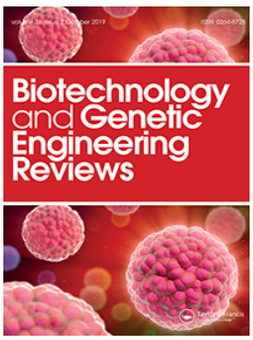

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Glycoconjugate vaccines: some observations on carrier and production methods

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ABSTRACT

Glycoconjugate vaccines use protein carriers to improve the immune response to polysaccharide antigens. The protein component allows the vaccine to interact with T cells, providing a stronger and longer-lasting immune response than a polysaccharide interacting with B cells alone. Whilst in theory the mere presence of a protein component in a vaccine should be sufficient to improve vaccine efficacy, the extent of improvement varies. In the present review, a comparison of the performances of vaccines developed with and without a protein carrier are presented. The usefulness of analytical tools for macromolecular integrity assays, in particular nuclear magnetic resonance, circular dichroism, analytical ultracentrifugation and SEC coupled to multi-angle light scattering (MALS) is indicated. Although we focus mainly on bacterial capsular polysaccharide-protein vaccines, some consideration is also given to research on experimental cancer vaccines using zwitterionic polysaccharides which, unusually for polysaccharides, are able to invoke T-cell responses and have been used in the development of potential all-polysaccharide-based cancer vaccines.

A general trend of improved immunogenicity for glycoconjugate vaccines is described. Since the immunogenicity of a vaccine will also depend on carrier protein type and the way in which it has been linked to polysaccharide, the effects of different carrier proteins and production methods are also reviewed. We suggest that, in general, there is no single best carrier for use in glycoconjugate vaccines. This indicates that the choice of carrier protein is optimally made on a case-by-case basis, based on what generates the best immune response and can be produced safely in each individual case.

KEYWORDS

Glycoconjugate; polysaccharide; vaccine; protein; conjugate; carrier

Abbreviations: AUC: analytical ultracentrifugation; BSA: bovine serum albumin; CD: circular dichroism spectroscopy; CPS: capsular polysaccharide; CRM197: Cross Reactive Material 197; DT: diphtheria toxoid; Hib: *Haemophilius influenzae* type b; MALS: multi-angle light scattering; Men: *Neisseria menigitidis*; MHC-II: major histocompatibility complex class II; NMR: nuclear magnetic resonance spectroscopy;

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OMP: outer membrane protein; PRP: polyribosyl ribitol phosphate; PSA: Polysaccharide A1; Sa: *Salmonella*; St.: *Streptococcus*; SEC: size exclusion chromatography; Sta: *Staphylococcus*; TT: tetanus toxoid; ZPS: zwitterionic polysaccharide(s).

Introduction

Polysaccharides are attractive antigens to use as vaccines because they are often key exposed components of the bacterial capsule and tumours (Hütter & Lepenies, 2015; Zimmermann & Lepenies, 2015). The capsule protects bacterial cells from phagocytes (Finlay & Falkow, 1997) and is located on the cell surface, readily available to interact with cells of the adaptive immune system. In a similar way, glycans on the surface of cancer cells are useful strategic targets for developing cancer vaccines. B cells are able to bind to polysaccharides that possess suitable complementary binding sites, typically repeating patterns that cross-link multiple receptors (Maglione et al., 2014). The process depends on interaction with other immunogenic molecules such as lipopolysaccharides, to show that the non-self-polysaccharide is also a risk to the host body (Bretscher & Cohn, 1970; Snapper & Mond, 1996). Once activated, the B cell releases IgM isotype antibody (Maglione et al., 2014; Snapper & Mond, 1996), a pentameric antibody which gives it high avidity (Niles, Matsuuchi, & Koshland, 1995) but low affinity as no affinity maturation occurs (Eisen & Siskind, 1964).

A major histocompatibility complex class II (MHC-II) bound antigen can interact with CD4 + T cells and become activated (Janeway, 1989), causing isotype switching and affinity maturation in B cells. The B cells will now make high affinity IgG antibodies rather than IgM (Guttormsen et al., 1999), and memory cells will develop for a longer-lived immune response (Avci & Kasper, 2010). Without MHC-II binding, no T cells become involved and so these advantages of longer-lived immunity do not occur, and there is a less marked improvement on re-inoculation. This is a major issue when trying to use polysaccharides as vaccines which in general do not bind to MHC-II (see, for example, Zimmermann & Lepenies, 2015).

Protein antigens are taken up by antigen-presenting cells and digested. MHC II proteins fold in the endoplasmic reticulum where a complex is formed with a protein known asinvariant chain (Ii) (Roche & Cresswell, 1990). This precursor molecule is then transported to late endosomal compartments where the invariant chain is cleaved by cathepsin proteases. However, a short fragment is retained by the binding groove of MHC II. The fragment can be exchanged with higher affinity molecules in endocytic compartments, which will be derived from proteolytically degraded

antigens. Often a human leukocyte antigen initially binds and acts as a catalyst for further binding with pathogen antigens. The specific antigen that is exchanged depends on the charge distribution, geometry and hydrophobicity of the binding groove, as well as the catalyst currently in the binding groove (Wieczorek et al., 2017). The binding groove itself is highly variable as it is produced from multiple gene locations and codominantly expressed alleles (Janeway, Travers, Walport, & Shlomchik, 2001). Ideally there would be a simple method to predict this binding to allow more comprehensive control in vaccine design. However, while progress has been made developing bioinformatic algorithms to predict antigen binding to MHC II, these remain imperfect (Lyngaa et al., 2014; Paul et al., 2013). Antigen fragments are presented in the MHC-II on the cell surface where they can interact with CD4 + T cells to activate B cells triggering differentiation and clonal expansion. Therefore, unlike most polysaccharide antigens, the secondary response to infection is improved due to the presence of high levels of IgG, making proteins more useful as vaccines.

Unfortunately, for pathogens such as encapsulated bacteria, the only available surface antigens are polysaccharides (Kasper, 1986). As they generally cannot be processed using the MHC-II and T cells, with the exception of zwitterionic polysaccharides (ZPS), these are not optimal molecules to use as vaccines (Avci & Kasper, 2010; Guttormsen et al., 1999). Fortunately, there is a way to improve the immunogenicity of a polysaccharide antigen. Glycoconjugate vaccines add the positive traits of a protein antigen to a polysaccharide antigen by covalently binding the two together. When taken up by antigen-presenting cells, the conjugate molecule will be digested, and covalently-linked fragments containing both polysaccharides and proteins can bind to the MHC-II (Avci, Li, Tsuji, & Kasper, 2011). This is presented to T cells, triggering isotype switching and B cell differentiation into memory cells, thus acting as a good vaccine.

An alternative method may be available in which instead the vaccine triggers the production of carbohydrate specific CD8 + T cells, which being cytotoxic can kill malfunctioning body cells directly, which would be of immense importance in combatting cancer. Tumour marker gangliosides, sialic acid-containing glycosphingolipids with extracellular polysaccharide head groups, are expressed at high levels on cancer cells (Daniotti, Lardone, & Vilcaes, 2016; Daniotti, Vilcaes, Torres Demichelis, Ruggiero, & Rodriguez-Walker, 2013; Saragovi & Gagnon, 2002). This carbohydrate head at the cell surface is readily available to interact with cells of the immune system, and therefore acts as an antigen for cancer cells and so is a potential cancer vaccine target. However, a number of possible examples proved to be poor vaccines, as they were either insufficiently immunogenic or did not provide clinical benefits (Chapman et al., 2000; Kawashima et al., 2003; Kim et al., 2011; Kirkwood et al., 2001; Ragupathi et al., 2003, 2000). In

these instances, the vaccines worked by triggering the production of antibodies. However, Tong et al. (2019) have produced synthetic antigens of tumour marker gangliosides linked to polyamidoamine scaffolds to illicit responses in γδ T cell receptor and CD8+ phenotypes that seem to be therapeutic.

To produce a glycoconjugate vaccine, each part is produced individually then conjugated into one molecule. Only the polysaccharide part is unique for a given vaccine. Usually one of a small group of proteins is used, usually inactivated toxins, 'toxoids'. Toxins are produced by some pathogenic cells, which can be used as bio-factories (Chung et al., 2016; Nielsen, 1967). They are inactivated, often with formaldehyde (Nielsen, 1967), and are shown historically as strongly antigenic and safe, such as tetanus toxoid (TT), in use as a vaccine since 1924. It is also possible to use comparatively new non-toxic mutants of TT such as Cross Reactive Material 197 (CRM197) rather than diphtheria toxoid (DT) (Giannini, Rappuoli, & Ratti, 1984; Rappuoli, 1983). However safe a toxoid is known to be, use of a non-toxic mutant is inherently safer to produce than a toxoid and alleviates risk of a toxoid that is not fully inactivated, or from which formaldehyde has not been removed.

Pathways to conjugation

Figure 1 shows 3 pathways to glycoconjugate vaccine production (Jones, 2012). In Pathway (a) the glycoconjugate vaccine is produced by coupling monofunctional oligosaccharides (after depolymerisation of the parent polysaccharide) or bifunctional oligosaccharides at low coupling efficiency, and either through direct attachment to the carrier protein or indirect attachment (through a linker). Pathway (b) involves activation of higher molecular weight polysaccharides (without depolymerisation) and conjugation through non-specific chemistry to multiple carrier proteins to give a very high molecular weight complex of >1MDa size and (c) involving reduced mass polysaccharides with multiple activations coupled to LPS-depleted outer membrane protein (OMP) vesicles.

Although the bulk of protein carriers are related to toxins, other examples are known. As any antigen that is perceived as non-self will be sufficient to induce an immune response, there is a vast array of known proteins at our disposal to use as carrier. For example, keyhole limpet hemocyanin has been used as a carrier for glycoconjugate vaccines against *Candida albicans* (Liao et al., 2016) and even some cancers with distinct molecular markers (Song, Zheng, Liu, Zhou, & Ye, 2017). Bovine serum albumin (BSA) is a well-characterised protein that has also been used, for example against *Aspergillus fumigatus* (Komarova et al., 2015). All these proteins can be produced by growing cell cultures to high cell density and then purifying the protein. This is particularly easy for toxoids, as the toxin is naturally secreted

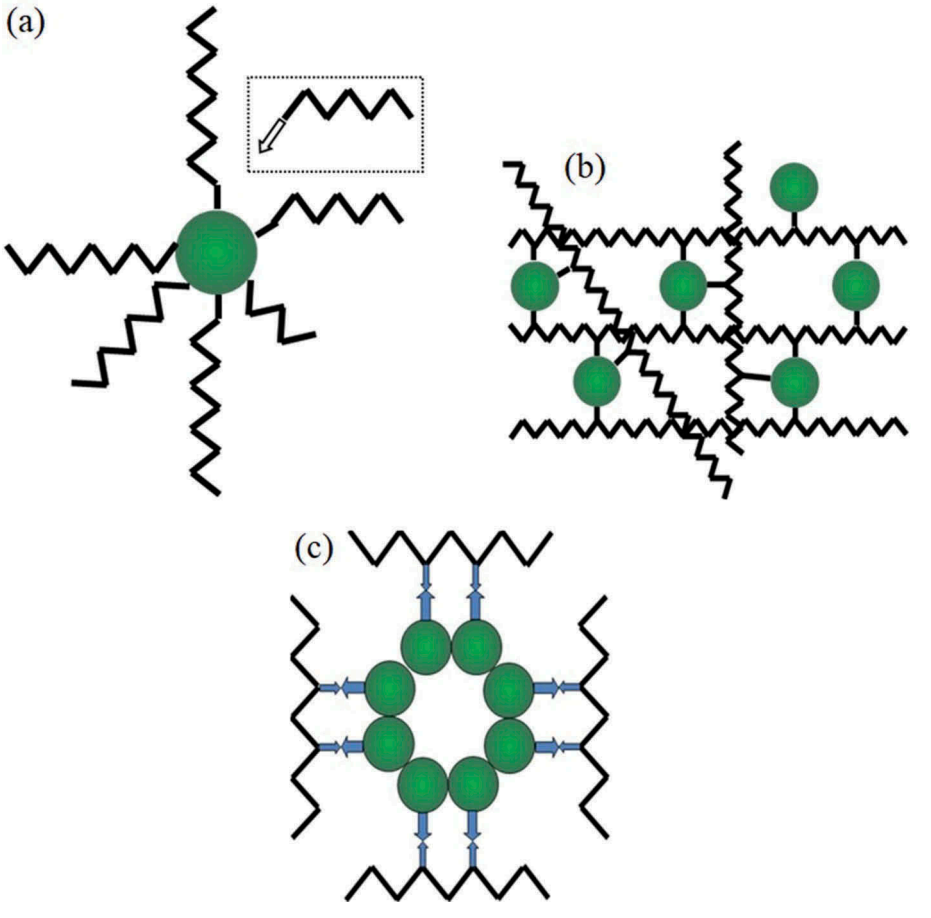

Figure 1. The three pathways to glycoconjugate vaccine production. The ellipsoid(s) shown in green represent(s) a carrier protein (popularly tetanus toxoid protein, diptheria toxoid protein or CRM197), the black zig-zags represent the capsular polysaccharide, and the white arrow the activated end region. (a) Glycoconjugates produced from oligosaccharides with a single attachment site, or limited crosslinking through two sites. (b) Glycoconjugates produced by multiple activation sites on high mass polysaccharides and multiple attachments to the carrier protein, producing crosslinked network. (c) Glycoconjugates produced by attachment of 'size-reduced' polysaccharides to outer membrane protein vesicles through multiple attachment sites. From Jones (2012) and reproduced by permission of the Royal Society of Chemistry.

by pathogens (Chung et al., 2016; Nielsen, 1967). However, it is also possible to apply cloning strategies in order to place the encoding gene of interest into a less pathogenic organism.

The polysaccharide portion of the glycoconjugate has in the past been more problematic to produce. Unlike proteins, polysaccharides are not produced from a direct set of instructions that give an identical structure every time. Instead, they are produced enzymatically, which gives variation or 'polydispersity'. This depends on the local kinetics, thermodynamics, and relative proportions of subunits and enzymes during the polysaccharide

production. This means that the easiest means by which to generate antigens that are similar to the wild type pathogen is to purify the polysaccharides from a bacterial culture. However, as with proteins, this is undesirable, as it sometimes requires large pathogen cultures to be grown. It is possible that small fragments could be synthesised *in vitro*, but even if the epitope has the same sequence it will also need the same three-dimensional structure to interact correctly with immune receptors. Nonetheless advances have been made in synthesis and fractionation making it possible to produce polysaccharide fractions which are near-monodisperse. Greater polydispersity arises from the variability in the extent of conjugation, rendering molecular weight (molar mass) distribution and other characterisation methods particularly important (Harding, Abdelhameed, Gillis, Morris, & Adams, 2015).

Once the components have been produced, they need to be linked together covalently. However, just as the synthesis of polysaccharides results in polydispersity, this step can also introduce variation. On the surface of each molecule there are a number of functional groups with the potential to generate a link under the right conditions. One way of limiting this polydispersity is to target the formation of specific bonds, as described by Adamo et al. (2014).

Once a vaccine is made, it must be tested to ensure it is of acceptable quality. Each part, as well as the link between them, must be stable. Of the two components, the most important to remain stable is the polysaccharide, as this must be able to mimic accurately the structure of the target antigen, while the protein only needs to be recognised as non-self. The link between the two molecules also needs to remain intact, although there must be an acceptable level of breakdown. As the entire vaccine must be produced on an industrial scale, care should be taken to predict manufacturing issues to prevent problems later in development. In this regard, physicochemical methods have been proven to be important in assaying the molecular integrity of conjugated vaccines.

Physico-chemical checks on the molecular integrity and stability following a production process

Besides traditionally used gel permeation chromatography or size exclusion chromatography (SEC) a number of physico-chemical techniques have been shown to be particularly useful as tools for monitoring macromolecular integrity after production/processing. High field nuclear magnetic resonance (NMR) spectroscopy has been shown to provide an important check on the chemical integrity of products after the industrial production of glycovaccines, and follows WHO (World Health Organisation) recommendations. NMR provides a routine release test for identity and testing conformity for the bulk monovalent, blended, activated and conjugated polysaccharide component in conjugates (Berti & Ravenscroft, 2015), and examples are quoted below. A complementary method to NMR is circular dichroism spectroscopy (CD)

which can monitor secondary structure changes in the carrier protein before and after conjugation processing.

Another technique which is becoming increasingly important is analytical ultracentrifugation (AUC) (Harding et al., 2015), now recognised as a gold standard technique for assessing the molecular integrity and freedom from aggregation of monoclonal antibody preparations, particularly at high concentration, and is a matrix-free analytical tool, avoiding problems of non-inertness. A combination of sedimentation velocity and sedimentation equilibrium methods from the AUC, reinforced by complementary methods such as SEC-MALS and viscometry are providing (i) absolute molar mass (molecular weight) information (mainly through the weight average molar mass, M_w) (ii) heterogeneity information either through the polydispersity index (M_z/M_w or M_w/M_n) , where M_z and M_n are the z- and number- average molar masses respectively, and/or also molar mass or sedimentation coefficient distributions (Figure 2) (iii) the overall conformation or shape of the carrier proteins (Figure 3) and (iv) conformational flexibility (in terms of persistence length or power-law parameters) of the glycoconjugate and the constituent polysaccharides (Figure 4). Table 1 shows the change in persistence length (a measure of flexibility) and the mass per unit length of the *Haemophilus influenzae* Type b (Hib) polysaccharide after activation and complexation with TT protein.

These methods can also provide interaction information: self-assembly and reversibility, aggregation phenomena (together with other methods like dynamic light scattering (Harding et al., 2015) and possible complex interactions with other molecules such as plasma proteins.

The above discussion gives a snapshot of the power of analytical tools in assessing macromolecular integrity of the vaccines after production. Before presenting further observations on research into glycoconjugate vaccine design and production, the following section on zwitterionic capsular polysaccharides (ZPS) considers an exception to the general idea that polysaccharides are unable to bind to MHC-II and thereby elicit long-term immunity. Because of their ability to invoke long-lived T-cell based immunity, ZPS have been exploited as carriers to explore the development of all-carbohydrate vaccines, particularly in the field of cancer vaccine development. The following section details the characteristics of some of these exceptional ZPS (with particular emphasis on bacterial capsular examples), how they activate T cells and how they have been used in glycoconjugate cancer vaccine research to date.

Zwitterionic capsular polysaccharides

Unlike most known naturally-occurring carbohydrate structures (which are either negatively charged or uncharged, and which are unable to activate T cells), some glycans possessing dual or zwitterionic charge motifs which appear to be able to activate the traditional MHC-II route to activate T cells

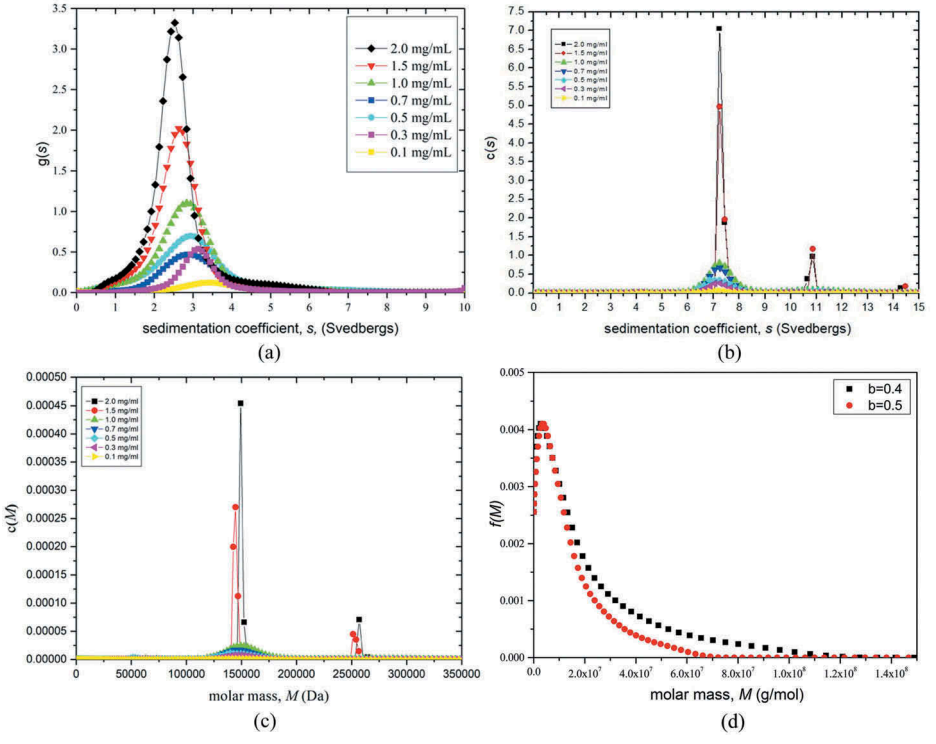

Figure 2. Heterogeneity/purification determinations using analytical ultracentrifugation (a) sedimentation coefficient distribution plots g(*s*) vs *s* showing continuous but unimodal distributions at different concentrations of a *Streptococcal pneumonia SP5* capsular polysaccharide preparation. From Harding et al. (2012) and reproduced with permission from Elsevier. (b) sedimentation coefficient distribution plot (corrected for diffusive effects) c(*s*) vs *s* of a tetanus toxoid preparation showing the presence of dimer (up to 14%) at higher concentration. (c) the corresponding molar mass distribution c(*M*) vs *M* for the tetanus toxoid preparation. From Abdelhameed et al. (2012) and reproduced by permission of Elsevier. (d) molar mass distribution *f*(*M*) vs *M* from sedimentation velocity of a large Hib PRP-TT conjugate for a plausible range of the conformation parameter *b* using the Extended Fujita approach of Harding et al. (2011). The size and distribution of the assembly would suggest a 'model c' (Figure 1c) type of assembly of Jones (2012). From Abdelhameed et al. (2016) and reproduced by permission of the Nature Publishing Group.

without the assistance of a carrier protein (Cobb & Kasper, 2005; Cobb, Wang, Tzianabos, & Kasper, 2004). Characteristically, these zwitterionic glycans possess both positive (such as free amine) and negative (such as carboxylate or phosphate) centres within a repeating molecular unit (Tzianabos, Onderdonk, Rosner, Cisneros, & Kasper, 1993; Tzianabos, Wang, & Lee, 2001). This shows a similarity to the repeating structure of peptides, which also contain amine and carboxylate groups, suggesting it is the interaction of these charged groups with the MHC II binding groove that allows ZPS to bind. A diverse range of bacteria produce ZPS including type 1 *Streptococcus (St.) pneumoniae* (the SP1 polysaccharide capsule) (Velez,

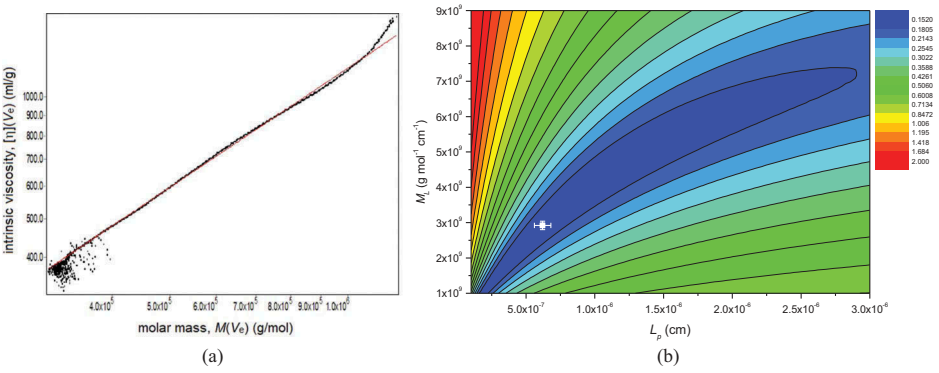

Figure 3. (a) Conformational 'power law' plot from SEC-MALS for a capsular polysaccharide from *Streptococcus pneumoniae* SP1. The slope or Mark-Houwink-Kuhn-Sakurada parameter $a = 0.8$, corresponds to a flexible coil polysaccharide. From Harding et al. (2012) and reproduced by permission from Elsevier (b) Plot of mass per unit length M_L versus persistence length L_p evaluation for the Hib PRP-native capsular polysaccharide. The plot yields $L_p \sim 7.0 \times 10^{-7}$ (cm) and $M_L \sim 3.8 \times 10^9$ (g.mol^{-1}.cm^{-1}) at the minimum target (error) function (indicated by the white cross). From Abdelhameed et al. (2016) and reproduced by permission from the Nature Publishing Group.

Lewis, Kasper, & Cobb, 2009), *Staphylococcus (Sta.) aureus* (the CP8 polysaccharide) (O'Riordan & Lee, 2004), and *Morganella morganii* (the O-chain antigen) (Young et al., 2011). Also, see the review of Neff et al. (2016) which gives examples from intestinal bacteria. However, the most intensively studied example of capsular bacterial ZPS is the polysaccharide A1 (PSA) from *Bacteroides fragilis* (Figure 5). PSA averages over 100 kDa in size, equivalent to more than 100 repeating units with both positive and negative charged groups.

B. fragilis is an obligately anaerobic bacterium which forms part of the natural human gut flora. However, it is also implicated (because it is the most commonly isolated bacterium) in formation of abscesses that result from secondary peritonitis arising due to surgery and bowel wounds (Kasper et al., 1977). The immune response that leads to abscess formation involves both innate and adaptive systems. The contribution from the adaptive immunity response depends on activation of $CD4^+$ T cells. In particular, the capsular polysaccharide (CPS) complex of *B. fragilis* with PSA is needed for activating the $CD4^+$ αβT cell receptor (TCR)-carrying T cells that are required for abscess formation (Tzianabos et al., 1993). Indeed, PSA elicits a clonal expansion of a subset of suppressive $CD45RB^{low}CD4^+$ effector memory T cells and thus a highly specific $CD4^+$ T cell response (Johnson, Jones, & Cobb, 2015a). It is also a toll-like receptor 2 (TLR-2) agonist (Wang et al., 2006).

The original suggestion of the involvement of T cells comes from several lines of compelling evidence reviewed by Cobb and Kasper (2005). The broad mechanism by which polysaccharides such as PSA activate T cells was

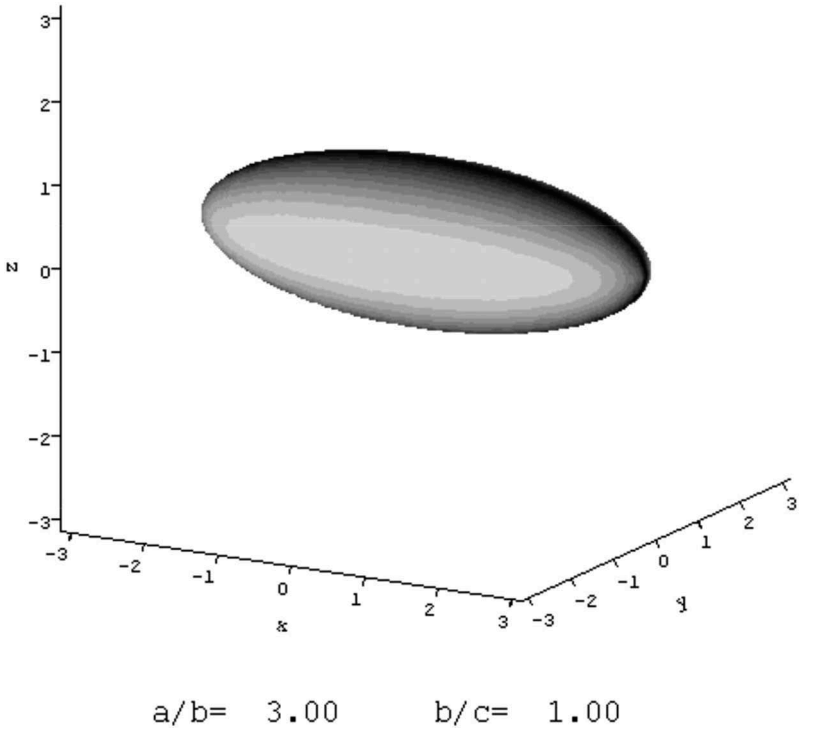

Figure 4. Ellipsoidal (prolate) representation from hydrodynamic analysis of the conformation of monomeric tetanus toxoid protein using the program ELLIPS1 (García de la Torre & Harding, 2013). The protein is shown to be asymmetric with an axial ratio ~ 3. Coincidentally this appears to reproduce the 'guessed' cartoon representation given earlier by Astronomo and Burton (2010). Reproduced from Abdelhameed et al (2010) by permission from Elsevier.

Table 1. Values for the mass per unit length M_L and the persistence length L_p from global hydrodynamic analysis (combination of SEC-MALS, AUC and viscosity of Hib capsular polysaccharide, before and after activation and conjugation with tetanus toxoid protein (Abdelhameed et al., 2016).

Sample	$10^{-7} \times M_L$ (g.mol^{-1}.cm^{-1})	$10^{7} \times L_p$ (cm)
PRP-native	380 ± 50	7.0 ± 1.0
PRP-ADH	600 ± 50	7.0 ± 1.2
PRP-TT	1070 ± 50	4.5 ± 0.3

first described by Cobb et al. (2004), who showed that ZPSs are first processed to low molar mass carbohydrates through a nitric acid-based mechanism and then presented to T cells that recognise the MHC/ZPS complex with αβTCRs, through the MHC II endocytic pathway. This was a ground-breaking finding, since it was believed at that time that only peptides (not polysaccharides) elicit T cell activation via MHCII antigen presentation; indeed, the work suggested a fundamental shift in the MHCII presentation paradigm (Cobb et al., 2004). The immunological events that

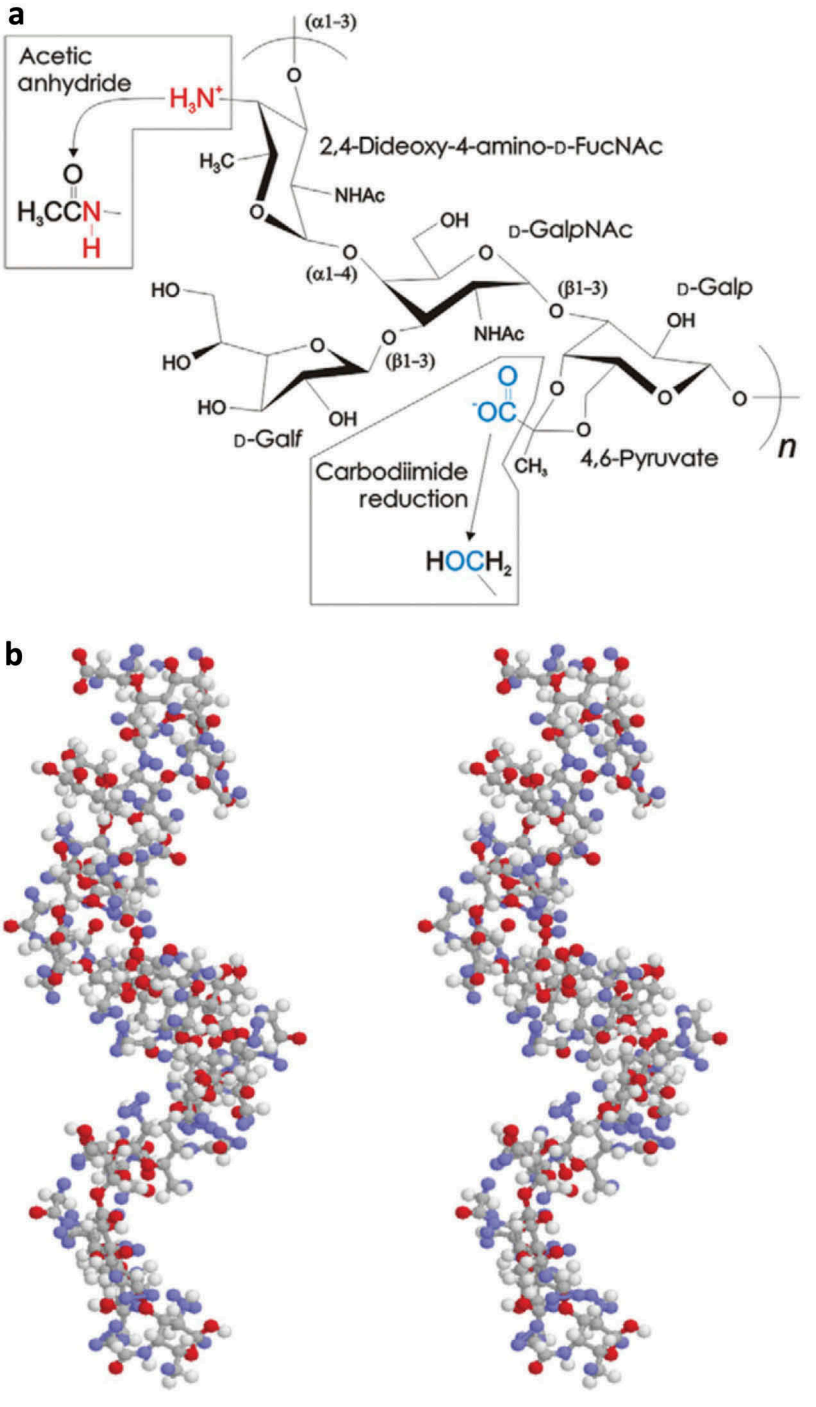

Figure 5. The molecular structure of *B. fragilis* PSA showing (A) one tetrasaccharide repeating unit (and the chemical modifications required to abolish the negatively-charged free amine (red) and the negatively-charged carboxylate (blue) groups); (B) NMR spectroscopy-derived average solution structure (double image for stereo view) which highlights the right-handed helical conformation (taken from Kreisman et al., 2007). Reproduced by permission from Oxford University Press.

result in abscess formation comprise: (1) activation of antigen-presenting cells (as the ZPS antigens are endocytosed) which in turn produces TNF-α and (2) activation of T cells produces cytokines and chemokines that amplify the response and recruit PMNs to the site of infection.

In addition to its dual charge, another unusual characteristic of PSA is that it adopts a stable extended helical conformation in solution with two repeating units per turn and a pitch of 20 Å, as revealed using total correlated spectroscopy and nuclear Overhauser spectroscopy NMR spectroscopy (NOESY) (Figure 2(b)). The carboxylate lies in repeated grooves every 15 Å whilst the amine groups lie on the outer surface of the glycan (Wang, Kalka-Moll, Roehrl, & Kasper, 2000). Whilst Cobb et al. (2004) established that oxidative processing of PSA to fragments of < 15 kDa is required in order to obtain successful MHCII presentation, another study by Kalka-Moll et al. (2000) demonstrated that fragments less than ~5 kDa exhibit severely reduced T-cell activation activities. These collective findings suggested that the epitope is more complex than merely one repeating unit and could involve a conformational component. When this possibility was investigated by Kreisman, Friedman, Neaga, and Cobb (2007), it was found that the functional epitope from PSA requires a specific conformation that is stabilised by adjacent repeating units, as well as the zwitterionic charge. These researchers interrogated the conformation of PSA using far-UV CD. Intriguingly the CD spectra confirmed a helical conformation and a similarity to the spectrum of myoglobin possessing a spectral minimum at 225 nm (but no dual minima characteristic of alpha helices) and a spectral maximum at approximately 205 nm (Figure 6), characteristic of a helical character, and bearing no resemblance to spectra of control non-helical carbohydrates or dextran polysaccharides that exhibit no optical activities in this wavelength range.

Interestingly, the CD spectra of ozone-cleaved PSA exhibited progressive loss of helical nature (revealed as shallowing of spectral peaks/maxima and minima) as smaller sized fragments were analysed. PSA fragments of 1 kDa (one repeating unit) or smaller exhibited no helical character at all. When these different sized fragments were compared for their ability to bind to MHCII protein, it was fragments in the 3–30 kDa range that exhibited maximum MHCII binding.

Fragment sizes greater than this bound only modestly to MHCII molecules whilst fragments less than this exhibited poor or no binding. The fragments in this size range also retained their helical character, suggesting conformation was important. Indeed, the authors suggest that the MHCII binding maximum correlated with the dominant fragment size of 3 kDa (three repeating units of the PSA structure) which may be larger than the twelve amino acid residues claimed previously for proteins. However, later studies claimed that the α-helical structural characteristic may not play as

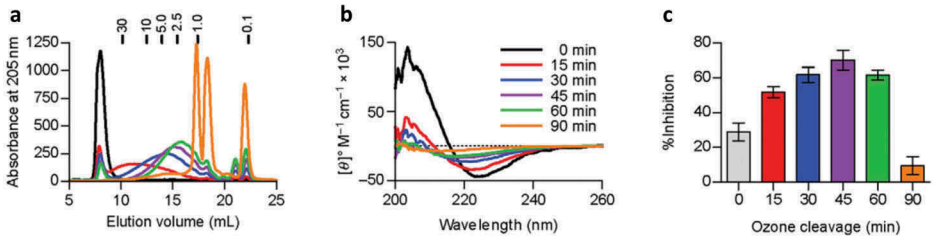

Figure 6. Effect of the size of PSA on its structural conformation and function. (a) overlaid Superdex-75 elution characteristics of ozone-cleaved PSA samples; (b) CD spectra of ozone-cleaved PSA, showing the progressive loss of helical character as more ozone-mediated breaks are introduced. Full-length PSA is shown at 0 min and exhibits greatest helicity; the smallest fragments are in 90-min incubated samples in which the shallow spectra indicate loss of helicity; and (c) PSA binding to MHCII protein, measured by comparison with a conventional peptide antigen. Colour coding of fragment sizes are retained through all panels. Taken from Kreisman et al. (2007). Reproduced by permission from Oxford University Press.

a significant role in immune activation after all. Experiments in which PSA was used as a carrier to the tumour-associated carbohydrate antigen/hapten Thomsen-nouveau (Tn = alpha-D-GalNAc-OSer/Thr) to make an entirely polysaccharide based glycoconjugate vaccine resulted in retained T-cell mediated immune responses (De Silva et al., 2012), but less α-helical conformation (Trabbic, De Silva, & Andreana, 2014). However, although helicity does indeed appear to be reduced at 217 nm, nevertheless the spectra indicate that a detectable level of helicity still remained in the conjugate (Figure 7).

Since the discovery by Cobb et al. (2004) that PSA is able to prompt T-cell mediated immune responses, there have been successful applications of this knowledge towards development of all-carbohydrate vaccines, especially cancer vaccines (2012; De Silva, Wang, Chidley, Appulage, & Andreana, 2009; Nishat & Andreana, 2016; Trabbic et al., 2014), and regarding potential strategies for preventing inflammation using PSA-experienced T cells (Johnson, Jones, & Cobb, 2015b, 2018), highlighting the considerable future potential of using ZPS to control immune responses.

Comparison of glycoconjugate and polysaccharide vaccines

The act of conjugating a polysaccharide to a protein may alter some aspects of its structure, and its effect on the immune system. For this reason, wherever possible, our review includes comparisons of unconjugated polysaccharide vaccines with glycoconjugate vaccines in which the same antigenic molecules have been used. This has previously been reviewed in a meta-analysis by Mitchell, Kelly, Pollard, and Trück (2014), which focused on the mechanism of action of each vaccine in terms of which immune system cells were stimulated by each vaccine. The results generally show that immediately after vaccination, glycoconjugate vaccines show an almost

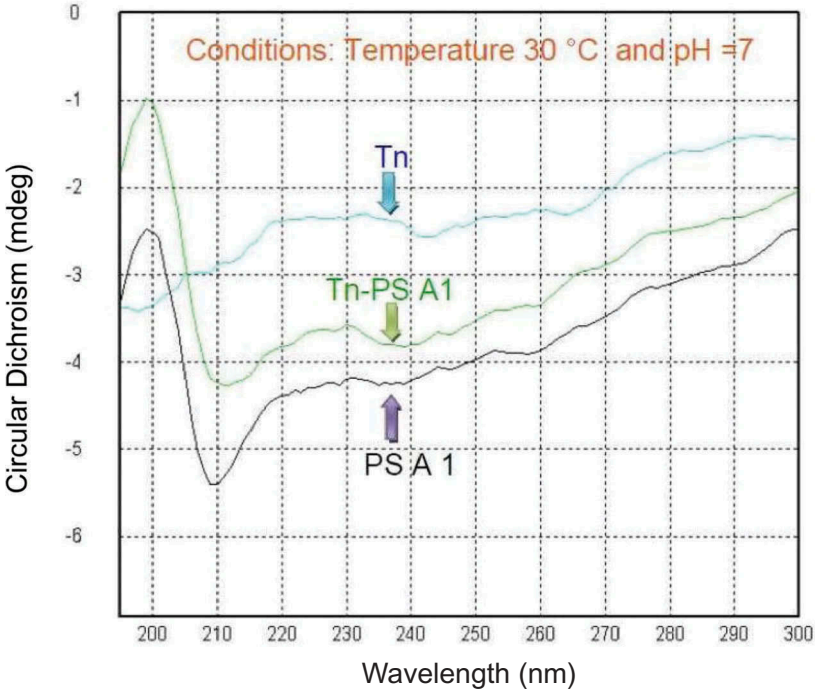

Figure 7. CD spectra obtained for PSA (dark blue spectrum), the Tn-PSA conjugate (turquoise) and the Tn amino-oxy sugar (sky blue). Adapted from Trabbic et al. (2014). Reproduced by permission of the Royal Society of Chemistry.

identical response to polysaccharide vaccines. However, they also found that upon a second vaccination, in the majority of cases a stronger response was seen in the groups that had received glycoconjugate vaccines. This is consistent with theories that glycoconjugate vaccines stimulate B memory cell development.

Two other studies investigated vaccines to Hib using polyribosyl ribitol phosphate (PRP) as the polysaccharide antigen. PRP is a CPS, and in both studies it was compared on its own and conjugated to CRM197. First, Rothstein et al. (1991) tested different combinations of conjugated/unconjugated vaccines on 7- to 15-month old infants with inoculation performed three times. Their findings showed that if all three vaccinations used the conjugated vaccine, or if only the last one was replaced with pure polysaccharides, the responses gave similar mean antibody levels. These levels were only reduced if two of the three inoculations were replaced with polysaccharide only. This suggests that polysaccharide vaccines may not always be inappropriate for use as booster vaccinations. Steinhoff et al. (1991) looked specifically at the effect of the vaccine on individuals immunocompromised by HIV. The population studied were split into four groups depending on severity of infection (HIV-, HIV+, asymptomatic HIV+, and

AIDS). It was found that the conjugate vaccine gave a three-fold increase in antibody titre over the pure polysaccharide vaccine unless infection had progressed to AIDS, at which point they responded poorly to both vaccinations. This suggests that glycoconjugate vaccines may be more suitable for use in moderately immunocompromised patients.

Commercially available *Neisseria menigitidis* (Men) vaccination typically include CPS from serotypes A, C, W, and Y, all conjugated to CRM197. This vaccine was used in the following three studies. Jacobson et al. (2013) studied the persistence of immunity after 5 years with either polysaccharide or conjugate vaccines. It was found that while the polysaccharide vaccine gave a 'less robust' response, it was still adequately protective. This suggests that whilst a conjugate vaccine may have increased protection, it is not always necessary, as the polysaccharide vaccine may still be protective. However, this does not take into account how the response may vary between different groups (e.g. immunocompromised) and the paper suggests that the response may wane to unsafe levels beyond the five years tested. Ramasamy et al. (2014) attempted to explain the differences observed in antibody titre observed after testing conjugated versus polysaccharide vaccine. It found that the polysaccharide vaccine gave better protection against serotype C strains, whilst the conjugate vaccine gave better protection against strains belonging to serotype W. The differences may be due to a lower titre of polysaccharide C conjugated to the CRM197 protein. However, the mechanism by which the response to serotype W is reduced in the pure polysaccharide vaccine is unclear, although it is suggested that there may be some sort of negative feedback mechanism present when polysaccharide vaccines are used. This may be explained by O'Connor et al. (2017), who found that as well as being less effective at eliciting a response, polysaccharide vaccines might take a role in actively depleting B memory cells when used as a booster vaccine, a mechanism that may also explain the findings of Rothstein et al. (1991).

Borja-Tabora et al. (2015) and Holme et al. (2015) studied a meningococcal vaccine (Men) with the serotypes A, C, W and Y polysaccharides either linked to TT as a glycoconjugate or as a polysaccharide-only vaccine. Holme et al. (2015) examined immediate differences between the two vaccines, while Borja-Tabora et al. (2015) studied the difference in the persistence of immunity after 5 years. Both studies found that antibody titres were higher and persisted in a greater number of patients when they were inoculated with the conjugate rather than polysaccharide-only vaccine.

All comparative studies of conjugated versus unconjugated vaccines against *St. pneumoniae* used CRM197 as the carrier protein. In each case, a 23-valent polysaccharide vaccine was compared to either a 7- or 10-valent conjugate, with comparisons being drawn only for those serotypes that were present in both vaccines. Simple comparisons of the effectiveness of

conjugated and unconjugated vaccines were performed for specific populations. Jackson, Gurtman, van Cleeff, Frenck et al. (2013) showed that in people aged 60–64, increased antibody titres were seen for the majority of serotypes analysed when the conjugate vaccine was used. Similarly, in a Japanese population aged over 80, a higher titre was seen with the conjugate vaccine (Namkoong et al., 2015). While in this case all vaccines were safe, there was a slight increase in swelling/redness from the conjugate vaccine. While this was mild, it is interesting to note that an increase in side effects seems to be linked to an increase in response. In patients with systemic lupus erythematosus, both conjugate and polysaccharide vaccines for pneumonia-causing *St. pneumoniae* were found to be safe, but neither was superior in terms of the protection they provided (Grabar et al., 2017).

It has been shown that booster vaccinations may play a significant role in improving vaccination protection success of the frail hospitalised elderly against *St. pneumoniae* (MacIntyre et al., 2014). It was found that immediately after vaccination with a 7-valent conjugate vaccine (PCV7) there was a significant increase in titre of antibodies against serotypes V, and 23F strains, whilst the 23-valent polysaccharide vaccine (23vPPV) had increased titres for serotypes 3, 19F and 19A strains. No major benefit of one vaccine over the other was seen, as all vaccines were protective initially. If a booster polysaccharide vaccination was administered to the conjugate vaccine group, antibody titres were increased for serotypes 6A, 9V, 18C and 23F, but waned in the group without booster vaccinations.

This result was corroborated by Jackson, Gurtman, van Cleeff, Jansen et al. (2013), who also found that a booster vaccination with a polysaccharide vaccine after a glycoconjugate vaccine gave a greater antibody response than polysaccharide vaccine alone. Vandecasteele, De Bacquer, Caluwe, Ombelet, and Van Vlem (2018) found that a conjugate vaccine was less able to provide a boosting effect on vaccination against *St. pneumoniae* in patients previously inoculated with unconjugated vaccine, but at the 1-year endpoint all vaccines studied provided acceptable levels of protection. Conversely, other papers found that booster vaccination may be more effective if they are glycoconjugate, particularly in populations immunocompromised by HIV (Lu, O'Halloran, Williams, & Harpaz, 2017). In addition, in asplenic patients, a difference is also seen depending on the order of conjugated/unconjugated vaccinations. Rezai, Ghaffari, Mahdavi, Bahari, and Ala (2017) reported that for asplenic patients, inoculation with a conjugate vaccine is more effective when it acts as a complimentary booster vaccine to an unconjugated vaccine, based on IgG titres observed in each case.

Two other examples of these types of comparisons have been reported. One report investigated unconjugated versus glycoconjugated cholera vaccines (Alam et al., 2014), and found that in a mouse model, unconjugated

vaccine did not induce the production of IgG. However, IgM production was comparable between the two vaccine types. Finally, a typhoid vaccine is currently being studied by Jin et al. (2017). While this trial is ongoing, it appears that the titre of antibodies is increased using conjugate vaccines, but that protection levels are essentially the same as comparable unconjugated vaccine.

Comparison of carriers used in glycoconjugate vaccines

If a polysaccharide antigen can be conjugated to multiple carriers with equal ease and stability, tests must be performed to determine which compound is the best at producing an immune response. The following section describes some of the recent advances in this area.

A range of vaccines have been studied by Gao, Lockyer, Burkin, Crane, and Bolgiano (2014) to establish common features in their stability, such as the effect of storage at 2-8°C, −20°C, and repeated freeze/thaw cycles. While it was found that there were no significant differences in the majority of cases, storage at −20°C did have a negative impact on the stability of glycoconjugate vaccines made with CRM197.

A number of studies have been performed on vaccines for Hib. It has been found by Vella and Ellis (1991) that CRM197 conjugates were poorly immunogenic in comparison to OMP conjugates. The CRM197 conjugates only became effective when they were administered along with DT, which had an adjuvating effect. However, as commercial vaccines were used with different polysaccharides (OMP-PRP vs CRM197-Hib oligosaccharide) in this study, the comparison is not direct. The currently licensed Hib vaccine contains TT, but studies have been performed to challenge its effectiveness with new vaccinations. When TT and CRM197 were compared as carriers for PRP (Togashi et al., 2016), no significant differences were found between the two carriers in terms of their immunogenicity, although the CRM197 conjugate had an increased local reaction. In contrast, it has been found by Akeda et al. (2018), that CRM197 conferred an increased bactericidal activity compared to the TT.

In a study on MenACWY conjugated to TT or CRM197 by Bona et al. (2016), no significant differences were found in the responses between the two vaccines, despite having subtle variations in polysaccharide compositions in those cases.

Two other studies have made direct comparisons of Hib and meningococcal (Men) vaccines. Otto, Burkin, Amir, Crane, and Bolgiano (2015) compared their ability to interact with aluminium-based adjuvants rather than comparing the activity of the vaccines. It was found that $AlPO_4$ was less able to interact with the vaccines than $Al(OH)_3$, but it was also found that the degree of interaction varied depending on the protein carrier. TT

interacted more with the adjuvants than CRM197, regardless of whether the Hib or MenC polysaccharides were bound. Booy et al. (2015) sought to make a bivalent vaccine on TT and compared its efficacy to that of Hib-TT and MenC-CRM197 conjugates. No significant differences in immunogenicity were observed between the bivalent vaccine and either of the other vaccines when considering the relevant disease. Therefore, the bivalent vaccine may be more useful due to the ease of application, assuming that production costs are sufficiently low.

Finally, Ali, An, Cui, Haque, and Carbis (2014) compared the effects of the same basic carrier, DT, on the effectiveness of the O-specific polysaccharide of *S. enterica* serovar *partyphi* A with or without a linker between the two components. It was found that a higher antibody titre was produced when the linker was present compared to the simple glycoconjugate. A number of factors were suggested to be the underlying basis for the differences observed, including the increased distance between the polysaccharide and toxoid permitting better access to the antigen sites in the conjugate with the spacer. However, it is also suggested that the conjugation event was merely more efficient when the linker is present.

Polysaccharide production

Another important factor that affects vaccine design is the reliable production of the individual parts. The protein portion is usually easier to define and is common between vaccines: the use of proteins in glycoconjugates has recently been considered in for example Bröker, Berti, Schneider, and Vojtek (2017) including some relatively new carriers. However, most proteins are chosen for use in a glycoconjugate based on their historic safety and strong immunogenicity, and so there have been relatively fewer new developments in protein production. Therefore, this section reviews only polysaccharide production.

One method to produce polysaccharides for vaccines is to culture the relevant bacterium and purify the desired molecules. If this is the goal, it is first necessary to produce a high yield culture by controlling growth conditions (Momen, Siadat, Akbari, Ranjbar, & Khajeh, 2016). In the case of Gram-negative bacteria such as *Salmonella (Sa.) enterica* serovar *typhi*, one suitable method reported involves use of hot phenol to extract lipopolysaccharide, and core hydrolysis to extract O-antigen to produce vaccine components (Salman et al., 2015). Another method involves the breaking down of the extracted CPS of *Acinetobacter baumannii* into repeating units using bacteriophage Phi AB6 tailspike protein (Lee et al., 2017). One scheme by Zanardo et al. (2016) considered the whole process from start to finish, involving cell separation by tangential microfiltration, concentration by tangential ultrafiltration, diafiltration

with SDS, precipitation with trichloroacetic acid, precipitation with ethanol, and finally anion exchange chromatography. This method was found to give acceptably pure polysaccharides for use as vaccines for *St. pneumoniae* serotype 14. However, as any method that involves growing a large culture of potentially deadly bacteria is, understandably, not ideal, alternative methods for *in vivo* production have been suggested, as we now consider.

One study found that it was possible to transfer the polysaccharide synthesis machinery from Men serotype X into *Escherichia coli* to produce polysaccharides with the same properties as the native polysaccharide (Muindi et al., 2014). Similarly, it has been found for MenX that it is possible to produce a trisaccharide in *E. coli* that remains immunogenic, that could be used rather than the native pentadecasaccharide (Morelli et al., 2014). Another study found a method of triggering glucosylation of lipopolysaccharides of *Raoulterra terrigena* by introducing bacteriophage lysogenic enzymes to improve the accuracy of antigen production in *E. coli* (Mann, Ovchinnikova, King, & Whitfield, 2015). It has also been possible to perform such transformation on plant cells; the genes for *St. pneumoniae* CPS from serotype 3S were transformed into *Nicotina tabacum* using a pCambia2301 vector with *Agrobacterium tumefaciens* mediated gene transfer, and the resulting product was found to be immunogenic in mice (Smith et al., 2014).

Such *in vivo* production systems are not limited to single serotypes. A method has been found to produce a trisaccharide common to B and C serotypes of *Cryptococcus neoformans* (Guazzelli, Ulc, & Oscarson, 2015), and CPS for *St. pneumoniae* serotype 6 that are sufficiently small to react with *St. pneumoniae* serotypes 6A, 6B and 6D (Park et al., 2015). Work has also been performed to produce CPS from four *St. pneumoniae* serotypes *in vivo* in the same *E. coli* cells (Kay, Yates, Terra, Cuccui, & Wren, 2016). There has even been some work done to produce a live vaccine for *Sa. enterica* serovar *typhi* that produces the O-antigen from *Shigella* spp., and is therefore able to act as a vaccine for two infectious diseases at once (Dharmasena et al., 2016).

It is also possible to produce polysaccharides *in vitro* by producing enzymes that synthesize the polysaccharides. For example, CsaA, CsaB and CsaC enzymes have all been combined *in vitro* to produce MenA CPS that was identical to the native form (Fiebig, Freiberger et al., 2014). A similar method has been used to produce MenX CPS (Fiebig, Berti et al., 2014) which has also been conjugated to CRM197 (Fiebig et al., 2016).

Another strategy has been to produce polysaccharides synthetically in blocks. The exact number can vary, such as a di- and tetra-saccharides (Budhadev & Mukhopadhyay, 2015), di- and tri-saccharides (Mandal, 2014; Mondal, Liao, Mondal, & Guo, 2015; Si & Misra, 2016), three disaccharides (Scott et al., 2016) and two tri-saccharides (Seeberger, Pereira, &

Govindan, 2017). However, it should be noted that it is possible to compare assembly methods and one study revealed that linear assembly may be more effective than block assembly (Dhara & Misra, 2015; Seeberger et al., 2017).

Other methods have been described to produce polysaccharides linearly such as an automated glycan assembly (Hahm, Hurevich, & Seeberger, 2016) or solid phase synthesis using cyanopialoyl ester as a protecting group (Geert Volbeda et al., 2017).

For *E. coli*, methods have been described to generate a pentasaccharide with a propyl-amino linker at one end to allow easy conjugation to CRM197 (Shang et al., 2015) or use of a heat labile p-methyloxybenzyl protecting group (Ghosh & Misra, 2016).

To develop a vaccine for *Leishmania*, glycosyl 1-phosphate was synthesised using fluoridated analogues and solid phase phosphoramidite chemistry to make repeating structures (Hara et al., 2018). A repeating trisaccharide unit for *Sta. aureus* has been produced, despite the difficult chemistries involved in alpha-fucosylation and beta-mannosylation, by using careful choices of protecting groups (Gagarinov, Fang, Liu, Srivastava, & Boons, 2015).

A number of polysaccharides have been produced to act as vaccines for different strains of *St. pneumoniae*. For serotype three strains, a tetrasaccharide (Parameswarappa et al., 2016) or glucuronic acid-linked trisaccharide (Weishaupt et al., 2016) has been used. For serotype 6a, a capsular repeating unit has been produced in a one-pot method despite the relatively challenging chemistry needed to produce 1,2-*cis* glycosidic linkages. Finally, Schumann et al. (2017) have designed an automated glycan assembly that has produced serotype 8 polysaccharides. They have also uncovered the minimum epitope needed for protection, and it was conjugated to CRM197 for effective protection in rabbits.

Polysaccharides can also be produced that are effective in the treatment of some cancers. Thomsen-Friedenreich tumour associated carbohydrates have been produced by 11 step synthesis using D-GalN(3) acceptor carrying pre-installed alpha-N-hydroxysuccinimidyl moiety, prepared using D-GalN (3)-thioglycoside donor with N-hydroxysuccinimide (Bourgault, Trabbic, Shi, & Andreana, 2014).

Conjugate production

The final factor affecting glycoconjugate vaccine design which we would like to conclude our review with is the actual linking together of the components.

One method of producing a glycoconjugate vaccine is to use glycosylation enzymes to transfer polysaccharides onto protein carriers. Typically, this involves using *E. coli* as a recombinant expression vector to produce the

enzymes, since the glycosylation machinery is not native to *E. coli*. The gene for *Campylobacter jejuni* oligosaccharyltransferase PglB was transformed into *E. coli* to express PglB and thus perform the glycosylation for *Burkholderia pseudomallei* OPS II conjugation to glycoprotein AcrA (Garcia-Quintanilla, Iwashkiw, Price, Stratilo, & Feldman, 2014). It was also used to generate a vaccine for *Sta. aureus* using CPS from serotypes 5 and 8 conjugated to detoxified *Pseudomonas aeruginosa* exoprotein A, which was then shown to be protective in mice (Wacker et al., 2014). A vaccine was made in this way for both *Sa. enterica* serovar *typhi* O-antigen and *Sta aureus* CPS 5 with various proteins (Ihssen et al., 2015). Another vaccine developed using this scheme is that for *Shigella flexneri*, in which *S. flexneri* O-polysaccharides were linked to *Pseudomonas aeruginosa* EPA-2a (Kämpf et al., 2015).

Perhaps most interestingly, it has also been possible to synthesise *St. pneumoniae* polysaccharides covalently liked to CRM197 with multiple antigens, giving a 13-valent glycoconjugate produced *in vivo* (Herbert et al., 2018).

E. coli is not the only possible recombinant host, and as already discussed can be unsuitable due to its lack of O-linked glycosylation machinery. A glycoconjugate vaccine against Men has been constructed using a *Shigella* spp. O-linked glycosylation system (Pan et al., 2016). However, it is also possible to make a recombinant form using the original pathogen that expresses introduced O-linked glycosylating enzymes, as demonstrated using *Sa. enterica* serovar *paratyphi* A, in which the O-polysaccharide was successfully transferred onto an antigenic peptide (Sun et al., 2018).

Schemes have also been devised for conjugation to non-protein carriers *in vivo*. Serotype 14 polysaccharides from *St. pneumoniae* have been linked to liposomes, which are highly immunogenic when administered with a suitable adjuvant (Deng et al., 2014). Similar work has also been performed for *E. coli* vaccines using the O-antigen and outer membrane vesicles (Chen et al., 2016).

It is also possible to perform conjugation *in vitro*. A vaccine for *Bordetella pertussis* has been synthesised by linking oligosaccharides with terminal trisaccharides to aminooxylated BSA via terminal ketodeoxyoctanate residues (Robbins et al., 2014).

Conjugation of MenA CPS to TT has been performed by activating groups to make conjugation more likely (Frasch, Kapre, Lee, & Preaud, 2015). Linking *St. pneumoniae* and *Sa. enterica* serovar *typhi* to DT and TT respectively can be achieved using an isocyanide-based multicomponent process with a Ugi-multicomponent reaction (Méndez et al., 2018).

As with the manufacture of individual components, it is important to ensure that conjugation has occurred successfully and is stable. A number of

proposals for more effective conjugations have been reported. One such report by Möginger et al. (2016) finds that the more common binding sites are at the termini of the protein, suggesting that these bonds form more easily and should be considered targets for conjugation as they will produce more stable glycoconjugates.

Adamo et al. (2014) produced a glycan-based antigen from *Candida albicans* and conjugated it to CRM197, using tyrosine-directed ligation. This gave a higher anti-glycan IgG levels than if a lysine residue was used. A particularly interesting feature of this study was the improvement seen if directed conjugation was used rather than allowing conjugation to occur at random, which suggests that reducing the polydispersity may improve immunogenicity. Conversely, Stefanetti et al. (2014) found that random conjugation improves the bactericidal activity of antibodies produced in response to *Sa. enterica* serovar *typhimurium* O-antigen conjugated to CRM197. This may be logical, as if the conjugation always occurred on the same place in a polysaccharide, very little would be 'seen' of the rest of the antigen after digestion and presentation on the MHC-II.

An experiment was performed to establish if ELISA was a suitable test to act as quality control in glycoconjugate synthesis. Hib PRP antigens were conjugated to TT, and ELISA used to assess if the PRP was pure and if the two components were successfully linked. It was found that ELISA was suitably precise and cheap for use as a test by others for similar purposes (Hamidi & Kreeftenberg, 2014).

For *Sa. enterica* serovar *typhi*, the Vi-polysaccharide of different lengths were assessed as an antigen conjugated to a number of different carriers. Short chain Vi polysaccharide was less able to develop a robust immune response than long chain, but only when conjugated to DT or CRM197, and only on primary infection (Arcuri et al., 2017). Quantification of free Vi polysaccharide has also been used to assess the stability of conjugates (Giannelli et al., 2017).

Efficacy of a number of factors, in particular the pH, were used to find a conjugation method for lipopolysaccharide from *Vibrio cholerae* to either BSA or TT (Xu et al., 2017). However, such covalent linkages may not be necessary. It has been found that simply cross-linking a polysaccharide to a protein matrix may be sufficient to trigger a T cell dependent response (Thanawastien, Cartee, Griffin, Killeen, & Mekalanos, 2015).

Perspectives

Immunity is complex, but the ability to combine different antigens to produce beneficial effects is clearly a great advantage for pharmacologists. It has also borne huge potential for the development of polysaccharide vaccines, as most polysaccharides (except ZPS) by themselves exhibit poor

ability to invoke long-term immunity. There are now many examples of successful long-term, T-cell mediated immunological responses to conjugated polysaccharides. However, in the process of glycoconjugation, there does not appear to be a single 'best' carrier or carrier pathway (Figure 1) and studies suggest that carriers must be tested on a case-by-case basis. Indeed, based on these previous studies, it seems likely that the best carrier in a given situation can be chosen for reasons of practicality rather than immunogenicity alone. Key elements to consider when choosing a carrier antigen are the ease with which it can be made reproducibly on an industrial scale, how stable the final product is, and how safe it is to produce. While the methods for synthetic polysaccharide production are complex, it is clear that production *in vitro* is much safer than purification from a pathogen culture. As the repertoire of methods for tailored polysaccharide production, fractionation and characterisation grows (Harding et al., 2017), it will become easier to produce efficient, effective and consistently reproducible glycoconjugate vaccines.

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