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Review

NMR assays for carbohydrate-based vaccines

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Abstract

Antibodies against the cell surface carbohydrates of many microbial pathogens protect against infection. This was initially exploited by the development of purified polysaccharide vaccines, but glycoconjugate vaccines, in which the cell surface carbohydrate of a microbial pathogen is covalently attached to an appropriate carrier protein, are proving the most effective means to generate this protective immunity. Carbohydrate-based vaccines against *Haemophilus influenzae* Type b, *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Salmonella enterica* serotype Typhi (*S.* Typhi) are already licensed, and many similar products are in various stages of development. For many of these vaccines, biological assays are not available or are inappropriate and NMR spectroscopy is proving a valuable tool for the characterisation and quality control of existing and novel products. This review highlights some of the areas in which NMR spectroscopy is currently used, and where further developments may be expected.

Keywords: Carbohydrate-based vaccines; Glycoconjugate; Capsular polysaccharide; Identity; *O*-acetylation; Meningitis; Pneumonia; Typhoid

Contents

Abbreviations: dn/dc, refractive index increment; Hib, *Haemophilius influenzae* type b; HPSEC, high performance size exclusion chromatography; MALLS, multi-angle laser light scattering; MRSA, methicillin-resistant *Staphylococcus aureus*

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1. Vaccines based on the cell surface carbohydrates of microbial pathogens

1.1. Cell surface carbohydrates

The cell surface polysaccharides of many bacteria – capsular polysaccharides (CPSs) and the O-chains of lipopolysaccharides (LPSs) – and the CPSs of some fungi such as *Cryptococcus neoformans* have a key role in protecting the pathogen whilst it establishes an infection. The CPS hides cell surface proteins from the immune system, inhibits phagocytosis and protects the bacterium from killing if phagocytosis occurs [1]. Many of these encapsulated and gram negative bacteria are major causes of death and morbidity, particularly for children in developing countries, as highlighted in Table 1. Antibodies against cell surface polysaccharides are often protective against infection [11]. Hence CPS and CPS- or LPS-derived conjugates can be used to stimulate protective immunity against these pathogens—as vaccines, that is. The challenge in the development of polysaccharide-based vaccines has been to produce an immunogen, which stimulates a strong, long-lasting, high avidity antibody response with appropriate complement activating antibody isotypes present and with induction of immunological memory. Whilst pure polysaccharide vaccines are still in use, against pneumonia, meningococcal meningitis and typhoid, better immune responses and responses in target populations who could not otherwise be protected, can be achieved by covalently attaching the polysaccharide, or an oligosaccharide fragment derived from it to a suitable carrier protein. These are the glycoconjugate vaccines [12].

1.2. Carbohydrate-based vaccines

At present, purified polysaccharide vaccines are available for protection against *Salmonella enterica* serotype Typhi (formerly *S. typhi*), against two, three or four serogroups of *Neisseria meningitidis* and against 23 serotypes of *Streptococcus pneumoniae* [13]. The first licensed glycoconjugate vaccine was against *Haemophilus influenzae* type b (Hib) infections and a number of variants are now available, differing in the carrier protein, conjugation chemistry, and whether the saccharide chain is a high molecular weight polymer, 'size reduced polysaccharide', or an oligosaccharide. Conjugates against *Neisseria meningitidis* Group C and multiple pneumococcal serotypes have also been licensed, whilst conjugate vaccines against other meningococcal serogroups, typhoid, methicillin-resistant *Staphylococcus aureus* (MRSA), and Group B Streptococcus and *Cryptococcus neoformans* are

Table 1
Estimated annual number of cases and death from selected pathogens for which carbohydrate-based vaccines are believed feasible: for under 5 s unless indicated otherwise

Organism (bacterial)	Disease	Death rate or morbidity	Carbohydrate-based vaccine?	Reference for mortality data
Streptococcus pneumoniae	Acute respiratory infections and meningitis	>1 million, deaths 1–2 million deaths (plus many with neurological damage)	Conjugate vaccines becoming available, but are currently very expensive	[2] [3]
Haemophilusinfluenzae (mainly type b)	Acute respiratory infections and neonatal meningitis	400,000 to 700,000 deaths (of which 8000 from meningitis)	Type b conjugate vaccine available	[4]
Neisseria meningitidis	Meningitis and bacteraemia	500,000 cases: 50,000 deaths (plus ca. 60,000 left with neurological damage)	Conjugate vaccines against Group C available: other serotypes coming soon	[5]
Shigella	Shigellosis – diarrhoea	164.7 million cases, 1,100,000 deaths	Conjugate vaccines shown to be feasible in clinical trials	[6]
Salmonella enterica serotype Typhi	Typhoid (all ages)	21.6 million cases: 216,500 deaths (2004 estimate) 16 million cases: 600,000 deaths (1984 estimate)	Polysaccharide vaccine available: conjugates in development	[7,8]
Staphylococcus aureus	Sepsis	Important cause of neonatal death, but clear figures not available	Vaccine under development, to be targeted at hospital-acquired infections in developed countries	[9]
Vibrio cholerae	Cholera (all ages)	120,000	Conjugate vaccines in development	[10]

in development [14]. The coming decade promises to be an extremely exciting time in this area of science.

Due to a combination of their relative simplicity and the lack of good in vivo assays which mimic responses in human patients, quality control of polysaccharide-based vaccines relies heavily on physicochemical methods to determine structural identity and other factors crucial for vaccine efficacy.

1.3. The structures of bacterial polysaccharides

CPSs and LPS O-chains are usually assembled by polymerisation of preformed oligosaccharide units, and have a strict repeating unit of between one and about ten sugar residues [15]. These repeat units may be linked through phosphodiesters or alditol phosphates, and may be branched or linear [16]. Various substituents such as glycerol phosphate, pyruvate ketals or *O*-acetyl groups may be present. The *O*-acetyl groups are labile and migrate between adjacent hydroxyl groups on the same sugar ring or, less frequently, to sterically available hydroxyl groups on adjacent sugar residues, or may be lost from the polymer by hydrolysis. This is often the only source of structural heterogeneity present and is observable in the spectrum. Bacterial polysaccharides are a rich source of unusual sugar residues [17]. Because of this repeating structure the NMR spectra of bacterial polysaccharides are relatively simple. The structures of some important bacterial CPSs used in vaccine manufacture are shown in Table 2 (containing Refs. [18–37]). Although bacterial polysaccharides have high molecular weights, they give relatively simple and high quality NMR spectra (Fig. 1), especially at elevated temperature. As they have limited tertiary structure, relaxation is dominated by internal mobility. Thus spectral line widths for resonances tend to depend on the structure of the repeat unit and the presence of a particularly flexible linkage, rather than the overall molecular weight of the CPS [38]. In a branched polysaccharide, residues in mobile sidechains can have different relaxation properties than a residue forming part of a rigid backbone.

2. NMR tests for the identity and purity of polysaccharides used in vaccine manufacture

A key quality control test for polysaccharides used in vaccine production is to ensure that they are what they are supposed to be—which is a combination of their identity and their purity. Within the context of this review, quantification of the *O*-acetyl content will be considered as an

Table 2
Structures of the repeating units of some of the polysaccharides used in vaccine production

Polysaccharide	Repeat unit	Reference
Haemophilus influenzae Type b ('PRP')	→3)-β-D-Rib*f*-(1→1)-D-Ribitol-(5→OPO_3→	[18,19]
Neisseria meningitides		
Group A	→6)-α-D-Man*p*NAc(3OAc)-(1→OPO_3→	[20]
Group C	→9)-α-D-Neu5Ac(7/8OAc)-(2→	[21]
Group W135	→6)-α-D-Gal*p*-(1→4)-α-D-Neu5Ac(9OAc)-(2→	[22]
Group Y	→6)-α-D-Glc*p*-(1→4)-α-D-Neu5Ac(9OAc)-(2→	[22]
Salmonella enterica Typhi Vi	→)-α-D-Gal*p*NAcA(3OAc) -(1→	[23]
Streptococcus pneumoniae		
Type 1	→3)-D-AAT-α-Gal*p*-(1→4)-α-D-Gal*p*A(2/3OAc)-(1→3)-α-D-Gal*p*A-(1→	[24]
Type 2	→4)-β-D-Glc*p*-(1→3)-[α-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→2)]-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)β-L-Rha*p*-(1→	[25]
Type 3	→3)-β-D-GlcA-(1→4)-β-D-Glc*p*-(1→	[26]
Type 4	→3β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*2,3(S)Py-(1→	[27]
Type 5	→4)-β-D-Glc*p*-(1→4)-[α-L-Pne*p*NAc-(1→2)-β-D-Glc*p*A-(1→3)]-α-L-Fuc*p*NAc-(1→3)-β-D-Sug*p*-(1→	[28]
Type 6B	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→*P*→	[29]
Type 9N	→4)-α-D-Glc*p*A-(1→3)-α-D-Glc*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-(1→	[30]
Type 14	→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→	[31]
Type 18C	→4-)β-D-Glc*p*-(1→4)-[α-D-Glc*p*(6OAc)-(1→2)][Gro-(1→*P*→3)]-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)β-L-Rha*p*-(1→	[32]
Type 19A	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→*P*→	[33]
Type 19F	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→*P*→	[34]
Type 23F	→4)-β-D-Glc*p*-(1→4)-[α-L-Rha*p*-(1→2)]-[Gro-(2→*P*→3)]-β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→	[35]
Staphylococcus aureus		
Type 5	→4)-β-D-ManNAcA(3OAc)-(1→4)-α-L-FucNAc-(1→3)-β-D-FucNAc-(1→	[36]
Type 8	→3)-β-D-ManNAcA(4OAc)-(1→3)-α-L-FucNAc-(1→3)-β-D-FucNAc-(1→	[37]

AAT is 2-acetamido-4-amino-2,4,6-trideoxygalactose, Gro is glycerol, Pne is 2-acetamido-2,6-dideoxytalose, and *P* is phosphate in a phosphodiester linkage.

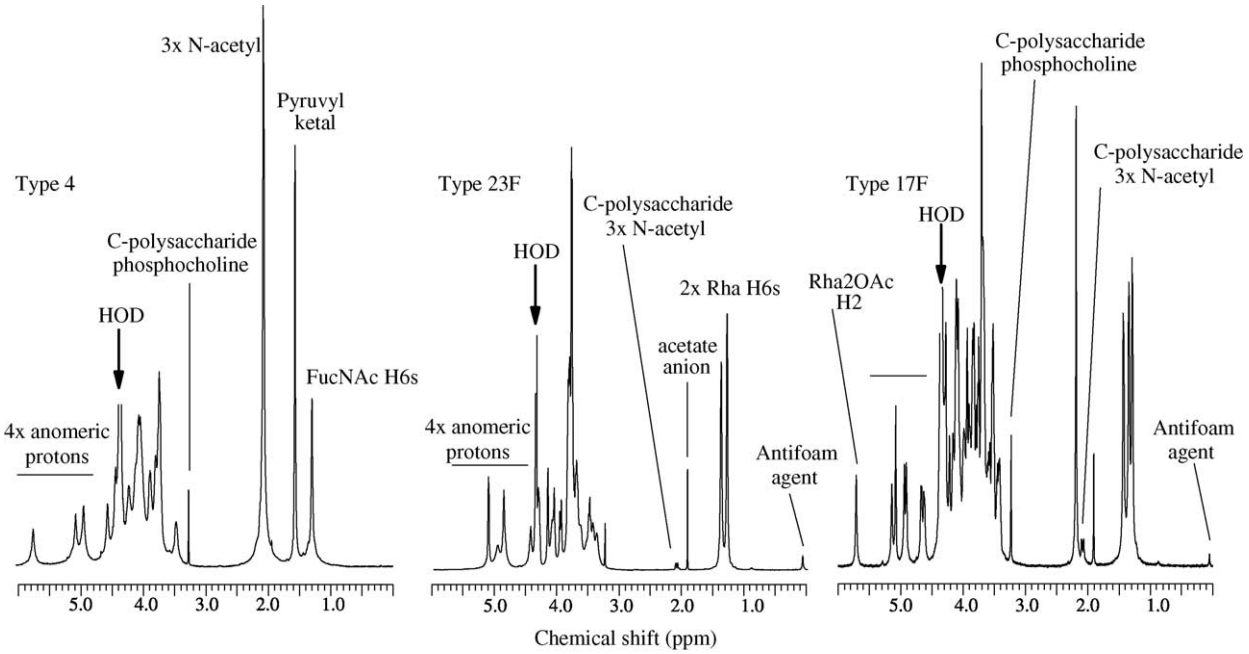

Fig. 1. Partial 500 MHz ^{1}H NMR spectra of several CPSs used in vaccines, emphasising differences in line width between different samples: (a) pneumococcal Type 4; (b) pneumococcal Type 23F CPS; (c) pneumococcal type 17F CPS. All spectra were collected at an indicated probe temperature of 70 °C.

aspect of 'identity', as some immunologically distinguishable CPSs differ only the presence or not of *O*-acetyl groups (such as the pneumococcal Types 9A and 9V), and the immune responses evoked by these CPSs are not necessarily cross-protective.

2.1. Traditional wet chemical approaches

Methods to establish the identity and purity of the CPSs were developed when the structures of most of the repeat units were undefined, and these remain the pharmacopoeial methods [39–41]. These are a combination of colorimetric methods for different saccharide types (uronic acids, aminosugars and methylpentoses) or substituents (*O*-acetyl groups). It is not usually known if, for example, the response factor for a 2,6-dideoxy-2-amino sugar is the same as for a 'simple' amino sugar or 'simple' 6-deoxy sugar in these assays. Assays for phosphate were sometimes required. Coverage is incomplete, with no specification for the *O*-acetyl content for some serotypes now known to be so substituted. Limit specifications on, for example, phosphorus in pneumococcal CPSs which do not contain phosphate groups are, consequently, an indirect limit on the content of C-polysaccharide (a cell-wall associated teichoic acid which is a ubiquitious contaminant of pneumococcal CPSs). Wet chemical assays are complemented by an immunological test with specific antiserum, as several of the different pneumococcal serogroups differ only in inter-sugar linkages, not composition. Additional tests are used to limit protein and nucleic acid contamination.

2.2. Polysaccharide identity determination by NMR spectroscopy

Over the past few years we and others have developed NMR spectroscopy as a means to control the identity of CPSs used in vaccine manufacture. NMR spectroscopy provides a fingerprint characteristic of the structure and is sensitive to the small structural differences, such as changes in a single inter-sugar linkage (Fig. 2). The spectra of all of the CPSs used in vaccine manufacture that we have studied to date (approximately 32) are quite distinct. The spectrum of an individual serotype is sensitive both to the degree of substitution and location of *O*-acetyl groups, and other labile substituents (Fig. 3). When using high field instruments, NMR approaches require relatively little material (typically 2 to 3 mg in our laboratory, but sometimes 0.5 mg), and no calibration against reference compounds. When the spectra have been collected, there is no need for reference materials, although published data at several field strengths would be desirable. Application and validation of this approach has been published for the CPSs from *Haemophilus influenzae* type b (Hib) [42], four *Neisseria meningitidis* serogroups [43], 23 *Streptococcus pneumoniae* serotypes [44] and the *S.* Typhi Vi CPS [45]. These approaches are being incorporated into the more recent WHO Recommendations on the production and quality control of the different glycoconjugate vaccines [46–48]. A different approach, intermediate between the traditional wet chemical methods and the NMR fingerprint method, is to use the NMR spectrum to calculate the proportions of different sugar residue types

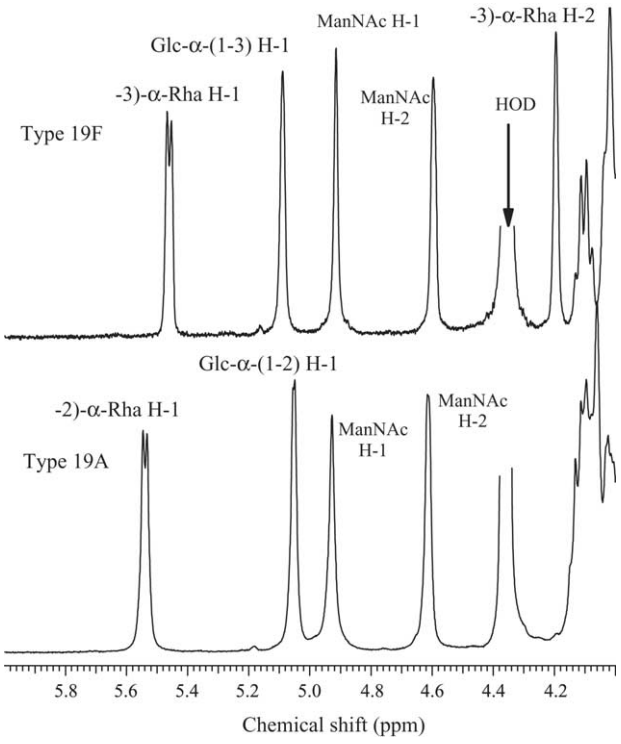

Fig. 2. Partial 500 MHz ^{1}H NMR spectra of the anomeric regions of the spectra of pneumococcal Types 19F and 19A CPS. These CPSs have the same composition and differ only in the linkage between the Glc and Rha residues, which is α(1→3) in the Type 19A and α(1→2) in the Type 19F. The NMR spectra are clearly different and identity can be assigned by visual inspection or by numerical analysis.

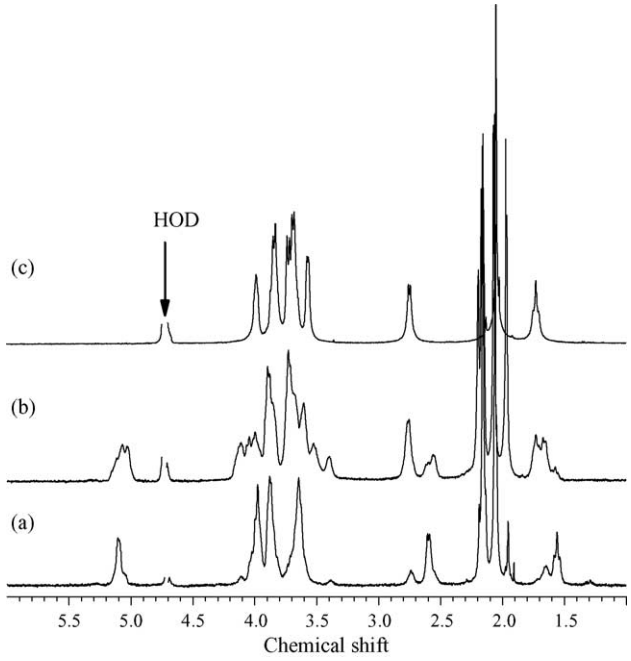

Fig. 3. Partial 500 MHz ^{1}H NMR spectra of the meningococcal Group C polysaccharide: (a) the *O*-acetylated CPS with the *O*-acetyl group principally located on the Neu5Ac *O*-8; (b) with the *O*-acetyl group located principally on the Neu5Ac *O*-7, the thermodynamically favoured form; (c) chemically de-*O*-acetylated material. Spectra were collected at an indicated probe temperature of 30 °C.

from the intensities of characteristic resonances in the spectrum.

The methodology has been validated using variable amounts of polysaccharide between 0.5 and 5 mg, by variation in temperature ±20 °C from the target temperature, by the addition of salt into the solutions up to 200 mM. These variations of conditions caused, for the Hib PRP spectrum extremely small variations in the chemical shifts of the resonances (typically 0.005 ppm between 30 and 50 °C), apart from the ribitol H-3, which moves by 0.02 ppm over the same temperature range. 'Feasible' errors in shimming or setting of the 90° pulse caused no spectral variation which could compromise the assignment of the CPS identity.

In most cases, comparison of the NMR spectrum of the test sample and the reference spectrum has been by visual inspection. The group at Merck, however, calculate correlation coefficients between these spectra [44], using only the anomeric region of the spectrum, and set a specification that the correlation coefficient should be higher than 0.95. This was validated by calculating a correlation matrix of all the possible combinations of the 23 serotypes. It is not clear how sensitive this approach is, for example, to elevated levels of pneumococcal C-polysaccharide in the sample. More sophisticated mathematical approaches, based on principal component analysis, for example, are obviously possible. Merck also add reference compounds to the sample to allow quantification of the saccharide content of the sample, and purity can be assessed by comparison of this figure with the dry weight of the sample.

Whilst not essential, full assignment of the NMR spectrum obviously gives greater confidence, and the development of the methodology has been accompanied by an effort to gain the assignment data. In several cases, this has indicated that the published structures are incorrect, and revised structures reported.

We have observed by NMR spectroscopy the presence of a variety of minor contaminants not previously recognised, including antifoam agents, formate and phenol (Fig. 4) which has, at time required a re-evaluation of the manufacturing process.

2.3. NMR analysis of blends and CPSs in the presence of excipients

Control testing of the CPSs is usually performed at the monovalent bulk stage, but high field instruments have sufficient resolution to obtain meaningful data on the blended bulks and final formulations (since no adjuvants or excipients are usually included in polysaccharide vaccines). Spectra are deconvoluted to demonstrate that all the CPSs are present and, potentially, provide relative quantification. This approach highlighted the presence of the pneumococcal type

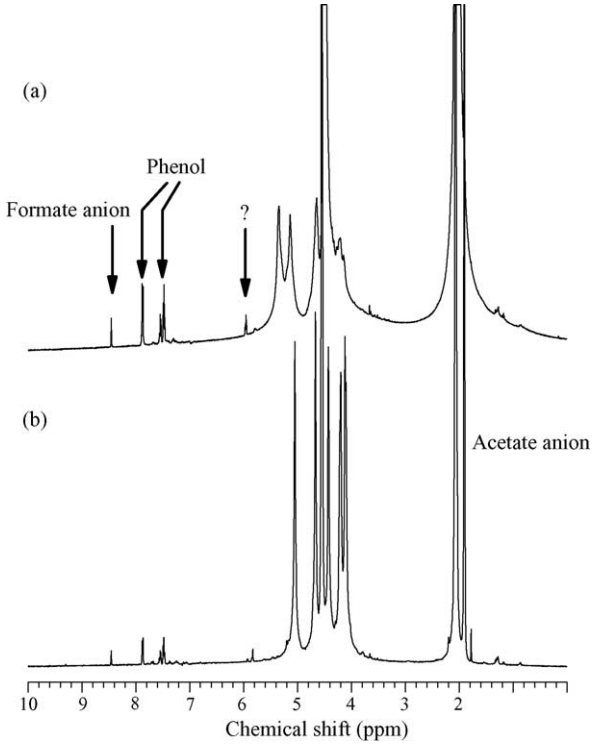

Fig. 4. Partial 500 MHz NMR spectra of (a) native *O*-acetylated Vi CPS from *Salmonella enterica* serotype Typhi, at 50 °C, and (b) the same material after a short treatment in situ with 200 mM NaOD. Resonances arising from various impurities such as phenol and silicone-based antifoam agents are highlighted.

17A CPS in a 23-valent pneumococcal vaccine, rather than the expected Type 17F.

2.4. Quantitation of the O-acetyl contents of CPSs

Variation in the degree and position of the *O*-acetyl groups can complicate this and make validation impossible. The most extreme example of this is the meningococcal Group C CPS, where slow spontaneous migration of the *O*-acetyl group from the Neu5Ac *O*-8 to the Neu5Ac *O*-7 results in very great spectral changes (Fig. 3). The solution to this problem, and of how to quantify the degree of *O*-acetylation, is to obtain two spectra on each CPS sample, firstly the spectrum of the native CPS, then to add NaOD in D_2O to the sample in the NMR tube to a final concentration of 200 mM, and to collect the spectrum after de-*O*-acetylation. The spectrum of the de-*O*-acetylated material is characteristic of the polysaccharide backbone, and the degree of *O*-acetylation in the original sample is calculated from the integrals of the acetate anion and an appropriate resonance arising from the saccharide backbone (Fig. 4). Validated methods have been published for the meningococcal and *S.* Typhi Vi CPSs [43,45], and assays for pneumococcal and staphylococcal CPSs are under development. In our experience, it has not proven necessary to correct for the presence of acetate anion in the original sample. Currently, specifications for the CPSs typically quote a minimum quantity of '*O*-acetyl residues' (measured by a Hestrin assay) per gram dry weight of CPS, but the increasing use of NMR methods will lead, I hope, to adoption of a simpler specification based on the proportion of repeat units which are *O*-acetylated. The base treatment has other beneficial effects, in leading to reductions in linewidths for some of the more viscous CPSs, such as the *S.* Typhi Vi [45].

2.5. Further work in this area

Although there are no formal direct pharmacopoeial limits for the content of contaminants such as C-polysaccharide in the pneumococcal CPSs, they are a concern and are markers of the consistency of the production process. This information is present in the NMR spectrum. For the C-polysaccharide content, we compare the intensity of the C-polysaccharide phosphocholine resonance with known CPS resonances. Whilst there are known differences in the number of phosphocholines per C-polysaccharide repeat unit in some serotypes (which can be defined) and loss of phosphocholine can occur during manufacture [49], this approach has the advantages of simplicity and sensitive proton detection. The major problem is to correct for the degree of phosphocholine substitution, typically 80%, which might be by running a separate ^{31}P spectrum on the same sample [50], or by reference to historic data. No authoritive report to validate this approach has been published. Alternatively, an internal reference compound containing both resolved proton and ^{31}P resonances could be added to quantify the CPS (through the ^{1}H spectrum) and the C-polysaccharide through the relative intensity of the in-chain phosphodiester resonance. This is a less sensitive approach, but will be unaffected by loss of phosphocholine substituents. In some cases, where the CPS contains a phosphodiester linkage, direct quantification is feasible. The decision between these approaches is likely to depend on the required accuracy, the required sensitivity, and the time needed for validation. Non-carbohydrate impurities which have been observed in these polysaccharides include trace quantities of silicone-based antifoam agents, acetate anion, formate anion and phenol. There are suggestive resonances which may arise from the lipid anchors postulated to be present on the meningococcal and Hib CPSs [51,52]. If NMR spectroscopy is to be used to identify of the CPS, the same assay can be used to determine of the saccharide content of the sample by adding known amounts of reference compounds, providing information on sample purity [44].

3. Identification of end groups as markers of polysaccharide degradation

For unconjugated polysaccharides, immunogenicity depends on molecular weight/size, and only high mass CPSs are immunogenic. Practically, this has limited the number of CPS vaccines which are feasible, and the isolation of pure CPS without depolymerisation drove much of the early de-

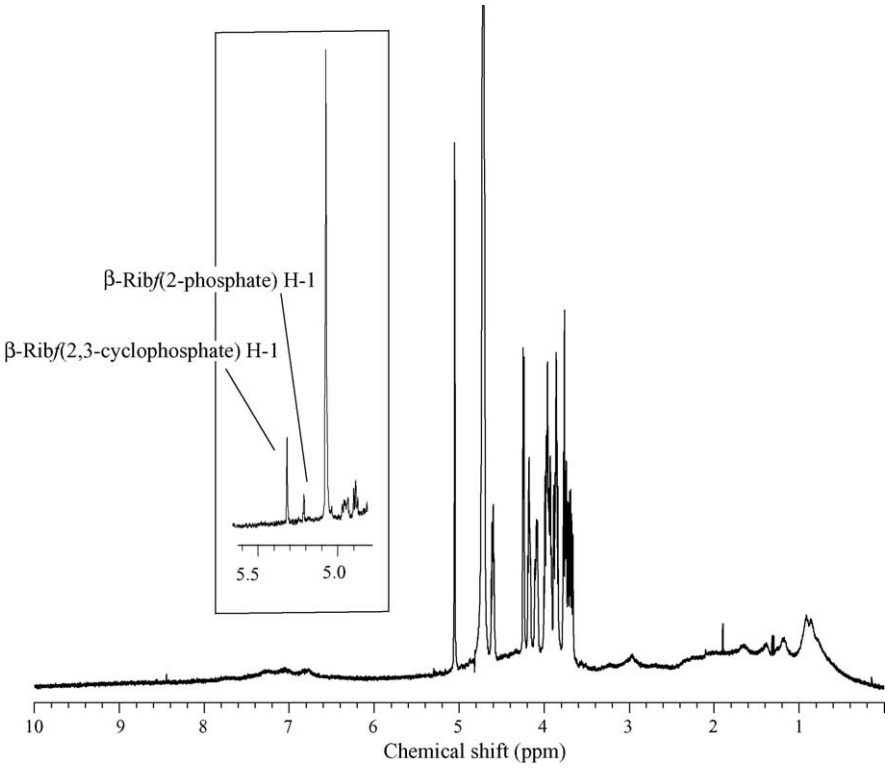

Fig. 5. Partial 500 MHz ^{1}H spectrum of a Hib-CRM197 glycoconjugate vaccine, obtained at 30 °C. The inset shows the 'anomeric' region of the spectrum of a deliberately degraded sample, with resonances arising from the newly formed ribose-2,3-cyclophosphate endgroups highlighted.

velopmental work. Estimation of molecular weight is a key control test, and extremely rare breaks are sufficient to reduce vaccine immunogenicity and efficacy by a clinically significant degree. Whilst chain cleavage leads to the formation of characteristic NMR resonances from newly formed end group, the sensitivity and dynamic range of NMR spectroscopy to observe small signals in the presence of intense ones means that it is not an appropriate technique. The methods used are soft gel chromatography or HPSEC coupled to MALLS detection.

However, the earliest glycoconjugate vaccines against *Haemophilus influenzae* type b and *Neisseria meningitidis* Group C infections were, coincidentally, produced from some of the least stable polysaccharides used in vaccine manufacture, and many of these conjugates are produced from relatively short glycan chains. Degradation/depolymerisation of the glycan chains and release of free saccharide is the most significant method by which these vaccines lose immunogenicity, and this process can be monitored either by NMR spectroscopy (as the newly formed end groups give rise to characteristic signals) or by quantifiable changes in the amount of free unconjugated saccharide present. The latter method is used in routine quality control. Hib PRP degrades by cleavage of the either side phosphodiester linkage, with initial formation of five-membered cyclophosphate intermediates that, under basic conditions, can open to one of two different monophosphate esters. The major cyclophosphate is on *O*-2 and *O*-3 of the ribofuranosyl residue, and can open to either the 2- or the 3-*O*-phosphomonoester. The minor cyclophosphate intermediate is the ribitol-4,5-cyclophosphate. The terminal β-Rib*f* 2,3-cyclophosphate residue has characteristic low-field H-1, H-2 and H-3 resonances which are well resolved from those of the intact repeat units (Fig. 5) and which can allow quantification [53]. Full NMR assignments of the endgroup residues formed during degradations have been obtained (Jones and Lemercinier, unpublished data). On the other hand, the Group C meningococcal CPS degrades by hydrolysis of the labile ketosidic linkage, allowing migration of the *O*-acetyl group to the thermodynamically favoured *O*-9 position at the non-reducing terminus and anomerisation to form the favoured β-anomer at the new reducing terminus (Fig. 6). Both of these groups give rise to identifiable signals in one- and two-dimensional NMR spectra [54].

4. Characterisation of activated intermediates in vaccine manufacture

A common feature of the production of any glycoconjugate vaccine is the need to activate the polysaccharide for conjugation to the carrier protein. In some cases, the carrier protein is also activated to provide a complementary reactive group. Although it is conceivable, for example, to couple a carboxylate-containing polysaccharide to an amino-group

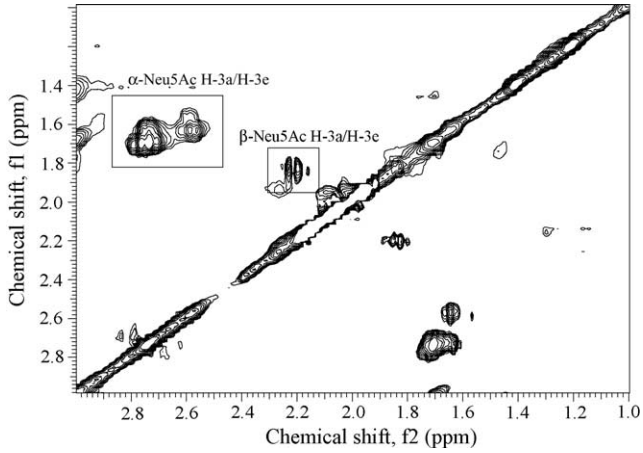

Fig. 6. Partial 500 MHz TOCSY spectrum of a deliberately degraded Men C-CRM197 conjugate, showing Neu5Ac H-3a/H-3e crosspeaks from in-chain residues in the α-anomeric configuration, and reducing terminal Neu5Ac residues in the β-anomeric configuration.

containing protein through direct amide formation, in practice there are always too many competing reactive groups present to allow the use of such a simple strategy. Two general strategies are in use. Firstly, a depolymerisation step is used (periodate oxidation, acid hydrolysis, deamidation or radiation induced damage) which generates one or two reactive sites at the chain terminus/termini which can be used directly in the conjugation or which allow attachment of an appropriately activated linker. The resulting 'neo-glycoconjugate' vaccine tends to be monomeric (although low degrees of crosslinking often occur), and may be similar to a typical plasma protein in size and proportion of glycan, apart from the identity of the glycan chains. The second approach is random multiple activation at sites along the 'intact' polymer chain, with cyanogen bromide, periodate or amide formation at carboxylate groups. Reactive groups present on the linker, or introduced as a secondary step, react with complementary groups on the carrier protein to form complex crosslinked network vaccines.

4.1. Combined activation and depolymerisation using periodate oxidation or acid hydrolysis

Glycoconjugate vaccines stimulate immunity through an entirely different immunological mechanism than unconjugated polysaccharides. This pathway, present in even very young infants, involves T cells and leads to avidity maturation, induction of complement-recruiting antibody isotypes, and induction of immunological memory. Another consequence of exploiting a different mechanism of immunogenicity is that these vaccines can be manufactured using relatively short glycan chains, less than 10 sugar residues are necessary. For some products, therefore, oxidative depolymerisation of the Hib and Group C meningococcal CPSs to oligosaccharides with concomitant activation for conjugation is a key manufacturing step, and quality control procedures aim to ensure that successive batches of oligosaccharides are consistent in molecular weight (i.e. that the degree of activation has been consistent). Although there are several approaches to obtain these data, including HPLC-MALLS, ion exchange chromatography and combinations of wet chemical assays, NMR can provide a mean value for the degree of polymerisation (but not a profile) and is useful as a reference method as no assumptions about response factors or reference compounds are required. Periodate oxidation leads to the formation of terminal (hydrated) aldehydes with low-field proton resonances, whilst acid hydrolysis gives rise to low-field anomeric resonances, and these resonances are usually resolved and quantifiable [55]. NMR has been used to monitor the conjugation process from polysaccharide through activated intermediates to the final conjugate [56].

4.2. Random activation without depolymerisation

A second common approach is to randomly activate the polysaccharide for conjugation through free hydroxyl or carboxylate groups. Activation of hydroxyls uses cyanogen bromide, or a crystalline variant such as CDAP [57], and attachment of a bifunctional linker, whilst addition of a suitable bifunctional linker, such as cysteamine, to the carboxylate groups using a water-soluble carbodiimide [58] produces glycan suitable for selective attachment to a (suitably activated) carrier protein. Again, key control tests are to quantify the degree of activation of the polysaccharide, and to ensure that the manufacturing process is consistent. As the linkers contain resonances well resolved from the usual saccharide resonances, NMR provides a means to quantify the proportion of repeat units which have been modified, although wet chemical methods are more commonly applied in routine quality control. A recent publication validated NMR spectroscopy for the quantification of the degree of activation of Hib PRP and indicated that 1,1′-carbonyldiimazole shows no significant specificity for different hydroxyl groups within the repeat unit [59].

5. Identity tests for polysaccharide components in glycoconjugate vaccines

As for the polysaccharide components, the identity of the glycan chains in the glycoconjugate vaccine needs to be determined. This should include assessment of the degree of *O*-acetylation, as labile substituents might be lost during the manufacturing process. For many glycoconjugate vaccines, principally but not exclusively those vaccines using CRM197 as carrier protein and either oligosaccharide or polysaccharide glycans, we have succeeded in obtaining one- and two-dimensional NMR spectra of sufficient quality to assess the integrity of the glycan chains. The spectrum of the glycan moieties is essentially the same as that of the un-

conjugated saccharide, whilst the carrier protein gives rise to broad peaks (Fig. 5). The glycan signals are, therefore, easily recognised and the glycan identity can be obtained by comparison with the spectrum with that of the free saccharide. We have applied this approach to glycoconjugate vaccines prepared from Hib PRP, four meningococcal serogroups, multiple pneumococcal serogroups and *Staphylococcus aureus* vaccines. Samples which have failed to produce acceptable spectra have been crosslinked Hib conjugate vaccines using tetanus toxoid as the carrier protein and the Hib vaccine using meningococcal OMPs vesicles as the 'carrier' protein (Merck's PedVaxHib®).

The role of *O*-acetyl groups in the stimulation of protective immunity is a matter of debate. Their importance is probably serotype specific and may be different in polysaccharide and conjugate vaccines. Appropriate biological assays should measure functional antibody responses in animal models vaccinated with conjugates differing solely in the *O*-acetylation status of the glycan, which may be difficult to obtain. We have not yet quantified *O*-acetyl content by treatment of the glycoconjugate vaccine in the NMR tube with base and comparison of the integrals of the acetate and an appropriate glycan resonance.

5.1. Detection of polysaccharide degradation in glycoconjugate vaccines

The most important mechanism by which glycoconjugate vaccines lose immunogenicity appears to be degradation and depolymerisation of the glycan chains. As commented on above, the first glycoconjugate vaccines were produced from some of the least stable saccharide chains, Hib and the meningococcal Group C. NMR spectroscopy provides a means to monitor glycan degradation in the final bulk conjugate through the observation of the minor resonances arising from newly formed endgroups. In the Hib conjugate, the major degradation pathway is the formation of a ribofuranosyl-2,3-cyclophosphate intermediate, with breaking of the glycan chain, and this cyclophosphate can open to form either the 2- or 3-monophosphate (Fig. 5). The anomeric resonance from the 3-phosphate coincides with that of the ribofuranosyl residue in the intact glycan chain, but that of the 2-phosphate is resolved. This process is catalysed by adsorption of the conjugate onto aluminium hydroxide adjuvants [60]. Depolymerisation of Men C occurs by hydrolysis at the labile glycosidic linkage, which allows migration of the *O*-acetyl group to now-available hydroxyl at the Neu5Ac *C*-9 position, the thermodynamically favoured position, and mutarotation of the reducing terminal residue to the β-anomeric form. The H-9 resonances of the 9-*O*-acetylated non-reducing terminal residues are observable in one-dimensional ^{1}H spectra, and the H-3a/H-3e crosspeak of the newly formed β-anomers are observed in the TOCSY spectrum (Fig. 6). We have not, to date, used this approach to quantify the degradation of the saccharide chains.

5.2. Determination of polysaccharide-protein ratio in glycoconjugate vaccines

The ratio of polysaccharide to carrier protein is a key test in vaccine development and quality control. If the ratio is low, excessive amounts of carrier protein are present for the required saccharide dose, whilst conjugates whose saccharide content is too high tend to be less immunogenic, possibly due to inhibition of the proteolysis which generates the peptides for display by MHCII to initiate T cell involvement. Three basic approaches have been used. Firstly, separate wet chemical analyses for the polysaccharide and protein components can be used. Secondly, gel permeation chromatography with dual monitoring of the UV absorbance, for protein, and refractive index for total material. Finally NMR methods are available. In the first approach, the saccharide content is measured by a colorimetric approach or HPAEC after hydrolysis, which works well for Hib and the meningococcus CPSs that are easily and quantitiatively degraded, but is problematic for pneumococcal CPSs where some serotype CPSs fail to degrade cleanly, and is unlikely to be applied to the *S.* Typhi Vi or *Staphylococcus aureus* conjugates, where the CPS is extremely resistant to hydrolysis. Protein content is determined by colorimetric methods or UV absorbance. The methodology is simple, but requires careful validation of many individual steps that introduce errors. The second approach provides a ratio more directly, but depends upon an accurate knowledge of the extinction coefficient for the carrier protein at a wavelength where the CPS has negligible absorbance, values for *dn/dc* (the refractive index increment) for the protein and the polysaccharide, and assumptions that *dn/dc* for the conjugate is a linear combination of those of its components. As polysaccharide degradation is not required, this approach should be applicable to any type of conjugate. On the other hand, NMR methods are relatively difficult to implement and only applicable to conjugates prepared from pure carriers such as CRM197, but they directly provide a ratio by integration of sets of resonances from the protein and polysaccharide. Practically, the carrier protein must be denatured by the addition of GnHCl to a final concentration of 5 M and by obtaining the spectrum at elevated temperature [56]. This removes the sequence-specific chemical shift variation for the resonances from the sidechains of the aromatic amino acids and promotes rapid deuterium exchange of the His H-2. The resonances from the aromatic sidechains (ignoring the lower field resonances from Trp H-4 and H-7) provide a measure of the protein content, and one of more of the sugar-derived resonances can be integrated to determine the saccharide content. This is illustrated in Fig. 7. Although, to the best of my knowledge, this approach has never been formally validated, the well understood physical basis for NMR spectroscopy and the fact that the saccharide and protein moieties are quantified simultaneously make this approach suitable as a primary method for measurement of polysaccharide-protein ratios, against which other methods

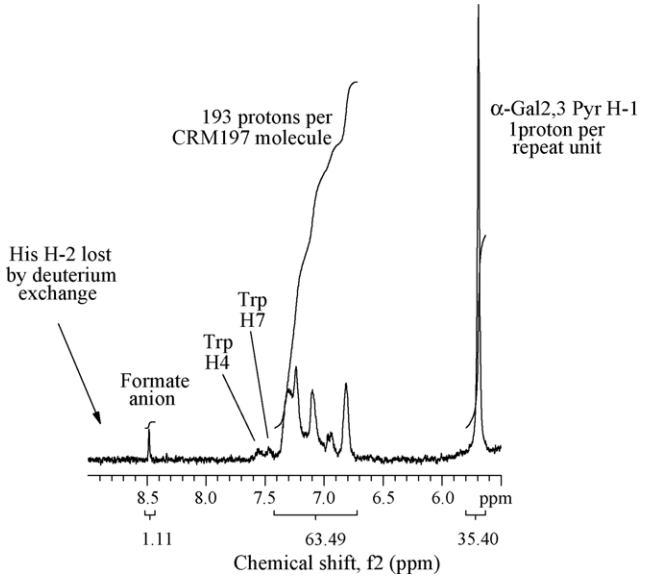

Fig. 7. Partial 500 MHz ^{1}H NMR spectrum of a denatured pneumococcal Type 4 glycoconjugate vaccine with CRM197 as carrier protein, showing the region of the spectrum containing resonances from the sidechains of aromatic amino acids and the anomeric protons of the sugar residues. The sample is dissolved in 5M deuterium-exchanged guanidinium hydrochloride in deuterated water, and the spectrum collected at a nominal probe temperature of 70 °C. Chemical shifts are referenced against internal acetate anion at 1.908 ppm. Key resonances are identified and the integrals of the resonances from the sidechains of the aromatic amino acids and the anomeric resonances shown. From the data the molar ratio of polysaccharide repeat units to protein can be calculated, and hence the weight ratio. In this sample the molar ratio of saccharide repeat units to carrier protein is approximately 110:1, which equates to a polysaccharide:protein weight ratio of 1.56:1.

can be calibrated or validated. For each glycoconjugate vaccine analysed by NMR spectroscopy it is, of course, necessary to validate that the resonances integrated arise solely from the desired hydrogens.

6. Conclusions

The first applications of NMR spectroscopy to polysaccharide and glycoconjugate vaccines were for structure determination and for identity testing of the glycan components, where NMR is a single generic technology able to substitute for a wide variety of disparate wet chemical approaches whilst providing more precise data. The NMR spectra also identify and quantify known impurities previously controlled only through indirect approaches, or not suspected to be present. It soon became apparent that in many cases the same approaches could also be applied to the characterisation of manufacturing intermediates and final conjugates, and that a single technology – capital and expertise intensive admittedly – provides precise and quantifiable data which would otherwise require the application of multiple approaches. Quantification of polysaccharide purity and, for example, polysaccharide-protein ratios in final conjugates are clearly possible, although there is little published data in the field so far. Even when NMR is not an appropriate commercial option as a routine test, it offers many advantages as a means to establish reference preparations with defined properties to allow calibration of wet chemical approaches, as these usually require multiple subsidiary measurements and assumptions about response factors. As some of the new glycoconjugate vaccines, such as those against typhoid and *Staphylococcus aureus*, utilise extremely stable polysaccharides, NMR approaches may prove the only viable methods. Progress to date has been slow because there are few laboratories working in this field.

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