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Review

Synthetic Glycans to Improve Current Glycoconjugate Vaccines and Fight Antimicrobial Resistance

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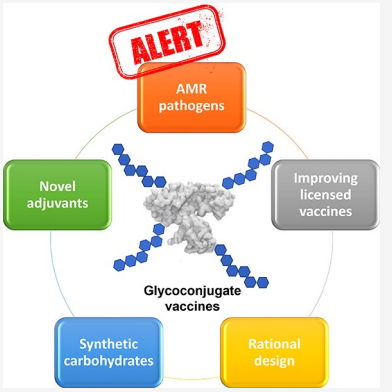
ABSTRACT: Antimicrobial resistance (AMR) is emerging as the next potential pandemic. Different microorganisms, including the bacteria *Acinetobacter baumannii, Clostridioides difficile, Escherichia coli, Enterococcus faecium, Klebsiella pneumoniae, Neisseria gonorrhoeae, Pseudomonas aeruginosa,* non-typhoidal *Salmonella,* and *Staphylococcus aureus,* and the fungus *Candida auris,* have been identified by the WHO and CDC as urgent or serious AMR threats. Others, such as group A and B *Streptococci,* are classified as concerning threats. Glycoconjugate vaccines have been demonstrated to be an efficacious and cost-effective measure to combat infections against *Haemophilus influenzae, Neisseria meningitis, Streptococcus pneumoniae,* and, more recently, *Salmonella typhi.* Recent times have seen enormous progress in methodologies for the assembly of complex glycans and glycoconjugates, with developments in synthetic, chemoenzymatic, and glycoengineering methodologies. This review analyzes the advancement of glycoconjugate vaccines based on synthetic carbohydrates to improve existing vaccines and identify novel candidates to combat AMR. Through this literature survey we built an overview of structure−immunogenicity relationships from available data and identify gaps and areas for further research to better exploit the peculiar role of carbohydrates as vaccine targets and create the next generation of synthetic carbohydrate-based vaccines.



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1. INTRODUCTION

Vaccination is one of the most impactful medical strategies protecting human health, and it has been shown to be the most effective intervention to control infectious diseases.[1] Carbohydrates, surrounding the surface of bacteria and fungi, play important roles in the establishment of infection, acting as

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Table 1. Glycoconjugate Vaccines Licensed by the FDA, EMA, and WHO

Pathogen	Commercial trade name/manufacturer	Carrier protein	Saccharide chain length	Conjugation chemistry	Approved by
Haemophilus influenzae type B	ActHIB/Sanofi Pasteur (monovalent)	TT	Native polysaccharide	Information not available	FDA/EMA
	Hexyon/Sanofi Pasteur* (Hib-TT ads, DT, TT, aP, hepatitis B rDNA and inactivated poliomyelitis at 6 months)				
	Hexaxim/Sanofi Pasteur* (Hexavalent composition of Hib-TT ads, DT, TT, aP, HBsAg and inactivated poliovirus indicated as boost)				
	Hiberix/GSK	TT	Native polysaccharide	Reductive amination	FDA/EMA
	Quinvaxem/Berna* (Pentavalent fully liquid formulation containing DT, TT, PT, HBsAg and Hib conjugate ads)*	CRM_{197}	Oligosaccharide	Active ester chemistry	FDA
	PedvaxHIB/Merck	OMPC	Native polysaccharide	Information not available	FDA
	Vaxelis/Sanofi Pasteur & Merck* (Hexavalent fully liquid composition of DT, TT, Bordetella pertussis antigens: PT, filamentous haemagglutinin, pertactin, fimbriae Types 2 and 3; HBsAg; poliovirus (inactivated): Hib-OMPC conjugate	OMPC	Native polysaccharide	Information not available	FDA/EMA
	Infanrix/GSK* (Hexavalent composition of Pediarix-DPT, TT, acellular pertussis, rDNA HBV, inactivated IPV- and Hib-TT on AlOH)	TT	Native polysaccharide	Reductive amination	EMA
	QuimiHib/CIGB	TT	Synthetic oligosaccharide	Thiol-maleimide addition	WHO
Neisseria meningitidis serogroup C	NeisVac-C/Pfizer	TT	Native polysaccharide	Reductive amination	FDA/EMA
	Meningitec/Nuron Biotech	CRM_{197}	Oligosaccharide	Reductive amination	FDA/EMA
	Menjugate/GSK	CRM_{197}	Oligosaccharide	Active ester chemistry	FDA/EMA
	Menitorix/GSK (with Hib)	TT	Native polysaccharide	Information not available	FDA/EMA
Neisseria meningitidis serogroup CY	MenHibrix/GSK (with Hib)	TT	Native polysaccharide	Information not available	FDA/EMA
Neisseria meningitidis serogroup ACWY	MenQuadfi/Sanofi Pasteur Fully liquid formulation	TT	Replaced Menactra that was based on depolymerized polysaccharide. It could be now based on a longer PS	Information not available	FDA/EMA
	Menveo/GSK Fully liquid under approval	CRM_{197}	Oligosaccharide	Active ester chemistry	FDA/EMA
	Nimenrix/Pfizer	TT	Native polysaccharide (microfluidized)	CDAP-ADH-EDAC	FDA/EMA
Streptococcus pneumoniae PCV10, serogroup (1, 4, 5, 6B, 7F, 9 V, 14, 18C, 19F, 23F)	Synflorix/GSK	NTHi PD, DT, TT	Native polysaccharide (microfluidized)	CDAP	FDA/EMA
Streptococcus pneumoniae serogroup 1, 3, 4, 5, 6A, 6B, 7F, 9 V, 14, 18C, 19A, 19F, 23F	Prevnar PCV13/Pfizer	CRM_{197}	Native polysaccharide	Reductive amination	FDA/EMA
Streptococcus pneumoniae serogroup 1, 3, 4, 5, 6A, 6B, 7F, 8, 9 V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, 33F	Prevnar PCV20/Pfizer	CRM_{197}	Native polysaccharide	Reductive amination	FDA (approved in June 21)
Streptococcus pneumoniae serogroup 1, 3, 4, 5, 6A, 6B, 7F, 9 V, 14, 18C, 19F, 19A, 22F, 23F, 33F	Vaxneuvance PCV15/Merck	CRM_{197}	Native polysaccharide	Reductive amination	FDA approved in Jul 21

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Table 2. Glycoconjugate Vaccines Currently under Clinical Development[a]

Pathogen	Commercial trade name/ manufacturer	Carrier protein	Saccharide chain length	Conjugation chemistry	Clinical development phase
Streptococcus pneumoniae Serogroup 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9 V, 10A, 11A, 12F, 14, 15B, 177, 18C, 19A, 19F, 20B, 22F, 23F, 33F	Affinivax	Rhizavidin fused protein	Native polysaccharide	MAPS technology	Phase II
Streptococcus pneumoniae Serogroup 1, 5, 6B, 14, 18C, 19F, 23F	QuimiVio/CIGB	TT	Native polysaccharide	Ugi reaction	Phase III
Extraintestinal pathogenic *Escherichia Coli* (ExPEC) Serogroup O25B, O16, O75, O2, O1A, O15, O18A, O6A, O4 and/or O8	J&J	EPA	Oligosaccharide	Bioconjugation	Phase III
Group B *Steptococcus* Serotype Ia, Ib, II, III, IV, V	Pfizer	CRM_{197}	Native polysaccharide	Reductive amination	Phase II
PNAG (*Staphylococcus aureus, Klebsiella pneumoniae. Escherichia Coli, Neisseria gohorrheae, Neisseria meningitidis, Streptococcus pneumoniae*)	Alopexx	TT	Synthetic oligosaccharide	Thiol-maleimide addition	Phase II
Shigella 2a and 4 V	LMTB-GSK	EPA	Oligosaccharide	Bioconjugation	Phase I
Shigella 2a	Pasteur Institute	TT	Synthetic oligosaccharide	Thiol-maleimide addition	Phase I
Klebsiella 4 V O-antigen based	LMTB-GSK	EPA	Oligosaccharide	Bioconjugation	Phase I

[a]Abbreviations: TT, tetanus toxoid; CRM_{197}, cross-reactive material 197; OMPC, outer membrane porin C; PD, protein D; DT, diphteria toxoid; MAPS, multiple antigen presentation system; EPA, *Pseudomonas aeruginosa* exotoxin A. *Glycoconjugate vaccines in multivalent combination with other vaccines.

virulence factors but also hiding the microorganisms from the immune system of the host and activating inflammatory responses. For these reasons, cell surface polysaccharides have been targeted for the development of bacterial vaccines. A first generation of polysaccharide-based vaccines against *meningococcus*, *pneumococcus*, and *Haemophilus influenzae* type b (Hib) was licensed between the '70s and the early '80s and demonstrated to be efficacious in adults. However, these vaccines failed to provide adequate protection in infants and children under two years of age, which are the populations at higher risk of infections.[2,3] The introduction of glycoconjugate vaccines, composed of a carbohydrate covalently linked to a carrier protein, has overcome this limitation, by raising a carbohydrate directed T-cell-dependent immune response,[4] ensuring efficaciousness in children and the elderly. Today commercial glycoconjugate vaccines are available to prevent bacteremia, pneumonia, and meningitidis caused by *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, and *Neisseria meningitidis* in infants. Recently, the FDA approved the PCV15 and PCV20 pneumococcal vaccines from Merck and Pfizer,[5,6] containing 15 or 20 different serotypes, that have shown an impact in reducing noninvasive illnesses such as acute otitis media, nonbacteremic pneumonia, and sinusitis.[7] A conjugate vaccine against typhoid fever caused by *Salmonella* Typhi,[8] showing high protectivity and a sustained immune response in children as young as six months of age, has also been licensed. The glycoconjugate vaccines licensed to date by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) are shown in Table 1. All marketed vaccines are based on the chemical conjugation of capsular polysaccharides, and because of their capacity to control a variety of bacterial infections, more vaccines targeting unmet medical needs are either under development at a preclinical level or in clinical trials (Table 2).[8] Over the last two years the world has seen the SARS-Cov2 pandemic become a major scourge, and the preparedness for future pandemics has become a priority for governments. The emergence of antimicrobial resistance (AMR) can be considered a silent pandemic that could lead to consequences far more deadly than COVID-19.[9] The threat of AMR undermines progress in health care, food production, and life expectancy and can have a major impact on society. Reports from the Center for Disease Control (CDC)[10] and the European Antimicrobial Resistance Surveillance Network (EARS-Net)[11] have identified different microorganisms, including the bacteria *Acinetobacter baumannii, Clostridioides difficile, Escherichia coli, Enterococcus faecium, Klebsiella pneumoniae, Neisseria gonorrhoeae, Pseudomonas aeruginosa*, non-typhoidal *Salmonella*, and *Staphylococcus aureus* and the fungus *Candida auris*, as serious AMR threats.[12] Others, such as group A and B *Streptococci*, are classified as concerning threats. As the diseases associated with these pathogens are currently not prevented by vaccination, the identification of novel candidates is becoming an urgent need.[13] Vaccination appears to be less vulnerable to genetic evolution and, consequently, to resistance, as compared to antibiotic treatment, and it can also provide protection for individuals who are not vaccinated through herd immunity.[14,15] Different from the currently commercialized glycoconjugate vaccines, which are generally administered to healthy individuals to prevent the disease, vaccines against AMR pathogens should provide a fast and robust immune response also in a nosocomial setting or in subjects with underlying conditions, for example to combat the carbapenem-resistant *K. pneumoniae*, that often occurs in hospitalized older adult patients.[16]

Historically, vaccines were constructed using polysaccharides isolated from pathogens and were developed primarily based on empirical evidence. Recent times have seen enormous progress in methodologies for the assembly of complex glycans and glycoconjugates, with developments in synthetic, chemoenzymatic, and glycoengineering methodologies.[17] Synthetic carbohydrate chemistry offers a unique tool to obtain well-defined, thoroughly characterized carbohydrate antigens of high purity and devoid of any potential microbial contaminant. Fully synthetic antigens reached the clinic by the synthetic *Haemophilus influenzae* type b (Hib) vaccine in Cuba,[18] and more recently a vaccine to fight shigellosis has progressed to phase II clinical trials.[19,20] The use of synthetic glycans with modern structural glycobiology approaches, such as glycan arrays, X-ray crystallography, surface plasmon resonance, and

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saturation transfer difference NMR, can nowadays be used for rational rather than trial-and-error-based glycoconjugate vaccine design.[21,22] This review aims at discussing the advancement of glycoconjugate vaccines based on synthetic carbohydrates to improve existing vaccines and identify novel candidates to combat AMR. Through this literature survey we intend to build structure−immunogenicity relationships from available data and identify gaps to be covered to advance synthetic carbohydrate-based vaccines to fight AMR.

1.1. Carbohydrates in Gram-Positive and Gram-Negative Bacteria

The bacterial cell wall is surrounded by a dense layer of polysaccharides and glycoproteins, named the glycocalyx, which allows bacteria to survive in the surrounding environment, while permitting the exchange of nutrients from the outside and waste products from the inside. Generally, the outermost surface of both Gram-positive and Gram-negative bacteria presents capsular polysaccharides (CPS),[23,24] which can have different structures within the same bacterial species and can, therefore, be used for serotyping. Gram-negative bacteria present an outer membrane (OM) to which lipopolysaccharides (LPS), CPS, and proteins are attached (Figure 1). A thin layer of peptidoglycan is

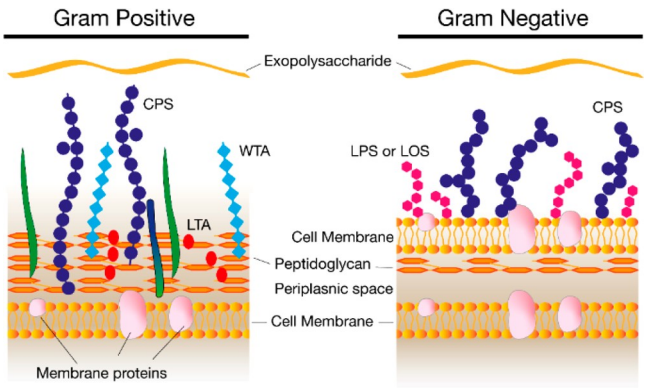

Figure 1. Surface glycans commonly found on Gram-positive and Gram-negative bacteria. Gram-positives lack an outer membrane and present a thick peptidoglycan (PG) layer, to which lipoteichoic (LTA) and wall teichoic acids (WTA) are connected. The Gram-negative bacterial cell wall features two membranes and a thin PG layer. Both can be surrounded by capsular polysaccharide (CPS). The Gram-negative outer membrane is decorated with lipopolysaccharide (LPS) or a truncated form lacking of the O-antigen, termed lipooligosaccharide (LOS).

located between the OM and the inner cytoplasmic cell membrane. In contrast, Gram-positive bacteria lack an OM and are surrounded by a much thicker layer of peptidoglycans as compared to Gram-negative bacteria.[8] CPS are known virulence factors and are typically made of highly hydrophilic, often negatively charged, long-chain polysaccharides, anchored to the cell membrane, to the peptidoglycans, or to membrane components.[25] Their hydrophilic character protects microorganisms from desiccation during transmission from host to host. CPS may also mediate adhesion to abiotic surfaces and to each other, promoting the formation of biofilms, conferring protection against specific and nonspecific host immunity. They display a vast structural diversity, that is brought by numerous different composing monosaccharides, varying types of glycosidic linkages, and different substituents such as acetyl groups, phosphates, and pyruvate ketals. Examples of the enormous structural diversity of CPS include the 80 different capsular serotypes of *E. coli*, the more than 90 serotypes of *S. pneumoniae*, and the 70 capsular serotypes reported for *K. pneumoniae*.[26]

The OM of Gram-negative bacteria is an asymmetrical lipid bilayer, which has phospholipids on the inner side while the outside is densely functionalized with lipopolysaccharide (LPS),[27] which is responsible for the endotoxic shock associated with septicemia.

LPS consists of three parts: lipid A, the core oligosaccharide, and the O-specific side chains known as O-antigens (OAg) or O-polysaccharides. Lipid A is the most conserved part, shares structural similarity between most LPS, and consists of a glucosamine disaccharide substituted with fatty acids, whose acyl chains enable tight packing of the OM, contributing to its barrier function. Lipid A carries the endotoxic properties of the LPS. The core oligosaccharide is divided into two regions: the inner core, consisting of 2-keto-3-deoxy-octulosonic acid (Kdo) and L-glycero-D-mannoheptose residues, that is highly conserved, and the outer core, which displays some structural diversity and which consists mainly of hexose sugars. The OAg domain is made up of repeating units of one or more sugar residues and exhibits remarkable structural diversity. Variations in its composition are often the basis for serotype classification. The OAg is an important virulence factor and helps the bacterium to escape the complement system and to resist environmental stresses.[28] Some bacterial species, such as *Neisseria* spp., are unable to synthesize OAg but produce a form of LPS which is referred to as lipooligosaccharide (LOS), that contains an inner core from which one or more oligosaccharide branches extend.[29]

Differently from Gram-negative bacteria, Gram-positive bacteria lack the OM but are surrounded by a thick layer of peptidoglycan (PG). PG has numerous important functions, the most important being stabilization of the cell wall, enabling it to withstand high internal osmotic pressure. PG is made up of repeating units of the disaccharide *N*-acetylglucosamine-*N*-acetylmuramic acid, which are cross-linked by pentapeptide side chains.[30] Gram-positive bacteria expose teichoic acids (TAs), zwitterionic glycopolymers containing phosphodiester-linked polyol repeating units.[31] TAs play a crucial role in cell shape determination, regulation of cell division, and other aspects of Gram-positive bacterial physiology. They can be divided in two major classes: lipoteichoic acids (LTAs), anchored in the bacterial membrane via a glycolipid, and wall teichoic acids (WTAs), covalently attached to PG. WTAs consist of two parts: a disaccharide unit highly conserved across bacterial species, which is connected at the reducing end to the PG via a phosphodiester linkage, and a main chain polymer composed of phosphodiester-linked polyol repeating units, most commonly 1,5-D-ribitol phosphate (RibP) or (1−3)-*sn*-1-glycerol-phosphate (GroP).[32] The structural diversity of WTA can derive from the presence or absence of substituents attached to the backbone, including cationic D-alanine esters and a variety of mono- or oligosaccharides, most commonly glucose (Glc) or *N*-acetyl glucosamine (GlcNAc).[33] LTAs typically consist of a poly-*sn*-3-glycerolphosphate chain that can be decorated with D-alanine residues as well as different carbohydrates.[34]

Numerous pathogenic bacteria also synthesize and secrete exopolysaccharides as part of the biofilm matrix, a slime composed of extracellular material embedding the bacterial cells. Biofilms protect the microbial community from environ-

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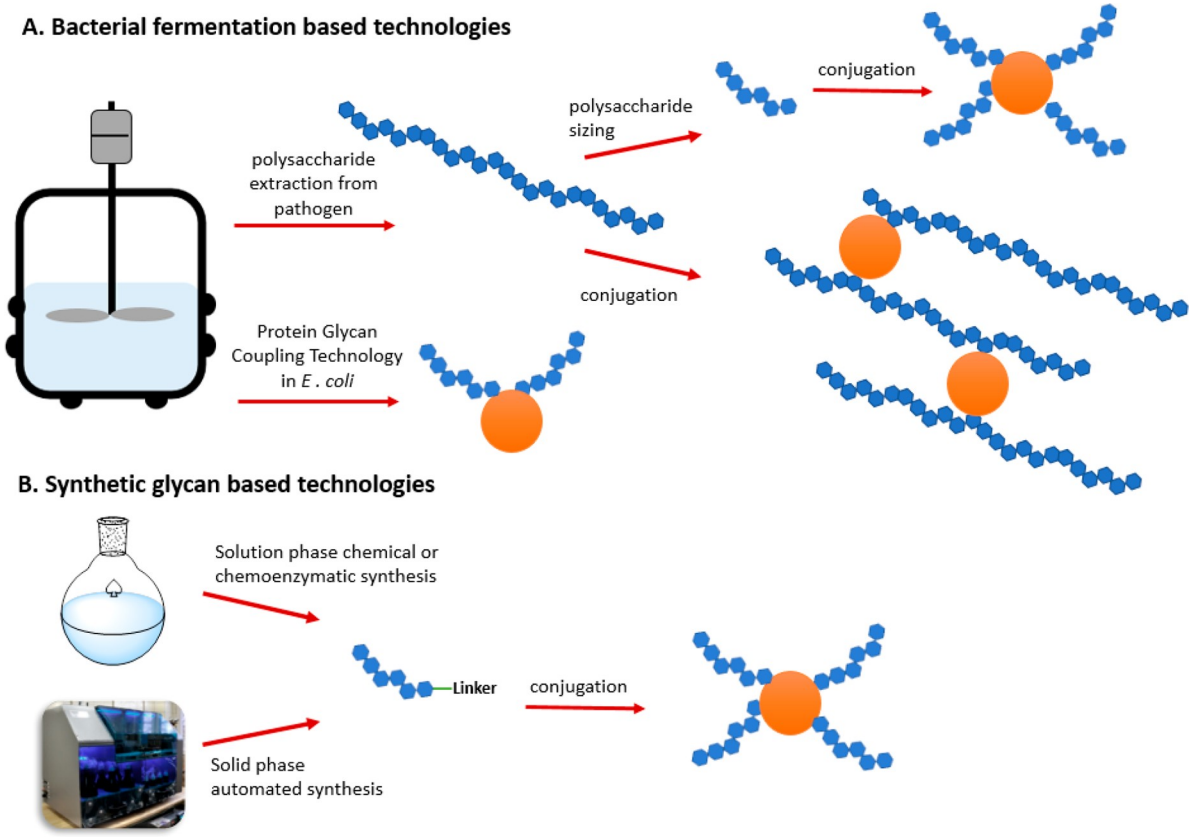

Figure 2. Approaches for manufacturing of glycans for vaccine development based on (A) bacterial fermentation, including classic polysaccharide extraction from pathogens and Protein Glycan Coupling Technology (PGCT) and (B) total synthesis of oligosaccharides via solution phase chemical/chemoenzymatic approaches or solid phase automated synthesis.

mental stresses, allowing resistance to bacteriophages, amoebae, host immune responses, and antibiotics.[35]

Many extracellular polysaccharides are polyanionic, such as alginate, xanthan, and colanic acid. Polycationic exopolysaccharides also exist, such as PNAG, a polymer of β-(1−6)-*N*-acetylglucosamine secreted by many pathogens including *Staphylococcus aureus* and *Staphylococcus epidermidis*.[36] *Pseudomonas aeruginosa* is one of the most studied biofilm forming pathogens, and it produces at least three distinct exopolysaccharides which are involved in biofilm formation: alginate, Pel, and Psl. Alginate is high molecular weight, unbranched heteropolymer consisting of (1−4)-linked β-D-mannuronates and α-L-guluronates, and it is involved in the establishment of microcolonies at the beginning of biofilm formation. Pel is a cationic galactosamine-rich polysaccharide, essential for biofilm formation, while Psl consists of a repeating pentasaccharide containing D-mannose, D-glucose, and L-rhamnose and has been shown to be involved in the adherence to biotic and abiotic surfaces.[37] Carbohydrates are also abundant on the surface of fungi. Similar to bacteria, fungal cell walls are composed of chemically different carbohydrate polymers forming a robust scaffold that incorporates a variety of proteins and other surface components, conferring flexibility. The majority of fungal cell walls is constituted by a conserved inner layer and a more heterogeneous outer layer. The inner layer is a core of chitin and β-(1−3) glucan polymers with β-(1−6) branches (about 60%) forming intrachain hydrogen bonds.[38] The outer layer of the fungal cell wall comprises proteins and/or other polysaccharides whose composition may vary with the fungal species. For example, the outermost glycan layer of *Candida albicans* is composed of mannans.[39]

1.2. Technologies for Manufacturing Glycoconjugate Vaccines

Glycoconjugate vaccines are most commonly prepared using polysaccharides, which are extracted from bacteria, purified, and coupled to a carrier protein through random linkages along the saccharide chain (Figure 2A). This results in the formation of high molecular weight, cross-linked, heterogeneous, and rather undefined structures.[4,40,41] Carriers currently used in licensed vaccines include tetanus toxoid (TT), diphtheria toxoid (DT), CRM_{197}, the outer membrane protein complex of meningococcus B (OMPC), Protein D from *H. influenzae*, and the recombinant exotoxin A of *P. aeruginosa*.[42−45] One of the most commonly used chemistries for conjugation involves $NaIO_4$ oxidation of the polysaccharides, generating aldehydes from *cis*-diols, that can be used for reductive amination with amine groups of the protein.[46−48] Also 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) chemistry is often exploited to randomly activate hydroxyl groups of polysaccharides for subsequent direct condensation with amines of the protein or the incorporation of linker moieties, such as dihydrazine adipate, for further reaction with carboxylic acid residues of the protein.[49]

Better defined glycoconjugate vaccines can be generated by using shorter oligosaccharides, obtained by chemical (using acidic or oxidative conditions) or mechanical polysaccharide fragmentation, followed by size fractionation. These fragments can be linked directly to the protein either through the end-

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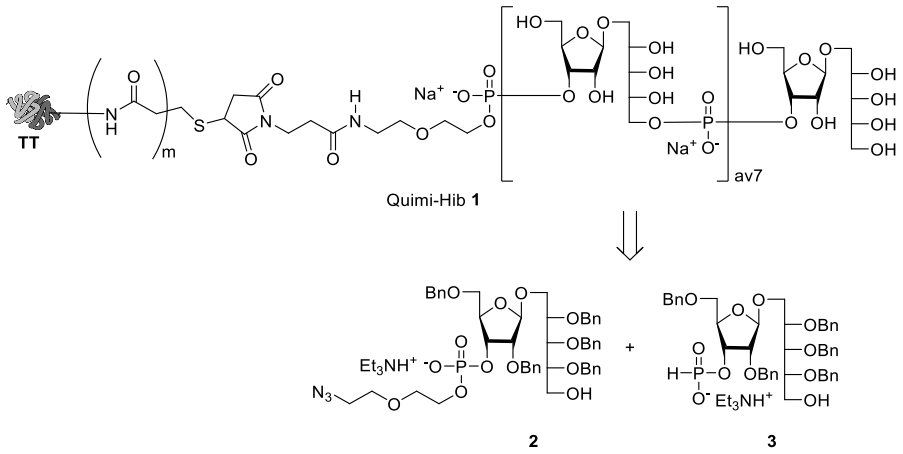

Figure 3. Synthetic polyribosyl-ribitol-phosphate (PRP) antigen of Quimi-Hib, the first synthetic glycan-based vaccine for prevention of *Haemophilus influenzae* type b, is produced by a controlled polycondensation of a H-phosphonate building block.

reducing sugar or via a spacer (Figure 2A).[50−52] Classic and emerging methods for conjugation of poly- or oligosaccharides to protein carriers have been recently thoroughly reviewed.[53]

Other approaches to obtain glycoconjugate vaccines include the so-called "protein glycan coupling technology" (PGCT) or "bioconjugation",[54] which is based on the genetic engineering of the *E. coli* Wxx-Wzy biosynthetic pathway to express a glycoprotein containing a heterologous polysaccharide. This is achieved by incorporating the genome clusters of choice in *E. coli*, along with the oligosaccharyl transferase PglB from *Campylobacter jejuni* and a plasmid or gene encoding for the carrier protein carrying the N-glycosylation consensus sequence D/E-N-X-S/T, where X can be any amino acid except proline.[55−57]

This methodology has been successfully applied to advance vaccines against *Shigella* spp. (Limmatech Biologics)[58] and extra intestinal pathogenic *E. coli* (Janssen/J&J).[59] The translational machinery of *E. coli* has also been engineered to express proteins with unnatural (i.e. not naturally found or encoded) amino acids (uAAs) to provide specific groups for conjugation.[60] The inverse demand Diels−Alder reaction between tetrazine functionalized glycans and genetically encoded trans-cyclooctene or bicyclononyne-modified uAAs has been used for conjugation, either in cellular media or in lysates.[61] Based on the "XtractCF" system, which enables the production of proteins displaying uAAs for subsequent click chemistry with carbohydrates, Vaxcyte (former Sutrovax) is currently developing a pneumococcal 24-valent conjugate vaccine.[62]

Another emerging approach is based on the protein capsular matrix vaccine (PCMV; Matrivax) technology to connect the glycan and protein components in a nonconventional manner. In this approach a biotinylated polysaccharide and the carrier protein fused with a streptavidin domain are crosslinked in a matrix.[63] This technology has been used by Affinivax for a prototype 24-valent pneumococcal vaccine, which was demonstrated to provide protection against invasive disease and carriage in mice[64] and has been advanced to phase II clinical trials, where it was shown to be well tolerated and immunogenic in an adult population (Table 1).[65]

Regardless of the approach used, the immunological activity of the glycoconjugates is influenced by a variety of factors, including the nature and length of the carbohydrate, the substitution pattern, and the number of glycans attached to the protein (i.e. the saccharide/protein ratio).[4]

This review will focus on glycoconjugates obtained from synthetic glycans (Figure 2B). Because of their well-defined length and composition, synthetic glycans offer clear advantages in the elucidation of the parameters impacting the immunogenicity of glycoconjugate vaccines.[66] In addition, synthetic glycans are typically assembled with a linker preinstalled at the downstream end of the oligosaccharides, allowing conjugation to the protein with preservation of the integrity of all sugar moieties. Numerous methodologies for fast assembly of oligosaccharides, including one-pot protocols[67] in solution phase, enzymatic approaches,[68] and solid phase automated synthesis,[69−71] have now become available. Site-selective conjugation methods have also been developed to direct this step to preselected sites on carrier proteins, which enables investigations into the impact of the position of the glycan on the protein carrier on the activity of the glycoconjugates.[72,73] We will first provide examples how synthetic chemistry can be used to improve existing vaccines and then continue to outline the progress that has been made to generate vaccines to combat AMR.

2. IMPROVING EXISTING VACCINES

2.1. *Haemophilus influenzae*

H. influenzae is a Gram-negative coccobacillus part of the nasopharynx microbiota in healthy adults, children, and infants.[74] Serotype b (Hib) causes severe diseases in humans such as epiglottitis, sepsis, pneumonia, and meningitis. The Hib CPS is made up of poly(ribosyl ribitolphosphate) (PRP) repeating units (RUs) and represents the most important virulence factor.[75] PRP was conjugated to a diphtheria toxoid (DT) protein in 1983, resulting in the first glycoconjugate vaccine ever developed.[76,77] Currently, different Hib glycoconjugate vaccines with different carrier proteins are available on the EU and US market, either in single form or in combination with other vaccines (Table 1).[78,79] Quimi-Hib (**1**, Figure 3) is based on a synthetic PRP and has been licensed in Cuba in 2004. It is composed of a synthetic antigen with an average of seven RP repeating units conjugated to a thiolated TT carrier protein through a 3-(maleimido)-propylamide linker.[18] This vaccine represents the first licensed glycoconjugate vaccine based on a synthetic carbohydrate antigen. The large scale production of the PRP fragments was achieved through a one-step polycondensation reaction using H-phosphonate chemistry,

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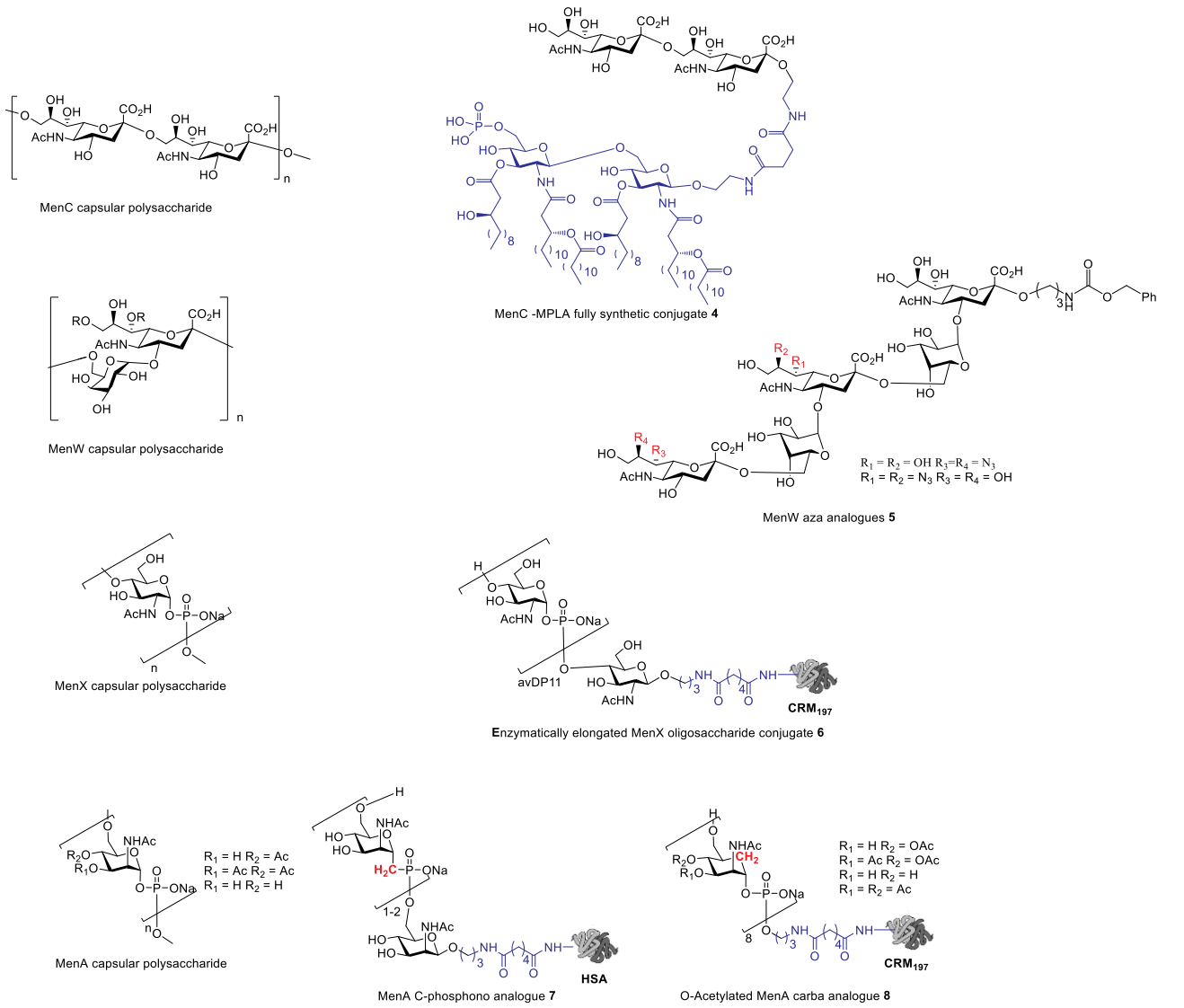

Figure 4. Meningococcal capsular polysaccharide structures and related synthetic conjugates. On the right the structure of the repeating unit of the natural CPS is described. Synthetic structures include copies of the natural polysaccharide (**4**−**6**) and analogues (**7** and **8**). In conjugate (**6**) the 11-mer molecule was obtained in a one-pot fashion using the enzymatic elongation of a synthetic acceptor.

where the spacer containing end residue (**2**) and H-phosphonate derivative (**3**) were oligomerized in a pivaloyl chloride mediated polycondensation.

Over the years, different methods have been developed to obtain Hib oligosaccharides by depolymerization of natural polysaccharide[48,77,80,81] and by chemical synthesis.[82−84]

Recently, Baek et al. synthesized oligomers ranging in length from a tetramer up to a decamer, using a [2+2] elongation strategy in an iterative approach involving a ribosyl-ribitol acceptor, with the ribitol C5-OH acting as the nucleophile, and a ribosyl-ribitol C-3′-H-phosphonate aminopentyl donor, in line with the approach taken to construct the Quimi-Hib vaccine antigen.[85] The CRM_{197} conjugates of the obtained oligosaccharides were used to immunize Zika-rabbits, and the sera were analyzed using a glycan array.

The tetramer and octamer revealed the highest immunogenicity although no linear or marked length-dependent trend could be shown. This study suggests that four repeating units would fully cover the Hib polysaccharide epitope and represent an optimal length for vaccine design.

Similarly to other phosphodiester bridged polymers, Hib polysaccharide suffers from intrinsic lability, especially in the presence of metal cations. The hydrolysis of the phosphodiester linkages has been explained with a mechanism involving the 2-OH of the ribose moiety;[86] therefore, analogues with different functional groups (*e.g.* -OCH_3 and -F) to replace the 2-OH have been developed, resulting in higher stability.[87] In the post-Hib vaccine era, other serotypes and nontypeable strains (NTHi) are emerging as a significant cause of invasive infections and meningitis.[88] *H. influenzae* serotype a (Hia) represents the second most virulent capsular serotype of *H. influenzae*, especially in children aged six months to two years, and it can cause meningitis, pneumonia, septic arthritis, and bacteremia.[74] The Hia capsule is a polymer of glucose and ribitol connected by phosphodiester linkages. The synthesis of oligomers up to two repeating units was reported in 1988 and was achieved through condensation of an α-D-glucopyranosyl bromide derivative with 5-*O*-allyl-1,2,3-tri-*O*-benzyl-D-ribitol, followed by phosphorylation and removal of protective groups.[89] Development of Hia vaccines should be technically feasible, but a limited market

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would likely require donor support to incentivize a manufacturer.[90] An approach similar to the Hib oligomers can be envisaged. Considering the structural and infectious similarities between Hia and Hib serotypes, the success of Hib vaccination could suggest a rational background for the development of a Hia glycoconjugate vaccine protective both in adults and infants.[91] Indeed, a CPS-based glycoconjugate vaccine has shown promising results, and it is at an early stage of development.[92] In silico studies have recently shown important conformational differences between the serotype a and b polysaccharides, explaining the lack of cross-reactivity, justifying the need for a separate conjugate vaccine to target Hia.[92]

2.2. *Neisseria meningitidis*

Efforts in the generation of meningococcal vaccines have been directed to the pathogen-free production of structures identical to the natural glycans and to the design of glycomimetics with improved stability. The serotype A CPS is composed of (1−6)-linked *N*-acetyl-α-D-mannosamine-1-phosphates with acetyl esters at C-3 or C-4 (Figure 4).

The synthesis of a nonacetylated MenA CPS up to a trimer has been achieved using a 2-azido mannosamine H-phosphonate building block using pivaloyl chloride mediated condensations followed by iodine oxidation of the intermediate phosphites.[93] A 3-*O*-acetylated MenA tetramer was successfully assembled using a 3-*O*-acetyl-2-azido-4-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-mannopyranosyl H-phosphonate.[94] The TT conjugate obtained incorporated an *S*-acetylthioglycolic linker for subsequent thiol-maleimide addition and inhibited the binding of anti-MenA PS serum to the capsular PS.

Synthetic routes have also been exploited for other serotypes. The MenX CPS is composed of (1−6)-linked *N*-acetyl-α-D-glucosamine-1-phosphates (Figure 4), and a MenX CPS trimer was synthesized from a 2-azide glucose building block using H-phoshonate chemistry.[95] Fragments ranging in length from a monomer to a trimer were obtained and conjugated to CRM_{197} using active ester chemistry. Only the trimer conjugate induced bactericidal anti-MenX PS antibodies in mice.[96] Subsequently a tetramer fragment was generated,[97] and a competitive ELISA showed that the corresponding TT conjugate was a much better competitor than the unconjugated form, indicating the need for longer length CPS fragments to sufficiently emulate the PS epitope. Recently, the structural epitope of the MenX CPS was mapped using a Fab derived from a murine bactericidal monoclonal antibody (mAb) obtained against the conjugated MenX PS.[98] Surface Plasmon Resonance (SPR), isothermal titration calorimetry (ITC), and STD-NMR showed binding for a fragment longer than 5 RUs. In silico docking with a hexamer and the structure of the Fab resolved by X-ray crystallography confirmed binding through a network of salt bridges and hydrophobic interactions. Upon conjugation, 5 RUs were sufficient to elicit functional antibody levels at a level comparable to a longer 10 RU fragment, suggesting that glycoconjugates with relatively short structures can be used for vaccine development.

Recent progress in the construction of the challenging α-sialic acid linkage has enabled access to polysialic acid MenC structures.[99] An *N*5,*O*4-carbonyl-protected α-sialyl phosphate donor was a key intermediate for the assembly of α-(2−9)-linked oligoasialic acids up to a dodecamer.[100,101] Conjugates with KLH (Keyhole Limpet Hemocyanin) induced murine antibodies recognizing MenC cells in ELISA. Recently, a synthetic MenC tetramer and octamer were assembled and conjugated with TT, inducing in mice bactericidal IgGs at a level comparable to a licensed vaccine.[102] A fully synthetic MenC-glycan antigen linked to a monophosphoryl lipid A (MPLA) carrier (**4**) has also been generated and shown to elicit good levels of bactericidal antibodies.[103] The MenW CPS is composed of →6)-α-D-Gal-(1−4)-α-D-Neu5Ac-(2→ repeats, and the synthesis of MenW CPS fragments up to the length of a decamer was accomplished using a sialylated galactosyl disaccharide thiophenyl building block.[104] Conjugated oligomers induced an immune response in mice against themselves, with the octamer providing the most bactericidal serum against a MenW135 strain. Differently from ACWYX capsular polysaccharides that have been successfully advanced as vaccine antigens, MenB CPS, composed of a →8)-α-D-Neu5Ac-(2→ repeating unit, exhibits high similarity with gangliosides expressed during fetal development (GD3) and involved in neural growth.[105] Although an *N*-propionyl sialyl conjugate vaccine generated in a preclinical model[106] antibodies not or minimally cross-reactive with purified human glycans,[107] safety concerns have prevented the use of MenB CPS for clinical applications.

As an alternative to the use of CPS, the use of a highly conserved LPS portion has been considered. A model vaccine, constructed by conjugation of a deacetylated truncated LPS form to CRM_{197} through the Kdo residue was able to elicit anti-LPS antibodies in both rabbits and mice.[108] However, the raised sera cross reacted only with de-*O*-acylated LPS of MenB strains but not with the native fully acylated LPS. Recently, the core tetrasaccharide composed of two heptose residues and two Kdo saccharides (Hep2Kdo2), a common motif shared by different pathogenic strains of *N. meningitidis* but also *P. aeruginosa* and *E. coli*, has been synthesized.[109] The related CRM_{197} conjugate raised murine antibodies, which recognized in vitro the inner core of the LPS. Combination of the antibodies with an inhibitor of capsular polysaccharide transport enabled bacterial killing of MenB strains. This shows that the LPS is effectively masked by CPS, and its use as a vaccine target remains challenging. Today vaccines based on the outer membrane vesicle (OMV) and the proteins, Bexsero and Trumemba, are available against MenB,[110] as well as a bivalent MenB/MenC vaccine composed of MenB, OMV, and MenC CPS (VamengocBC).[111]

Enzymes involved in the biosynthesis of natural polysaccharides have also been explored as powerful tools to simplify and accelerate manufacturing of meningococcal CPS oligomers. The biosynthesis of the MenA CPS consists of three steps: (i) the epimerization of UDP-GlcNAc to UDP-ManNAc by the epimerase CsaA; (ii) the condensation of this UDP donor at the C-6 of an ManNAc-1P acceptor by the polymerase CsaB; and (iii) the acetylation of the assembled polymer by the acetyl transferase CsaC. By cloning and expressing a soluble form of these three enzymes, the biosynthesis of the MenA CPS was replicated in vitro, providing structures identical to the native polysaccharide, in terms of both structure and immunological activity.[112] A synthetic disaccharide was required for priming the polymerase reaction and allowing the enzymatic elongation of MenA CPS.

The mechanism of *O*-acetylation has been further detailed by structural studies showing that the CsaC selectively transfers an acetyl ester to the O3 position; however, part of the acetyl esters migrates to the O4 position at neutral pH.[113] This generates the typical acetylation pattern of MenA CPS that is important for its immunogenicity. Similarly, the production of MenX oligomers

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has been explored. The polymerase CsxA enabled elongation of naturally derived acceptors and generation of oligomers that upon conjugation to CRM_{197} elicited an immunogenic response in mice comparable to the conjugated natural PS.[114,115] Subsequently, an efficient chemoenzymatic approach was developed by combining the chemical synthesis of a trisaccharide acceptor, equipped with a linker for conjugation, and the enzymatic elongation through a column immobi-lized truncated form of the polymerase CsxA.[68]

By passing the growing oligosaccharide together with an immobilized truncated form of the polymerase CsxA through a column[68] with GlcNAc-UDP, a structure with an average length of 12 RUs (**6**) was assembled and conjugated to CRM_{197}. Vaccination of mice elicited a high level of bactericidal antibodies comparable to conjugates made from extracted material. Compared to conventional synthetic protocols, this methodology proved very expeditious and drastically reduced the number of purification steps to generate the oligomers. A chemoenzymatic method has also been developed for the synthesis of the MenC CPS. A spacer equipped with synthetic lactosides was modified with two sialic acid residues with a *C. jejeuni* transferase for further elongation with the MenC sialyltransferase. A TT conjugate of the obtained oligo-(2−8)-sialyl oligomers elicited a MenC CPS-specific immune response in mice.[116]

Recently, studies on the MenW capsular polymerase (NmSiaDW) have identified 4-azido-4-deoxy-*N*-acetylmannosamine and 6-azid-6-deoxy-*N*-acetylmannosamine as suitable substrates for the synthesis of MenW oligosaccharides containing 4,7-di-*N*-acetylneuraminic acid and 4,9-di-*N*-acetylneuraminic acid.[117] Through these azide intermediates an enzymatic strategy was developed to assemble the *N*-Ac-oligosaccharides up to a length of a pentasaccharide (**5**). Overall, these studies suggest that chemoenzymatic approaches can be very versatile and they can likely be expanded to other sero groups.

The MenA polysaccharide suffers from instability in solution because the anomeric phosphate is relatively easily cleaved, as the neighboring *trans*-oriented acetamide can participate in its displacement. Therefore, the development and manufacture of a MenA glycoconjugate vaccine in liquid formulation remains a challenge.[118]

Typically, this issue is solved by using lyophilized formulations, which are reconstituted with saline before use or by storage at 2−8 °C. This solution, however, implies extra costs for production and distribution of the vaccine, particularly in areas where maintaining the cold chain is not trivial, such as emerging and poor countries. Therefore, stabilized analogues are desirable.

Analogues of the MenA CPS have been designed by replacing the phosphodiesters with a more stable C-phosphonate (Figure 4, compound (**7**)).[119,120]

The critical installation of the interglycosidic phosphomonoester linkages was accomplished using a Mitsunobu coupling of the glycosyl C-phosphonate building block with the 6-OH moiety of the mannosamine acceptor.[119] C-phosphonate bridged oligomers up to the trimer length (**7**) were conjugated to human serum albumin (HSA), stimulating in vitro T-cell proliferation (CFSE method) and IL-2 release (ELISA) and inducing in vivo anti-polysaccharide-specific IgGs.[120] In an alternative approach, so-called "carba-analogues", in which the mannosamine ring oxygen is replaced by a methylene group, have been explored.[121] These analogues are stabilized because the lack of the ring oxygen precludes the formation of an oxocarbenium ion-like species that would form upon cleavage of the anomeric C−O phosphate bond and appear more suitable for large scale manufacturing since phosphoramidite chemistry can be used for the construction of the phosphate linkages.[122] The key step in the formation of the carbacycle analogue of mannosamine is the Claisen rearrangement of a 6-methylene glucal. Short oligomers of the carbaMenA analogues, containing up to three repeating units, were initially generated using carba H-phosphonates (Figure 4, compound (**8**)).[122] The carbaMenA trimer induced low levels of antibodies, that cross-reacted with the native MenA CPS and showed bactericidal activity. To explain the behavior of the carba analogues, conformational modelling studies were undertaken. Molecular dynamics simulations indicated the MenA CPS conformation to be a flexible random coil which becomes less conformationally defined as the length increases.[123] Acetylation would provide structural rigidity to the MenA backbone. Also, the carbaMenA was predicted to present a more random extended conformation as compared to the natural MenA.[124] A more thorough investigation was performed by combining theoretical calculations and NMR spectroscopy experiments, using chemically synthesized 1- or 6-phophorylated monomannosamines and their carba analogues as chemical probes.[125]

The data confirmed that in spite of a high conformational freedom of the key pseudoglycosidic and aglycon torsion angles in the carba analogues, one of the favorable geometries corresponds to that of the natural monomer.

To improve the immunogenicity of the carbaMenA oligomers, more recently, structures up to eight repeating units in length were assembled.[126] Non-*O*-acetylated 6- and 8-mers conjugated to CRM_{197} elicited in mice anti-MenA CPS antibodies but still at suboptimal levels. However, the 8-mer showed the unique capacity to recognize a bactericidal murine mAb that previously had been shown by STD NMR and X-ray crystallography to bind to an *O*-acetylated trisaccharide epitope.[127] The introduction of *O*-acetyl groups in the carbaMenA octamer (**8**) through a controlled random acetylation reaction led to an O-acetylation degree of 75%, similar to the natural polysaccharide. Conjugation of this antigen led to a model vaccine, that induced bactericidal antibodies to a level comparable to the MenA vaccine benchmark.[126] This work represents an important proof-of-principle for the use of glycomimetics as more stable alternatives for polysaccharide-based vaccines.

Novel concepts have been tested with natural CPS, and these could be further exploited for the synthetic structures. Conjugation of oligosaccharides from two diverse sero groups, namely MenA and C, has been shown to induce high titers of bactericidal antibodies against the two different sero groups after the third administration.[128]

The MenA CPS seems to suffer from carrier epitope suppression as a result of preexisting immunity to the protein carrier, in particular CRM_{197} and TT.[45] The use of alternative carriers, such as the pneumococcal Spr96-2021, has been explored.[129] Genetically detoxified outer membrane vesicles (GMMA) from MenB have also been introduced as a potent carrier for MenA and MenC polysaccharides, giving levels of murine bactericidal antibodies superior to CRM_{197} already at the first dose..[130] Finally, MenC polysaccharide conjugated to full-length hepatitis B core (HBc) antigen virus-like particles using heterobifunctional polyethylene glycol linkers generated 10-fold higher anti-carbohydrate IgGs than the unconjugated poly-

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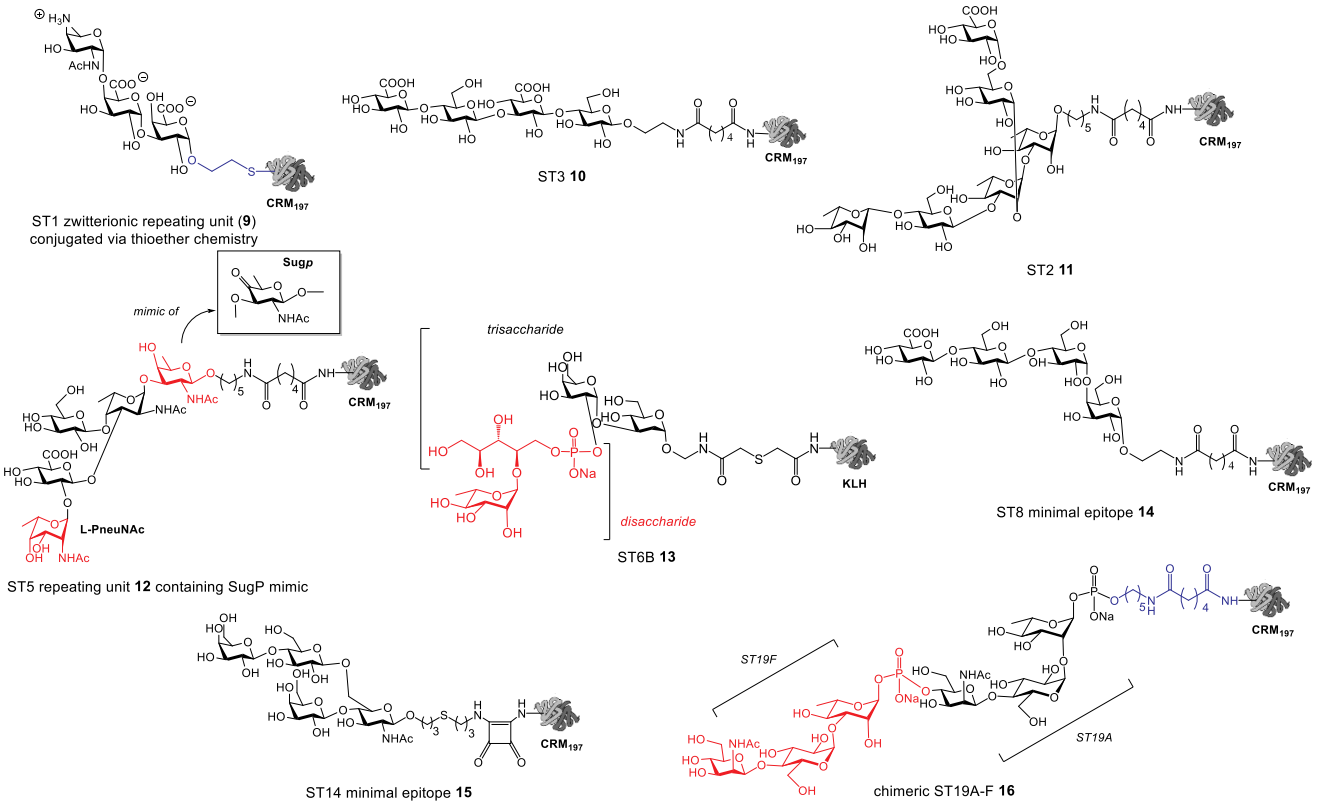

Figure 5. Pneumococcal vaccine candidates based on synthetic glycans. Synthetic oligosaccharides mimicking several *Streptococcus pneumoniae* serotypes (ST) have been synthesized and tested at the preclinical level to identify key glycan epitopes.

saccharide and increased IgG2a subclass production with a shift to a Th1 cellular immune type response.[131]

2.3. *Streptococcus pneumoniae*

Despite vaccines being available in developed countries, the Institute for Health Metrics and Evaluation (IHME) estimates that *S. pneumoniae* accounts for 1.2 million deaths and approximately 900,000 disabled patients annually from pneumonia and meningitis. In addition, *S. pneumoniae* is responsible for an estimated 36% of the global burden of pneumonia and 27% of the global burden of otitis media. A priority for pneumococcal vaccination is to increase the global distribution in poor and emerging countries. Some years after introduction of PCV7, a clear impact of vaccination on community acquired pneumoniae was observed;[132] however, an increase of invasive pneumoniae disease (IPD) of non-PCV7 serotypes was observed. Although these events were far below the magnitude of the reduction in IPD caused by the vaccine serotypes,[133] this highlights the need for broader coverage in the countries where a vaccine is available.

Additional challenges associated with pneumococcal vaccination have been the low immunogenicity of some glycoconjugates in the complex multivalent formulation (*e.g.* serotype (ST) 3) and the unexpected lack of cross protection between some structurally similar polysaccharides (*e.g.* ST19A and 19F).

Synthetic chemistry has been a fundamental means to generate glycans for the identification of key polysaccharide epitopes and the improvement of pneumococcal vaccines, particularly for the most challenging serotypes. This topic has been recently thoroughly reviewed,[134−137] so we will here only give an overview of the most relevant concepts developed through synthetic carbohydrate derived glycoconjugates to obtain vaccine candidates at the preclinical level. The seminal work of Vliegenthart and Kamerling was pivotal to demonstrate that the immunogenic character of long and complex polysaccharides can be related to short defined glycan epitopes. KLH conjugates of di-, tri-, and tetrasaccharide fragments of serotype 6A and 6B were synthesized and shown to elicit high levels of ST6B antibodies in rabbits, that facilitated type-specific phagocytosis (Figure 5).[138] The rabbit antisera raised against the tri- and tetrasaccharide also reacted with the 6A PS in an ELISA assay and promoted phagocytosis of 6A pneumococci. All rabbit antisera passively protected mice against a ST6B challenge. In mice, however, phagocytic and protective anti-ST6B antibodies were only induced by the tetrasaccharide conjugate, and these antibodies did not cross-react with ST6A PS in an ELISA. Overall, these studies have shown that the tetrasaccharide (**13**) was capable of inducing ST6B-specific, fully protective antibodies in two animal models. Likewise, through a library of ST14 derived structures, a branched tetrasaccharide repeating unit (**15**) was shown to be the functional minimal epitope in a mouse model.[139,140] This small epitope, that recently has been readily assembled also by automated synthesis,[141] has become a model to obtain a fully synthetic vaccine based on gold nanoparticles where T-cell help is provided by a synthetic peptide.[142] A polysaccharide mimic composed of a sequential synthetic tetrasaccharide unit connected through a linker has been made and shown to be immunogenic in mice.[143] A hexasaccharide conjugated to BSA induced apparently both innate (through TLR2 activation) and humoral responses in mice, with longer antibody persistence when aluminum hydroxide was used as adjuvant. However, the TLR2 expression on spleen cells from immunized mice seems not specific and caused by cytokine action rather than being the

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outcome of a ligand−receptor interaction.[144] A synthetic ST14 tetrasaccharide was site selectively linked via a thiol−maleimide coupling to four different pneumococcal surface adhesin A (PsaA) mutants, each harboring a single cysteine mutation at a defined position.[145] Antibody responses to both the carbohydrate and PsaA protein antigens were generated in mice. Various other pneumococcal structures have more recently been synthesized. Synthetically made zwitterionic polysaccharides from ST1 have been generated by different research groups.[146−149] This serotype has attracted attention for its property to elicit T-cell responses without conjugation.[150] An elegant strategy for insertion of a benzylthio linker in the trisaccharide of ST1, to release a thiol group for conjugation at the end of the assembly under Birch debenzylation conditions, enabled the chemoselective conjugation to a carrier protein as well as a glycan array surface, preserving potential protective epitopes.[148] The CRM_{197} conjugate of the trisaccharide (**9**) induced a robust antibody response in rabbit compared to the PCV13 commercial vaccine, that was used as a control, and it reduced the blood bacterial load of mice in a passive immunization approach using pooled rabbit serum.[148] The assembly of structures up to four repeating units showed that the longer synthetic structures adopt helical structures with approximately eight monosaccharides completing a full turn. It was also shown that the oligomers encompassing three RUs (nine monosaccharides) bound monoclonal antibodies, raised against the deacetylated native CPS, much better than shorter fragments. There, thus, seems to be a correlation between the secondary structure and antibody binding activity. An outstanding issue remains the introduction of *O*-acetyl groups, that have been shown to be an important structural feature, but the introduction of these groups, alongside the carboxylic acids, acetamides, and free amines in the structure, represents a significant synthetic challenge.

Conjugates of synthetic fragments of serotype 3 PS of different lengths (up to a tetramer) have been prepared and conjugated to different carriers, including TT, KLH, and CRM_{197}.[151−153] The CRM_{197} conjugates of the di-, tri-, and tetrasaccharides of ST3 were shown to be protective in mice against a challenge with ST3.[151] Parameswarappa et al. showed that a tetrasaccharide exhibited the highest recognition of human anti PS serum on a glycan array, outperforming shorter fragments, and this structure was selected for conjugation to CRM_{197}, to deliver a model vaccine (**10**) that induced protection against ST3 in a mouse model.[154]

Oligosaccharides deviating from the serotypes included in the PCV13 vaccine have been targeted, and this has aided in gaining relevant structural information on key epitopes of these glycans. A synthetic hexasaccharide repeating unit of the ST2 CPS was selected from a set of different structures, based on glycan microarray screening.[155] Vaccination with the hexasaccharide linked to CRM_{197} (**11**) stimulated a T-cell-dependent B-cell response and induced CPS-specific opsonic antibodies in mice, resulting in killing of encapsulated bacteria by phagocytic activity. In addition, the neoglycoconjugate reduced the bacterial load in lung and blood of mice, transnasally challenged with the highly virulent ST2 strain NCTC 7466.

The combination of automated glycan assembly (AGA) and glycan microarray-based monoclonal antibody (mAb) binding studies has been used to identify a protective glycan epitope from ST8.[156] Out of four structures, a tetrasaccharide frameshift was shown to be preferentially recognized by a protective anti-natural PS mAb and this structure was conjugated to CRM_{197}. The ST8 neoglycoconjugate (**14**) induced an antibacterial immune response also in coformulation with the commercial PCV13, demonstrating the feasibility of a vaccine with extended coverage based on mixed synthetic and naturally derived carbohydrates.

A combination of ST2, 3, 5, 8, and 14 glycoconjugates (sPCV5), all obtained by chemical synthesis, has been shown to give high levels of specific protective antibodies against the different serotypes in rabbits.[157] All STs in sPCV5 induced immune responses comparable to the licensed control vaccines, except ST14, which led to a lower response. Coformulation with PCV13 and PCV10 also led to a robust protective immune response in this animal model, highlighting the potential of a fully synthetic carbohydrate-based multivalent vaccine as well as the combination of conjugates from chemical synthesis and polysaccharide extraction.

Stabilization of conjugates susceptible to degradation has been proposed as a way to improve vaccine performance. The ST5 repeating unit contains two rare sugars, the ketoamino sugar 2-acetamido-2,6-dideoxy-D-xylose-hexos-4-ulose (Sug*p*) and *N*-acetyl-L-pneumosamine (l-PneuNAc). The keto group of Sug*p* can cause degradation of the natural polysaccharide.[158] A set of structures equipped with a linker was synthesized, and through a glycan array, a penta- and a tetrasaccharide both containing the immune dominant L-PneuNAc and a terminal L-FucNAc (thus lacking the vulnerable C-4-ketone) were selected for conjugation.[158] Studies in rabbit showed that a pentasaccharide (**12**), presenting an additional β-Glc, induced a higher level of opsonic antibodies in comparison to the conjugated tetrasaccharide. Notably, the identified oligosaccharide epitope is devoid of the residues that are responsible for the product instability.

The two structurally similar polysaccharides 19A and 19F have also attracted a lot of attention because of the unexpected lack of cross-reactivity observed with PCV17, containing ST19F, with ST19A PS. Along with the synthesis of the individual serotype fragments,[159] the identification of cross-protective epitopes has been pursued. A chimeric oligosaccharide antigen (**16**) containing the repeating units of ST19A and ST19F has been assembled, and its glycoconjugate induced good levels of antibodies in rabbits that killed ST19A and ST19F bacteria in vitro.[160] By testing a series of synthetic short glycans, a shared ManNAc-β-(1−4)-Glc epitope, carrying a phosphate at the ManNAc-C4 has been identified,[161] although the immunogenicity of this epitope was not investigated.

Gold nanoparticles have also been used to combine glycans from two different serotypes onto the same carrier. Vetro et al. described the functionalization of gold nanoparticles with the tetrasaccharide epitope of ST14, the trisaccharide repeating unit of ST19F along with a T-helper peptide and a D-Glc, as a hydrophilic "filler" molecule.[162] This conjugate induced specific IgG antibodies in mice against the type 14 polysaccharide but not against the ST19F. This could either indicate that the conjugated ST19F fragment was suboptimal or that immune interference between the two glycans led to suppression of the response against this serotype.

3. EMERGING AMR TARGETS

3.1. *Acinetobacter baumannii*

A. baumannii, a commensal Gram-negative bacterium, is an important source of hospital acquired infections, and extremely drug-resistant strains have emerged.[163] Intensive care patients

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and patients receiving mechanical ventilation are at risk of developing lung infections, urinary tract infections, bacteremia, endocarditis, skin infections, and meningitidis.[164] The high mortality and morbidity of *A. baumannii* infections are a strong incentive for the development of alternative treatments, such as active or passive vaccination strategies, and whole cell vaccines, outer membrane vesicles (OMVs), as well as protein-based vaccines are under development.[165] *A. baumannii* shows an extremely diverse CPS repertoire, with many different serotypes expressing unique structures. Passive immunization strategies using a monoclonal antibody against the K1 capsular polysaccharide,[166] a polymer built up from [→3)-*β*-D-QuiNAc4NR-(1−4)-*α*-D-GlcNAc6OAc-(1−4)-*α*-D-GalNAcA-(1 →] repeats, with the quinovose residue carrying an acetyl or 3-hydroxybutyrate residue at the C-4-amine, are under development.[167]

The extraordinary biodiversity of the *A. baumannii* polysaccharides,[168] of which more than 40 different structures have been reported, poses tremendous challenges to their syntheses. Although they serve as a source of inspiration for the development of new synthetic methodologies, conjugation-ready *A. baumannii* oligosaccharides with application in initial immunology studies have not been reported. Wu and co-workers have reported that the natural polysaccharide of strain 54149 (a strain that was isolated from IC patients) could be depolymerized by the tail spike protein of bacteriophage ΦAB6TSP to generate an octasaccharide, comprising two tetrasaccharide repeating units of the polymer. These repeating units are built up of [→3)-*β*-GalNAcp-(1−3)-[*β*-Glcp-(1−6)]-*β*-Galp-(1→] repeating units with the characteristic pseudaminic acid residues at the C-6 of the glucose residue.[169] This octasaccharide (**17**) was conjugated to CRM_{197} by formation of the anomeric hemiaminal, reaction with dithiobis-(sulfosuccinimidyl propionate) (DTSSP), dithiothreitol (DTT) mediated reduction of the disulfide, and addition to maleimide functionalized CRM_{197} (Figure 6). Immunization of rabbits with conjugate (**18**) (4 boosts in 2 weeks) gave serum capable of recognizing *A. baumannii* 54149 CPS and (**17**). Cross-reactivity of this serum with other strains was less than the cross reactivity of sera raised using the native polysaccharide. The pseudaminic acid residue was shown to be a crucial binding motif for the antibodies in the sera, which showed adequate bactericidal activity.[169]

3.2. *Clostridioides difficile*

Clostridioides difficile, also known as *C. difficile*, is a Gram-positive species of spore forming bacteria that causes severe diarrhea and inflammation of the colon such as colitis.[170] *C. difficile* infection (CDI) is a global health threat with a high incidence in both hospitals and communities. It is estimated that *C. difficile* causes nearly half a million infections in the US annually with associated health care costs over $5 billion.[171] CDI is associated with a high risk of recurrence (approximately 60% after three or more episodes of infection), even in the absence of additional risk factors, such as antibiotics or exposure to the bacterium.[172]

The main risk factor for CDI is the imbalance of the microbiota, which leads to the disruption of its barrier effect, mainly due to systemic exposure to antibiotics. CDI affects elderly people with comorbidities, patients undergoing surgery, long-term hospitalized patients, and immunocompromised patients. The main virulence factors of *C. difficile* are two large exotoxins, toxins A and B (TcdA and TcdB, respectively),

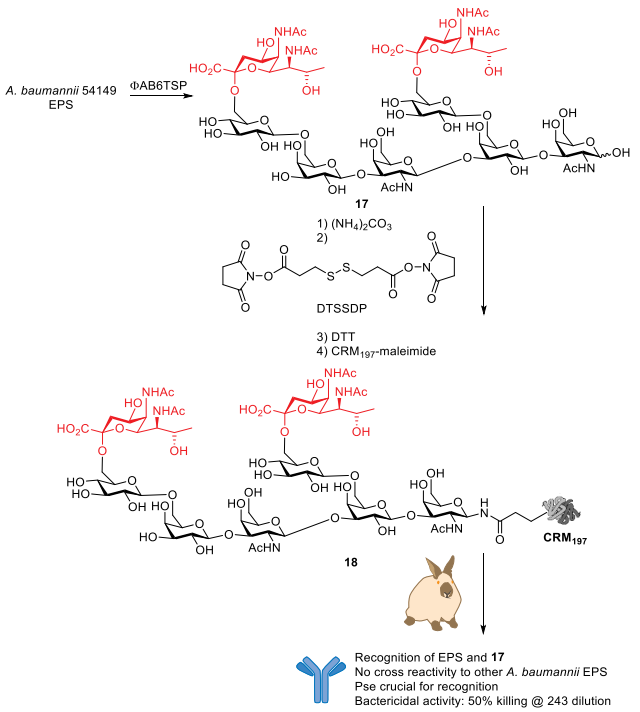

Figure 6. Pseudaminic acid (marked in red) containing octasaccharide conjugated to CRM_{197} as vaccine candidate against *A. baumannii*. Raised antibodies recognized the exopolysaccharide (EPS) and induced bacterial killing.

which are important mediators of intestinal damage and disease. In addition to these toxins, several surface components have been characterized as colonization factors, and these have been shown to be immunogenic.[173]

On the cell surface of *C. difficile*, three cell-wall polysaccharides, PSI, PSII, and PSIII, that are essential for bacterial survival and virulence, were identified and structurally characterized.[174,175] PSI is expressed at low levels on the cell surface, and it is unclear whether it is present in different *C. difficile* strains, while PSII and PSIII appear to be conserved surface polysaccharide antigens across the majority of *C. difficile* strains.[176] PSI is a polymer of branched pentasaccharide phosphate repeats, composed of rhamnose, glucose, and phosphate. PSII is made up of a hexasaccharide phosphate repeat of glucose, mannose, and *N*-acetyl-galactosamine constituents. PSIII is a water-insoluble lipoteichoic acid-like glycan, composed of phosphate, *N*-acetyl-glucosamine, and glyceric acid with minor repetitions of glucose and glycerol portions in the reducing regions.[174] In 2013 Martin et al. synthesized the pentasaccharide repeating unit of PSI and oligosaccharide substructures by applying a linear synthesis strategy from monosaccharide building blocks following the strategy schematically depicted in Figure 7.[177] The synthetic PSI repeating unit conjugated to CRM_{197} was shown to be capable of inducing immunoglobulin class switching as well as affinity maturation in mice. A disaccharide motif was identified as the minimal epitope, recognizing antibodies from serum and stools from CDI patients.

The synthesis of the PSI pentasaccharide repeating unit and conjugation to a subunit of *C. difficile* exotoxin B to yield a potential dual vaccine were reported by Monteiro's team.[178] Sera from healthy horses were shown to contain natural anti-PSI IgG antibodies that detected both the synthetic nonphosphory-

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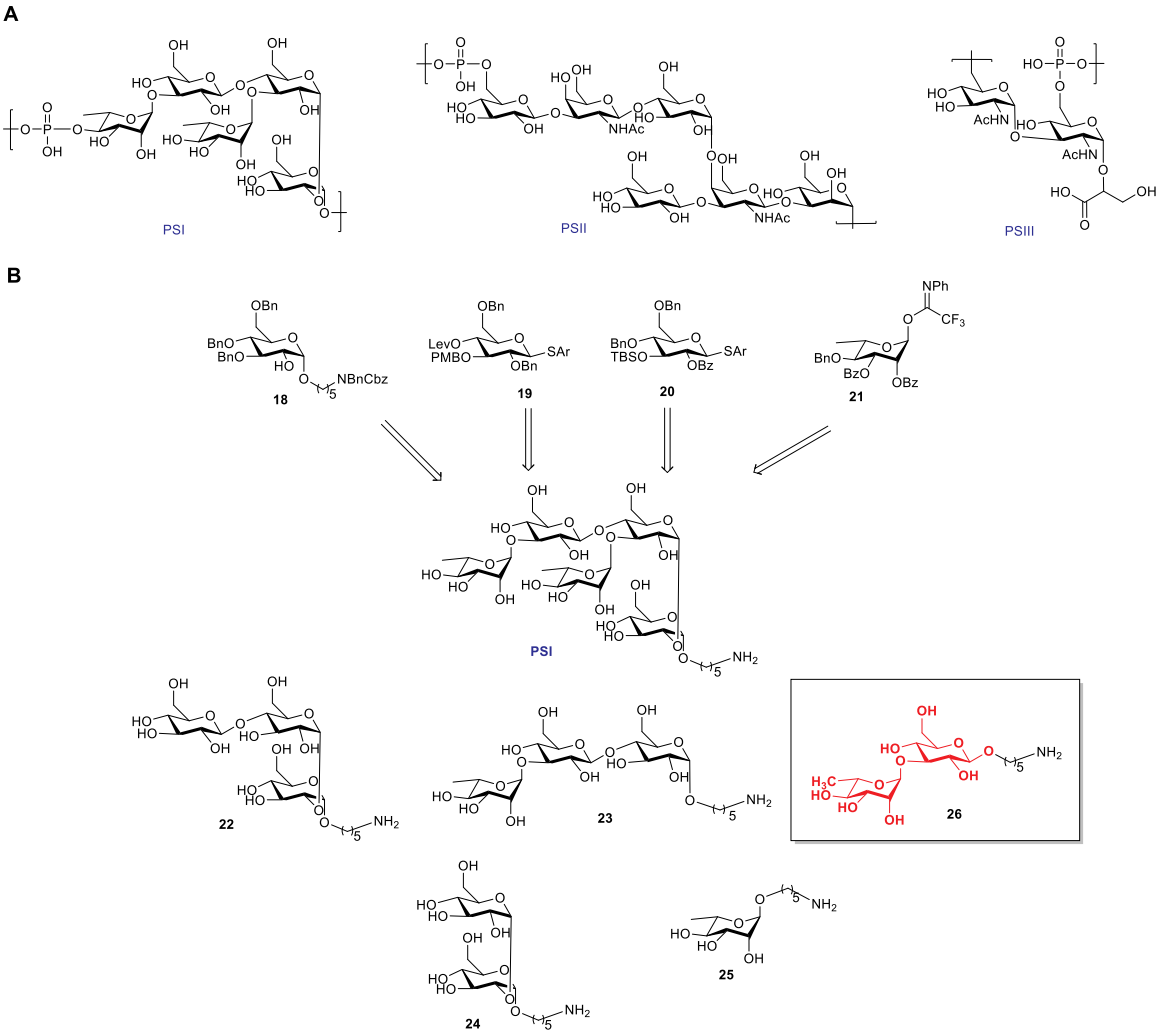

Figure 7. (A) Chemical structures of *C. difficile* PSI, PSII, and PSIII. (B) Synthetic routes for PSI and related fragments have been developed enabling identification of the disaccharide epitope (**26**).

lated PSI repeat and the native PSI polysaccharide, with a slightly higher recognition of the native PSI polysaccharide. Adamo and co-workers synthesized the hexasaccharide repeating unit of PSII, with and without the phosphate group at the nonreducing end, using a convergent [4+2] approach.[179] The synthetic hexasaccharides were conjugated to CRM_{197} carrier protein and evaluated in mice, showing that the hexasaccharide with the phosphate group was able to elicit IgG antibodies that recognized PSII on the surface of *C. difficile* cells, while the nonphosphorylated hexasaccharide induced neither IgG nor IgM antibodies (Figure 8).[180] The synthesis of the nonphosphorylated hexasaccharide has also been achieved by Seeberger and co-workers, who conjugated the glycan to CRM_{197} using squaric acid chemistry. The conjugate was capable of inducing carbohydrate-specific antibodies in mice, which bound to the hexasaccharide immobilized on a glycan array.[181]

Antibodies in stool from patients were also probed. It was observed that not all stool samples showed binding. This could be explained by varying amounts of antibodies in the stool samples, occurrence of CDI from a different serotype, or the need of the phosphate group for higher affinity binding as reported above. In 2013, Seeberger's group reported the synthesis of phosphodiester bridged oligomers of the PSIII

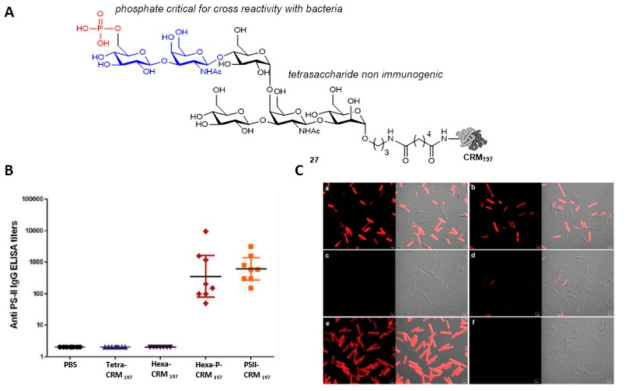

Figure 8. (A) Synthetic fragments of PSII from *C. difficile*. (B) Recognition of PSII on ELISA by the conjugated fragments showed the importance of the phosphate group for cross-reactivity with the natural PS. (C) Recognition of PSII on the bacterial surface assessed by confocal microscopy with anti-PSII (left part) and anti-(**27**) antibodies (right part). Adapted with permission from ref 180. Copyright 2012 American Chemical Society.

repeating unit. A synthetic PSIII monomer and dimer, synthesized using phosphoramidite chemistry, were immobilized on a glycan array and used to detect anti-glycan antibodies

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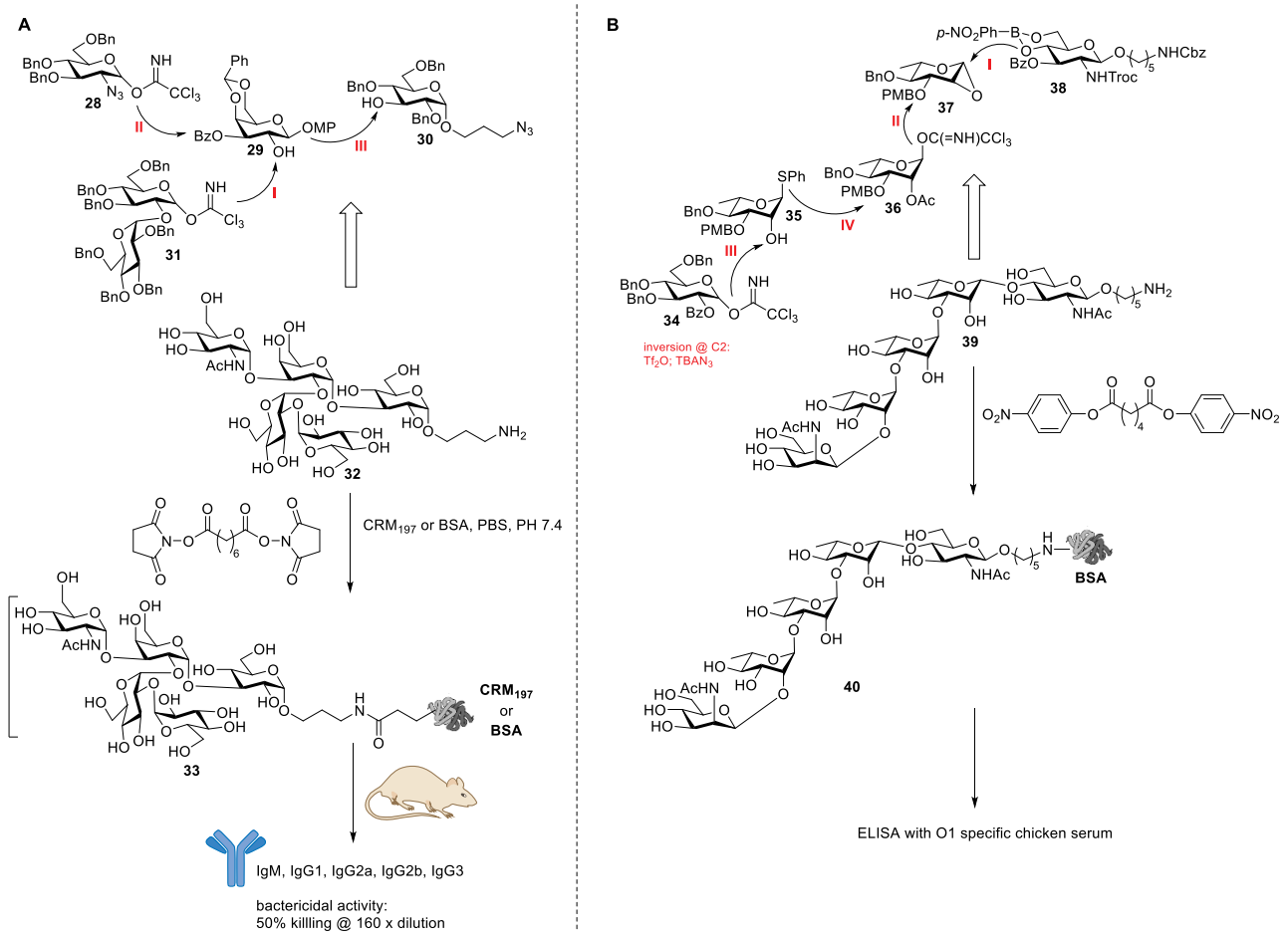

Figure 9. Synthetic routes for conjugates of the *E. coli* O-antigen core pentasaccharide (A) and repeating unit of O1 (B). A conjugate of the core pentasaccharide was shown to induce functional antibodies. The conjugate of the O1 fragment was recognized by anti-O1 chicken serum. The β-Rha-(1−3)-GlcNAc linkage was successfully generated by boron mediated insertion of the GlcNAc building block in the rhamnose-1,2-epoxide intermediate. The assembled structure was deprotected and conjugated to BSA (**40**). This conjugate was shown to recognize chicken anti-O1 serum, using an ELISA assay.

in the sera of CDI patients.[182] The PSIII dimer conjugated to CRM_{197} proved to be immunogenic and capable of protecting mice from a challenge with *C. difficile*.[183,184]

Later, the same team evaluated the protective efficacy of various glycoconjugates prepared by linking the various synthetic glycans to CRM_{197}, demonstrating that PSI and PSIII conjugates can reduce colonization in a murine model more effectively than an antitoxin vaccine candidate.[185] However, no binding to *C. difficile* cells could be demonstrated by flow cytometry, indicating that expression of the glycans was different in the used in vitro setup, that binding was too weak, as described above, or that cellular immune responses (as opposed to antibody mediated effects) were responsible for the protective effect. Furthermore, this study showed that the PSI, PSII, and PSIII glycoconjugates did not significantly affect the murine gut microbiota, indicating that the aroused antibodies are highly specific for *C. difficile*.

3.3. *Escherichia coli*

E. coli is a Gram-negative bacterium of which most strains are harmless commensal species. It is the most studied prokaryote and the "work horse bacterium" in molecular biology, microbiology, and biotechnology. Pathogenic *E. coli* strains, however, are causative agents of diarrhea disease, urinary tract infections, and pyelonephritis and neonatal meningitidis,[186] and pathogenic strains are emerging as the primary cause of bacteremia and sepsis in the US[187] and are associated with increasing AMR.[188] *E. coli* strains can be serotyped using different antigens, including their O-antigens (based on the partial structure of the lipopolysaccharides, of which currently >180 have been established) and K-antigens (based on the structures of the capsular polysaccharides, of which there are currently ~80 reported).[189]

Particularly, *E. coli* O25 ST131, producing extended-spectrum beta-lactamases (ESBLs), has spread globally, becoming the dominant type among extraintestinal isolates in many parts of the world.[190]

The development of vaccines against different strains has taken several approaches, including the use of attenuated pathogens and the development of protein- and peptide-based vaccines.

Polysaccharides have been used in the development of glycoconjugate vaccines, that have been generated through conjugation of native polysaccharides to carrier proteins using classic chemical conjugation or the in vivo bioconjugation (PGCT) approach. A nine-valent bioconjugate vaccine (ExPEC 9 V) is currently approaching phase III studies, after promising immunogenicity data of nine out of ten conjugates tested in phase II (Table 2).[191] The broad structural variety of the *E. coli* O- and K-antigens has been an inspiration for the development

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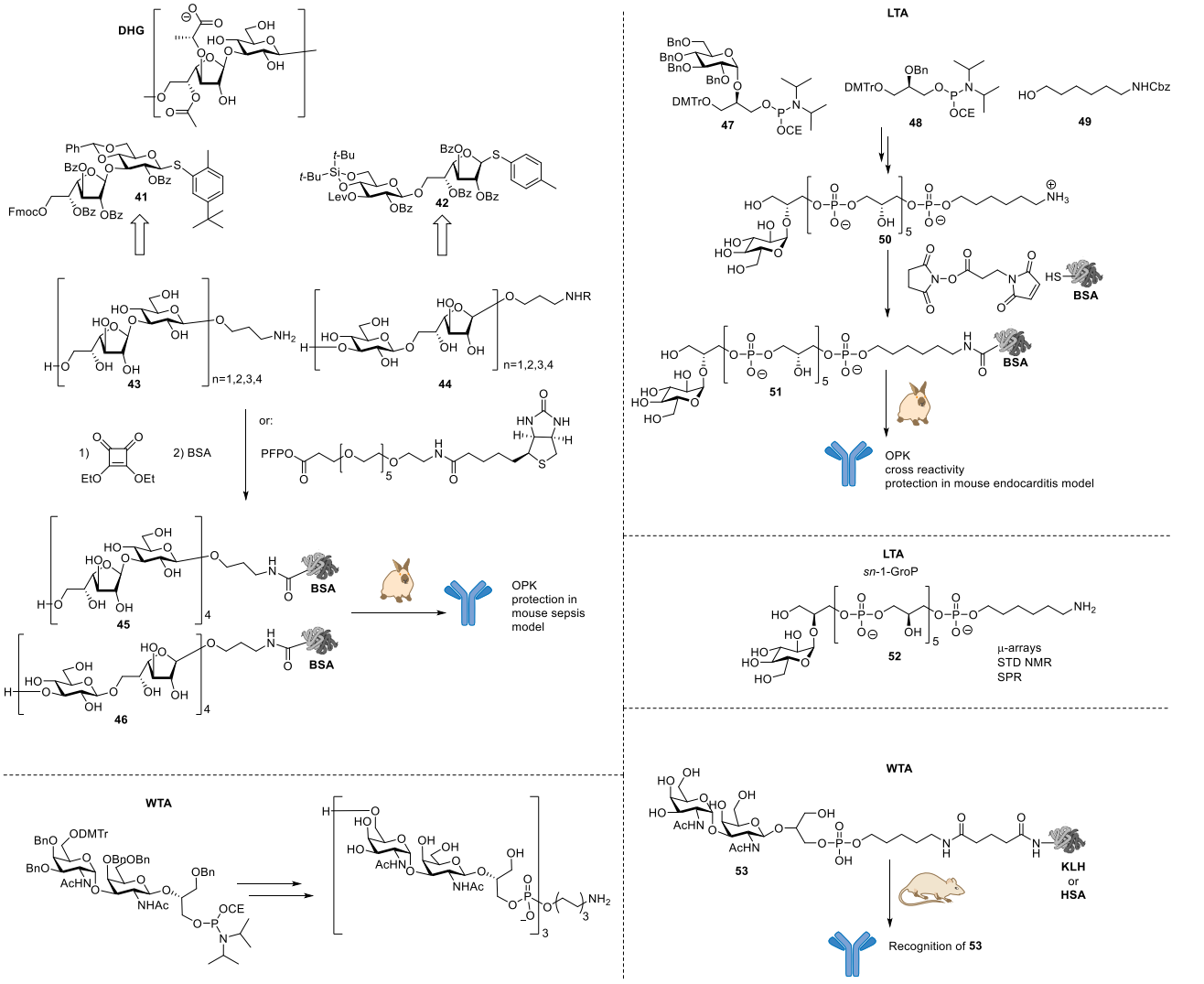

Figure 10. *Enterococcus faecalis* and *faecium* synthetic antigens, including capsular polysaccharides (DHG), as well as lipoteichoic acids (LTA) and wall teichoic acids (WTA). Glycoconjugates of synthetic DHG (**45** and **46**) and LTA (**51**) fragments elicited antibodies in rabbit, inducing bacterial opsonophagocytic killing (OPK) in vitro and providing protection from bacterial colonization in vivo.

of effective syntheses to generate well-defined fragments of these polysaccharides. The vast majority of these synthetic efforts, however, generated stand-alone saccharides, not suitable for the generation of glycoconjugate vaccines. Li and co-workers reported on the assembly of a core R3 pentasaccharide that was used to generate a conjugate vaccine.[192] The core oligosaccharides of *E. coli* lipopolysaccharides show limited variation—only five are known, termed R1, R2, R3, R4, and K12—and therefore represent attractive targets against which to direct the immune system. Figure 9 shows the structure and synthetic strategy of the conjugation-ready R3 pentasaccharide (**32**), built up from the common monosaccharides galactose, glucose, and glucosamine.

Conjugation of this glycan, using a disuccinimidyl suberate linker, to the common carrier protein CRM_{197} delivered a model vaccine (**33**) carrying ±18 oligosaccharides per protein. Immunization of mice in a mixture with Freund's complete adjuvant (FCA), led to the generation of sera with increased IgM, IgG1, IgG2a, IgG2b, and IgG3 levels. The sera were shown to have bactericidal activity against the *E. coli* O157:H7 strain, with 50% killing at 160-fold dilution.

Recently, Toshima's group reported on the synthesis of a pentasaccharide from the LPS of avian pathogenic *E. coli* O1, which is considered problematic because of its zoonotic potential.[193] The structure (**39**) presents a β-ManNAc residue at the upstream end and a β-Rha linked to the downstream GlcNAc (Figure 9). The synthesis was achieved through a [2+3] strategy, wherein the β-ManNAc-(1−3)-Rha was built using a Glc donor, followed by inversion of configuration at the Glc-C2 via an azide displacement of a triflate intermediate.

3.4. *Enterococci* spp

Enterococcus faecalis and *faecium* are Gram-positive bacteria that are associated with infections worldwide. *Enterococci* account for 11% of nosocomial bloodstream isolates, and an increasing trend has been recorded over the last decades.[194,195] In the US about 66,000 enterococcal infections occur each year, of which 20,000 are due to multiple drug-resistant strains, with about 1,300 deaths per year.[196]

E. faecalis is particularly worrisome because of vancomycin resistance, which also occurs in the community, with risk factors including non-heme residence, chronic skin ulcers, previous invasive procedures, exposure to antibiotics, and the presence of

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indwelling devices.[197] Four different serotypes (CPS-A to CPS-D) have been described for *E. faecalis* by mapping their immunoreactivities with ELISA and the opsonophagocytic capacity of antisera raised against the different CPS of prototype strains combined with analysis of their CPS locus.[198] One of the capsules produced by the CPS locus is diheteroglycan (DHG), which is present in CPS-C and CPS-D strains. DHG masks LTA in the bacteria and prevents opsonization by anti-LTA antibodies. The structure of DHG from the *E. faecalis* type 2 strain has been described by Theilacker and co-workers, where the polysaccharide repeating unit [→6)-*β*-D-Gal*f*-(1−3)-*β*-D-Glc*p*-(1→] is decorated at C-5 of the Galf residue with an O-acetyl and at C-3 with an R-lactic acid (Figure 10).[198,199]

Recently, Laverde et al. described the synthesis of a library of DHG-oligomers up to an 8-mer in length, lacking the *O*-acetyl and lactic acid residues.[200] The fragments (**43** and **44**) were built up using either Gal*f*-Glc*p* or Glc*p*-Gal*f* dimers to explore whether the type of frameshift played an important role in immunogenicity. The assembled library of DHG-fragments was functionalized with a biotin handle and printed on a streptavidin glycan array. It was revealed that the longer fragments were more immunoreactive with the frameshifts having the "non-self" galactofuranose terminus appearing as better antigens. The octasaccharides were selected for conjugation, and the two octamer frameshifts were coupled to BSA through squarate chemistry (**45** and **46**). Antibodies raised in mice with the two conjugates showed opsonophagocytic killing activities of *E. faecalis* type 2 strains and CPS-C and CPS-D strains expressing DHG. In a mouse sepsis model, it was shown that the raised sera offered protection in a passive immunization strategy. As the evaluated synthetic fragments were not decorated with acetyl or lactic acid substituents, the role of these functional groups remains to be established. The cell wall of *E. faecalis* A and B is not protected by a CPS, and the LTA of these species is thus more exposed. LTA is built up from a poly(1−3)-glycerol phosphate (GroP) backbone and has attracted attention for its potential as an universal antigen as it is shared with other Gram-positive pathogens such as *S. aureus*.[201−203] Initial studies conducted with isolated LTA from *E. faecalis* showed that opsonophagocytic antibodies could be raised against type 1 LTA, and cross-reactivity was observed against *E. faecalis*, *E. faecium*, *S. aureus*, and *S. epidermidis* strains.[204] Different synthetic approaches toward well-defined LTA fragments have been developed using both solution phase[205] and solid phase chemistry,[206,207] and these have been recently reviewed.[203] *E. faecalis* GroP LTA can be decorated with *α*-D-glucosyl or *α*-kojibiosyl residues at the C-2 of the GroP residues. This position can also carry labile D-alanine esters. Building on contemporary DNA chemistry, Hogendorf et al. assembled a small library of LTA fragments, out of which (**50**) was identified as a potent antigen, able to inhibit the opsonic killing of antibodies raised against LTA from *E. faecalis* 12030.[208] The BSA conjugates of (**50**) induced specific IgG antibodies in rabbits, that were directed to the native LTA. These antibodies showed cross-reactivity against other types of Gram-positive strains and could be used in a passive immunization scheme to protect mice in an endocarditis (heart valve infection) model.

To generate a broader and more diverse library of TA oligomers presenting different carbohydrate appendages at different positions on the TA chain, van der Es et al. developed a second generation automated solid phase approach using a universal linker system and fluorous-tagging strategy.[209] These TA fragments were used to create a TA glycan array to characterize the binding specificity of sera raised against isolated LTA from *E. faecalis* and the well-defined TA conjugate (**51**). The arrays revealed that the serum raised against the native LTA bound various TA fragments and no specificity for any carbohydrate pattern became clear. The serum raised against conjugate (**51**) proved to be very selective in binding, showing that it is possible to selectively raise antibodies to recognize specific and well-defined TA epitopes, which is not possible for TAs isolated from bacterial sources.

The effect of the *α*-glycosylation has been recently more thoroughly explored.[210] Using an improved method for the assembly of the glycosylated GroP building block, a series of glucosylated GroP hexamers was assembled, varying in the position of the glucose residue. Besides, a series of glucosylated GroP of the opposite stereochemistry (sn-1-GroP) was generated as well. Using glycan array binding studies, it was revealed that antibodies raised against (**52**) selectively recognized the sequence to which they were raised with binding decreasing as the glucose moiety moved down the chain. The GroP hexamers of the opposite stereochemistry were poorly recognized. The serum raised against the native LTA bound the opposite stereochemistry GroP better as it better resembles how LTA is anchored in the bacterial cell wall. A follow-up study elucidated the interactions of a mAb generated against (**52**) and a library of LTA structures through a combination of glycan array, ELISA, SPR-analyses, and STD-NMR spectroscopy to show that the number of GroP residues was important for affinity of the mAb and that binding was indirectly effected by the presence of the glucose residue.[211] This study confirmed the importance of the backbone chirality.

Enterococci also express different types of WTA, where several type 2 WTAs have been described for *E. faecalis*. A WTA from *E. faecium* strain U0317 was found to consist of →6)-*α*-GalNAc-(1−3)-*β*-D-GalNAc-(1−2)-GroP-(3→ repeating units, and this complex WTA can shield the LTA from opsonophagocytic antibodies.[198,203,212] While the biosynthesis of LTA includes GroP units consisting of phosphatidyl glycerol having sn-1-glycerol phosphate stereochemistry, the WTA is generated using cytidine sn-3-glycerol phosphate. The stereochemistry of the GroP moiety from the WTA was unambiguously established by van der Es et al.[213] through the synthesis of two sets of oligomers using either an sn-glycerol-1-phosphate or an sn-glycerol-3-phosphate constituent. From comparison of the NMR spectra of the synthesized fragments and naturally occurring WTA, the stereochemistry of *E. faecium* WTA GroP was established to be sn-3-glycerol phosphate.

Zhou et al. also reported a synthesis of the *E. faecium* U0317 repeating unit for investigation of a WTA-based vaccine. However, this report did not describe the stereochemistry of the GroP moiety. The target structure was synthesized using H-phosphonate chemistry to insert the phosphodiester bridged linker for conjugation. The trimer was conjugated with the carrier proteins KLH and HSA (**53**) using a bifunctional glutaryl ester, and the conjugates could be used to generate antibodies that were capable of recognizing the sequence to which they were raised. Binding to naturally sourced WTA or *E. faecium* was not reported, nor were any opsonic properties evaluated.[214]

3.5. Group A *Streptococcus*

Streptococcus pyogenes, commonly known as group A *Streptococcus* (GAS), is a Gram-positive *β*-haemoliticum bacterium that can cause a variety of diseases, varying from minor illnesses (pharyngitis and cellulitis) to very serious (glomerulonephritis,

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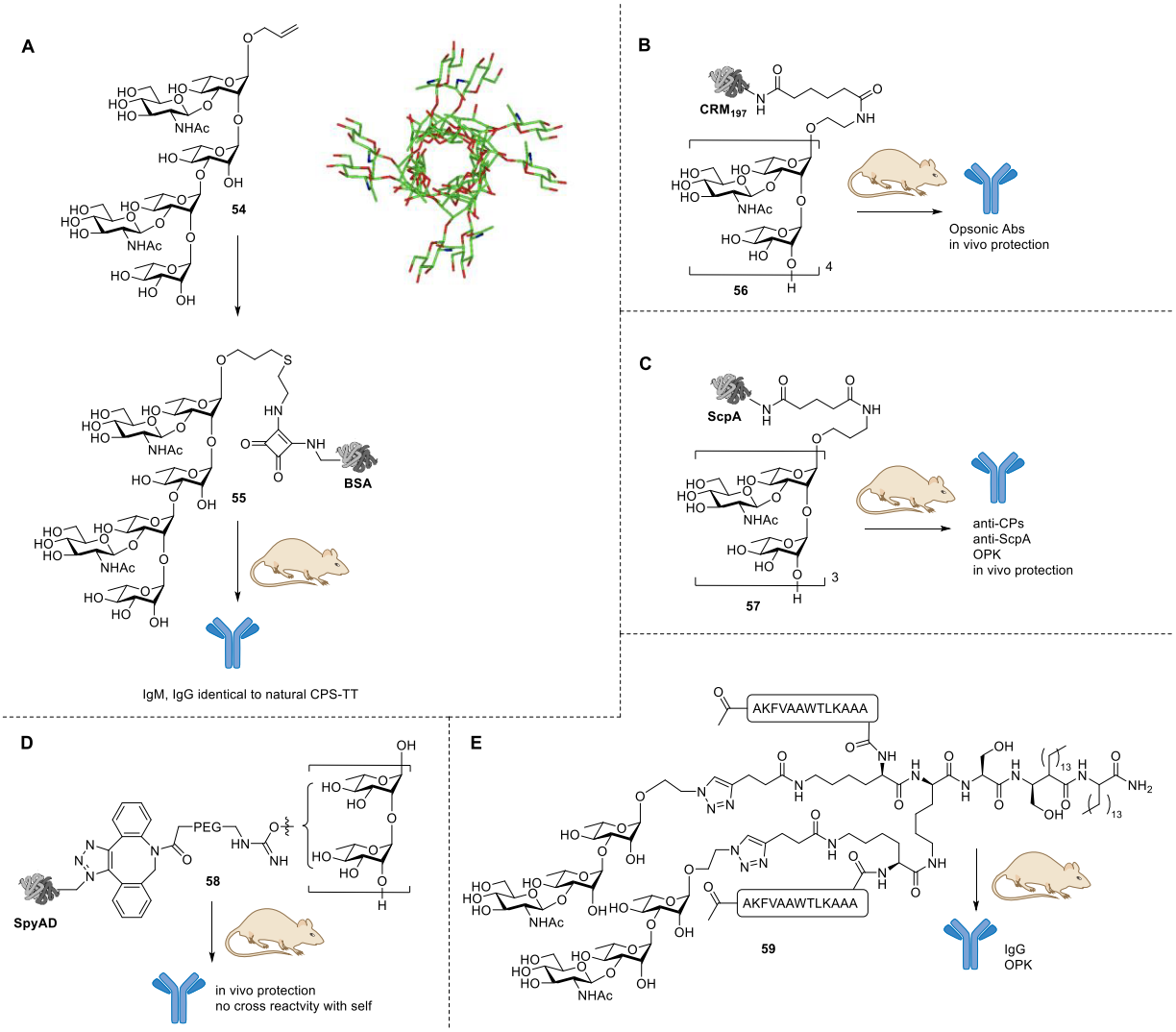

Figure 11. Synthetic strategies for group A *Streptococcus* antigens and collected immune data. (A) Immunization with a GAS cell wall hexasaccharide conjugated to BSA through squarate chemistry induced antibodies identical to a natural CPS-TT conjugate. In silico conformational analysis of the immunogenic fragment suggested that the polyrhamnose backbone forms a helix with the GlcNAc moieties on the periphery. Adapted with permission from ref 230. Copyright 2004 Elsevier). (B) The GAS dodecasaccharide conjugated to CRM_{197} elicited a high titer of anti-GAS opsonic antibodies. (C) Chemoenzymatically assembled nonasaccharide conjugated to the GAS protein ScpA (GAS C5a peptidase) induced antibodies against both antigens. (D) Site-selective conjugation of natural GAS polysaccharide to the streptococcal protein SpyAD induced antibodies able to provide in vivo protection in mice. (E) GAS trisaccharide conjugated to a synthetic peptidolipid, containing the T-cell peptide epitope PADRE.

necrotizing fasciitis, and rheumatic fever) and deadly diseases (sepsis).[215] It, thus, represents a significant threat to human health.

Currently GAS infections are treated with antibiotic therapy, but the increased emergence of antibiotic resistance renders new effective treatment and prevention strategies urgent.[216] The GAS cell wall is decorated with a surface polysaccharide, classified as the Lancefield antigen,[217] that consists of trisaccharide repeating units of [→3)-α-L-Rha-(1−2)-[β-D-GlcNAc-(1−3)]-α-L-Rha-(1→]. This polysaccharide is a bacterial virulence factor and has been recognized as a promising target to develop GAS vaccines.[218] Its expression is conserved across a variety of bacterial strains,[219] and protective properties of anti-GAS-PS antibodies have been demonstrated.[220] Pinto and co-workers have reported several syntheses of GAS-PS fragments and their protein conjugates.[221−226] To gain insight into the epitope of GAS-PS, oligosaccharides were used to study interactions with an IgG3 mAb generated against the natural polysaccharide. It was found that β-D-GlcNAc-(1−3)-[α-L-Rha-(1−2)]-α-L-Rha and an extended surface were the key components for epitope recognition.[227] Homology-based molecular modeling of the Fv region of the mAb indicated that two pockets could accommodate two GlcNAc residues on adjacent trisaccharide units, which was further supported with data from STD-NMR studies.[228]

Using naturally sourced CPS in combination with well-defined synthetic fragments, it was revealed that polyclonal rabbit serum and serum of children recovered from a GAS infection showed different binding specificities.[229]

The human sera required a hexasaccharide binding motif with at least two GlcNAc residues for adequate binding, while the rabbit sera also bound well to shorter fragments. Likely, repeated exposure to the bacterium led to affinity maturation of the human antibodies and a much narrower binding specificity. The GAS PS adopts a helical structure with the backbone

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polyrhamnose forming a helix placing the GlcNAc residues on the periphery.[230]

Based on these detailed structural and binding studies it was proposed that an effective oligosaccharide conjugate vaccine would require the presentation of a contiguous series of GAS-PS helical motifs and would likely be comprised of, at least, a doubly branched hexasaccharide. Indeed, when the immunogenicity of TT conjugates of a synthetic hexasaccharide, conjugated through squarate chemistry (**55**), and the native cell-wall polysaccharide (CWPS) were compared in mice, similar IgG titers were elicited (Figure 11 panel A).[231] In a more recent work by Costantino and co-workers, a series of GAS oligosaccharides with different frameshifts were chemically synthesized and the immunogenicities of their CRM_{197} conjugates were tested in mice.[232] All the structures induced robust production of functional IgGs, and the dodecasaccharide (**56**) with a terminal Rha residue induced the highest opsonophagocytic titer. A similar trend was observed in a GAS infection model, indicating the correlation of opsonic antibodies with protection. Gu and co-workers have tested synthetic GAS-PS with different carrier proteins, including BSA, CRM_{197}, TT, and GAS C5a peptidase (ScpA, **57**).[233] The use of a streptococcal protein could increase protection and could avoid potential epitope suppression with CRM_{197},[234] as this latter protein is used in many commercial vaccines. Mono-, di-, and trimers of the trisaccharide repeating unit of GAS PS were synthesized by a convergent and efficient strategy and conjugated to an enzymatically inactive ScpA mutant through a bifunctional glutaryl linker.[233] The ScpA-neoglycoconjugate induced an anti-carbohydrate immune response in mice, that was comparable to the one elicited by the related CRM_{197} and TT conjugates. In addition, robust ScpA193-specific antibodies were observed, making the ScpA193−oligosaccharide conjugates promising bivalent anti-GAS vaccine candidates.

In another study a more in-depth immunological characterization of the ScpA193 conjugates was conducted. Of the tri-, hexa-, and nonasaccharides, the latter two nonasaccharide−ScpA193 conjugates in a mouse model revealed that the latter conjugate could effectively protect structures, leading to a better in vitro recognition of GAS cells and opsonophagocytosis.[235]

Evaluation of the hexasaccharides and the animals from GAS challenge and GAS-induced pulmonary damage showed significantly increased animal survival. Further studies suggested that the two ScpA193 conjugates could function through activation of CD4+ T-cells and promotion of T-cell help, which would trigger differentiation into antigen-specific Th1 and Th2 cells, which, in turn, would provoke strong B-cell activation and IgG production.

It has been proposed that antibodies directed at the immunodominant GlcNAc residues may give rise to auto-immunity directed at GlcNAc residues on self-tissue. Therefore, polyrhamnose has been proposed as a possible alternative to GAS PS,[236,237] and it has been shown that an immune response can be invoked against the polyrhamnose backbone, lacking the GlcNAc branches, and that sera raised against this backbone PS can promote opsonophagocytic killing of multiple GAS strains to offer protection against a systemic GAS challenge after passive immunization.[236] Nanoparticles generated by conjugation of a rhamnohexaose, corresponding to three consecutive GAS PS epitopes, devoid of the GlcNAc moieties, to gold beads have been shown to inhibit binding of anti-GAS murine serum in an ELISA setup, indicating that this hexasaccharide may be an adequate antigen in a synthetic anti-GAS vaccine.[238] Naturally sourced GAS-polyrhamnose has been conjugated to the streptococcal protein SpyAD (**58**) and explored as a vaccine modality.[239] Conjugation was achieved in a site-selective fashion by expressing the protein in the so-called Xpress cell-free expression system, to incorporate a non-natural amino acid enabling click chemistry of the polysaccharides. The conjugated SpyAD-GACPR elicited antibodies that bound the surface of various GAS strains and promoted opsonophagocytic killing by human neutrophils. Active immunization of mice with a multivalent vaccine consisting of SpyAD-polyrhamnose, together with the candidate vaccine antigens streptolysin O and C5a peptidase, protected against a GAS challenge in a systemic infection model and localized skin infection model. No evidence for cross-reactivity to human heart or brain tissue epitopes was found, indicating this approach can circumvent potential safety concerns of the GlcNAc carrying GAS PS. Furthermore, the inactive mutant of SpyAD is suited for site-selective conjugation, thereby preserving critical protective immune epitopes of the carrier protein.

An innovative design has been proposed by Stephenson and co-workers, who conjugated small GAS PS fragments to a synthetic peptidolipid, containing the PADRE peptide, known to trigger T-cell responses.[240] By this approach a fully synthetic nanoparticle vaccine (**59**) of 300−500 nm was formulated. The di- and trirhamnosyl as well as the GlcNAc dirhamnosyl lipopeptides were able to produce statistically significant GAC-specific IgG responses in comparison to unconjugated structures.[241] Mice were immunized with the glycopeptidolipids, and the serum raised against the di- and trirhamnoside constructs showed opsonic activity.

3.6. Group B *Streptococcus*

Streptococcus agalactiae, also known as group B *Streptococcus* (GBS), is a Gram-positive pathogen that colonizes the recto-vaginal tract and represents a major cause of neonatal and maternal infections.[242] There is an estimate of 2.6 million stillbirths each year, many of which are due to GBS infections, especially in low and middle income contexts.[243] The bacterial capsule is surrounded by sialylated branched polysaccharides, which share great structural similarity to one another, based on which 10 serotypes are defined (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX).

All GBS polysaccharides are sialylated, and most of them share the same monosaccharide residues.[244,245] The GBS CPS is a fundamental virulence factor and is promising for the development of a vaccine for maternal immunization,[242] in which the vaccine would be administered to pregnant women to elicit serum antibodies that are then transferred to the baby through the placenta. Data on the global distribution of GBS clinical isolates show that a hexavalent vaccine including serotypes Ia, Ib, II, III, IV, and V could provide a protection over 95% for all virulent GBS strains, and multicomponent CRM_{197} conjugates have been tested in phase I and II clinical trials.[246,247] Vaccines currently under development exploit polysaccharides extracted from bacteria conjugated to CRM_{197}, which yield complex heterogeneous mixtures.

Synthetic GBS oligosaccharides have been prepared and evaluated at a preclinical level, being mainly used to understand the chemical features correlating with immunogenicity. Among all GBS serotypes, type III has been most studied, because of its seroprevalence.[243]

Various approaches to produce short PSIII fragments have been based on either depolymerization or approaches employ-

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Table 3. Synthetic GBS Structures and Corresponding Synthetic Strategies

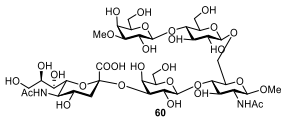
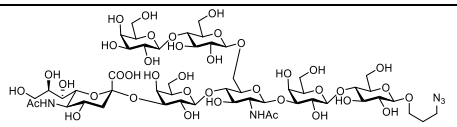
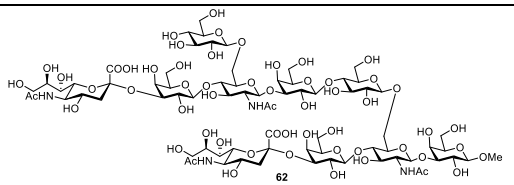
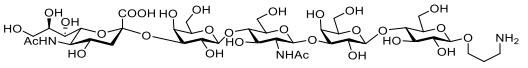
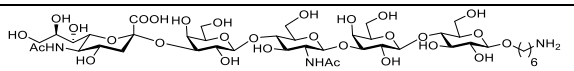
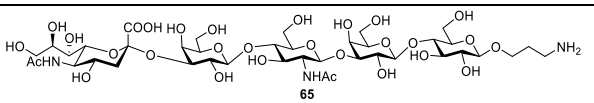
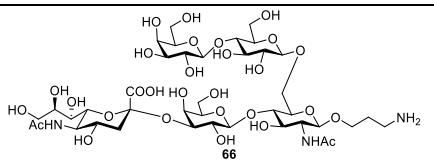
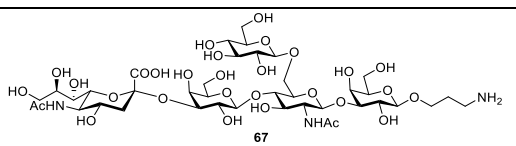
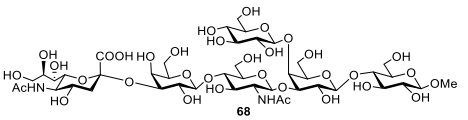
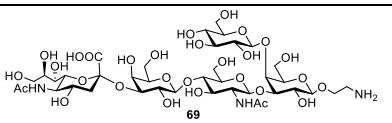
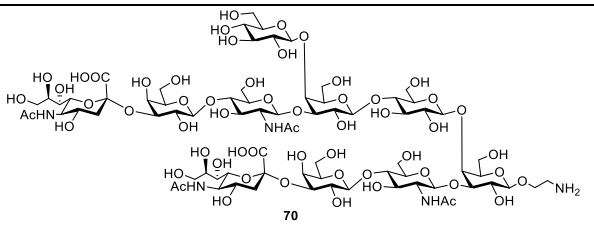
Entry	Structure	Synthetic method	Serotype	Ref
1	 60	Chemoenzymatic sialylation of a tetrasaccharide with a rat liver sialyltransferase	III	263
2	 61	[3+2+2] convergent synthesis	III	253
3	 62	Double enzymatic sialylation of octasaccharide acceptor	III	261
4	 63	Reactivity-based one-pot glycosylation	III	99
5	 64	[2+3] convergent approach	III	250
6	 65  66  67	[2+3] convergent approach	III	257
7	 68	Enzymatic syalylation of a pentasaccharide using CMP-Neu5Ac as donor	Ia	261
8	 69	[2+3] convergent approach	Ia	262
9	 70	One pot iterative glycosylation followed by dual glycosylation	Ia	264

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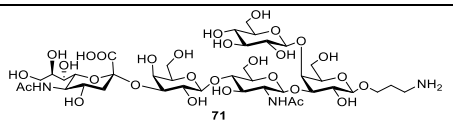
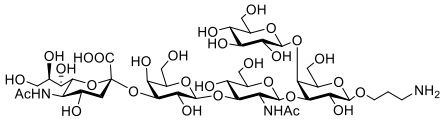
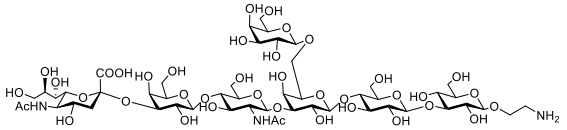
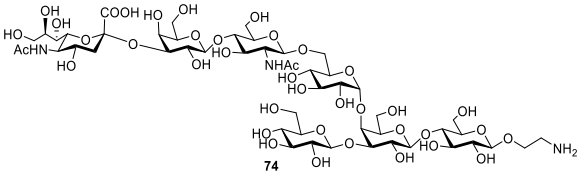
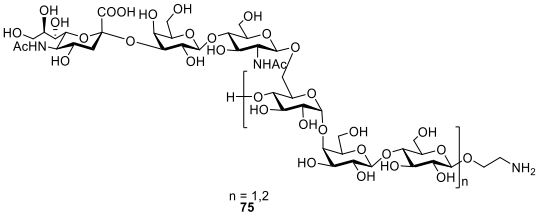
Table 3. continued

Entry	Structure	Synthetic method	Serotype	Ref
10	 71	Galactose 3-OH regioselective glycosylation	Ia	265
	 72		Ib	
11	 73	Convergent [4+2+1] glycosylation strategy	II	266
12	 74	Pre-activation based one-pot [2+1+4] glycosylation strategy	V	267
13	 n = 1,2 75	Pre-activation based iterative one-pot glycosylation based on the optimization of the stereoselective α-glucosylation	VII	263

ing organic synthesis or chemoenzymatic tools.[243] GBS type III PS has for a long time been regarded to be a length-dependent conformational epitope. Molecular dynamics simulations and NMR studies[248,249] showed that the GBS PSIII is able to form extended helical structures formed of at least four repeating units (RUs), that are stabilized by the presence of the charged sialic acids.[47]

This hypothesis has been based on the length-dependent recognition of the PS polymer with a high affinity anti-PSIII mAb. The presumed conformational epitope has hampered the development of anti PSIII vaccines based on short synthetic glycans, which have only recently emerged as useful tools to effectively mimic the GBS PSIII in its immunogenicity.

Syntheses of structures related to the linear form of the GBSIII repeating unit, a glycan that has also been found as part of viral glycans, have been reported;[99,250] however, these linear structures have been found to be poorly immunogenic. The synthesis of GBS structures poses several challenges, one of which is the α-sialylation of the Gal residues. Significant advances have been made to achieve stereoselective sialylations with the development of new building blocks and glycosylation methodologies, summarized in Table 3.[251]

An early synthesis of a more complex branched GBSIII oligosaccharide was based on a chemoenzymatic approach where a tetrasaccharide fragment[252] was sialylated using a specific rat liver sialyltransferase to obtain the branched repeating unit (**60**).

A completely chemical assembly of a PSIII heptasaccharide (**61**) using a highly convergent strategy has been described by Demchenko and Boons,[253] who used a highly convergent strategy which also provided the hexasaccharide fragment without the sialic acid.[254] Zou et al. used a chemoenzymatic approach to assemble the decasaccharide, representing two repeating units of GBSIII.[255]

The octasaccharide precursor was enzymatically sialylated by reaction with an α-(2−3)-sialyltransferase and CMP-Neu5Ac as donor. Using the same octasaccharide, an *N*-propionyl substituted sialic acid analog of the GBSIII dimer was also prepared. A similar enzymatic approach has been utilized to transform oligosaccharides from pneumococcal ST14 to their PSIII counterparts.[256] More recently, a thorough study on the immunogenicity of the three different repeating unit frameshifts has been conducted by Adamo and co-workers.[257] A branched (**66**) and "Y-shaped" (**67**) pentasaccharide, along with the linear frameshift (**65**), bearing a linker for conjugation to carrier proteins were generated through a convergent [3+2] approach employing a sialogalactosyl imidate donor.

The pentasaccharides were coupled to CRM_{197} and the conjugates bound to polyclonal antibodies in anti-PSIII murine serum in an ELISA. The branching β-(1−6) glucose, linked to the GlcNAc residue, proved to be an important motif for antibody recognition. Next, structural studies, including STD-NMR and X-ray experiments, indicated that a hexasaccharide fragment spanning across two repeating units is responsible for interactions with a protective mAb.[258]

It was found that oligosaccharides, obtained from nitrosylation of the polymer, with a length between 2 and 6 repeating units and conjugated to CRM_{197}, were able to elicit a functional immune response, comparable to CRM_{197}-PSIII, when conjugated with high glycodensity.[259] Following these studies, the

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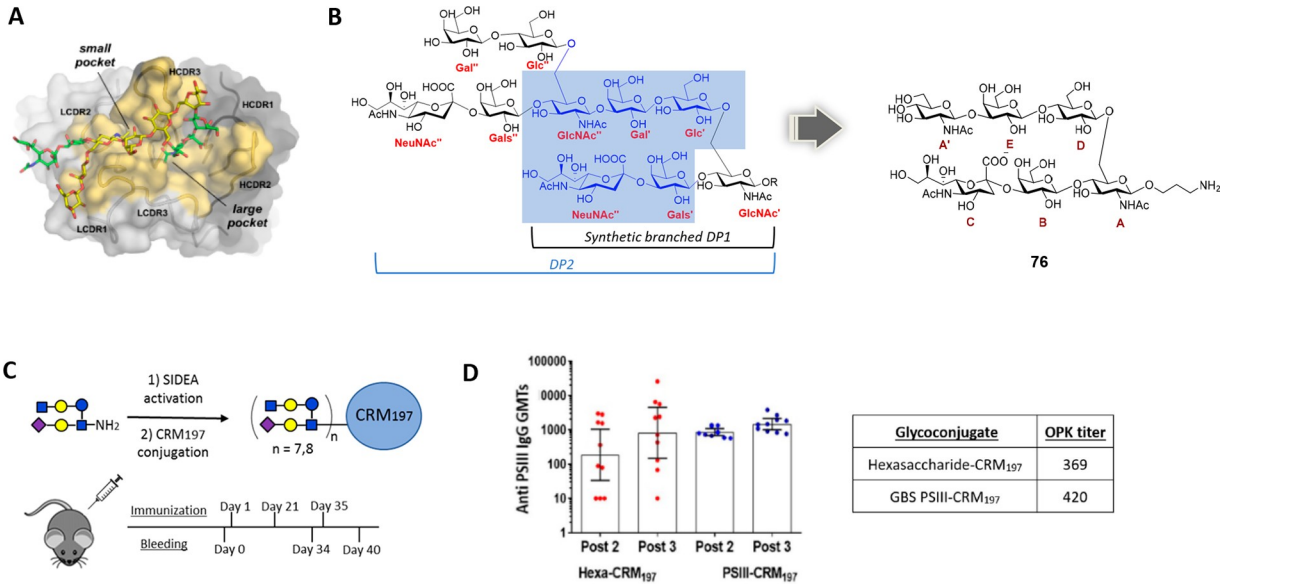

Glycoconjugate	OPK titer
Hexasaccharide-CRM_{197}	369
GBS PSIII-CRM_{197}	420

Figure 12. (A) X-ray crystallography of semisynthetic GBS III-DP2 (**76**) complexed with an anti PSIII rabbit Fab. (B) Combining data from STD-NMR and X-ray crystallography led to the identification of GBSIII structural minimal epitope highlighted in blue corresponding to hexasaccharide (**76**), which was synthesized accordingly. (C) The hexasaccharide (**76**) was conjugated to CRM_{197} carrier protein through the SIDEA activation chemistry and tested in mice. (D) Hexa-CRM_{197} conjugate elicited functional antibodies comparable to the positive control PSIII-CRM_{197}.

synthetic hexasaccharide (**76**) corresponding to the identified minimal structural epitope was synthesized using a regioselective glycosylation approach (Figure 12), and this structure was conjugated to CRM_{197} for in vivo evaluation. The resulting neoglycoconjugate elicited a strong protective immune response, similar the GBSIII conjugate that is currently in clinical trials, confirming that the hexasaccharide is the minimal GBS serotype III epitope and indicating the potential of the synthetic carbohydrate-based vaccine targeting these complex polysaccharides.[260]

Over the years, various syntheses of other GBS serotype glycans have been reported, with a focus on serotype Ia, which—after type III—is one of the most prevalent serotypes. The GBSIa hexasaccharide (**68**) was prepared using a chemoenzymatic synthesis starting from a synthetic pentasaccharide, using an enzymatic sialylation.[261]

In 2015 a total chemical synthesis of the pentasaccharide corresponding to the branched repeating unit of GBSIa (**69**) was accomplished using a convergent [2+3] route using a sialogalactosyl thioglycoside donor and a branched trisaccharide acceptor.[262] An investigation on the reactivity of the galactose acceptor in the glycosylation reaction showed that the presence of a sugar at the 4-O-position had a big impact on the reactivity of the C-3-hydroxy group, since it made the 3-O position inaccessible for further glycosylation.

Glycosylating the 3-*O*-position had a smaller impact on the 4-*O*-position, and for this reason the most effective sequence to glycosylate the galactose C-3 and C-4 hydroxy groups was to glycosylate the 3-*O*-position before introduction of a glycan to the C-4-OH. This approach was extended to the synthesis of a GBSIa decasaccharide (**70**), corresponding to two repeating units, which was prepared using a highly convergent strategy. First, a key intermediate trisaccharide was constructed via a one-pot iterative glycosylation; then, it was elongated to provide the hexasaccharide intermediate to which two side chains were attached by dual glycosylation with a sialogalactoside donor.[264]

Both the GBS Ia monomer and dimer were conjugated to CRM_{197}, and the resulting glycoconjugates were evaluated in mice, showing that both conjugates induced robust IgM and IgG antibodies, which were cross-reactive with both the monomeric and dimeric haptens, offering the first proof of principle for the use of synthetic glycans for an anti GBS Ia vaccine. Another convergent synthesis of branched GBS Ia and Ib repeating units ((**71**) and (**72**), respectively) has recently been described by our group, along with their linear frameshifts. NMR studies of these structures in combination with molecular dynamic simulations on the PSIa and PSIb polysaccharides were used to explain the origin of the highly specific immune response against these structurally very similar PSs, which differ only in one linkage in the repeating unit.

These conformational studies indicated that they place the disaccharide Neu5Ac(2−3)Gal motifs in a different spatial orientation, leading to the presentation of different epitopes.[265] Other GBS serotypes have also been synthesized, but data on their immunogenicity are not yet available. Gao et al. described the first synthesis of a GBS type V repeating unit, consisting of a heptasaccharide fragment.

The target structure (**73**) was prepared using a preactivation-based one-pot [2+1+4] glycosylation strategy, which not only resulted in the reduction of the number of steps to the final oligosaccharide but also minimized manipulations of the glycosyl donor.[267] A GBS type II branched heptasaccharide (**74**), corresponding to a frameshift of the repeating unit, was prepared by a convergent [4+2+1] glycosylation strategy.[266] Recently, Guo and co-workers also assembled a hexasaccharide repeating unit of GBS type VII and the corresponding dimer (**75**). The synthesis was achieved by means of a preactivation-based iterative one-pot glycosylation protocol, which was made possible by optimization of the challenging stereoselective α-glucosylation reaction.[263]

These synthetic efforts will pave the way for the preparation of even more complex GBS oligosaccharides from different serotypes. These structurally well-defined oligosaccharides can be used to map the relevant epitopes expressed by the capsular polysaccharides and be used as potential antigens in GBS glycoconjugate vaccines.

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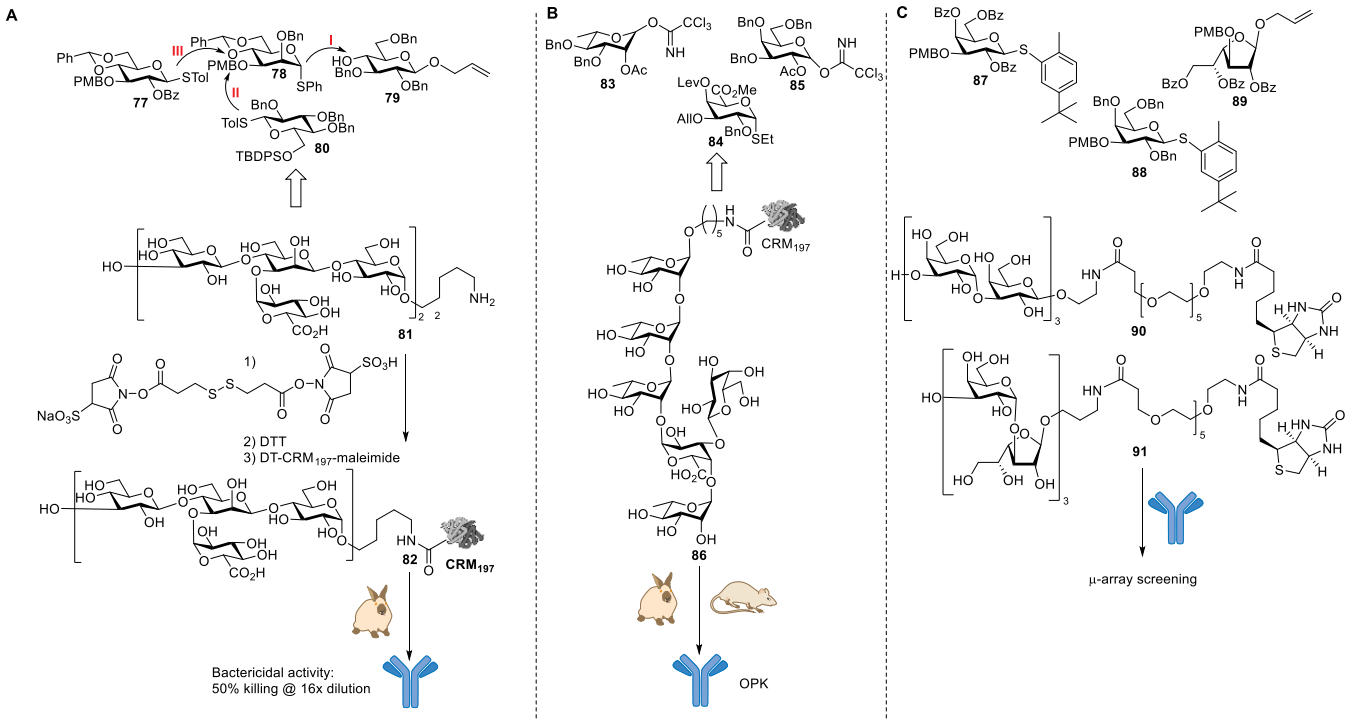

Figure 13. Synthetic routes for *K. pneumoniae* glycans for immunological testing: (A) Design of conjugates from synthetic fragments of the K2 capsule used for immunization of rabbits. (B) Conjugates from Kp ST238 CPS, that have been tested in rabbits, providing antibodies with opsonophagocytic killing (OPK) activity in vitro. (C) Structures related to O1 and O2 O-antigens used in the glycan array.

3.7. *Klebsiella pneumoniae*

K. pneumoniae (Kp) is a commensal Gram-negative bacterium that can cause chronic urinary tract and soft tissue infections, pneumonia, and sepsis, particularly in elderly and immunocompromised subjects.[197,268,269] *Klebsiella* spp. are the third most frequent cause of healthcare-associated infections, and the occurrence of multidrug-resistant Kp strains is considered as an urgent threat worldwide.[269] Carbapenem-resistant (CR-Kp) and extended spectrum β-lactamase (ESBL-Kp) Kp are major causes of concern due to the lack of therapeutic options; therefore, prevention of Kp infections by vaccination has become of paramount importance.[270] Synthetic vaccines targeting the sugar armory of Kp are currently under preclinical development. Kp surface oligosaccharide molecules, the capsular polysaccharide (CPS, K-antigen), and the lipopolysaccharide (LPS, with the O-antigen as the main target) are major virulence factors and promising targets for the development of vaccines.[271]

The CPS is mainly involved in colonization, adhesion, perpetuation, and proliferation in the host, while the LPS plays a main role in conferring resistance to the bactericidal activity of the complement.[272]

Kp K-antigen is anchored to the outer membrane through a lipid A tail. The structural variability of the CPS is extremely high, with more than 77 serotypes identified. This heterogeneity challenges the development of vaccines based on the CPS, which should comprise at least 24 serotypes to ensure adequate coverage against the most virulent strains. Proof of concept of the use of the K-antigen as a vaccine target has been provided in late '80s and '90s, when a six-valent and a 24-valent vaccine based on different Kp CPS were tested in humans, showing it to be safe and to induce serospecific IgGs in all vaccines.[273] Due to the complexity in manufacturing these vaccines, the development was discontinued. It did, however, provide the proof of principle for the use of K-antigens in anti-Kp vaccines. Besides the use of full-length, high molecular weight extracted polysaccharides, in 1985 Zigterman et al. demonstrated the efficacy of a vaccine based on an octasaccharide, obtained by depolymerization of the Kp capsular polysaccharide serotype 11.[274] K1 and K2 are the most virulent Kp serotypes, often present in antibiotic resistance strains, and these have, therefore, been recently targeted for vaccine design through different technologies. In 2019, expression of K1 and K2 polysaccharides conjugated to EPA was achieved in *E. coli*, and the bioconjugates proved to be immunogenic in mice and provided protection from lethal infections by two hypervirulent Kp strains.[275] Recently, the synthesis of oligosaccharides derived from K2 CPS was described and the obtained oligosaccharides were used as tools to elucidate the structure of the minimal epitope.[276] The repeating unit of the K2 antigen is built up from a [→3)-β-D-Glc-(1−4)-β-D-Man-(1−4)-α-D-Glc-(1→] backbone carrying α-D-GlcpA branches at the O3 of the mannoses. The main challenge of the synthesis is presented by the two 1,2-*cis* linkages (Figure 13A). First, the tetrasaccharide repeating unit was assembled; then, it was elongated through [1+4], [2+4], [3+4], and [4+4] glycosylations to obtain the penta-, the hexa-, the hepta-, and the octasaccharides. The α-selective glycosylation, key for the elongation process, was achieved using glycosyl fluoride donors using Cp_2HfCl_2/AgOTf mediated catalysis. The synthetic hexa-, hepta-, and octasaccharides (**81**) were conjugated to DT and tested in mice. Sera against the heptasaccharide were able to cross-react with all synthetic glycans, including the longer octasaccharide (**81**), and antisera against the hepta- and the octasaccharide DT conjugates showed a high titer of functional antibodies in a serum bactericidal assay.

A synthetic approach to the repeating unit of Kp ST238 CPS, a carbapenem-resistant (CR) strain which has been responsible for several outbreaks in hospitals in the US,[277] has been

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described by Seeberger's team.[278] The target hexasaccharide was synthesized using a [3+3] convergent approach based on three monosaccharide building blocks, of which the GalA building block was obtained by a de novo route (Figure 13B). The synthetic hexasaccharide (**86**) and shorter fragments were anchored to a glycan array and assayed against the protective mouse IgM mAb 1C9, raised against the CR-Kp CPS. The specific recognition of the hexasaccharide was demonstrated by inhibition of the binding of the mAb preincubated with Kp CPS. The hexasaccharide was then conjugated to the carrier protein CRM_{197} and tested in mice in two adjuvanted formulations, one with the Freund adjuvant (FA) and the other with Alum, which both elicited antibodies reactive against the hexasaccharide. However, only the conjugate formulated with FA induced antibodies that were cross-reactive with the Kp CPS. The formulation with Alum was also tested in rabbits and induced antibodies that were able to bind Kp cells and promoted the uptake and killing by phagocytic cells, indicating that the hexasaccharide-CRM_{197} conjugate represents a promising vaccine against hypervirulent CR-Kp. Two mAbs, 17H12 and 8F12, binding the synthetic glycan epitope, were shown to agglutinate all clade 2 strains and were also shown to promote inhibition of biofilm formation and extracellular killing of these bacteria, by complement deposition and the deployment of neutrophil extracellular traps.[279] The mAbs also promoted opsonophagocytic and intracellular killing of Kp by human derived neutrophils and cultured murine macrophages, indicating that this structure can be targeted to develop therapeutics.

The Kp LPS is composed of a conserved core oligosaccharide linked to the terminal lipid A portion and to the O-antigen, whose composition is highly variable across different strains. As opposed to the K-antigens, only 11 O-antigens have been identified, and these have been used for Kp typing; four of these O-antigens (O1, O2, O3, and O5) are found in clinically relevant strains, and therefore, it has been proposed that a vaccine comprising these four serotypes would ensure a 80% coverage against Kp infections.[280] O1 and O2 consist of D-galactose and D-galactofuranose, while O3 (including the related serotype O3b) and O5 are composed of D-mannoses. The O1 polysaccharide is the most prevalent serotype in clinical isolates, followed by the O2 and the O3 polysaccharides; therefore, most preclinical development of potential vaccines focused on these serotypes.

The O1-antigen is built of two polysaccharide types: Galactan (Gal) I, located at the inner part of the O-chain, shared also with other Kp serotypes (O2 and O2ac) and formed by a [→3)-Gal*p*-β-(1−3)-Gal*f*-α-(1→] repeating unit, and Galactan II, constituting the capping sequences and formed by a [→3)-Gal*p*-β-(1−3)-Gal*p*-α-(1→] repeating unit.[281] Extracted O-antigens have been tested in animal models as stand-alone polysaccharide antigen[282] or conjugated to Tetanus Toxoid[283] and *P. aeruginosa* flagellins FlaA and FlaB.[284] The syntheses of O-antigens belonging to serotypes O1 and O2 have been described. The first synthesis of the Galactan I disaccharide β-Gal*f*-(1−3)-Gal*p*, corresponding to the repeating unit of O2 LPS, was reported by Wang et al.[285] Longer Gal I oligosaccharides (a tetra- and hexasaccharide), bearing a *p*-methoxyphenyl aglycone at the reducing end, were prepared by Zhu et al.[286] The synthetic design was based on the preparation of a Gal*f*-β-(1−3)-Gal*p* disaccharide intermediate bearing a *p*-methoxyphenyl group at the reducing end and a levulinoyl group to mask the Galf 3-OH. The key step in the synthesis was the α-selective galactopyranosylation of the 3-OH of the di- and tetrasaccharide acceptor in solvent controlled glycosylation reactions. Further advances in the synthesis of Gal I (**90**) and II (**91**) hexasaccharide fragments were reported by Nifantiev and co-workers (Figure 13C).[287,288] A [2+2+2] synthetic approach was developed, and a pyranoside-into-furanoside (PIF) rearrangement was used to generate the galactofuranose building block. The synthesized di-, tetra-, and hexasaccharides of both serotypes were biotinylated for immobilization on a streptavidin-coated glycan array plate to screen sera against Kp O1 of serotypes K1, K2, and K16. All tested sera showed significant concentration-dependent reactivity with the Gal II hexasaccharide while less interaction was observed with the Gal II tetrasaccharide and the disaccharide was poorly recognized. The Gal I tetra- and hexasaccharides were specifically recognized by antibodies in sera against the Kp type 16, but not types 1 and 2, showing domination of Gal II antibodies over anti Gal I antibodies.

O1 and O2 oligosaccharides (and related subtypes O2afg and O2ac) with a length ranging from one to eight repeating units were synthesized recently using a highly convergent strategy based on a key di-galactose building block with a 3-Nap protecting group. Oligosaccharides from these serotypes were conjugated to CRM_{197} and tested in vivo in rabbit and mice. After immunization, functional antibodies against the homologous oligosaccharides used for the immunization and against the natural O-polysaccharides of Kp serotypes O1, O2, and O2ac and carbapenem-resistant ST258 were detected, indicating the potential of these vaccines to confer protection against Kp infections.[289]

O3, O3b, and O5 polysaccharides represent other clinically relevant Kp O-antigen serotypes which should be included in a vaccine to ensure adequate coverage against circulating strains. The repeating units of these polysaccharides are composed of mannoses and vary in the length of the repeating unit and the linkages between the monosaccharides. Syntheses of oligosaccharides from O3 to O3b to O5 have been recently described both in solution phase and by automated glycan assembly on a polymer matrix. O3 oligosaccharides spanning from one to four RUs (from pentamer to 20-mer), O3b hexa- and dodecasaccharides (corresponding to two and four RUs), and O5 tri-, hexa-, and nonasaccharides (one, two, and three RUs) were assembled and equipped with a chemical handle suitable for conjugation. Key building blocks for the solid phase synthesis were the two mannoses reported in bearing temporary protections on 2-OH and/or 3-OH, giving access to all the target O-antigen structures. The O3 pentasaccharide and O5 hexasaccharide were activated as *p*-nitrophenyl esters and conjugated to CRM_{197} and BSA. The CRM_{197}-conjugates were formulated with Alum for mice immunization and incomplete Freund adjuvant for rabbit immunization. Sera from immunized rabbits cross-reacted in ELISA with the corresponding O-antigens as BSA conjugates, while sera from mice were able to selectively recognize the homologous LPS.[289]

3.8. *Neisseria gonorrhoeae*

N. gonorrhoeae is a Gram-negative diplococcus bacterium closely related to other human *Neisseria* spp. *N. gonorrhoeae* only infects humans and most commonly leads to urethritis in men and cervicitis in women.[290] It causes the sexually transmitted genitourinary infection gonorrhea and other forms of gonococcal disease including disseminated gonococcemia, septic arthritis, and gonococcal ophthalmia neonatorum. Among these, the sexually transmitted infection (STI)

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gonorrhea remains a major global public health concern, causing 87 million cases globally and being the second most commonly reported infection in the United States.[291] To date, there is no vaccine for gonorrhea in humans, and efforts to develop it are becoming increasingly important, given the growing threat of gonococcal antimicrobial resistance (AMR) and increased incidence of STI gonorrhea. Recent studies have shown that vaccines against a closely related pathogen, such as the *N. meningitidis* serogroup B vaccine Bexsero, containing outer membrane vesicle (OMV) components can reduce the incidence of gonorrhea.[292] Several approaches for the identification of a candidate vaccine from outer membrane or pilin proteins are currently in preclinical development.[293]

N. gonorrhoeae lipooligosaccharides (LOS) play a major role in pathogenesis by inducing host inflammatory responses, including resistance to complement, adhesion, and entry into cells, and also enabling evasion of host innate immunity through sialylation.[294] The LOS is located in the outer membrane of the pathogen and constitutes about 50% of its mass. LOS are composed of three oligosaccharide (OS) chains, made up of glucose, galactose, and *N*-acetylglucosamine, which are linked to two heptose residues, which in turn are attached to lipid A through two molecules of 2-keto-3-deoxy-mannooctulosonic acid (Kdo) (Figure 14).

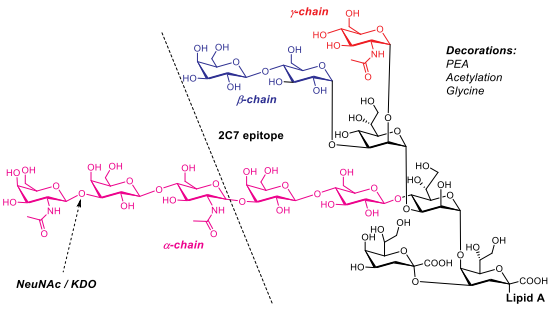

Figure 14. Chemical structure of *N. gonorrhoeae* LOS, composed of three characteristic α, β and γ chains. Highlighted is the epitope identified through the C27 mAb.

One OS chain, the α-chain, extends from the first heptose (Hep-I), while the second and third chains, the β- and γ-chains, are attached to the second heptose (Hep-II) (Figure 14). The compositions of the oligosaccharides are highly variable, in length, carbohydrate content, and number of chains. Phosphoethanolamines, acetyl esters, glycines, *N*-acetylneuraminic acid, and Kdo can also be present, resulting in heterogeneity of the LOS and different antigenic epitopes.[295,296]

In the last decade the so-called peptide mimotope approach has been applied to resemble the carbohydrate epitope structure, recognized by the opsonophagocytic mAb 2C7.[297] By screening a random peptide library, the Octa-MAP1 peptide was identified as an immunological surrogate of the 2C7-oligosaccharide epitope. The 2C7 epitope mainly includes the α-lactoside on Hep and may also contain a β-lactoside on Hep-I. In vivo evaluation of the Octa-MAP1 peptide in a mouse model demonstrated that it is capable of generating cross-reactive anti-LOS antibodies with complement-dependent bactericidal activity against gonococci.[295]

3.9. *Pseudomonas aeruginosa*

P. aeruginosa (PA) is a ubiquitous, Gram-negative bacterium that can grow under both aerobic and anaerobic conditions. Serious infections with PA are primarily encountered in the hospital setting,[298] such as fulminant and acute ventilator-associated pneumonia.[299,300] PA causes slowly progressive deterioration of pulmonary function in cystic fibrosis (CF) patients[298] as well as non-CF bronchiectasis in COPD patients.[301,302] Antibiotic resistance of PA is a major concern, and consequently, attempts have been made to obtain a vaccine, amongst others targeting the OAg. There are 20 different OAg structures, of which about 11 are expressed in the majority of PA clinical isolates.

The most commonly isolated serotypes in acute infection are O1, 6, and 11, although there are also isolates lacking the OAg.[303] A challenge associated with the OAg is the structural variability of the lipid A in the transition from acute to chronic infections, when the LPS changes from smooth (with OAg) to rough (without OAg). An eight-valent vaccine consisting of OAgs conjugated to exotoxin A (EPA), Aerugen, was initially demonstrated to be safe and immunogenic in plasma donors, as well as bone marrow transplant and noncolonized CF patients.[304−307] In a larger trial in European CF patients, however, Aerugen showed good safety but no significant difference in clinical outcome compared to the placebo control group.[308] In bronchiectasis patients, eliciting high titers of IgG2, specific against the OAg, resulted in impaired serum mediated PA killing,[309] which could explain the inconsistent results of LPS-based vaccination approaches.

Synthesis of fragments lacking Lipid A contaminants could be a means to better understand the reasons behind this inconsistent data. PA expresses two distinct lipopolysaccharide (LPS) molecules, also known as the A-band and B-band.[310] The B-band is the serospecific LPS, while the A-band is the common LPS antigen composed of a D-rhamnose O-polysaccharide chain. Ghosh's group constructed synthetic routes toward the trisaccharide repeating unit [→2)-α-D-Rha-(1−3)-α-D-Rha-(1−3)-α-D-Rha-(1→] of the A-band polysaccharide.[311] The total synthesis of the PA 1244 pilin trisaccharide α-5N-βOHC4-7N-FmPse-(2−4)-β-Xyl-(1−3)-FucNAc was achieved by Liu[312] and co-workers. Kulkarni and co-workers have accomplished the challenging synthesis of the PA O11 trisaccharide β-D-Glc-(1−3)-α-L-FucNAc-(1−3)-β-D-FucNAc,[313] where the highly stereoselective 1,2-*cis* incorporation of the L-fucosamine and D-fucosamine residues was achieved using fucosazide thioglycosyl building blocks.

PA also expresses exopolysaccharides, such as Psl, which is found in 76% of all analyzed clinical isolates. Its expression occurs in the primary infecting strains but seems to play a role also in establishing a persistent infection.[314−316] Psl is responsible for the formation and maintenance of biofilms, and an anti-Psl monoclonal antibody exhibited opsonophagocytic killing of a number of strains and conferred significant protection in multiple animal models.

Boons and co-workers reported the synthesis of a Psl tetra-, penta-, hexa-, and decasaccharide[317] (Figure 15) to map the epitope of three different classes of mAbs, developed as therapeutics to treat PA infections.[318] The synthetic strategy enabled the efficient stereoselective installation of the challenging β-mannosyl linkages as well as the construction of the very crowded mannoside that is glycosylated at C-1, C-2, and C-3.[317] The class II mAb reacted potently with each oligosaccharide, indicating its epitope to reside within the tetrasaccharide, indicating no need for a branched mannoside. The class III antibody well recognized the hexasaccharide and weakly interacted with the decasaccharide, while no binding was

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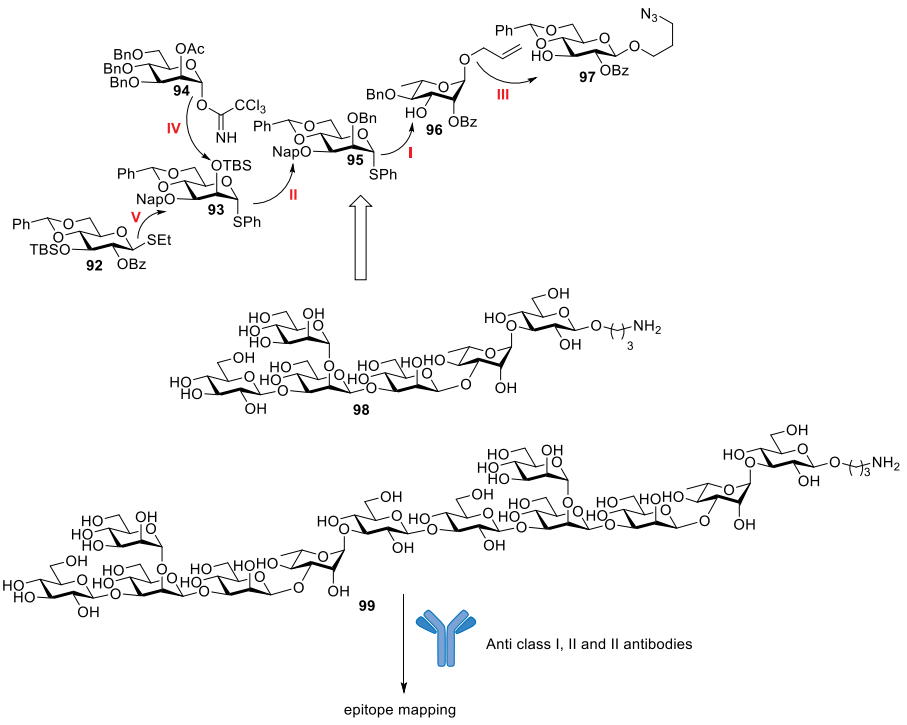

Figure 15. Synthetic strategy for *P. aeruginosa* Psl oligosaccharides and immune evaluation with class I, II, and II mAbs. Of these three mAbs, while class II and III allowed epitope mapping, class I did not map any epitope, suggesting that the structure is lacking motifs relevant for antibody recognition.

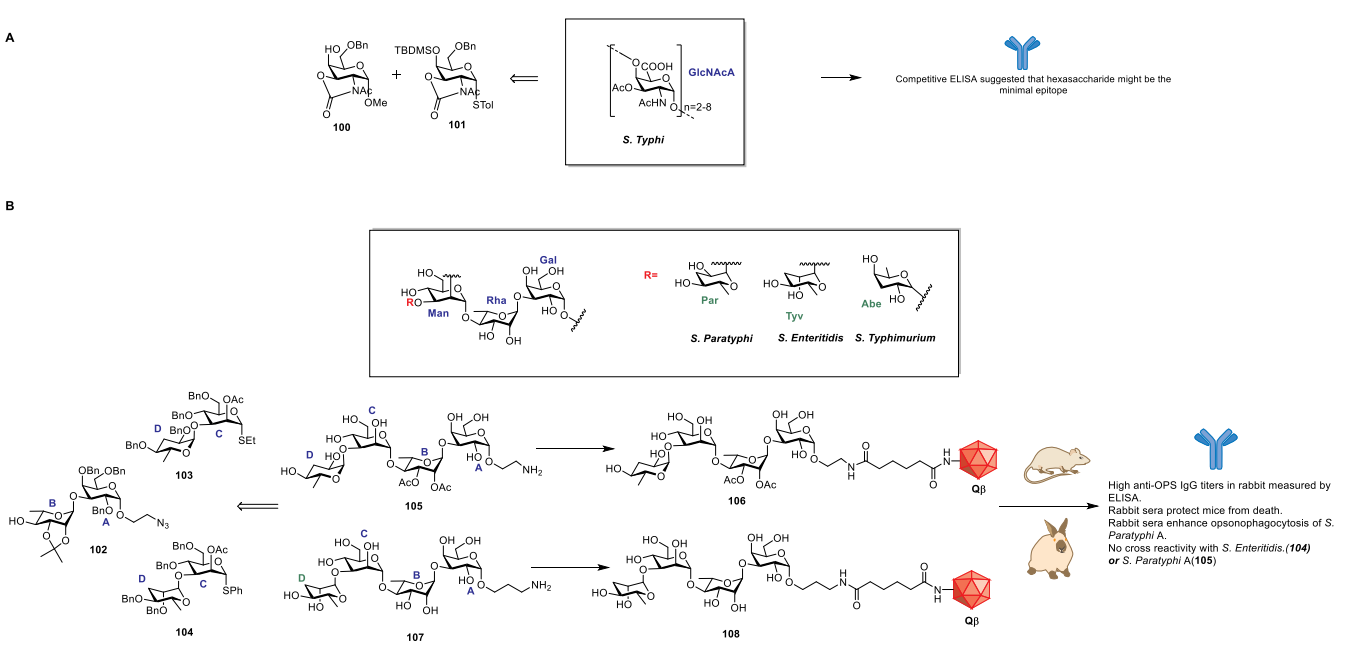

Figure 16. Synthetic approaches for conjugates from *Salmonella* oligosaccharides: (A) Fragments of *S.* Typhi capsular polysaccharide used to identify the minimal epitope length. (B) Design of conjugates against *S.* Enteritidis and Paratyphi and immunological evaluation in animal models.

observed with the tetra- or pentasaccharide, suggesting a terminal glucoside as a requirement for mAb recognition. Finally, the class I mAb did not react with any of the oligosaccharides, suggesting that some missing structural feature is needed for binding.

3.10. *Salmonella enterica*

Salmonella enterica is a Gram-negative bacterium that is most widespread in developing countries, and it is responsible for severe gastroenteritis and systemic illnesses.[319]

Human infections are generally due to the ingestion of contaminated food or water and can generally be attributed to four key serovars: *S.* Typhi and *S.* Paratyphi (Typhoidal Salmonellae), the causative agents of typhoid and paratyphoid fever, and *S.* Typhymurium and *S.* Enteritidis (non-typhoidal Salmonellae, NTS), responsible for gastroenteritis and bloodstream infections.[320,321] Conventionally, *Salmonella* infections are treated with antibiotics, but the rise of multidrug-resistant strains demands the development of effective vaccines.[322]

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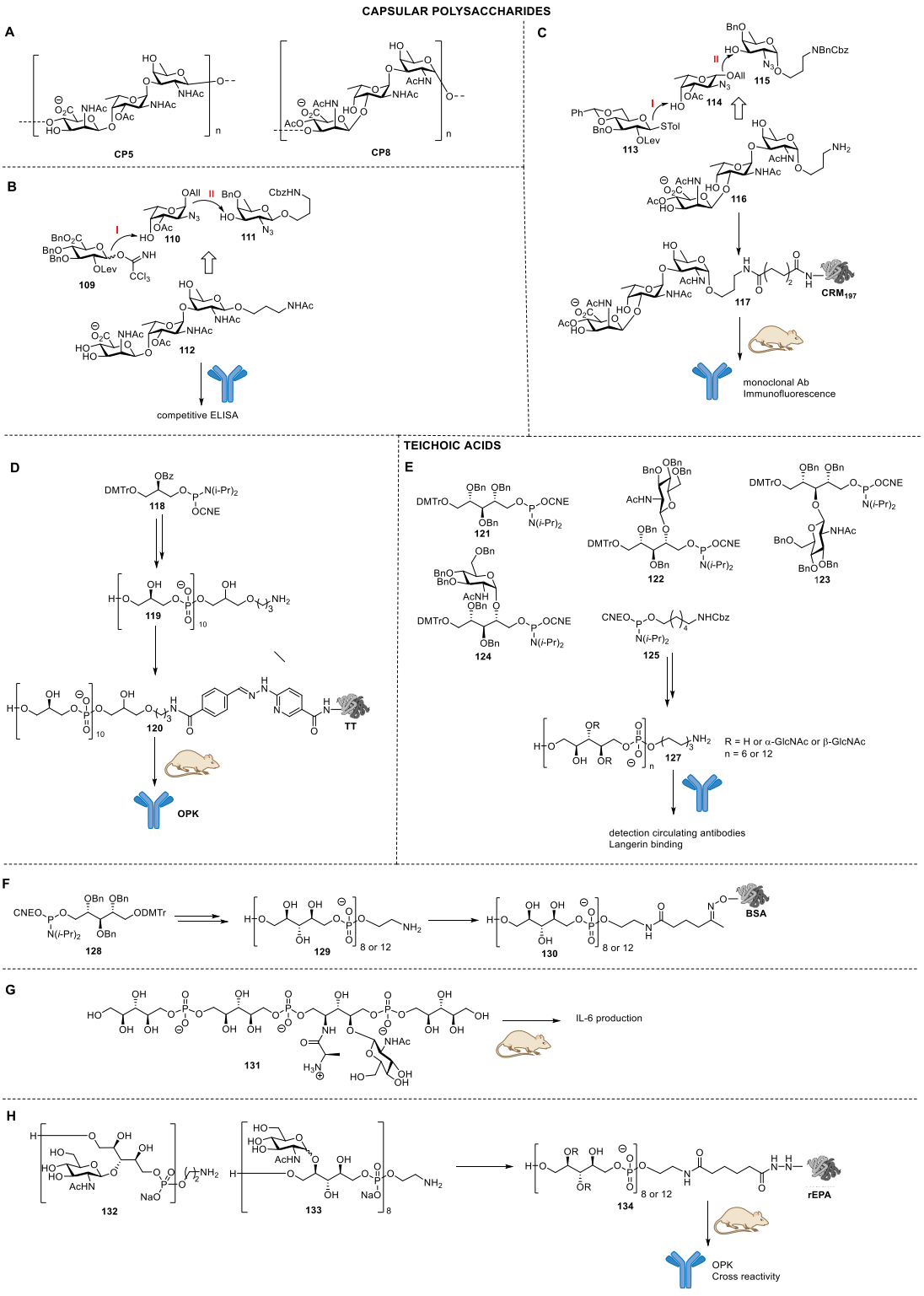

Figure 17. Assembly and immunological evaluation of staphylococcal surface carbohydrates, including capsular polysaccharides and teichoic acids. (A) Repeating unit structure of capsular polysaccharides CP8 and CP5. (B) Synthesis of CP5 trisaccharide, which was recognized by murine anti-CP5 serum. (C) Synthesis of the CP8 trisaccharide repeating unit, which, conjugated to CRM_{197}, elicited anti-glycan antibodies in mice. (D) Assembly of a 10-mer glycerol phosphate (GroP) polymer and conjugation to TT for immunological evaluation. (E) Synthesis of ribitol-phosphate (RboP) oligomers. (F) Solution phase synthesis of 8-mer and 12-mer RboP oligomers and conjugation to BSA. (G) Example of WTA RboP substituted tetramer tested in mice. (H) Wall teichoic acid (WTA) fragments conjugated to recombinant *P. aeruginosa* exotoxin A (rEPA) induced murine antibodies providing in vitro opsonophagocytic killing (OPK).

The Vi capsular polysaccharide (Vi PS) is the most important virulence factor of *S.* Typhi, making it the principal target of vaccines. A Vi polysaccharide-based vaccine[323] and an oral typhoid vaccine, Ty21a,[324] consisting of an attenuated strain of *S.* Typhi, have been available for many years, but they are poorly immunogenic in young children, where the burden of invasive

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salmonella disease is highest.[320] More recently, a conjugate vaccine against typhoid fever caused by *S. typhi* has been licensed in India.[319] A variety of glycoconjugate Vi vaccines differing in the carrier protein (TT, DT, CRM_{197}, and EPA) have been developed and have been tested at different clinical stages.[325] The Vi PS is a linear homopolymer of [→4)-α-D-GalNAcA-1→], predominantly O-acetylated at C_3-OH_3,[326] and the synthesis of Vi oligomers is challenging because of the presence of the 1,2-*cis* glycosidic linkages.[327] Yang et al. reported an effective synthesis involving the use of a carbamate protecting group masking the C-2-NH_2 and C-3-OH, that allows for stereoselective glycosylation reactions and easy removal.[327] This protective group was used in syntheses of both acceptor (**100**) and donor (**101**) di-, tri-, and tetrasaccharides bearing an extra acetyl group in the C4-OH position of the terminal repeating unit (Figure 16A). It has been shown that short oligosaccharides having an acetyl group are considerably more immunogenic than the methylated analogs previously reported in the literature,[328] suggesting that the OAc groups are an important antigenic determinant.[329] In a subsequent study, the authors used the same approach to extend the oligosaccharide chain of the Vi antigen. Penta-, hexa-, hepta-, and octasaccharides were synthesized, and a competitive ELISA showed that the hexasaccharide could represent the minimal epitope of the Vi antigen.[330] Despite numerous efforts to develop typhoid vaccines, there are no approved products against the other serotypes.[331,332]

Over the years, studies have been performed to identify protective antigens and evaluate the best strategy to develop an efficient vaccine. *S.* Paratyphi, as well as *S.* Enteritidis and *S.* Typhimurium, does not express the Vi antigen, leaving the cell surface-exposed lipopolysaccharide (LPS) as a promising vaccine target. The OAg portion of LPS is responsible for *Salmonella* serovar specificity and is considered to be an excellent protective antigen.[333] The OAg moiety of *S.* Paratyphi A that is composed of [→2)-α-D-Man-(1−4)-α-L-Rha-(1−3)-α-D-Gal-(1→] repeats with an α-D-paratose branch at the mannose C3 was proven to be immunogenic in mice (Figure 15B).[334] Acetylation of the rhamnose C-3-OH is known to be an important factor for antigenicity.[335] The first synthesis of a tetrasaccharide representing the *S.* Paratyphi A repeating unit (**105**) has recently been achieved following a stereoselective [2+2] glycosylation strategy.[336] After conjugation to the bacteriophage Qβ-carrier (**106**), its immunogenicity was tested in mice and rabbits, highlighting the importance of the paratose residue and *O*-acetyl modifications on the backbone for antibody recognition. It was demonstrated that one repeating unit is sufficient to elicit protective antibodies, suggesting a promising direction for the development of synthetic antigen-based *Salmonella* vaccines.[336] Vaccines are under development against other *Salmonella* species, and a bivalent *S.* Typhimurium and *S.* Enteritidis vaccine formulation based on vesicle-based technology that involves the use of generalized modules for membrane antigens (GMMA), naturally expressing the OAg, has shown promising results in mice.[337]

The *S.* Enteritidis and *S.* Typhimurium OAg share the same trisaccharide backbone of *S.* Paratyphi but differ in the structure of the mannose C-3 side chains, in glucosylation, and in *O*-acetylation. *S.* Enteritidis presents two oligosaccharides corresponding to two representations of the repeating unit →3)-α-D-Galp-(1−2)-[α-D-Tyv-(1−3)]-α-D-Man-(1−4)-α-L-Rha-(1→ and α-D-Tyv-(1−3)-α-D-Man-(1−4)-α-L-Rha-(1−3)-α-D-Gal-(1→.[338] Both structures present a rare tyvelose monosaccharide which was shown to play an important role in pathogenesis.[339] The first synthesis of the α-D-Gal-(1−2)-[α-D-Tyv-(1−3)]-α-D-Man-(1−4)-α-L-Rha tetrasaccharide repeating unit was achieved by using a key dodecylthioglycosyl donor.[340] Recently, Huang's group reported the synthesis of the tetrasaccharide frameshift α-D-Tyv-(1−3)-α-D-Man-(1−4)-α-L-Rhap-(1−3)-α-D-Gal (**107**) following a [2+2] glycosylation strategy. The tetrasaccharide conjugated to Qβ (**108**) proved to be immunogenic in mice and rabbits, suggesting that one repeating unit is sufficient to produce anti-OPS antibodies.[341] A synthetic approach was also reported for a methyl 3-*O*-(3,6-dideoxy-α-D-xylopyranosyl)-α-D-mannopyranoside disaccharide from *S.* Typhimurium, which upon conjugation was immunogenic in rabbits.[342] Subsequently, this synthetic disaccharide and tetra-, octa-, and dodecasaccharides, corresponding to one, two, and three repeating units, respectively, generated by fragmentation of *S.* Typhimurium OPS, were conjugated to BSA to evaluate the influence of glycan size on antibody response in rabbits and mice.[343,344] It was proposed that an optimal *S.* Typhimurium antigen should be composed of an octa- or dodecasaccharide and that the conjugate vaccine carries approximately 20 saccharide molecules per protein molecule.[344]

3.11. *Staphylococcus aureus*

S. aureus is a commensal Gram-positive bacterium and one of the most important hospital pathogens. Multidrug-resistant strains pose a significant health threat, and *S. aureus* invasion can lead to toxic shock, bacteremia, endocarditis, and osteomyelis. Glycopolymers are prime constituents of the *S. aureus* cell wall, as part of the biofilm, capsular polysaccharides, wall teichoic acids, and lipoteichoic acids and the thick peptidoglycan layer. All these glycopolymers have been found to be promising antigen candidates.

To date, 13 different serotypes of capsular polysaccharides (CPs) have been identified from clinical isolates, with capsular polysaccharide type 5 (CP5) and type 8 (CP8) being the most abundant.[345] These two polysaccharides have been targeted for vaccine development, and conjugates of the natural polymers have been tested in clinical trials,[346−348] unfortunately without demonstrated efficacy. A pentavalent vaccine is currently in a Ph1 clinical trial.[349] The structures of CP5 and CP8 are quite similar. The RU of CP5 is composed of →(4-β-D-ManNAcA-(1−4)-α-L-FucNAc(3-*O*-Ac)-(1−3)-β-D-FucNAc-(1→ and the RU of CP8 of →(3-β-D-ManNAcA(4-*O*-Ac)-(1−3)-α-L-FucNAc-(1−3)-β-D-FucNAc-(1→ units. The syntheses of CP5 and CP8 are very challenging because of the presence of the 1,2-*cis* glycosylic linkages and the presence of unusual amino sugars as well as the critical O-acetyl group. In 2012 Adamo et al.[350] reported the first synthesis of a CP5 trisaccharide (Figure 17). The β-ManNAcA residue was generated using a 2-O-levulinoyl glucuronate donor (**107**), which was used to first create a β-glucuronic acid linkage, after which a C-2- azide was introduced through a substitution reaction at C-2. Although a spacer was introduced at the reducing end terminus, this linker could not be used for conjugation purposes as an acetamide (**111**) was generated during the deprotection steps. Competitive ELISA and dot blot studies, with murine anti-CP5 serum, generated against the conjugated polysaccharide, showed recognition of the synthetic structure, representing a first proof that the trisaccharide contains the minimal elements for antibody binding.

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However, in comparison to the natural polysaccharide, significantly weaker interaction was observed, suggesting that longer fragments will be necessary to function as adequate synthetic antigens. To address the need for longer conjugation-ready structures, different approaches have been reported. Demchenko and co-workers[351] developed another synthetic route, based on inversion of configuration of a glucose building block to obtain the ManNAc residue, while Boons,[352] Codée,[353] and Kulkarni[313] exploited a direct β-mannosylation reaction. However, no further immunological evaluation has been reported.

The synthesis of a CP8-fragment has been achieved by Demchenko's group, who managed to synthesize both a trisaccharide and a protected hexasaccharide (Figure 17),[354,355] equipped with capping methyl groups at the points of propagation of the polysaccharide sequence. No immunological evaluation has thus far been reported. In 2020 Hu et al.[356] reported a synthesis of a CP8 trisaccharide (**116**) and the initial immunological evaluation thereof. The introduction of the β-ManNAcA moiety was achieved by a late-stage epimerization of a glucose precursor. The carboxylic acid functionality was introduced at the trisaccharide level. To test the immunogenicity of the trisaccharide, it was conjugated to the carrier protein CRM_{197}. After immunization of mice with the glycoconjugate, a glycan array showed the induction of anti-glycan IgGs. Hybridoma development and subcloning of the secreted antibodies was used to prepare mAbs. The purified mAbs and serum antibodies were analyzed for recognition of bacteria by immunofluorescence. Confocal laser scanning microscopy showed recognition of the bacterial surface of *S. aureus*. This data suggests that conjugates from the synthetic structures could be tested for functional activity and in infection models to assess their potential as vaccine candidates. Not only the CPs but also the teichoic acids (TA) of *S. aureus* have been considered to be promising vaccine candidates.[357] TAs are anionic glycopolymers, which are either covalently attached to the peptidoglycan, as in WTA, or anchored in the lipid bilayer through hydrophobic interactions, as in LTA. *S. aureus* WTA is composed of 20−-40 ribitol phosphate (RboP) repeats, which can be substituted with either α- or β-linked GlcNAc residues or D-alanine esters.[358,359] The most common type of LTA consists of a GroP backbone randomly decorated with D-alanine or α-GlcNAc moieties. Schmidt and co-workers have reported a significant amount of work to generate LTA fragments to probe their innate immune-stimulation activity,[360−362] finding that the glycolipid anchor and positively charged alanine esters on the GroP units were required for full innate immune-stimulation activity. The potential effect of these fragments in an adaptive immune response setting has not been investigated. In 2013 Snapper and co-workers reported a synthesis of a GroP oligomer for potential use in a conjugate vaccine.[363] A 10-mer (**119**) was synthesized taking a solid phase approach using a glycerol phosphoramidite building block (**118**), where a dimethoxytrityl (DMTr) was used to protect primary alcohols and a benzoyl (Bz) group to mask the secondary alcohols, even though these groups can readily migrate to the neighboring primary hydroxy groups under both basic and acidic reaction conditions. No characterization data have been provided for the generated structure.[364] The crude product was conjugated to a TT carrier protein to generate a model vaccine.

Administration in mice of the unconjugated and the TT-conjugated GroP oligomer adjuvanted with CpG-ODN, a strong TLR9 activator, showed that a high IgG level was only induced by the conjugate form. The pooled serum was able to mediate opsonic killing of both the Lowenstein and the USA300 strains of *S. aureus*.

An approach leading to a set of glycosylated LTA fragments has been reported by Codée's group as described above.[210] Cross-reactivity of serum generated against a glucosylated GroP hexamer, designed to mimic enterococcal LTA, was shown.

The glycosylation pattern of RboP-based WTA has been found to be critical for the fitness and virulence of bacteria. Different approaches toward the generation of well-defined WTA fragments have been investigated including solution and solid phase syntheses. In 2006 Pozsgay and co-workers successfully synthesized a Rbo-P-8-mer and a Rbo-P-12-mer using solution phase chemistry. The solid phase assembly of these fragments proved unsuccessful. The octamer and dodecamer were conjugated to a BSA carrier protein via an ethanol amine linker introducing 10 to 18 oligomers per protein (**130**).[365] However, the immunogenicity of the conjugates was not studied. Another approach for the synthesis of *S. aureus* WTA RboP was reported by Lee et al.[366] Four building blocks were used to enable the incorporation of different GlcNAc and D-Ala substitution patterns. The very labile D-Ala ester linkage was replaced in these structures by a robust amide linkage. The syntheses were achieved using a solution phase strategy, because attempts to use a solid phase nucleotide synthesizer required prohibitively large amounts of monomers. The synthesized tetramers (i.e. **131**) were tested in BALB/c mice, where increased levels of interleukin-6, as a marker of innate immunity activation, were measured.

Recently, Codée et al. reported a method to synthesize well-defined unsubstituted RboP oligomers using both solution and automated solid phase synthesis approaches.[367] The assembled library of structures consisted of oligomers with and without carbohydrate substituents, varying in length from hexa- to dodecamers. The phosporamidite building blocks (**121**−**123**) were used for synthesis with dimethoxytrityl (DMtr) protecting groups to mask the sites for elongation, in line with contemporary DNA synthesis. The use of mild acidic deprotection conditions enabled the solid phase assembly of RboP WTA fragments. For conjugation purposes a phosphoramidite hexanolamine spacer (**125**) was used.

Binding studies with the obtained molecules were conducted by conjugating the hexamers through a biotin handle to streptavidin-coated magnetic dynabeads M280 followed by interrogation with monoclonal antibodies. For this purpose mAbs were generated toward the α-GlcNAc functionalized RboP WTAs (clones 4461 and 4624) or β-1,4-GlcNAc carrying RboP WTA (clones 4497 and 6292).[368] The antibodies selectively recognized the glycosylated WTA fragments. Cross-reactivity of mAbs generated against β-(1−4)-GlcNAc RboP WTA against the corresponding β-1,3-GlcNAc RboP WTA could be demonstrated. The nonglycosylated WTA fragments could also be used as substrates for the *S. aureus* glycosyl transferases TarM, TarS, and TarP, capable of introducing α-(1−4)-GlcNAc, β-(1−4)-GlcNAc, or β-(1−3)-GlcNAc residues. The magnetic bead assay with these enzymatically glycosylated fragments was used to interrogate human sera to probe the presence of circulating anti-WTA antibodies. The β-(1−4)-GlcNAc- and β-(1−3)-GlcNAc-functionlized WTAs were found to be the most important targets for circulating antibodies, with significantly less recognition of the α-(1−4)-GlcNAc WTA. The nonglycosylated RboP WTA was not bound at all by the sera used.

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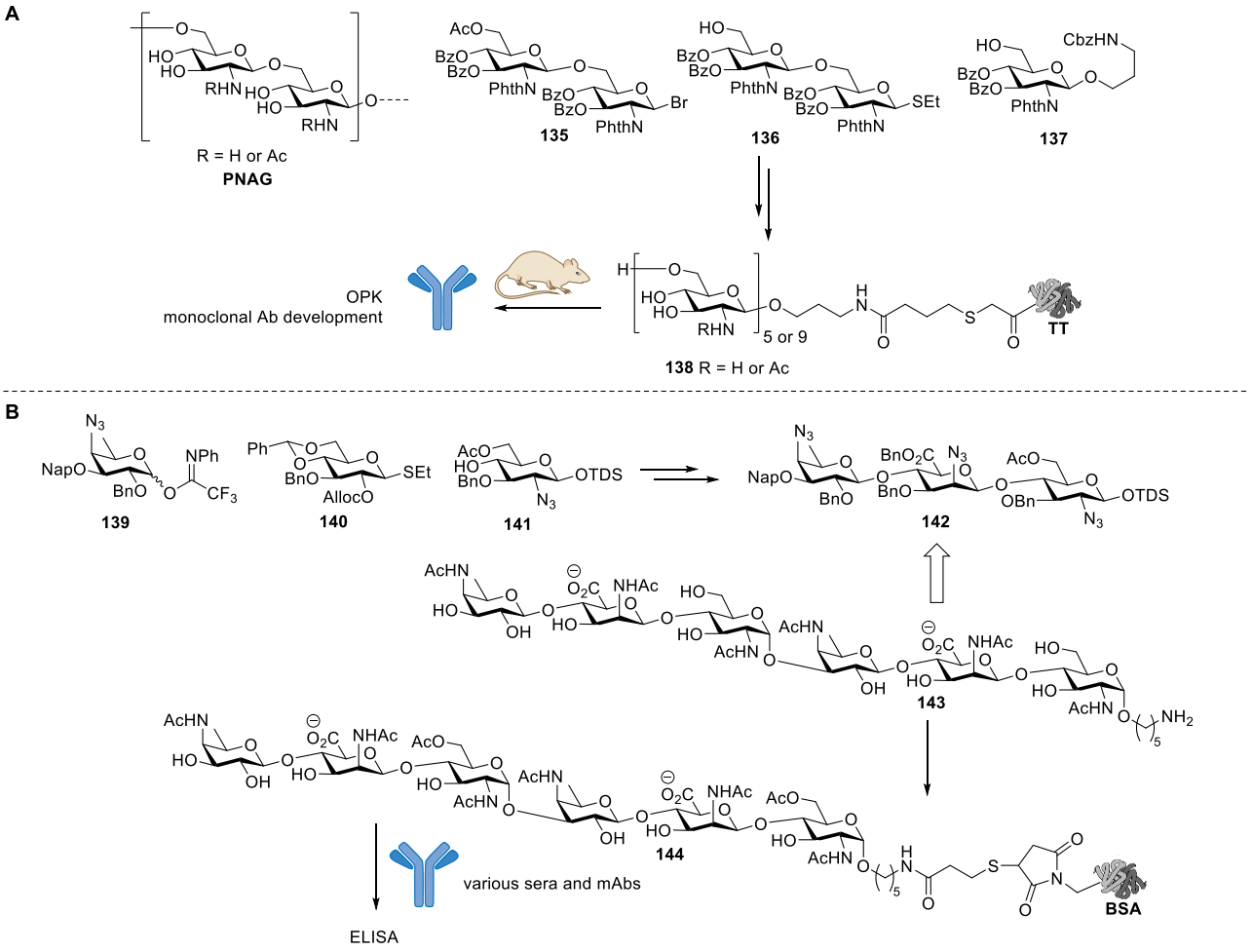

Figure 18. (A) PNAG structure and design of a universal PNAG-based universal vaccine and therapeutic mAb. (B) Synthetic route to the enterobacterial common antigen (ECA) conjugate used to generate specific sera and mAbs.

These findings somewhat contrasted the findings of Gerlach et al.,[369] who reported that β-(1−3)-GlcNAc WTA was significantly less immunogenic than β-(1−4)-GlcNAc WTA in a mouse model. The enzymatically glycosylated WTA magnetic beads were also used to investigate Langerin binding.[370] This C-type lectin receptor can be found on Langerhans cells, that play an important role in sensing the environment in the skin and kick-start an immune response against invading pathogens. The synthetic WTA fragments carrying β-GlcNAc substituents bound Langerin effectively, leading to cellular uptake in an in vitro model, showing that WTA may be a target for immune surveillance by Langerhans cells.

A team from Sanofi Pasteur generated three different WTA fragments, using a highly convergent synthesis approach, having either an α-(1−4)-GlcNAc (**133**), β-(1−4)-GlcNAc (**133**), or β-(1−3)-GlcNAc (**132**) substituent on each RboP monomer.[371] These fragments were conjugated to detoxified *S. aureus* α-hemolysin (HladM) or *P. aeruginosa* detoxified recombinant exotoxin A (rEPA) using hydrazide conjugation chemistry. Immunization using the AF04 adjuvant provided a strong immune response against the conjugates, while the use of nonconjugated WTA provided a poor response. The use of the adjuvant was important to raise a high level of antibodies. Interestingly, the sera raised against the β-(1−4)-GlcNAc-WTA conjugate proved to be more cross-reactive against different *S. aureus* strains than the α-(1−4)-GlcNAc-WTA and β-(1−3)-GlcNAc-WTA conjugate. The immune response against the latter conjugate was not cross-reactive. The exact reason for the different binding specificities of the raised antibodies remains unclear and will require detailed structural studies with well-defined synthetic WTA fragments.

Overall, the β-(1−4)-GlcNAc-RboP WTA appears to be a promising candidate for further development. The role of the labile D-Ala residues in immunogenicity remains to be established. These positively charged groups may play an important role as they introduce zwitterionic moieties in the WTA that may render these glycopolymers' T-cell-independent antigens, in line with the zwitterionic polysaccharides described above.

4. "UNIVERSAL" CARBOHYDRATE ANTIGENS

Cross-reactive antigens have been explored for their capacity to target multiple bacteria, thereby "killing more birds with one stone". The cell wall of *S. aureus* as well as other bacterial species, fungi, and protozoal parasites produces the cell surface polysaccharide poly-β-(1−6)-*N*-acetylglucosamine (PNAG, Figure 18A), which in the past decade has been investigated as a potential application in vaccine development (Table 1).[36] The structure of PNAG consists of β-linked *N*-acetylglucosamine, of which 10−20% of the amino groups are not *N*-acetylated (dPNAG).[372] Animal models using native PNAG and chemically deacetylated dPNAG have shown that antibodies

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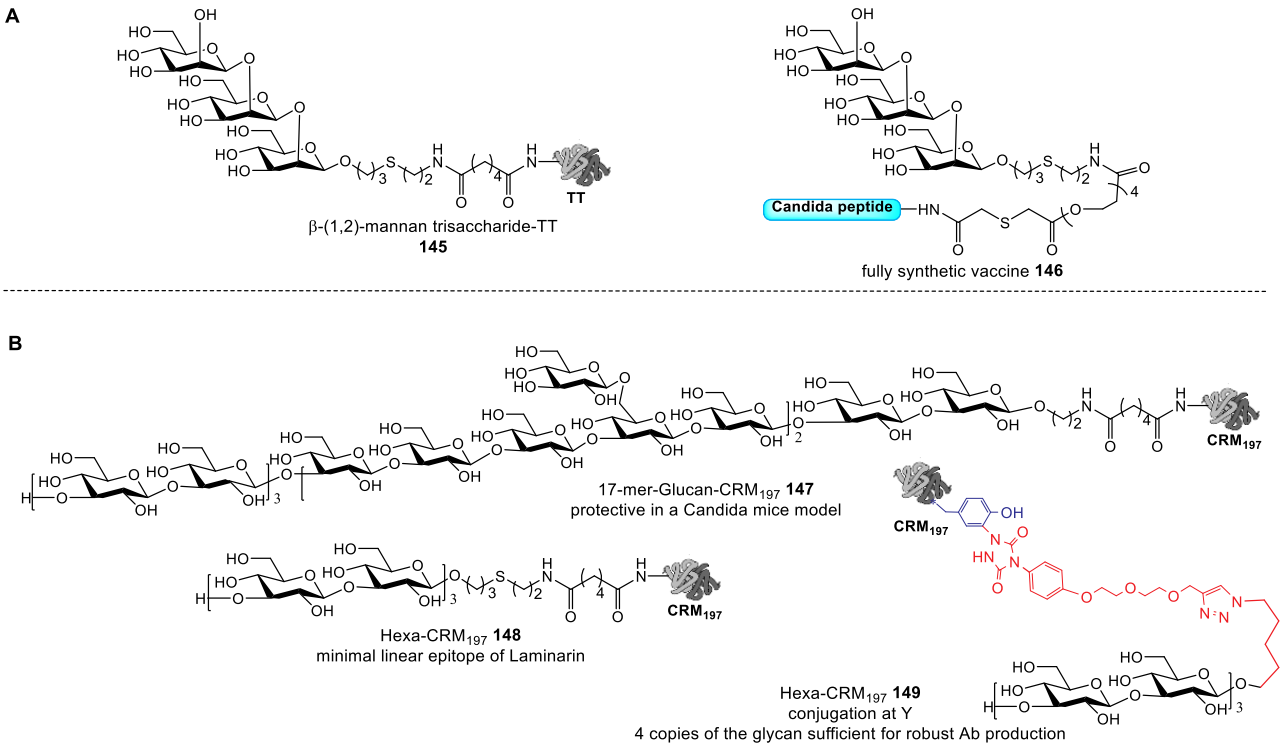

Figure 19. Vaccine candidates against *Candida* infections: (A) Different types of synthesized *β*-(1−2)-mannan conjugates used tested in animal models. (B) *β*-(1−3)-Glucan conjugates of different lengths enabling identification of a minimal hexamer epitope.

against dPNAG were more effective in mediating opsonophagotic killing[373] and protected mice from staphylococcal infection.[374] Chemically synthesized well-defined structures could be used to define the structure−immunogenicity relationship and, thus, find the best candidate for a vaccine. In 2007 Nifantiev and co-workers reported a route based on building blocks (**133**−**135**) toward well-defined GlcNAc and GlcN fragments up to the undecamer level.[375] These were equipped with an aminopropyl linker for functionalization. The synthesis relied on three building blocks, where an *N*-phthalimide directed the stereochemistry of the glycosylation reactions via neighboring group participation. For the immunogenicity studies the acetylated and non-acetylated pentasaccharide and nonasaccharide were coupled to TT (**138**).[376] Sera from mice immunized with the conjugates of the non-acetylated oligomers showed higher opsonic activity than the sera obtained from the acetylated ones. A conjugate of the pentamer has been tested in a phase I clinical trial, demonstrating the scalability of this synthetic conjugate for vaccine development.[377] Antibodies to dPNAG have been shown to be highly cross-reactive with numerous species including AMR pathogens such as *A. baumannii*, *C. difficile*, *E. coli*, and *K. pneumoniae* and, therefore, are very attractive to further develop as therapeutic monoclonal antibodies.[378−380] The mAb F598 is currently in clinical development, and it has been tested in a human challenge model against *N. gonorrhoeae* infection. The X-ray structure of the Fab fragment of the mAb in complex with synthetic dPNAG oligomers has elucidated that F598 recognizes PNAG through a large groove-shaped binding site that traverses the entire light and heavy chain interface and accommodates at least five GlcNAc residues, defining the dPNAG structural epitope.[381]

E. coli shares with all *Enterobacteriaceae* a polysaccharide known as the enterobacterial common antigen (ECA), which is composed of →3)-*α*-D-Fuc4NAc-(1−4)-*β*-D-ManNAcA-(1−4)-*α*-D-GlcNAc-(1→ repeating units that are substoichiometrically (∼70%) decorated at the GlcNAc C-6 with acetyl esters.[382] ECA is important for cell envelope integrity and has been suggested as a virulence factor, since bacteria that cannot produce ECA exhibit attenuated infections in oral and intraperitoneal mouse models.[383] Therefore, ECA represents an attractive target for the development of cross-protective vaccines or mAbs. The challenging synthesis involves the formation of multiple 1,2-*cis*- glycosyl linkages and the presence of rare monosaccharide constituents. Boons and co-workers achieved the synthesis of an ECA hexasaccharide through a [3+3] strategy, using an *N*-phenyltrifluoroacetimidate donor readily attainable from (**142**) (Figure 18B). The assembled oligosaccharide (**143**) was conjugated to BSA (**145**) and shown to recognize antibodies in sera of mice, exposed to different *K. pneumonia* strains as well as a mAb, SM250-1A5, raised against a mixture of six *Shigella* strains. These studies have revealed the potential of the ECA trisaccharide and hexasaccharide in the development of a conjugate vaccine and their potential to raise potentially cross-reactive mAbs. The effectivity of the ECA antigens remains to be assessed with respect to capsule and O-antigen expression of the different bacterial strains.

5. FUNGAL GLYCANS: *CANDIDA* SPP. AND EMERGENCE OF *C. AURIS*

5.1. *Candida* spp

Candida spp. are fungi (yeasts) that are part of the microbiome on human skin, mucous membranes, the female genital tract, and the gastrointestinal tract. Worldwide, recurrent vulvovaginal candidiasis affects about ∼140M women annually, with the highest prevalence on the 25−34 years age group and an increasing trend of incidence.[384] *Candida* spp. are also among the major causes of nosocomial infection, with invasive

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candidiasis gaining increasing prevalence worldwide in parallel with mortality rates ranging from 10 to 49%.[385] The epidemiology of candidemia has changed due to the increased number of patients receiving transplants and immunosuppressive therapy, use of broad spectrum antimicrobials, and number of AIDS patients.[386] Particularly, invasive candidiasis was mainly caused by *Candida albicans*, but in the past decade there has been a shift toward *Candida* spp. non-albicans, essentially due to the advent of *Candida auris*, identified in Japan in 2009 and recently recognized as an emerging multidrug-resistant pathogen that poses a major global health threat, including in the US. *C. auris* can cause invasive infections and outbreaks with high mortality rates in hospitalized patients, particularly among patients with multiple comorbidities and who have been admitted to intensive care or other special care facilities.[387−389]

Polysaccharides on the fungal surface have been targeted for vaccine discovery,[39] and carbohydrate synthesis has become a powerful tool to provide pure and well-defined glycan as opposite to highly variable structures from purification and aid elucidating protective epitopes and obtaining glycoconjugate vaccines with an optimized immunological response.[390,391]

Many syntheses of mannan oligomers[392−394] are reported which have allowed identification of the structure and dimensions of the protective epitope required for the development of promising vaccine antigens.[395,396] Synthetic conjugates of trisaccharide β-(1−2)-mannan to TT (**146**) showed a robust secondary antibody response in rabbits but poor immunogenicity in mice (Figure 19).[397] A conjugate with a synthetic 14-mer Fba peptide, derived from the *C. albicans* cell wall protein, elicited a strong antibody response and provided protection against a lethal challenge of *C. albicans*.[398] A tricomponent vaccine where the β-(1−2)-mannan was conjugated along with the β-glucan Laminarin, as immunopotentiator, was also constructed and shown to favor the conjugate's uptake from dendritic cells in mice, thus increasing its immunogenicity.[399] Lately, a fully synthetic conjugated vaccine (**146**) (Figure 18A) was constructed from a β-(1−2)-linked mannose trisaccharide conjugated to a T-cell peptide and an asymmetric dendrimer component bearing four copies of a β-(1−3)-linked hexaglucan dendritic cell epitope by click chemistry, yielding a conjugate vaccine that induced antibodies to all three epitopes of the fully synthetic construct.[400] Other researchers investigated a series of synthetic oligo α-mannosides containing branches or not. These synthetic structures were conjugated to BSA via squarate chemistry, and the immunological evaluation revealed that the linear oligomannoside structure could influence the quality of the antibody response.[401] Recently, synthetic biotinylated α-mannooligosaccharides mimicking *Candida* antigenic factors using RAW264.7 macrophages have been used to demonstrate their ability to induce a release of Th1, Th2, Th17, and Treg cytokine signature patterns.[402] It should be noted that α-mannooligosaccharides decorate human cells and their immunogenicity in humans could not be optimal. Subsequently, the mannose trisaccharide conjugated to β-glucan is an essential component of the *Candida* cell wall and has attracted attention for vaccine development because of its accessibility to antibodies.[403] Antibodies directed to β-glucan have been shown to confer protection to mice following challenge with *C. albicans*,[404] and the linear β-(1−3) glucan with sporadic β-(1−6) branches, Laminarin, which is extracted from the brown alga Laminaria digitata, has been proposed as an alternative to fungal glycans in order to avoid any possible contamination from the microorganism.[404,405] Laminarin conjugated to CRM_{197} induced in mice antibodies which recognized *C. albicans* and protected it from lethal challenge with fungal cells.[405] Synthetic oligosaccharides have been used to elucidate the structural features needed for optimal immunogenicity. By comparing a linear 15-mer with a branched 17-mer (**147**) it was observed that β-(1−6) branches need to be separated by at least six β-(1−3) residues for efficient antibody production (Figure 18B).[405] Based on this data, a linear synthetic β-(1−3)-glucan hexamer (**148**) was synthesized and conjugated to CRM_{197}, which was shown to elicit in mice anti β-glucan IgGs in a comparable manner to Lam-CRM_{197}.[406] Further investigations were carried out with the β-(1−3) glucan hexamer regioselectivity conjugated via tyrosine-selective alkynylation with a 4-phenyl-1,2,4-triazoline-3,5-dione spacer followed by a click chemistry mediated carbohydrate installation (**149**),[407] which was compared to a conjugate obtained by controlled reaction at the four more surface exposed lysine residues and the random conjugate (**148**). In vivo studies showed that conjugation to four tyrosines is sufficient for a strong production of antibodies that inhibit the adhesion of *C. albicans* to human epithelial cells.[408,409] More recently, synthetic octa-, deca-, and dodeca-β-glucans were synthesized and, after conjugation to KLM, shown to be more immunogenic than the hexamer with the octamer being the best among these lengths.[410]

C. auris shares a cell wall glycan composition with the other *Candida* spp. However, mannans seem to avoid triggering the antifungal activity of neutrophils, which would evoke the innate immune cells to respond to fungal infections, which could explain a different mechanism of immune evasion for this pathogen.[411] In a recent study, passive transfer of C3.1, a mAb that targets β-(1−2)-mannotriose (β-Man3) in the A/J mouse model, which mimics disseminated infection of human candidiasis, demonstrated a significantly extended survival and reduced burdens from *C. auris* in kidney, brain, and heart.[412] The data underpins the potential of glycan targeting for the design of therapeutics against multidrug-resistant *C. auris*.

Also, unlike *C. albicans*, which forms a heterogeneous architecture of biofilms combined with blastoconidia and hyphae embedded within the extracellular matrix, *C. auris* produces thin biofilms composed mostly of blastoconidia and occasionally pseudohyphae embedded within a very limited extracellular matrix, which show less susceptibility to antifungals than those of *C. albicans*.[413] Adhesion plays a key role in *C. auris* virulence and biofilm formation, and proteins of the agglutinin-like sequence (Als), in particular Als3393, are involved in the adhesion of *C. auris*.[414] Recently, the efficacy of the NDV-3A vaccine,[415] already tested in women for the prevention of recurrent vulvovaginal candidiasis, has been reported to protect neutropenic mice from *C. auris* infection, with an additive protective effect when combined with micafungin mice.[416]

6. CONCLUSIONS AND FUTURE DIRECTIONS

The ongoing SARS-Cov2 pandemic has clearly highlighted the need for vaccines for pandemic preparedness, capable of inducing sterilizing antibodies and providing herd immunity.[417] Glycoconjugate vaccines represent preventive therapeutics that have been shown able to induce killing (opsonic or bactericidal) antibodies and to achieve herd immunity.[418] Carbohydrate targeting for the design of vaccines to combat the possible next pandemic, AMR, therefore, appears crucial. The combination of synthesis with structural glycobiology is rationalizing vaccine design, enabling the generation of a more focused immune

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response against the most relevant polysaccharide epitopes.[134,419]

Further advances in the synthesis of complex bacterial glycans will allow the faster generation of more and more complex glycans, making carbohydrate antigen production faster and more scalable.[71,420] Key challenges include not only further optimization of stereoselective glycosylation methodology and automated synthesis techniques (a technique that, if sufficiently matured, will also ensure reproducibility of oligosaccharide synthesis) but also the incorporation of functional groups, including labile esters, differently functionalized amines/amides, pyruvate ketals, and phosphates and phosphonates, which often provide microheterogeneity to the bacterial polysaccharide chains.[421] A clear advantage of organic synthesis is the improvement of specific properties of the glycans, such as stability (as shown above for carbaMenA,[126] Hib,[87] and ST5[158] pneumococcal analogues). In some examples, chemically derived structures have the potential to improve the vaccine design for those polysaccharides that have been proven less efficacious in humans (e.g. pneumococcal ST3[154]).

Due to the complexity of the synthetic routes for glycans antigens, large scale manufacturability has historically been considered a challenge associated with this approach. Today, the success of the Cuban Quimi-Hib conjugate and scalability for GMP production of *Shigella* and PNAG vaccines increase the confidence in chemical synthesis as a tool to supply carbohydrate antigens at the industrial level.[18,20] Also, the need for minimal amounts per vaccine dose, as compared to other chemical therapeutics and the increased availability of commercial building blocks resulting in reduced cost of goods are positively contributing to the large scale manufacturability of this approach. Finally, various glycomimetic drugs and a synthetic heparin (Fondaparinux) are manufactured at the industrial level and available in the market.[422]

A more effective synthesis methodology will enable the generation of larger and more complex glycans, which may adopt crucial three-dimensional structures presenting conformational epitopes that will further resemble the natural polymers. From this perspective, the use of enzymatic glycosylations will significantly speed up the assembly of naturally occurring bacterial oligosaccharides and close analogues thereof, as has been shown for the production of the MenX CPS.[68] At present, the vast majority of the reported enzymatic oligosaccharide syntheses have been directed at the assembly of mammalian glycans, featuring the most common monosaccharide donors. Bacteria use a much wider monosaccharide panel for the construction of their polysaccharides. Expression of the relevant biosynthesis enzymes (besides the transferases also the enzymes that generate the nucleotide donor monosaccharides) and their use in the assembly of bacterial glycan epitopes will streamline bacterial glycan assembly.

Novel nanosized carriers favoring multimeric and optimal spatial presentation of short glycans may improve the immunogenicity of glycoconjugate vaccines, increasing the antibody avidity or breadth of the immune response. Novel site-selective protein conjugation chemistries are emerging to achieve better control over the carbohydrate−protein linkages. Not only the number of glycans per carrier protein but also the position on the protein may impact vaccine efficacy. Improved, site-selective ligation chemistry will allow the generation of multivalent conjugate vaccines, in which several epitopes can be connected to a single protein carrier, which can be a pathogen derived protein, also playing the role of antigen.

Very few adjuvants have been so far tested with conjugated synthetic carbohydrates (Freund adjuvant, Alum salts, and MF59 to mention some), and the effect of adjuvantation on this type of structures has not been thoroughly explored. The combination with well-defined molecular adjuvants (such as TLR, NLR, RIG, and STING ligands) and targeting devices is expected to further improve vaccine efficiency.[423−425] In particular, there is a need for potent and effective adjuvants to be combined with glycoconjugate vaccines in elderly, hospitalized, and immunocompromised patients where the need for therapies counteracting AMR is higher.[426] In combination with better defined delivery vehicles, such as polymers or supramolecular architectures, another level of control is introduced. The availability of well-defined glycans is mandatory to allow optimization of all these important vaccine parameters.

Finally, synthetic chemistry, in combination with chemical biology techniques and advanced microscopy, will enable studies to provide insight into how conjugate vaccines are taken up and processed by the host immune system.[427] This will enable the rational optimization of all steps required in the immunization process to achieve optimal delivery, uptake, processing, and presentation to the right partners of the immune system alongside optimal activation of all players involved. We believe that all these advancements will render carbohydrates a major player to defeat emerging AMR. While the RNA platform will be used more and more for the expression of protein antigens, progress in carbohydrate synthesis and conjugation will allow better exploitation of the peculiar role of carbohydrates as vaccine targets and create the next generation of glycovaccines.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): L.D.B., F.N., M.R.R., and R.A. are employees of the GSK group of companies. Bexsero is a trademark from GSK. Trumemeba is a trademark of Pfizer. QumiHib is a trademark of CIGB.

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Biographies

Linda Del Bino obtained her Master's Degree in Chemistry and Pharmaceutical Technology at the University of Pisa (Italy) in 2014 with a thesis on organic chemistry. She joined for two years the AstraZeneca IMED Graduate Programme in Göteborg (Sweden). In 2020 she obtained her Ph.D. Program at the University of Leiden working in GSK Siena (Italy) within the framework of the Horizon 2020 Marie Curie International Training Network Glycovax. Linda is currently working as Scientist in the Glycoconjugate Synthesis and Analytical Lab in Preclinical R&D at GSK Siena, focusing on glycan and glycoconjugate synthesis.

Kitt Emilie Østerlid obtained her Master's Degree at University of Copenhagen (Denmark) in 2020. She is currently doing her Ph.D. at Leiden University under the supervision of Prof. Dr. J. D. C. Codée. Her current research focuses on synthesis of glycans from *S. aureus* for vaccine development.

Dung-Yeh Wu received his M.S. degree in chemistry from National Tsing Hua University in 2018. Presently, he is a Ph.D. student in Chemistry at Leiden University under the supervision of Dr. Jeroen Codée. His current research focuses on oligosaccharide syntheses and bacterial vaccine development.

Francesca Nonne studied Pharmaceutical Chemistry and Technologies at the University of Sassari (Italy), where she graduated in 2015. After a Master's Program in Drug Design and Synthesis at the University of Siena, she joined GSK first for an internship in carbohydrate synthesis and then for a Ph.D. Program in Chemical and Pharmaceutical Sciences in collaboration with the University of Siena. Since January 2022 she has been Associate Scientist at GSK Vaccines Institute for Global Health.

Maria Rosaria Romano obtained her Ph.D. in Pharmaceutical Sciences at the University of Pisa on the synthesis of glycal derivatives in 2006. In 2007 she joined Novartis Vaccines as an Associate Researcher, and in 2016 she became Head of Glycoconjugates Synthesis & Analytic Laboratory in GSK Vaccines. Her research is focused on polysaccharide derived glycoconjugate vaccines, generation of new vaccines from synthetic carbohydrates, nanoparticle technologies, and carbohydrate epitope characterization for optimized vaccine design.

Jeroen Codée obtained his Ph.D. from Leiden University in 2004. After a post-doctoral stay at the ETH in Zurich, he returned to Leiden, where he now is a Full Professor in Organic Chemistry. The research in his group revolves around the many aspects of carbohydrate chemistry, ranging from fundamental mechanistic studies to the assembly of complex bacterial glycans, that are used as biosynthesis probes and vaccine entities, and the generation of carbohydrate-based adjuvants and biosynthesis inhibitors.

Roberto Adamo obtained his Ph.D. in Pharmaceutical Science from the University of Catania (Italy) in 2003, working on the synthesis of biologically relevant inositols. After two post-doctoral fellowships at the NIH in Bethesda (USA) and at Utrecht University (The Netherlands), in 2007 he joined Novartis, where he was later appointed Head of the Carbohydrate Chemistry Laboratory and Leader of the Conjugation & Synthesis Platform. Following the company acquisition by GSK, he has covered the role of Conjugation Platform Leader and, currently, of Discovery Project Leader. His research interests vary from the synthesis of glycans, glycoconjugates, and glyconanoparticles to structural glycobiology for the design of carbohydrate-based therapeutics.

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