenic bacteria, into the realms of viruses, parasites, and cancer.

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Figure 1. Immunological mechanisms of antibody generation against carbohydrates

(A) Pure polysaccharides primarily elicit T-cell independent, low-affinity IgM antibodies from B-cells by cross-linking the B-cell receptor (BCR). (B) When a carbohydrate antigen is linked to a protein or peptide carrier, carbohydrate antigen-specific B-cells can internalize both components and present a peptide or glycopeptide on major histocompatibility complex class II (MHC II). Helper T-cells with a T-cell receptor that recognizes the peptide or glycopeptide provide molecular signals that facilitate the production of high-affinity carbohydrate antigen-specific IgGs [74]. (C) When a carbohydrate antigen is linked to a glycolipid carrier such as α -galactosylceramide (α -GalCer), carbohydrate antigen-specific B-cells present the carrier on CD1d. T-cell receptors on invariant natural killer T-cells (iNKTs) recognize the CD1d-bound lipid and provide molecular signals that facilitate the production of high-affinity carbohydrate antigen-specific IgGs.

Figure 2. Formulations that enhance immune responses to carbohydrate antigens

A number of unique formulations have been described for effectively enhancing the immunogenicity of carbohydrates including: (A) neoglycoconjugates; (B) lattice-type glycoconjugates; (C) lipid-based glycoconjugates; (D) glycOMVs; (E) synthetic liposomes with helper T-cell epitopes; (F) synthetic liposomes with iNKT cell epitopes; and (G) protein/polysaccharide matrices.

Figure 3. Glycoconjugate vaccine production using *E. coli* cells

(A) In PGCT, carbohydrate antigens are assembled on Und-PP in *E. coli*. The glycan is flipped to the periplasm via the native *E. coli* flippase Wzx and may be polymerized via Wzy/Wzz. Heterologous expression of PglB is necessary to transfer the glycan to an acceptor sequence in a target protein. (B) Carbohydrate antigens assembled on Und-PP can also be ligated to the core oligosaccharide of lipid A via WaaL, the native O-PS antigen ligase in *E. coli*. The lipid-linked antigen is then shuttled to the outer membrane where it subsequently becomes released in OMVs that are shed from the cell surface. The resulting glycoOMVs can be isolated from the bacteria and used directly for vaccination.