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REVIEW

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Classical and novel strategies to develop a *Shigella* glycoconjugate vaccine: from concept to efficacy in human

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ABSTRACT

Shigella are gram-negative bacteria that cause severe diarrhea and dysentery, with a high level of antimicrobial resistance. Disease-induced protection against reinfection in *Shigella*-endemic areas provides convincing evidence on the feasibility of a vaccine and on the importance of *Shigella* lipopolysaccharides as targets of the host humoral protective immune response against disease. This article provides an overview of the original and current strategies toward the development of a *Shigella* glycan-protein conjugate vaccine that would cover the most commonly detected strains. Going beyond pioneering "lattice"-type polysaccharide-protein conjugates, progress, and challenges are addressed with focus on promising alternatives, which have reached phases I and II clinical trial. Glycoengineered bioconjugates and "sun"-type conjugates featuring well-defined synthetic carbohydrate antigens are discussed with insights on the molecular parameters governing the rational design of a cost-effective glycoconjugate vaccine efficacious in preventing diseases caused by *Shigella* in the most at risk populations, young children living in endemic areas.

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Introduction

Following several decades of investigation, there is yet no broadly available vaccines against shigellosis. Progress in vaccine development has been challenging for several reasons, such as, among others, the absence of firm correlates of protective immunity and lack of suitable animal models, a limited understanding of the mechanism governing protection, or lack of commercial interest. Nevertheless, numerous strategies, including live attenuated oral, killed oral, and subunit parenteral vaccines, are actively being explored.[1–3] Renewed concern for accelerating the development of *Shigella* vaccines has emerged following the recent disclosure of several reports underlying shigellosis as a major global cause of diarrhea and dysentery in children under five years living in endemic areas.[4,5] Shigellosis-associated long-term complications in this population and general burden, the increasing spread of antibiotic-resistant *Shigella* clones, their introduction into a larger diversity of populations by means of the travelers' diarrhea, and their easy dissemination within epidemiological niches,[6,7] all call for a *Shigella* vaccine. The discrepancy in the potential protective capacity of the live, rationally attenuated, oral vaccine candidates when tested in western volunteers, and in individuals in endemic areas,[8] added to the absence of a definitely established cross-protective antigen, have emerged as major issues. In this context, the development of polysaccharide conjugates initiated under John B. Robbins and Rachel Schneerson's impulsion at the National Institutes of Health (NIH) in the early 1990s, and extensions thereof, is the object of increasing interest.[9]

This chapter summarizes the different concepts and strategies toward a carbohydrate-based conjugate vaccine against *Shigella*. Going beyond bacterial polysaccharide conjugates and bioconjugates, attention is also paid to synthetic oligosaccharide (OS)-protein conjugates. All three categories having entered clinical trial to date, data in human are discussed accordingly.

The surface polysaccharides of *Shigella* for vaccine development

Shigellosis can be caused by any serotype belonging to four groups: group A (*Shigella dysenteriae*), group B (*Shigella flexneri*), group C (*Shigella boydii*), and group D (*Shigella sonnei*). *S. dysenteriae* serotype 1 (SD1), the first *Shigella* species isolated, emerges as one of particular concern, due to its expression of the Shiga toxin. It was responsible for several severe periodic epidemics associated to high-case fatality rates in the 20th century, with the last major occurrence in the 1990s.[10] *S. flexneri* (SF) and *S. sonnei* are endemic worldwide. They are linked to most infections, causing an estimated two-thirds and one-fourth of all episodes, respectively.[11] Other *S. dysenteriae* (13 serotypes) and *S. boydii* (19 serotypes) account for approximately 10% of all shigellosis episodes. While *S. flexneri* is the leading cause of endemic diarrhea in low-income and lower middle-income countries (LMICs), *S. sonnei* incidence tends to increase in areas where living standards improve, therefore dominating as a single type in developed and some transitional countries. In contrast, although their prevalence and geographic distribution vary, most of the 15 established *S. flexneri* serotypes are known causes of disease. A number of atypical variants are also reported, some of which are continuously isolated in diverse geographical regions.[12] Actually, at least a total of 30 variants

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have now been recognized, and a significant extension of the serotyping scheme was suggested.[13] As a general trend, three to four serotypes account for some 75% of all episodes in a given country with *S. flexneri* type 2a (SF2a) being the dominant serotype.[11] Epidemiological data related to the *Shigella* burden have, over the years, directly impacted efforts toward the development of a vaccine as illustrated in the following.

The lipopolysaccharide as a target of host immunity

All *Shigella* are surrounded by a polysaccharide antigen present in the form of a lipopolysaccharide (LPS) anchored in the outer membrane by means of its lipid A moiety. LPS contributes to virulence and plays an important role in bacterial resistance to innate immunity.[14,15] Termed O-specific polysaccharide (O-SP) or O-antigen (O-Ag), the polysaccharide moiety of LPS is the component most exposed to the environment. It is involved in many aspects of *Shigella* interaction with the host. Connected to lipid A via the core, a short OS encompassing rather conserved heptose and 2-keto-3-deoxy-d-*manno*-octulosonic acid (Kdo) residues,[16] the O-Ag is also the LPS component with the most diverse composition. It is defined by a repeating unit (RU), which is made of two to six monosaccharides and determines serotype immunospecificity.[17] The ladder-type pattern observed in silver-stained SDS-PAGE LPS analysis reveals a heterogeneous distribution of the number of O-Ag RUs per LPS molecule as well as rather diverse chain lengths. For example, the SD1 O-Ag contains up to 27 RUs,[18] whereas SF2a produces LPSs with two preferred O-Ag chain lengths, a short one comprising on average 17 RUs and a very long one composed of typically 90 RUs. Both the chain length distribution of the SF2a O-Ag and the composition of its RU modulate virulence.[14,15] They were shown to be under growth-phase-dependent regulation.[19]

Although protection is not absolute and rather short lasting, there has long been convincing evidence that *Shigella* natural infection confers protection to subsequent exposures to the homologous serotype.[20–23] Observations converge to suggest that LPS, and more particularly the O-Ag, is an important target of the host humoral protective immune response.[21–26] However, the exact mechanism by which anti-LPS antibodies mediate protection against *Shigella* is still the subject of debate. Still, it was hypothesized in the early 1990s that serum antibodies to *Shigella* O-Ags could provide protection by transudation to the mucosal surface and inactivation of the inoculum in the intestine.[27,28] This pioneering assumption served as a basis to extensive investigation on the use of *Shigella* surface polysaccharides as components of parenteral vaccines.

Lipopolysaccharide-based vaccine candidates

In contrast to purified high molecular weight capsular polysaccharides, which are well-established licensed vaccines for use in human, endotoxicity precludes the use of LPS as a parenterally administered immunogen. Instead, formulations whereby LPS toxicity is masked as in Invaplex,[29] reduced and even abolished as in GMMA[30] or encompassing LPS derivatives devoid of toxicity, especially in the form of protein conjugates, were investigated.

The seminal observations that capsular polysaccharides, which behave as T-cell independent antigens, and nonimmunogenic antigens such as haptens, could be converted to T-cell-dependent immunogens by covalent coupling to a -protein,[31,32] pioneered impressive developments in the field of conjugate vaccines.[33] The *Haemophilus influenzae* b (Hib) prototype vaccine elaborated in the Robbins' group[34] evolved into a breakthrough in the antibacterial vaccination of young children, ultimately leading to the licensing of the first glycoconjugate vaccines in the late 1980s. The growing success of capsular polysaccharide-protein conjugate vaccines over the past decades led to the licensing of several formulations for use in young children, among which well-established multivalent vaccines such as the 2- and 4-valent *Neisseria meningitidis*, as well as the 7-, 10-, and 13-valent *Streptococcus pneumoniae* conjugate vaccines.[35] Wherever they have been introduced, polysaccharide-protein conjugates vaccines have had a major impact in reducing homologous infectious disease occurrence. The strategy applicability to non-encapsulated bacteria is also actively explored. Assuming that achievements with bacterial capsular polysaccharides could be adapted to LPS, the development of glycoconjugate vaccines derived from *Shigella* LPS was investigated actively in the Robbins and Schneerson's group.[9]

As a general trend, the target glycoconjugates consist of *Shigella* LPS-related carbohydrate B-cell antigens covalently linked to a protein or peptide antigen, which provides the T-helper contribution required to ensure the induction of a robust anti-LPS T-dependent immune response and immune memory.[36,37] In the following, the four main strategies in this category under investigation in the context of *Shigella* vaccine development are discussed (Table 1). They are differentiated based on the composition of the carbohydrate component, classified as detoxified LPS, sized O-Ag-core (O-AgC), O-Ag, and synthetic O-Ag fragment or synthetic oligosaccharide (OS).

With more than 50 known serotypes, *Shigella* features a large variety of surface polysaccharides. Whether linear or branched, neutral, acidic, or zwitterionic, the RUs of the O-Ags from the *Shigella* most studied in the context of vaccine development are extremely diverse (Figure 1).

Detoxified LPS-protein conjugates as lead vaccine candidates

With bacterial cell cultures as the only source of glycan antigens, the design of *Shigella* LPS-based conjugates was originally addressed in terms of nature of the carbohydrate components and protein carriers on the one hand, and conjugation chemistry on the other hand (Scheme 1).

Strategies to promising vaccine prototypes

From LPS to "lattice"-type polysaccharide-tetanus toxoid conjugate immunogens

As the whole LPS purified from cell cultivation could not be used as immunogen, LPS chemical detoxification was achieved by two methods: (i) hydrazine-mediated hydrolysis

Table 1. Strategies to a *Shigella* glycoconjugate vaccine.

Glycan source and vaccine category	Vaccine candidate	Phase of development	Reference
Detoxified LPS: "lattice"-type conjugate			
SD1	dLPS-TT pmLPS-TT	Phase I	[38]
SF2a	pmLPS-rEPA$_{succ}$	Phase I	[39,40]
	pmLPS-CRM9	Phase I	[39]
	pmLPS-CRM9$_{succ}$	Phase I	[39]
	pmLPS-rEPA	Phase III	[38,41–44]
	pmLPS-CfaEB	Phase I	[45]
S. sonnei	pmLPS-rEPA$_{succ}$	Phase I	[39,40]
	pmLPS-CRM9 pmLPS-CRM9$_{succ}$	Phase I	[39,40]
	pmLPS-rEPA	Phase III	[38,41–44]
O-AgC: "sun"-type conjugate			
SD1, SF2a,46 SF6	O-AgC-rEPA	Preclinical	[47]
S. sonnei	O-AgC-rEPA	Preclinical	[46]
Bioconjugates			
SD1	O-Ag-rEPA	Phase I	[48]
SF2a	O-Ag-rEPA	Phase I	[49]
		Phase II	Completed[50]
Synthetic glycans: "sun"-type conjugates			
SD1	OS-BSA	Preclinical	[51]
SF2a	OS-TT	Phase I	Completed[52,53]
SF3a	OS-TT	Preclinical	[54,55]
SF2a, SF3a	OS-TT	Preclinical	[55]

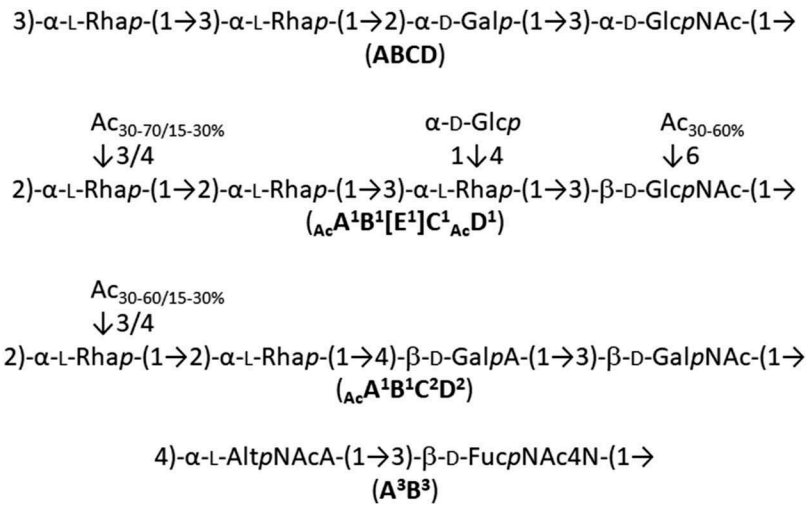
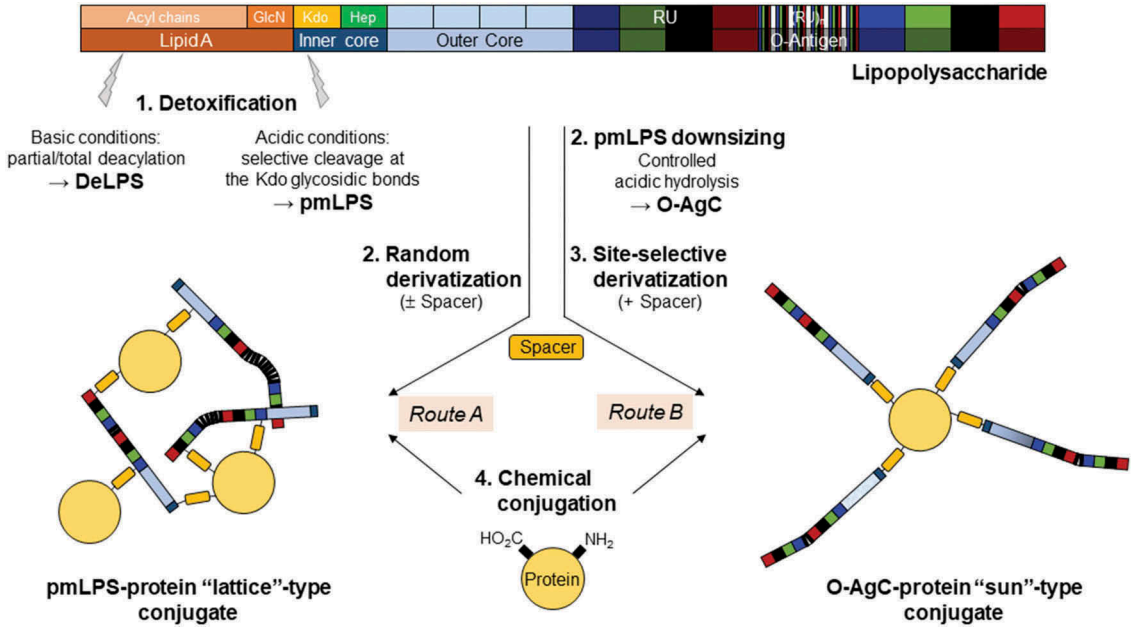


Figure 1. Biological RU of the O-Ags from SD1 (**ABCD**),[56] SF2a ($_{Ac}$**A**^{1}**B**^{1}**[E**^{1}**]C**$^{1}{}_{Ac}$**D**^{1}),[13,57] SF6 ($_{Ac}$**A**^{1}**B**^{1}**C**^{2}**D**^{2}),[57] and *S. sonnei* (**A**^{3}**B**^{3}).[58]

of the lipid A ester bonds, which provides partially deacylated LPS (DeLPS) and (ii) controlled aqueous acetic acid-mediated cleavage of the sensitive core Kdo glycosidic linkages, which releases the O-Ag appended to the residual core OS or LPS polysaccharide moiety (pmLPS). None of the detoxified species were immunogenic, which was ascribed to their low molecular weight and supported further investigations on polysaccharide conjugates.[59]

Using SD1 pmLPS as glycan antigen, adipic acid dihydrazide (ADH) as spacer, and tetanus toxoid (TT), a well-established protein carrier, three conjugation chemistries were investigated. Two methods were based on the site-selective introduction of ADH at the carbonyl group of the reducing end Kdo residue of SD1 pmLPS by reductive amination. Subsequent coupling of the ADH-equipped pmLPS at the carboxyl groups of aspartic/glutamic acids of TT by carbodiimide chemistry or at the amino groups of the TT lysine side chains by means of the thiol chemistry gave two families of "sun"-type glycoconjugates showing different loadings. The third method used the random CNBr-mediated derivatization of pmLPS with ADH and subsequent chemical conjugation to carboxyl groups of TT to provide sets of "lattice"-type conjugates, whereby the polysaccharide and protein carrier components are linked at multiple sites. Immunogenicity analysis of sera from mice injected subcutaneously three times, 2 weeks apart, with conjugate amounts corresponding to 2.5



Scheme 1. Strategies to detoxified-LPS protein conjugates as potential *Shigella* vaccines. "Lattice"-type conjugate (left) and "sun"-type conjugate (right). DeLPS: deacylated LPS, pmLPS: LPS polysaccharide moiety, O-AgC: sized O-antigen-core polysaccharide, RU: repeating unit.

μg carbohydrate equivalent indicated the superiority of the "lattice"-type conjugate following each injection.[60] Furthermore, subsequent evaluation of adjuvanted formulations of the latter revealed that the use of both alum and monophosphoryl lipid A (MPL) in an oil/water emulsion enhanced the induced anti-LPS serum antibody (Ab) response. The adjuvant effect of alum remained stable for at least 3 months.[60] With regards to conjugation chemistry, original work on SD1[60] paved the way to investigations on SF2a and *S. sonnei*.

In the search for the most immunogenic SF2a LPS-based conjugates, random CNBr-mediated derivatization of DeLPS and pmLPS with ADH and subsequent chemical conjugation to carboxyl groups of TT provided two different sets of conjugates, whereby the polysaccharide and protein carrier components are linked at multiple sites. The DeLPS material was of higher molecular weight than the corresponding pmLPS. In contrast, the saccharide:protein molar ratio was higher (3:1 to 5:1 versus 2:1) for conjugates made of a pmLPS component. Both conjugates elicited anti-LPS IgG Abs in mice after three subcutaneous injections of conjugate amounts equivalent to 2.5 μg saccharide. However, the SF2a pmLPS was turned into a superior T-cell-dependent immunogen through its covalent coupling to TT.[59] The LPS acid-mediated detoxification process was adopted in subsequent developments.

Toward improved SF2a and S. sonnei pmLPS-protein conjugate immunogens

As part of the early-stage developments, carriers other than those found in licensed vaccines were investigated. Novel SF2a pmLPS lattice-type conjugates featuring a 4:1 pmLPS: protein molar ratio were prepared from a non-toxic recombinant mutant of the *Pseudomonas aeruginosa* exoprotein A (rEPA). This protein was selected as carrier because it is serologically unrelated to *Shigella* and because there is evidence that Abs to *P. aeruginosa* exoprotein A confer protection against the homologous infection. In line with the above findings, the conjugates induced anti-LPS IgG Abs in mice.[38] Moreover, whereas the alum adjuvanted and nonadjuvanted conjugates induced similar anti-LPS IgM Ab levels, the alum-adsorbed conjugate elicited a higher level of anti-SF2a LPS IgG Abs in mice after the third injection.[38]

Similarly, the *S. sonnei* pmLPS-protein conjugates used random chemical conjugation, ADH as a spacer, and rEPA as the protein carrier. However, whereas hydroxyl groups were involved as ADH anchoring sites in the case of SD1 and SF2a pmLPS, ADH was directly attached to the carboxyl groups of the *S. sonnei* pmLPS. Immunogenicity analysis revealed a trend similar to that of the SF2a conjugates.[38]

In an attempt at increasing conjugate solubility, limiting aggregation and avoiding extensive intramolecular and intermolecular cross-linking, the conjugation process was modified. It was found that succinylation of the amino group of the protein carrier increased the conjugation yield and improved the immunogenicity of the conjugates in young outbred mice. In contrast, conjugates composed of succinylated proteins induced lower anticarrier IgG Ab levels than conjugates prepared with native proteins.[61]

Additional variations involved a deeper investigation on the choice of protein carrier. The immunogenicity of *Shigella* conjugates in mice was improved by introduction of CRM9, a genetically derived nontoxic mutant of *Corynebacterium diphteriae*.[61] Alternatively, SF2a pmLPS conjugates prepared with a succinylated nontoxic peptide from *Clostridium difficile* toxin A elicited high levels of anti-LPS and antitoxin IgG antibodies in mice, opening the way to multiple action vaccines in the field of enteric diseases.[62]

Toward a combined shigella-ETEC pmLPS conjugate vaccine

Pursuing along this line, a novel SF2a conjugate was described recently. Aiming at developing a multipathogen enteric vaccine, Laird et al. at the Naval Medical Research Center (Silver Spring, MD, USA) have explored the feasibility of developing a SF2a Enterotoxigenic *Escherichia coli* (ETEC) conjugate vaccine by using CFA/I (CfaEB), a recombinant ETEC adhesion protein as carrier for the SF2a pmLPS.[45] Also pertaining to the "lattice"-type family, these conjugates differ from those developed at the NIH. Besides the carrier, divergences reflect the conjugation chemistry and the absence of spacer. To this end, a minimal amount of carboxyl groups are generated by TEMPO-mediated oxidation of some of the pmLPS primary hydroxyl groups, in fact to an extent corresponding to an estimated 10% of RUs, to allow direct carbodiimide-mediated coupling onto the carrier amino groups.[45] Conjugates characterized by a nonoptimized 2:1 protein:carbohydrate ratio by weight were immunogenic in mice, when administered as single conjugate or in combination, subcutaneously, three times at 4-week intervals at doses equivalent to 5 μg carbohydrate in the absence of adjuvant. As envisioned, the SF2a pmLPS-CfaEB conjugate also elicited IgG Abs against the ETEC protein, which were capable of inducing hemaglutination inhibition of a CFA/I expressing ETEC strain. In contrast, no SF2a bactericidal response was detected in a pooled mouse serum evaluated in a recently established *Shigella* serum bactericidal assay (SBA).[45]

pmLPS-protein conjugates as promising vaccine prototypes in human

Apart from exploratory developments related to carrier selection, several SD1, SF2a, and *S. sonnei* pmLPS-protein conjugates have entered clinical trials. The first report on their immunogenicity in human was published in the early 1990s.[38] Adult volunteers received two doses of one of the three vaccine prototypes, SD1 pmLPS-TT, SF2a pmLPS-rEAP, and *S. sonnei* pmLPS-rEPA, with or without alum 6 weeks apart. All evaluated vaccine prototypes were shown to be safe and immunogenic in adult volunteers when administered once at a dose equivalent to 25 μg of pmLPS. In contrast to data in mice, there was no further significant increase in the vaccine-induced levels of anti-pmLPS IgM and IgG Abs after the second immunization. A possible explanation for the absence of boost was that all volunteers had preimmunization serum IgM, IgG, and IgA pmLPS Abs. Also diverging from data in mice, adsorption onto alum did not enhance the conjugate immunogenicity.[38] Nevertheless, in the case of SF2a and *S. sonnei*, the levels of postimmunization anti-pmLPS serum Abs were similar or greater than those of young adults, convalescent from naturally acquired shigellosis. A significant anti-LPS IgA and IgG response in urine was also observed in 60% of the recipients of the *S. sonnei*-rEPA conjugate.[26] The induced IgAs were shown to be of secretory origin. A high correlation was demonstrated between the urinary and serum *Ab* response.[26]

Follow-up studies in human for SF2a and *S. sonnei* used pmLPS-rEPA conjugates characterized by an average protein:saccharide w/w ratio of 3.[41] A phase II clinical trial in an area where *Shigella* is endemic demonstrated a significant rise in anti-*S. sonnei* pmLPS IgG and IgA Abs 2 weeks after immunization in 90% of young adult volunteers, who had received one dose equivalent to 25 μg pmLPS. Whereas the level of IgA Abs returned to prevaccination level after a year, the level of anti-pmLPS IgG Abs had also decreased but was still nine times higher than that of the pre-vaccination level 2 years post-immunization. There was no booster effect nor an increase in the number of responders in recipients of a second dose 6 weeks after the first one. A strong association was observed between a fourfold or greater IgA and IgG Ab rise on day 14 postimmunization and a positive ASC IgA or IgG response on day 7.[41] A similar trend was seen in recipients of the SF2a investigational vaccine, albeit with a lower number of responders (70%). Yet, elevated levels of serum IgG and IgA Abs induced by the conjugates against homologous LPSs, *S. sonnei*- and SF2a-specific, respectively, persisted for a longer duration than those following disease.[41]

A S. sonnei pmLPS-rEPA vaccine prototype conferred protection in young adults

Encouraging efficacy data were demonstrated during a *S. sonnei* outbreak for young adults, who had received a *S. sonnei* pmLPS-rEPA conjugate vaccine administered parenterally.[42] One dose of non-adjuvanted conjugate equivalent to 25 μg saccharide conferred 74% and 43% protection against outbreaks of culture-proven *S. sonnei* shigellosis occurring 70–155 and 1–17 days after vaccination, respectively. In agreement with previous findings suggesting a group-specific association between preexisting serum IgG Abs to *Shigella* LPS and resistance to infection, it was found that the vaccinees, who developed *S. sonnei* shigellosis had lower serum IgG and IgA responses to the *S. sonnei* LPS than those who did not.[42] Subsequent quantitative analysis of IgG class and subclass serum response to *S. sonnei* LPS following vaccination revealed that shortly after vaccination with a single dose of conjugate the concentration of IgG1 was significantly higher than that of IgG2. At 6 months and afterward, it had decreased to similar levels to those of IgG2, while the levels of specific IgG1 and IgG2 Abs above the prevaccination values persisted for at least 2 years.[63] In contrast, the IgG response elicited by the SF2a pmLPS conjugate was almost entirely represented by the IgG2 subclass. The observed patterns were similar to those produced after natural exposure to *S. sonnei* and SF2a, respectively.[23] Since rEPA was the carrier used in the two pmLPS conjugate vaccines it was concluded that the different pattern of IgG anti-LPS subclass response originated from the differences in the chemical structure of the two polysaccharides.[63] In particular, whereas the SF2a O-Ag is composed of frequently encountered neutral monosaccharides, the *S. sonnei* O-Ag is a zwitterionic polysaccharide featuring a disaccharide RU made of two unusual aminosugars (Figure 1). It is noteworthy that several zwitterionic

polysaccharides were since then shown to be potent immunodulators and to be capable of T-cell activation.[64]

As a follow up of the encouraging efficacy data in adults receiving the first generation *Shigella* pmLPS-rEPA conjugate and improved immunogenicity data observed in mice receiving conjugates prepared from CRM9 or succinylated protein carriers ($carrier_{succ}$), novel *S. sonnei* and SF2a pmLPS conjugates were assayed in adult volunteers.[39] All conjugates had protein:saccharide w/w ratio in the range of 2–2.5, demonstrating a higher pmLPS loading onto the carrier than in the first set of conjugates evaluated in humans. The corresponding pmLPS:carrier molar ratio of the novel conjugates were similar, ranging from 1.35 for *S. sonnei* pmLPS-CRM9 to 2.58 for SF2a pmLPS-$rEPA_{succ}$. The measured anti-LPS Ab levels after one and two doses of conjugates corresponding to 25 µg saccharide demonstrated trends in immunogenicity alike those observed previously. Whereas the three novel *S. sonnei* conjugates, prepared from CRM9, $CRM9_{succ}$, and $rEPA_{succ}$, induced similar anti-LPS IgG Ab responses, the SF2a $rEPA_{succ}$ conjugate was more immunogenic than the corresponding rEPA and CRM9 conjugates.[39]

Efficacy induced by a Shigella *pmLPS conjugate parallels its age-related immunogenicity in young children*

Evaluation in 4- to 7-year-old children of the first-generation *S. sonnei* and SF2a pmLPS rEPA conjugates, characterized by a 1:4 pmLPS:rEPA w/w ratio demonstrated that the two conjugates elicited homologous serum anti-LPS IgG, IgA, and IgM Abs.[43] With a fourfold increase in 96% and 98% of recipients receiving one dose of the *S. sonnei* or SF2a conjugate equivalent to 25 µg saccharide, respectively, the rise in anti-LPS IgG Abs was the highest and most sustained one. Revaccination induced a booster response in the case of SF2a only. In contrast, both vaccine candidates induced a booster response against the exoprotein A from *P. aeruginosa*. As observed in the case of adult volunteers, there was no heterologous anti-LPS response in either the *S. sonnei* or the SF2a conjugate recipients. Analogously, the anti-LPS IgG Ab titers measured at 6 months after the second dose were significantly higher than the prevaccination levels.[43]

Taking into account first-in-children immunogenicity data, synthetic challenges, and immunogenicity data in adults for improved investigational *S. sonnei* and SF2a conjugate vaccines, a *S. sonnei* pmLPS-CRM9 and SF2a pmLPS-$rEPA_{succ}$ conjugates characterized by protein:saccharide w/w ratio of 1.34 and 1.39 corresponding to 17 and 19 µg saccharide per dose, respectively, were selected for evaluation in 1- to 4-year-old children.[40] The immunization regimen—two doses administered 6 weeks apart—followed the protocol previously used for evaluation in older children. Consistent with previous studies, the two conjugates were safe and immunogenic. In contrast to observations in adults and in 4- to 7-year-old children, the *S. sonnei* conjugate induced a booster response whereas the increase in anti-LPS IgG Ab level induced by the second dose of SF2a conjugate was not statistically significant. A more than fourfold rise in type-specific IgG Ab levels over the preimmunization values was observed in 92% and 85% recipients of the *S. sonnei* and SF2a conjugates, respectively. Both investigational vaccines induced long lasting Ab responses against the homologous LPS. A statistically significant rise in the Ab responses against the homologous carrier was also observed, albeit without a boost, in the case of the *S. sonnei* CRM9 conjugate. This outcome was tentatively explained by the antidiphteria toxin hyperimmune state of the recipients.[40]

Subsequently, a double-blinded, randomized and vaccine controlled phase III clinical trial was performed in healthy 1- to 4-year-old children, for a *S. sonnei* pmLPS $rEPA_{succ}$ and SF2a pmLPS-$rEPA_{succ}$ conjugates.[44] Children received two doses of conjugates corresponding to 25 µg saccharide administered intramuscularly 6 weeks apart and were followed for 2 years. Owing to the low number of SF2a cases cultured in the frame of the study, no conclusion on vaccine efficacy could be established. However, a 2.7-fold difference was observed in the level of anti-SF2a LPS IgG levels measured in the 1- to 2- and 3- to 4-year-old groups, suggesting that immunogenicity was age-related. Otherwise, the study demonstrated a 27.5% overall efficacy of the *S. sonnei* investigational vaccine, which did not meet the primary aim of the study. However, analysis according to age subgroups revealed that protection conferred by this conjugate reached 71.1% and 35.5% in the 3- to 4- and 2- to 3-year-old cohorts, respectively, whereas there was no efficacy in the youngest.[44] Efficacy paralleled the age-related immunogenicity also observed in the case of *S. sonnei*. In this particular case, a 4.5-fold difference was observed in the level of anti-*S. sonnei* LPS IgG titers between the 1- to 2- and 3- to 4-year-old cohorts. As in adults, efficacy could be correlated to the anti-LPS serum IgG Ab level supporting the former hypothesis that a threshold in the IgG Ab level is required for protection.[27] A possible explanation for the reduced efficacy of the conjugate in the 1- to 3-year-old was that the vaccine acted as a boost in individuals likely to have been previously exposed, but was unable to prime an immune response in the naive ones, above all in children under 2 years of age.

A SF2a pmLPS conjugate induces protection against S. flexneri *serotype 6*

Although there were too few cases to assess efficacy against SF2a in the phase III trial performed in 1- to 4-year-old recipients of the SF2a investigational vaccine, 50% protection against *S. flexneri* serotype 6 (SF6) infection was observed in the 3- to 4-year-old cohort.[44] A study compiling sera remaining from SF2a vaccine recipients involved in previous clinical trials, demonstrated that 20% adults receiving one dose of conjugate responded with a ≥ fourfold rise in anti-SF6 LPS IgG Ab level. Similarly, 32% and 20% of the 4- to 7-year-old and 3- to 4-year-old recipients of two SF2a vaccine doses, respectively, had a ≥ fourfold rise in anti-SF6 LPS IgG Ab level.[65] This increase was beyond the age-related development of natural immunity to SF6 infection, which paralleled that to *S. sonnei* and SF2 infection in the 1- to 3-year-old cohorts. In contrast to observations with murine sera, inhibition assay of human SF2a and SF6 sera revealed 100% inhibition with the homologous LPS and about 30–40% inhibition with the heterologous material. An O-acetylated rhamnobiose ($_{Ac}\mathbf{A^1B^1}$) encountered on each one of the two O-Ags[57] (Figure 1) was suggested as the structural basis for the observed cross-reactivity between SF2a and SF6 LPSs.[65] This original finding supports further

investigation on the potential of a SF6 pmLPS conjugate at inducing protection against both SF2a and SF6 infection.

As a whole, the numerous available data on *Shigella* pmLPS conjugates in human suggest that vaccine efficacy could be predicted in recipients older than 3 years of age. However, protection was not demonstrated in the youngest.[44]

Need for alternatives to pmLPS-conjugate vaccine prototypes

Despite encouraging safety and immunogenicity data, the first generation of pmLPS protein conjugate vaccines did not fulfill all efficacy criteria. "Lattice"-type conjugates remain ill-defined complex constructs, owing to the use of randomly activated pmLPS. Partial loss of antigenicity may occur upon detoxification and conjugation, and accurate controls of these two crucial processes are compulsory. Likewise, requirements for always better-defined molecules for use in humans call for appropriate consideration. In that context, alternative strategies were investigated. While conceptually different in terms of saccharide component and design, they all converge in involving single-point attachment conjugation chemistries, yielding "sun"-type conjugates.

Sized O-Ag-core-protein conjugates toward improved immunogens

Interest in "sun"-type *S. sonnei* conjugates (Scheme 1) emerged in the Robbins and Schneerson's group with the finding that conjugates made of synthetic SD1 OSs of defined length bound by their reducing end to the carrier were more immunogenic in mice than the first-generation SD1 pmLPS conjugates (see below).[66] In the absence of relevant synthetic *S. sonnei* OSs, the use of low molecular weight O-AgC haptens was investigated.[46] The strategy takes advantage of the various forms of LPS, which may coexist in Gram negative bacteria: "rough" LPS consisting in the core-lipid A species, "semirough" LPS whereby the latter is attached to an OS made of a small number of O-Ag RUs and "smooth" LPS featuring a full length O-Ag.

O-AgC-protein conjugates demonstrate superior immunogenicity in mice

The O-AgC was isolated by size-exclusion chromatography (SEC) of the crude material resulting from *S. sonnei* LPS hot phenol extraction and mild acidic hydrolysis. It had an average of 3.5 RUs instead of 29 RUs for the *S. sonnei* O-Ag. Still, the O-AgC featured a rather heterogeneous composition in terms of number of O-Ag RUs and core molecular structure showing diverse substitution patterns (Scheme 1). Site-selective conjugation of the O-AgC to the protein carrier (bovine serum albumin (BSA) or recombinant diphtheria toxin), involved the formation of a stable oxime linkage between the unique keto group introduced at the reducing end Kdo residue upon LPS acid-mediated detoxification and an aminooxy spacer on the carrier protein.[67] The O-AgC conjugates were antigenic. Following three injections in mice, they were shown to induce Abs specific for the O-Ag and not for the core region.[46] All O-AgC conjugates induced significantly higher anti *S. sonnei* LPS Ab titers in young outbred mice than the "lattice"-type pmLPS conjugates.[46] The induced titers were dependent on the number of O-AgC covalently linked to the carrier suggesting the importance of vaccine design and careful control of the conjugation step.

Even though the isolated yields of O-AgC were low in the case of *S. sonnei* (17%), the production process was thought to be relatively easy. This observation added to improved immunogenicity in mice when compared to "lattice"-type pmLPS conjugates, encouraged to extrapolate the strategy to other prevalent *Shigella*.

Similarly in the case of SF2a, SF6, and SD1, SEC purification of the products of acid-detoxified LPS provided fractions corresponding to a 1RU-core OS, the targeted O-AgC, and pmLPS.[47] BSA conjugates derived from the SF2a 1RU-core O-AgC, whereby the core-linked RU is not glucosylated, were not antigenic.[47] In contrast, those made of O-AgCs containing an average of two or three RUs were antigenic. Conjugates featuring an average of eight O-AgCs per BSA induced anti-LPS antibodies with booster responses in mice receiving three injections equivalent to 2.5 μg of saccharide. Anti-LPS Ab levels were similar to those induced by conjugates comprising the full-length O-Ag.[47] Similarly, the SD1 O-AgC-BSA conjugates were antigenic and immunogenic in mice. Anti-LPS Ab levels measured after three injections of conjugate equivalent to 2.5 μg of saccharide were similar to those measured when immunizing mice with conjugates encompassing synthetic OSs (see below) and significantly higher than those induced by the "lattice"-type pmLPS conjugates.[47] In contrast to SF2a, SD1 conjugates made of an average of 23 1RU-core O-AgCs per BSA were antigenic and immunogenic.[47]

Four fractions were isolated following SF6 LPS acid-mediated detoxification. BSA conjugates featuring O-AgCs with an average of one RU and 2.5 RUs, respectively, were antigenic but poorly immunogenic. In contrast, those encompassing O-SPCs with an average of seven RUs bound at an average density of 3.5 per BSA induced anti-LPS Ab titers equivalent to those induced by a "lattice"-type pmLPS conjugate in mice receiving three injections of 2.5 μg OS-equivalent conjugate. In the latter case, the number of RUs per chain was in the range of 15–20.[47]

From the data available for O-AgC-protein conjugates targeting four different *Shigella* species and types, it was concluded that the conjugates were easy to prepare, characterize, and standardize.

A S. sonnei O-AgC conjugate prototype tested in human

The superior immunogenicity in mice of the first-generation *S. sonnei* O-AgC conjugates over the "lattice"-type pmLPS conjugates promoted their clinical evaluation. A *S. sonnei* vaccine prototype featuring an O-AgC component consisting of an average of 3.5 O-Ag RUs plus the core region, was selected for evaluation in adult volunteers. Expanding further the search for original protein carriers able to avoid possible immune interference,[68] the carbohydrate moieties are bound by their terminal Kdo residue to a recombinant nontoxic exoprotein B of *C. difficile* (rBRU) equipped with aminooxy linkers. rBRU may have a dual role, contributing as protective antigen (B cell epitopes) and carrier (T helper cell epitopes). IgG antibody levels induced in young outbred mice by this original *S. sonnei*

O-AgC conjugate were significantly higher than those elicited by the pmLPS conjugates. The immunization regimen consisted in volunteers receiving a single dose of conjugate equivalent to 25 µg sugar amount, as in previous clinical trials with *S. sonnei* pmLPS conjugates, or to 10 µg carbohydrate amount, which more closely resembles doses used in commercial polysaccharide conjugate vaccines. As part of the dose-escalation study protocol, vaccine-induced Abs were to be measured at 1 and 6 months after immunization, and compared to those elicited by *S. sonnei* pmLPS conjugate vaccines. The study is completed but data are yet to be disclosed (ClinicalTrials.gov Identifier: NCT01369927).

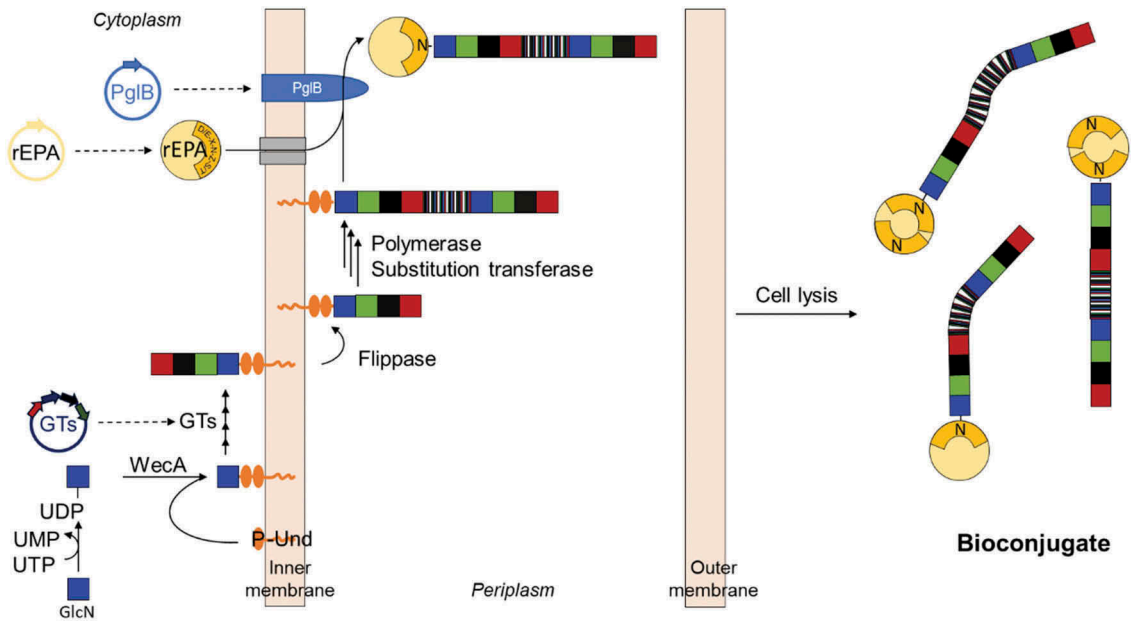
Bioconjugates or one-pot manufactured vaccine candidates

Polysaccharide-protein conjugate engineering in an Escherichia coli factory

In the strategies described above, the conjugate production consists in a combination of biological and chemical processes. The pmLPS and O-AgC components are produced either from the natural pathogen or from easier to cultivate bacteria expressing the same O-Ag, when available, before entering a chemical detoxification step. For example, *S. sonnei* and *Plesiomonas shigelloides* O17 produce the same O-Ag.[69] Since *S. sonnei* easily reverts to its rough form, *P. shigelloides* was used instead as the source of pmLPS in the "lattice"-type *S. sonnei* conjugates produced for clinical evaluation.[38] The protein carrier is produced separately, while the conjugation step is an independent chemical process. Despite ongoing efforts toward improvements, as exemplified by a recent report on SD1 LPS production and isolation,[70] or attempts at developing cost-effective strategies for isolating preferred O-Ag vaccine haptens overcoming limitations related to the natural heterogeneity of O-Ag chain length,[71] the full process to a glycoconjugate vaccine requires multiple purification and characterization steps.

As an attempt to simplify the production process of glycoconjugate vaccines, novel technologies were developed. Of particular interest is the *in vivo* synthesis of bacterial polysaccharide-protein conjugates, also named bioconjugates.[72] The strategy stemmed from the original identification of a general protein N-linked glycosylation machinery in *Campylobacter jejuni*, showing similarities with LPS biosynthesis in Gram negative bacteria.[73] The process is catalyzed by a single oligosaccharyl transferase named PglB, which exhibits relaxed specificity toward undecaprenyl pyrophosphate-linked glycan substrates. The pioneering demonstration by the Aebi laboratory (ETH, Zurich, Switzerland) that a functional PglB-dependent N-linked glycosylation pathway could be transferred to *E. coli*[73] has evolved the strategy into an engineered *E. coli* versatile platform for the making of "sun"-type glycoconjugates.[74] The technology was shown to be appropriate for the transfer a variety of O-Ags, bearing a 2-*N*-acetyl residue at their reducing end, to AcrA, a periplasmic protein originating from *C. jejuni*.[72] Paving the way to a larger variety of avenues, the subsequent identification of broad spectrum bacterial O-linked protein glycosylation processes and more diverse N-linked protein glycosylation systems has overcome the original glycan substrate limitation.[74]

Studies on SD1, were part of the early developments and involved PglB. As in *C. jejuni*, PglB-mediated glycan transfer to proteins in *E. coli* occurs at specific asparagine residues located within consensus sequences defined as D/E-*X*-**N**-*Z*-S/T, where *X* and *Z* can be any amino acid except proline.[75] With the aim of developing a SD1 bioconjugate investigational vaccine, the process originally established for AcrA was adapted to EPA (Scheme 2). To accommodate PglB protein substrate specificity, a modified version of the known carrier (EPA_{cs}) featuring two engineered consensus



Scheme 2. One-pot PglB-based bioconjugate engineering in *E. coli*. P: phosphate, Und: undecaprenyl, GT: glycosyl transferase, N: asparagine within the consensus sequence for N-glycosylation.

sequences was expressed in the periplasm of *E. coli* coexpressing PglB and the SD1 O-Ag synthesis genes. The O-Ag RU (Figure 1) was preassembled on undecaprenyl phosphate in the cytoplasm, flipped into the periplasmic space, polymerized into O-Ag chains varying in their number of RUs, which were then transferred onto EPA_{cs}. The process was adapted to some extent for production at a large scale, albeit with some limitations.[76] The high sensitivity of the rate-limiting N-glycosylation step to cultivation conditions was shed to light, resulting in low production yields whilst a high percentage of the carrier protein remained unglycosylated. Yet, fed-batch fermentation using a semidefined glycerol medium and a simple pulse feed strategy provided SD1 glycoconjugates, which were extracted out of the periplasm by osmotic shock and purified by anionic chromatography and SEC.[76] Using this technology, LPS detoxification and chemical manipulation are no longer required. Conjugate characterization and detailed analysis could be achieved by physicochemical methods, including NMR, compatible with quality control to ensure lot-to-lot consistency.[77]

Properly engineering of the consensus sequences within a protein carrier could *in fine* allow ideal control of the O-Ag:carrier loading and attachment site of the polysaccharide component. In the present case, detailed analysis of the bioconjugates demonstrated that although the newly engineered EPA_{cs} mutants could be glycosylated at either site when containing a single N-glycosylation consensus sequence located at two different positions, the use of engineered EPA_{cs} comprising two PglB-compatible consensus sequences resulted only in monoglycosylated SD1 EPA_{cs} conjugates.[76] With a molar O-Ag:EPA_{cs} ratio of 1 and O-Ag chains comprising 13–20 RUs, an average EPA_{cs}:sugar w/w ratio of 5:1 was measured for a SD1 O-Ag-EPA_{cs} conjugate,[77] which was found safe and immunogenic in a phase I clinical trial (GlycoVaxyn, Switzerland).[48]

Similarly to the SD1 O-Ag, the reducing end residue of the SF2a O-Ag is a 2-*N*-acetyl-D-glucosamine. Therefore, aiming at broadening vaccine candidate coverage against *Shigella*, the PglB-based engineered *E. coli* system was adapted to obtain a SF2a O-Ag-rEPA_{cs} conjugate.[78] Original developments of a fed-batch bioprocess tailored to SD1 glycoconjugate production had shed light on the complexity of the *in vivo* glycoengineering technology[76] and case-to-case process improvements were found necessary. In particular, a prerequirement for an efficient protein glycosylation *E. coli* factory consists in the constitutive expression of multiple functional proteins in the proper compartments in a coordinated manner to obey the dynamic of a tailored high yielding biosynthetic pathway.[78] Accordingly, a solid basis for further optimization studies was established in the case of a SF2a O-Ag-EPA_{cs} conjugate.[78] Several parameters were optimized for improved productivity, which enabled a 46-fold increase of the overall yield of the SF2a O-Ag-EPA_{sc} conjugate from shake flask conditions to high cell density cultures. As for SD1, Western blot analysis of the isolated SF2a bioconjugate revealed that glycosylation had occurred only at one of the two N-glycosylation consensus sequences available in engineered EPA_{sc}.[78] Consequently, the average EPA_{cs}:sugar w/w ratio was estimated to be approximately 5:1, and closely resembling that found for the SD1 investigational bioconjugate vaccine candidate.[77]

Bioconjugate vaccine prototypes: proof-of-concept in human

The GlycoVaxyn SD1 O-Ag-EPA_{sc} vaccine candidate, named SD133, was the first bioconjugate to enter a phase I clinical trial. GMP lots of the SD1 bioconjugate with a 5:1 protein:saccharide w/w ratio were formulated with or without 0.06% aluminum hydroxide in saline buffer, and administered twice 60 days apart to young adults, at doses corresponding to 2 and 10 μg saccharide, respectively. In the absence of placebo, the observed adverse events were found comparable to those reported in previous studies, and all formulations were found safe and immunogenic.[48] The vaccines were shown to induce functional Abs against the carrier, suggesting that the protein B-cell epitopes were preserved. All vaccines elicited statistically significant anti-LPS humoral responses, which were maintained for 5 months, the total duration of the study. Whereas the second vaccine dose did not contribute to any major increase in IgG Ab levels in the groups receiving the nonadjuvanted formulations, a slightly enhanced response was observed in subjects administered with the adjuvanted ones. Yet, there was no statistically significant difference in IgG Ab titers at any time point between groups, which precluded any conclusion on an optimal dose or adjuvant effect.[48]

As a follow up to demonstrate versatility for the engineered *E. coli* platform, a SF2a investigational bioconjugate vaccine, Flexyn2a (LimmaTech Biologics AG, formerly GlycoVaxyn, Switzerland), was evaluated for safety and immunogenicity in the frame of a phase I clinical trial,[49] following extensive analytical characterization as described for the SD1 bioconjugate vaccine candidate.[77] Groups of 12 healthy adult volunteers, prescreened for their preimmunization status showing low anti-SF2a LPS serum IgG titers, were administered the vaccines twice, via intramuscular injection, 28 days apart, and followed up to day 56. In contrast to the SD1 phase I trial, only the 10 μg saccharide-equivalent dose was used. The vaccines, which were formulated on-site with and without amounts of Alhydrogel adjuvant, corresponding to 0.02% Al^{3+} when present, contained 10 μg saccharide and some 50 μg protein in Tris-buffered saline pH 7.4. The vaccines were found safe with observed adverse events classified as mild, self-limiting, and comparable to those reported with licensed polysaccharide conjugate vaccines. Flexyn2a, whether adjuvanted or not, elicited robust anti-SF2a LPS IgG and IgA *Ab* levels after one dose compared to placebo recipients, with no statistically significant increase after the second dose. Analogously, analysis of Ab lymphocyte supernatants (ALS) isolated 7 days after the first and second shots, respectively, revealed that the majority of the response was detected after the first injection. These findings suggested that, with a capacity to induce a 16-fold and 14-fold increase in anti-LPS IgG and IgA Ab titers over baseline in 92–100% subjects, respectively, a single 10 μg saccharide equivalent dose may provide the required immunity. Still, data from a SBA, indicated that delivery of a second vaccine dose could impact the development of functional Abs. Besides, Ab functionality differed substantially between subjects and could not be directly

correlated to the anti-LPS IgG response.[49] As a major step forward, detailed data from the phase IIb, double blind, placebo-controlled, efficacy challenge study with the bioconjugate vaccine candidate Flexyn2a (ClinicalTrials.gov, Identifier: NCT02646371), a Controlled Human Infection Model (CHIM) study, shall be very instructive. Undoubtedly, information communicated to date[50] provides significant support to the bioconjugate technology. As a remarkable achievement, the Flexyn2a vaccine was shown to induce a protective immune response in naïve adult volunteers, who had received twice, 4 weeks apart, a 10 µg saccharide equivalent dose of nonadjuvanted Flexyn2a, and were challenged with 1500 cfu of the virulent SF2a strain 2457T 4 weeks after the second injection. These promising data pave the way to the production of a *Shigella* tetravalent bioconjugate vaccine candidate, featuring the *S. sonnei*, SF2a and SF6 components in combination with a *S. flexneri* 3a (SF3a) constituent.[49]

Bioconjugates versus detoxified LPS-based conjugates: O-acetylation

The SD1 and SF2a original "lattice"-type pmPLS-protein conjugates and the more recent "sun"-type bioconjugates resemble each other as both involve an EPA-related carrier and an O-Ag component of variable chain length. They differ in terms of manufacturing process, carbohydrate:carrier attachment, core implication and to some extent sugar:protein content. In addition, albeit barely highlighted, a constitutively relevant divergence between the two SF2a vaccine candidates resides in the specific O-acetylation pattern of their O-Ag component. Actually, the native SF2a O-Ag is O-acetylated at two sites in a non-stoichiometric manner to an extent, which may vary from one strain to another (Figure 1),[57,79,80] suggesting a potential for antigenic divergence. The contribution of O-acetylation in the functional immune response to licensed bacterial polysaccharide-based vaccines was shed to light in various instances as exemplified for *Salmonella typhi* Vi or *Neisseria meningitidis* serogroup A, but not in others, indicating a case-to-case specificity.[81,82] As outlined,[80] while not specifically quantified at the pmLPS-rEPA conjugate stage and a priori not particularly controlled although possibly affected in the course of the production process,[80,83] these non-carbohydrate substitutions are present in the first generation of SF2a conjugate vaccine candidates. In contrast, O-acetylation is absent in Flexyn2a, the bioconjugate vaccine candidate that has gone into clinical trial.[49] In support to this attractive option, no serological difference was observed between SF2a LPS and SF2a O-deacetylated LPS when tested against rabbit monovalent type II-specific and group (3,4)-specific sera or against polyvalent sera specific to type IV of *S. flexneri*, suggesting that O-acetyl substitutions do not interfere with Ab binding.[57] Yet, the frequent occurrence of related O-acetylation patterns in other *S. flexneri* O-Ags has led Knirel et al. to propose novel group O-factors, to account for additional antigenic diversity.[13] Since the two categories of SF2a polysaccharide conjugates stand as promising vaccine candidates, it may be worthwhile investigating further the possible contribution of the SF2a O-Ag O-acetyl groups to immunodominant epitopes, possibly shared across serotypes,[13,79] and their relevance to a functional immune response.[65] It is noteworthy that the sites and extent of SF2a O-Ag O-acetylation were first described for pmLPSs isolated from LPSs purified from the reference laboratory strain 2457T,[80] which was used for the preparation of pmLPS conjugate vaccines[59] and for immunoassays in clinical trials with the later.[38] The same strain was also used for challenge in several CHIM studies,[84] one in which the Flexyn2a bioconjugate has demonstrated a 30% efficacy.[84] Analysis of the recognition of a diversity of circulating SF2a strains by the vaccine-induced sera may contribute to a better perception of the potential efficacy of LPS-based vaccines in the field.

Synthetic oligosaccharide-based conjugates toward robust homogeneous vaccine candidates

Synthetic oligosaccharides as O-Ag surrogates

The concept of using well-defined synthetic OSs as surrogates of the bacterial polysaccharide antigens of interest has its foundations in the ground-breaking studies by W. F. Goebel, who reported in the late 1930s that protein conjugates of cellobiuronic acid, featuring the disaccharide repeating unit from *Streptococcus pneumoniae* type III capsular polysaccharide, induced sera that recognized the purified bacterial polysaccharide and conferred resistance to a challenge with the homologous bacteria in rabbits.[85] Ensuing fundamental support stemmed from seminal studies by Elvin Kabat and others,[86,87] revealing that anti-polysaccharide Abs could accommodate up to six or seven, occasionally eight monosaccharides in their combining site. Original investigation on the use of glycoconjugate immunogens derived from enterobacterial O-Ag fragments, as pioneered on *Salmonella typhimurium* by Alf Lindberg,[88,89] subsequently substantiated the idea that short OSs, featuring pertinent epitopes, covalently linked to an appropriate protein carrier could induce anti-LPS Abs conferring protection against challenge with virulent homologous bacteria.[90] When disaccharides employed in these early studies were of synthetic origin,[88] larger OSs were obtained by bacteriophage *endo*-rhamnosidase-mediated cleavage of alkaline-treated LPS.[89,91] It remains that single species O-Ag segments of defined length and composition are not easily available by detoxification and downsizing of the isolated biological material, suggesting the need for alternatives. In this respect, sustained progress in carbohydrate chemistry and conjugation chemistry, combined to advances in immunochemistry and structural biology, has permitted a better understanding of the fine specificity of bacterial polysaccharide recognition by monoclonal Abs (mAbs)[92] and deeper investigations in the field of synthetic carbohydrate-based conjugates.[93] Going beyond Ab binding, the ability of the latter conjugates to elicit Abs that recognize the natural surface polysaccharide antigens and protect against disease is a complex subject, also confronting interactions with cell surface receptors and antigen presentation. As of to date, a number of parameters, often interdependent, have been identified as exhibiting some influence on the immunological properties of such glycoconjugates designed as potential antibacterial vaccines (Figure 2).[94]

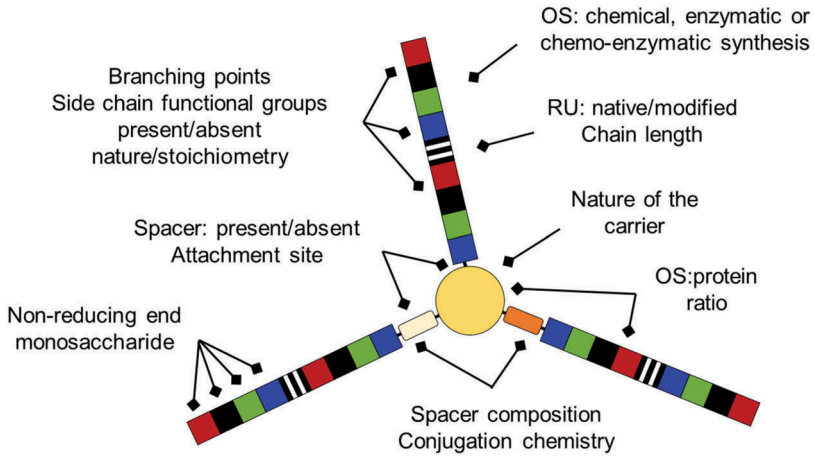


Figure 2. Synthetic carbohydrate-based vaccines. Key components and parameters possibly affecting immunogenicity. Abbreviation: OS: oligosaccharide, RU: repeating unit.

Undoubtedly, studies on the use of well-defined synthetic OSs as surrogates of *Shigella* O-Ags toward improved conjugate vaccines enabling long-term immunity in the pediatric population at risk have contributed to a substantial extent to recent developments toward better defined carbohydrate-based vaccines.

S. dysenteriae *type 1: "Sun"-type synthetic glycan conjugates override a "lattice"-type pmLPS conjugate in mice*

Besides pursuing active investigations on pmLPS conjugates as potential *Shigella* vaccines, studies at the NIH evolved along two orientations. Methyl glycosides representing part of and up to two O-Ag RUs, or analogs thereof, were chemically synthesized and used for epitope mapping toward elucidating the binding pattern of anti-SD1 murine mAbs to the O-Ag.[95,96] Conversely, the synthesis of chemically defined extended fragments of the O-Ag and their conversion to immunogens upon single-point attachment to protein carriers was tackled for *in vivo* evaluation.[97,98]

Molecular insights on the fine specificity of mAB binding to SD1 O-Ag

The SD1 O-Ag is a neutral heteropolymer defined by a linear tetrasaccharide RU (**ABCD**) composed of α-linked L-rhamnose (**A, B**), D-galactose (**C**), and *N*-acetyl-D-glucosamine (**D**) moieties (Figure 1). Using ligand-induced protein fluorescence change and a panel of chemically synthesized SD1 mono- to 8-mer, affinity measurement showed that murine IgM 3707 E9, isolated from immunization with heat-killed SD1,[95] recognizes internal segments on the O-Ag. Extension of the α-L-Rha*p*-(1→2)-α-D-Gal*p* sequence (**BC**) by flanking residues did not influence binding significantly. On that basis, Glaudemans and coworkers proposed disaccharide **BC** as the basic determinant of the SD1 O-Ag.[95] Chemical mapping based on the principle of hydroxyl group replacement revealed the fine network of molecular interactions governing Ab binding.[96] O-Ag binding to mAbs generated from immunization with a pmLPS-TT conjugate[18] was also analyzed. IgG 5338 H4 binding to the O-Ag was inhibited by all OSs containing the **BC** portion, even though it had only 50% sequence homology with IgM 3707 E9.[18] In contrast, despite extensive sequence homology with IgG 5338 H4, the other two Abs bound only to **ABCD**.[18] These observations corroborated independent findings released from inhibition studies that engaged six additional murine anti-SD1 LPS mIgMs.[99] It was surmised that an efficacious SD1 synthetic OS-based vaccine should include the **ABCD** tetrasaccharide.[18]

The above assumption was subsequently challenged as conformational analysis relying on NMR spectroscopy[97,100] and molecular modeling computation[101] revealed that the α-D-Gal*p*-(1→3)-α-D-Glc*p*NAc (**C-D**) linkage adopted two preferred conformations. The trend was in favor of a hairpin conformation for pentasaccharide **ABCDA'** and larger O-Ag portions, which evolved into a helix-type arrangement whereby the **C** residues protrude radially with partial exposure of the adjacent **B** and **D** monosaccharides.[101] This preferred conformation was thought to guide mAb recognition and possibly also the induction of a strong anti-O-Ag Ab response. NMR, immunochemical, and modeling data converged to hypothesize that O-Ag segments comprising at least the **ABCDA**' pentasaccharide would adopt a conformation more closely resembling that of the natural O-Ag.[100–102]

Influence of the synthetic OS chain length on the immunogenicity of "sun"-type SD1 conjugates

The disclosure of the first immunogenicity data for SD1 synthetic OS-protein conjugates represented a remarkable milestone in the field.[66] Accounting for the more viable OS block synthesis exploiting the **A-B** glycosidic linkage,[97] sets of conjugates encompassing synthetic [**BCDA**']$_n$ (n = 1–4) OSs attached at their reducing end to human serum albumin (HSA) by means of a 14-atom-long spacer were chemically synthesized[97] (Figure 3) and injected subcutaneously to mice three times at 2 weeks interval at a dose equivalent to 2.5 μg saccharide in the absence of adjuvant.[66] Consistent with assumptions stemming from structural investigation,[101,102] a {[**BCDA**']}$_{11}$-HSA conjugate elicited low levels of anti-SD1 LPS IgGs. Instead and beyond expectations, HSA conjugates

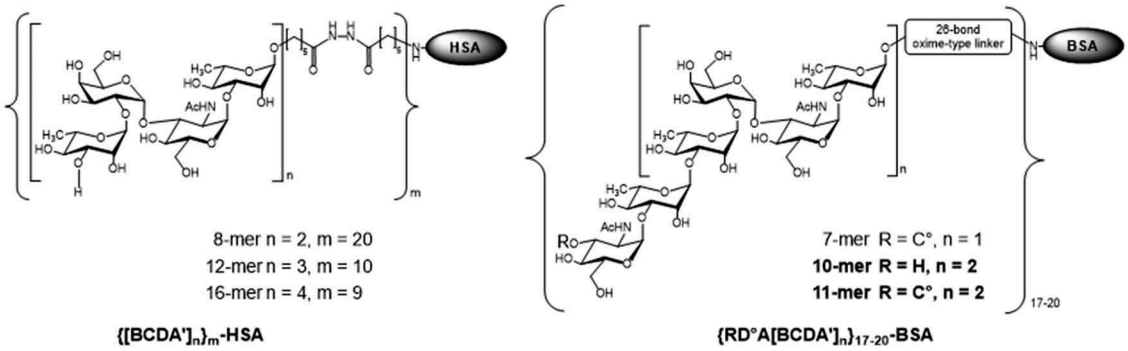


Figure 3. SD1 glycoconjugates demonstrating high immunogenicity in mice with emphasis on chain length (left) and nonreducing terminal residue (right). OSs selected for evaluation in a phase I clinical trial are marked in bold. The ° and ′ labels define residues occurring before and after the basic RU, respectively.

built from the [**BCDA**']$_2$ (8-mer), [**BCDA**']$_3$ (12-mer), and [**BCDA**']$_4$ (16-mer) haptens elicited significantly higher anti-LPS IgG *Ab* levels than that achieved by a "lattice"-type pmLPS HSA.[66] A statistically significant booster effect was observed with the third injection. OS length and OS density emerged as two interdependent variables governing conjugate immunogenicity. For conjugates featuring comparable OS densities, as determined by MALDI-TOF mass spectrometry, the 16-mer OS conjugate, was only slightly more immunogenic than its 12-mer OS counterpart. It was surmised that the use of longer OSs would result in little additional gain in immunogenicity. The 12-mer OS and 16-mer OS conjugates differing in their OS:carrier molar ratio induced significantly divergent anti-LPS IgG levels. Those characterized by a density of 9–10 OSs per carrier, corresponding to saccharide:HSA wt/wt ratios of 1:3.1 and 1:2.5, respectively, were the most immunogenic. It was hypothesized that for a given OS, a minimal density was required for efficient B-cell membrane receptor clustering whereas masking the carrier due to OS overload would reduce T-cell stimulation.[66] A maximal anti-LPS Ab response was achieved with a conjugate encompassing in average nine 16-mer chains per HSA molecule.

The synthetic OS nonreducing terminal residue dictates the immunogenicity of SD1 "sun"-type conjugates

Subsequent analysis of the anti-LPS serum Ab response induced by BSA conjugates comprising in average 17–20 synthetic 6- to 13-mer OSs differing by their non-reducing terminus, also underlined the importance of the non-reducing terminal residue.[51] In particular, immunogenicity data for the frame-shifted 10- and 11-mer conjugates differed markedly despite the similar size and loading of their OS component. They revealed the significant contribution of the terminal *N*-acetyl-D-glucosamine (**D**) and D-galactose (**C**) residues to the ability of short OS conjugates at inducing Abs cross-reactive with the SD1 LPS. Noticeably, upon conjugation, the 7- and 11-mer galactose-terminated haptens evolved into immunogens of comparable potency. These findings agreed with binding experiments[18,95,99] and molecular modeling studies[101] calling attention to the immunodominant residue **C** within the **ABCDA'** conformational epitope. They also provided the first demonstration that chemical synthesis and structural biology could open new avenues in the development of a *Shigella* glycoconjugate vaccine.

Instead of the originally envisioned [**BCDA**']$_n$ OSs,[66] the chemically synthesized **D°A[BCDA']$_2$** (10-mer) and **C°D°A[BCDA']$_2$** (11-mer) OS, featuring non-natural terminal residues (Figure 3),[103] were promoted for inclusion in experimental vaccines for phase I clinical evaluation.[104] While these achievements exemplify the interest of the synthetic approach to better understand the parameters contributing to glycoconjugate immunogenicity (Figure 2), no data in human are available for these improved SD1 glycoconjugate vaccine prototypes.

S. flexneri *serotype 2a: a chemical biology strategy toward the first-in human* Shigella *synthetic glycan conjugate vaccine*

In complement to promising achievements toward the development of a SF2a live attenuated oral vaccine candidate by Sansonetti *et al.*,[105] interests in synthetic glycans as O-Ag surrogates in the search for a potent, better characterized, SF2a glycoconjugate vaccine emerged at the Institut Pasteur in the late 1990s. A multidisciplinary strategy synergizing synthetic, immunochemical, structural and computational methods was implemented to advance our understanding of the parameters influencing the design of synthetic carbohydrate haptens as efficient functional mimics of the natural heterogeneous protective polysaccharide antigen.

Molecular insights on the fine specificity of murine mAb binding to SF2a O-Ag

The SF2a O-Ag is a neutral heteropolymer defined by a branched pentasaccharide RU ($_{Ac}$**A^{1}B^1[E^1]C^1**$_{Ac}$**D^1**) composed of α-linked L-rhamnose (**A^1, B^1, C^1**), and D-glucose (**E^1**) and E-linked *N*-acetyl-D-glucosamine (**D^1**) moieties (Figure 1). The α-D-Glc*p*-(1→4)-α-L-Rha*p* (**E^{1}C^1**) side chain common with the *S. flexneri* type 2b O-Ag is characteristic of type II O-Ags. In addition to the recently disclosed non-stoichiometric acetylation at O3$_{A1}$/O-4$_{A1}$ and O-6$_{D1}$, shared with O-Ags from several *S. flexneri* types and tentatively assigned group O-factor 9 and 10, respectively, group factor 3,4—associated to the O-Ag backbone—is variably expressed.[13] Five mAbs representing all IgG subtypes were isolated from mice immunized with killed SF2a bacteria and shown to protect passively against SF2a in a murine model of

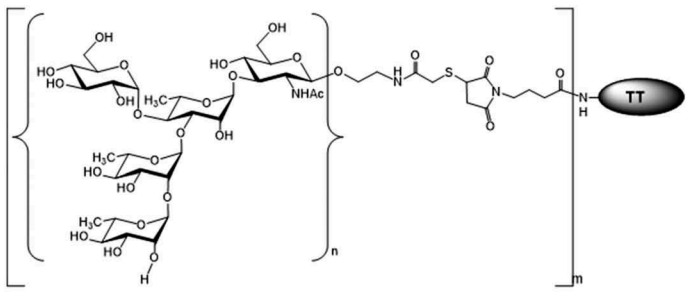
pulmonary infection, mimicking the disease-induced inflammation.[106] The detailed molecular analysis of the O-Ag recognition pattern by the five mIgGs was achieved by use of synthetic non-O-acetylated 2- to 15-mer glycosides corresponding to all frame-shifted sequences within the SF2a O-Ag RU and up to a 3RU O-Ag portion. In addition to highlighting the importance of the $\mathbf{E^1C^1D^1}$ trisaccharide for IgG binding, data revealed that the minimal sequence necessary for recognition by all five mAbs was the branched $\mathbf{B^1(E^1)C^1D^1}$ tetrasaccharide flanked by residue $\mathbf{A^1/A^{1'}}$ at either end.[106,107] Chain extension from 1RU- to 2RU-segments correlated with a significant increase in OS binding to all mAbs, which was not significantly influenced by further extension to OSs larger than a 10-mer. Analysis of Ab binding to the frame-shifted 10-mer segments $\mathbf{[A^1B^1(E^1)C^1D^1]_2}$ and $\mathbf{[D^{1^\circ}A^1B^1(E^1)C^1]_2}$ also revealed divergence between the IgG fine specificity suggesting that chain length is not the sole important parameter.[92] In this respect, the unique behavior of IgG F22-4, which was also the only mAb showing measurable affinity to $\mathbf{E^1C^1D^1}$, was outlined.

Crystallographic data for Fab F22-4 in complex with a synthetic 10-mer $\mathbf{[A^1B^1(E^1)C^1D^1]_2}$ and 15-mer $\mathbf{[A^1B^1(E^1)C^1D^1]_3}$, corresponding to a 2RU- and 3RU-segment of the SF2a O-Ag, respectively, provided structural evidence in support to $\mathbf{E^1C^1D^1}$ driving Ab binding.[108] The binding modes of the two OSs to IgG F22-4 were almost identical, showing a 9-mer epitope featuring six residues within two consecutive O-Ag RUs in direct interaction with the mAb. This original observation provided strong indication that synthetic glycans acting as O-Ag surrogates to be included in a SF2a glycoconjugate vaccine may require a minimum of two contiguous RUs. Molecular dynamics simulation supported by STD-NMR data revealed the ability of IgG F22-4 to recognize epitopes distributed along the O-Ag, albeit with a preference for the upstream O-Ag terminus.[107] In complement to structural data, conformational studies on the SF2a O-Ag showed that IgG F22-4 is likely to recognize a segment of its cognate O-Ag in a conformation favored when free in solution,[109] while NMR analysis of the O-deacylated SF2a O-Ag and of a synthetic 15-mer representing a 3RU non-O-acetylated O-Ag segment suggested comparable conformational behaviors.[109]

The SF2a O-Ag harbors non-stoichiometric labile acetyl groups to an extent varying in degree between strains in the ranges of 30–70%, 15–30%, and 30–60%, at $O3_{A1}$, $O\text{-}4_{A1}$, and $O\text{-}6_{D1}$, respectively,[57] generating antigenic diversity.[13] This observation calls for a better understanding of the possible influence of these O-Ag decorations on vaccine efficacy. Well-defined synthetic OSs embodying selected portions of the natural O-Ag, and used in its place, represent a promising strategy to address this issue. Taking advantage of the observed antigenic and conformational similarities between the synthetic 10-mer $\mathbf{[A^1B^1(E^1)C^1D^1]_2}$ and 15-mer $\mathbf{[A^1B^1(E^1)C^1D^1]_3}$ O-Ag fragments,[106,110] and exploiting the molecular diversity in reach owing to chemical synthesis, mapping of the binding site of the five available anti-SF2a mIgGs was achieved using three synthetic $\mathbf{[A^1B^1(E^1)C^1D^1]_2}$ analogs site-selectively mono- and di-acetylated at the internal $O3_{A1'}/O\text{-}4_{A1'}$ and/or $O\text{-}6_{D1}$, respectively.[110] Whilst the 6_{D1}-acetate did not significantly influence Ab recognition, the 3_{A1}-*O*-acetyl group strongly impaired OS binding to four mIgGs. In contrast, mIgG F22-4 was the only Ab able to recognize diversely O-acetylated segments of the SF2a O-Ag with micro- to submicromolar IC_{50}, which shed extra light on its unique recognition specificity.[110] Interestingly, the observation that none of the acetyl groups added significantly to broad Ab recognition substantiated previous conclusions on the absence of serological variation between the SF2a LPS and its O-deacetylated counterpart.[57] It comforted original findings suggesting that SF2a O-Ag mimicry was achieved by synthetic non-O-acetylated OSs. Moreover, the demonstration that O-Ag epitopes recognized by sera from patients naturally infected with SF2a were, to some extent, displayed in non-substituted OSs corresponding to a small number of RUs,[111] corroborated conformational, physicochemical and structural data unraveling the basis for accurate molecular mimicry of SF2a O-Ag by non-O-acetylated OSs featuring two to three consecutive RUs.

A synthetic 15-mer oligosaccharide-tetanus toxoid "sun"-type conjugate induces protective sera in mice

Guided by antigenicity data, a panel of conjugates derived from synthetic glycans, differing in terms of chain length and non-reducing terminal residue,[112,113] and attached at their reducing end to TT through a 13-atom-long spacer by means of the thiolmaleimide chemistry, were synthesized (Figure 4). Consistent with assumptions inferred from antigenic, conformational and structural investigation, a $\{[\mathbf{A^1B^1(E^1)C^1D^1}]\}_{12}$-TT conjugate elicited low levels of anti-SF2a LPS IgGs. At comparable OS density, those conjugates encompassing glycan chains



5-mer	n = 1, m = 12
10-mer	**n = 2, m = 12**
15-mer	**n = 3, m = 12**
15-mer	**n = 3, m = 14**
15-mer	n = 3, m = 4.6
15-mer	**n = 3, m = 8.5**
15-mer	**n = 3, m = 17**
15-mer	**n = 3, m = 26**

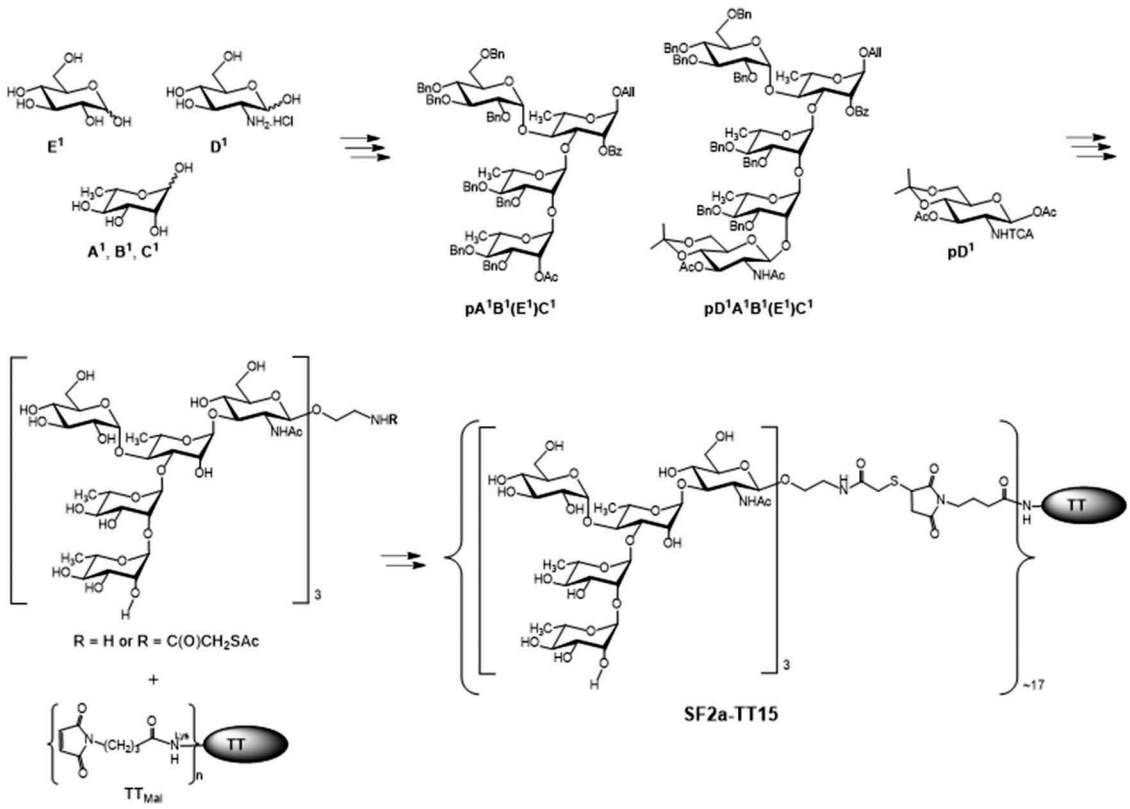
Figure 4. "Sun"-type SF2a conjugates featuring a synthetic glycan as hapten with emphasis on chain length and on sugar:protein mol/mol ratio. Composition yielding glycoconjugates demonstrating high immunogenicity in mice are highlighted in bold. Abbreviation: TT: Tetanus toxoid.

equivalent to 2RU and 3RU portions of the SF2a O-Ag were shown to induce a significant level of IgG Abs directed at the SF2a LPS when injected intraperitoneally to mice three times at 3-week interval followed by a fourth injection a month after the third one, at a dose equivalent to 10 μg saccharide in the absence of adjuvant.[106] As observed for SD1, immunogenicity increased with the number of RUs per OS in terms of measured anti-SF2a LPS Ab levels. Moreover, the number of responders was also affected, reaching 100% in mice receiving $\{[\mathbf{A^1B^1(E^1)C^1D^1}]_3\}_{12}$ TT.[106] Substantiating original findings, a SF2a glycoconjugate vaccine displaying a 15-mer density of 14 triggered a high and sustained anti-LPS Ab response, when administered four times at a dose corresponding to 2.5 or 1 μg of carbohydrate. Three immunizations were required to promote an optimal anti-LPS IgG response, with the fourth immunization only contributing to a minor increase in the anti-LPS Ab level.[111] The 3RU OS also showed promising, albeit less pronounced, immunogenicity when presented as B,T-diepitope liposomal constructs, displaying the glycan moiety and a universal T-helper peptide, individually attached to a Pam_3CAG TLR2 ligand contributing as anchor and adjuvant.[114] Unrelated to the nature of the immunogen, the induced sera exhibited protective capacity, underlining the potency of a synthetic OS corresponding to a non-O-acetylated 3RU segment of the O-Ag as functional mimic of the SF2a surface polysaccharide.

Remarkably, the recognition profile of the sera induced in mice by $\{[\mathbf{A^1B^1(E^1)C^1D^1}]_3\}_{14}$-TT and other 15-mer-TT conjugates attested an exquisite mimicry of LPS presentation at the bacterial surface by the conjugate.[52,111] As observed in naturally infected patients and in volunteers immunized with a SF2a pmLPS conjugate, the immune response induced in mice was of the Th2-type, mainly mediated by IgG1, and SF2a-oriented.[111] In agreement with findings for a SF2a pmLPS-rEPA conjugate,[38] immunization of mice three times with 2.5 μg saccharide equivalent doses of alum adjuvanted conjugates displaying in average 8.5 or 17 glycan chains per TT gave rise to anti-SF2a LPS IgG titers significantly higher and maintained longer—at least 14 months—than immunizations with their non-adjuvanted counterparts.[52] Moreover, whereas OS length and end-chain residue emerged as a pivotal variable in the studied range, the influence of OS density on the immunogenicity of the 15-mer conjugate was less striking.[111] Conjugates featuring a density of 8.5 and 17 OSs per carrier demonstrated comparable immunogenicity and protective capacity, with a non-statistically significant trend toward a slight decrease in immunogenicity for the highest density tested (26). Alternatively, a density of 5 resulted in a significantly less immunogenic conjugate, substantiating the need for a minimal loading.[52]

"Sun"-type synthetic glycan conjugate vaccine prototype: proof-of-concept in human

With only one licensed synthetic glycan conjugate vaccine (Quimi-Hib®) available to date,[115] the feasibility of using synthetic OSs as O-Ag surrogates for a potent *Shigella* vaccine remains to be established. In a sense, the SF2a synthetic glycan conjugates investigated in mice resembles Quimi-Hib® as they also involves TT as the carrier and the thiol-maleimide chemistry for the bioconjugation step. In this context, SF2a-TT15,



Scheme 3. Chemical synthesis of SF2a-TT15, showing starting materials, key synthetic building blocks featuring a convergent strategy emphasizing on the C-D linkage, the $\mathbf{[A^1B^1(E^1)C^1D^1]_3}$-$NH_2$ hapten, and its ready-for-conjugation counterpart. Abbreviations: Ac: acetyl, All: allyl, Bn: benzyl, Bz: benzoyl, p: fully protected, TT_{Mal}: maleimide-equipped TT.

a 15-mer-TT conjugate characterized by a molar ratio of $[\mathbf{A^1B^1(E^1)C^1D^1}]_3$ OSs per TT molecule of 17±5, corresponding to an average carbohydrate:TT wt/wt ratio of 1:3.6, was selected to establish the proof-of-concept in human.[52] The glycoconjugate is produced according to a two-stage process to achieve (i) the synthesis of the ready-for-conjugation 15-mer OS ($[\mathbf{A^1B^1(E^1)C^1D^1}]_3$-SAc), whether by a chemical route from three easily available monosaccharides (Scheme 3),[113] or a chemoenzymatic strategy,[116] and (ii) its controlled attachment to maleimide-equipped TT (TT_{Mal}). $[\mathbf{A^1B^1(E^1)C^1D^1}]_3$-SAc, which features a masked reactive thiol moiety, was obtained by means of an aminoethyl glycoside precursor itself synthesized by a convergent route from three fully protected building blocks—$\mathbf{pD^1A^1B^1(E^1)C^1}$, $\mathbf{pA^1B^1(E^1)C^1}$, and $\mathbf{pD^1}$—exploiting the more viable disconnection at the **A-D** glycosidic linkage.[113] The bioconjugation step is most critical, which calls for clear-cut robustness to ensure high yields and reproducibility. Key parameters, the control of which is essential for robustness, were identified and optimized. The established two-step process allows full control of the density of OSs per TT molecule. With an improved reproducible yield of 70% relative to $[\mathbf{A^1B^1(E^1)C^1D^1}]_3$-SAc, a comparable efficiency of the conjugation step relative to TT_{Mal}, and substantial margin in terms of applicable variables, the process complies robustness and efficiency criteria.[52]

As a major step forward, SF2a-TT15 (Institut Pasteur, Paris, France) has completed a single blind, dose-escalating, placebo-controlled phase I clinical trial (ClinicalTrials.gov, Identifier: NCT02797236). This investigational vaccine was evaluated for safety and immunogenicity in young healthy adult volunteers, administered the vaccines three times, via intramuscular injection, on days 0, 28, and 56, and followed up to 3 months post the third injection. Bridging the study with established data for existing similar vaccines—Quimi-Hib® is licensed for use of doses equivalent to 10 μg saccharide—or related studies—Flexyn2a was evaluated at the same dose—and going one step beyond, volunteers have received doses of SF2a-TT15 equivalent to 10 or 2 μg saccharide, the high dose and the low dose, with or without alum adjuvant, respectively. Undeniably, information communicated to date[53] provides considerable support to further development of the synthetic glycan conjugate strategy. Before all, they encourage the design of a *Shigella* tetravalent synthetic glycan conjugate vaccine candidate, featuring the *S. sonnei*, SF2a and SF6 components in combination with a *S. flexneri* 3a (SF3a) constituent. Active developments toward this aim are in progress at Institut Pasteur.[54,117,118]

Conclusions

Despite years of investigation and the large diversity of vaccine candidates having reached clinical trials in an attempt to develop a safe and effective *Shigella* vaccine enabling broad serotype coverage, a largely distributed licensed vaccine is not available yet. Despite some limitations, *Shigella* surface polysaccharides, especially the O-Ags, remain important targets for vaccine development. Going beyond pioneering achievements in the field of *Shigella* LPS-based conjugate vaccines, novel concepts bridging further glycoscience and molecular vaccinology, are attractive options to advance carbohydrate vaccine development and design better defined, more immunogenic, *Shigella* glycoconjugate vaccines. The rapidly evolving *in vivo* glycoengineering technology offers a novel and potentially cost-effective way to develop and produce vaccines against a major pathogen of global health importance. Alternatively, stepping on recent advancements in chemical biology and structural biology, the synthetic glycan-based strategy offers a unique opportunity for increasing our understanding of the immune parameters relevant to glycoconjugate vaccine-mediated protection, toward rationally designed improved *Shigella* carbohydrate antigens as surrogates of natural heterogeneous polysaccharides and tailored effective conjugate vaccines thereof. Moreover, the recent acknowledgment that the carbohydrate component dictates the mechanism of adaptive immune response to glycoconjugates,[119] opens new avenues toward highly protective knowledge-based carbohydrate-based vaccines. While fears related to manufacturing have hampered its early development, the synthetic glycan-based strategy is gaining attractiveness owing to established proof-of-concept at the industrial manufacturing scale,[115,120] and rapid ongoing methodology diversification including chemoenzymatic approaches, one-pot protocols, and automated solid-phase synthesis, besides constant progress in conjugation chemistry.[93,94] Monovalent *Shigella* vaccine candidates exemplifying the rapidly growing bioconjugate and synthetic glycan-based strategies have, in recent years, successfully completed various stages of clinical trial in western volunteers.[50,53] Despite possible concerns associated to novelty, the "sun"-type strategies currently under development offer clear advantages over the original approaches in terms of product manufacturing, characterization, integrity, control, and versatility toward efficacy. Yet, in the absence of a suitable animal model and clear-cut consensus on the critical correlates of protection, while increasing findings in the field suggest that multiple arms of the immune system are engaged in immunity to shigellosis, future progress will highly rely on (i) demonstrating the feasibility of vaccine candidates inducing an immune response against panels of representative circulating strains causing disease in the context of broad antigenic diversity and on (ii) clinical trials establishing vaccine immunogenicity in the targeted populations and providing insights on vaccine potency. In a context of renewed awareness of the burden of *Shigella*, effort intensification and resources from new funding partners have opened the way to rapid progress toward these aims while also stimulating synergic contributions from a large diversity of experts working toward combatting the *Shigella* burden.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Barry EM, Pasetti MF, Sztein MB, Fasano A, Kotloff KL, Levine MM. Progress and pitfalls in *Shigella* vaccine research. Nat Rev Gastroenterol Hepatol. 2013;10:245–55. doi:10.1038/nrgastro.2013.12.
2. Ashkenazi S, Cohen D. An update on vaccines against *Shigella*. Ther Adv Vaccines. 2013;1:113–23. doi:10.1177/2051013613500428.
3. Mani S, Wierzba T, Walker RI. Status of vaccine research and development for *Shigella*. Vaccine. 2016;34:2887–94. doi:10.1016/j.vaccine.2016.02.075.
4. Platts-Mills JA, Babji S, Bodhidatta L, Gratz J, Haque R, Havt A, McCormick BJ, McGrath M, Olortequi MP, Samie A, et al. Pathogenspecific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). Lancet Glob Health. 2015;3:e564–75. doi:10.1016/S2214-109X(15)00151-5.
5. Liu J, Platts-Mills JA, Juma J, Kabir F, Nkeze J, Okoi C, Operario DJ, Uddin J, Ahmed S, Alonso PL, et al. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. Lancet. 2016;388:1291–301. doi:10.1016/S0140-6736(16)31529-X.
6. Baker S, The HC. Recent insights into *Shigella*. Curr Opin Infect Dis. 2018;31:449–54. doi:10.1097/QCO.0000000000000475.
7. Kotloff KL, Riddle MS, Platts-Mills JA, Pavlinac P, Zaidi AKM. Shigellosis. Lancet. 2018;391:801–12. doi:10.1016/S0140-6736(17)33296-8.
8. Rahman KM, Arifeen SE, Zaman K, Rahman M, Raqib R, Yunus M, Begum N, Islam S, Sohel BM, Rahman M, et al. Safety, dose, immunogenicity, and transmissibility of an oral live attenuated Shigella flexneri 2a vaccine candidate (SC602) among healthy adults and school children in Matlab, Bangladesh. Vaccine. 2011;29:1347–54. doi:10.1016/j.vaccine.2010.10.035.
9. Robbins JB, Chu C, Watson DC, Szu SC, Daniels EM, Lowe CU, Schneerson R. O-specific side-chain toxin-protein conjugates as parenteral vaccines for the prevention of shigellosis and related diseases. Rev Infect Dis. 1991;13(Suppl 4):S362–5.
10. Levine MM, Kotloff KL, Barry EM, Pasetti MF, Sztein MB. Clinical trials of *Shigella* vaccines: two steps forward and one step back on a long, hard road. Nat Rev Microbiol. 2007;5:540–53. doi:10.1038/nrmicro1662.
11. Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, Marohn ME, Antonio M, Hossain A, Mandomando I, Ochieng JB, et al. *Shigella* isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis. 2014;59:933–41. doi:10.1093/cid/ciu468.
12. Shahnaij M, Latif HA, Azmi IJ, Amin MB, Luna SJ, Islam MA, Talukder KA. Characterization of a serologically atypical *Shigella flexneri* Z isolated from diarrheal patients in Bangladesh and a proposed serological scheme for *Shigella flexneri*. PLoS One. 2018;13:e0202704. doi:10.1371/journal.pone.0202704.
13. Knirel YA, Sun Q, Senchenkova SN, Perepelov AV, Shashkov AS, Xu J. O-antigen modifications providing antigenic diversity of Shigella flexneri and underlying genetic mechanisms. Biochem (Mosc). 2015;80:901–14. doi:10.1134/S0006297915070093.
14. West NP, Sansonetti P, Mounier J, Exley RM, Parsot C, Guadagnini S, Prévost MC, Prochnicka-Chalufour A, Delepierre M, Tanguy M, et al. Optimization of virulence functions through glucosylation of *Shigella* LPS. Science. 2005;307:1313–17. doi:10.1126/science.1108472.
15. Morona R, Daniels C, Van Den Bosch L. Genetic modulation of *Shigella flexneri* 2a lipopolysaccharide O antigen modal chain length reveals that it has been optimized for virulence. Microbiol. 2003;149:925–39. doi:10.1099/mic.0.26141-0.
16. Silipo A, De Castro C, Lanzetta R, Parilli M, Molinaro A. Lipopolysaccharides. In: Königs HC,H, Varma A, editors. Prokaryotic cell wall compounds. Berlin Heidelberg, Germany: Springer-Verlag; 2010. p. 133–53. doi:10.1007/978-3-642-05062-6_4
17. Liu B, Knirel YA, Feng L, Perepelov AV, Senchenkova SN, Wang Q, Reeves PR, Wang L. Structure and genetics of *Shigella* O antigens. FEMS Microbiol Rev. 2008;32:627–53. doi:10.1111/j.1574-6976.2008.00114.x.
18. Miller CE, Karpas A, Schneerson R, Huppi K, Kovac P, Pozsgay V, Glaudemans CP. Of four murine, anti-*Shigella* dysenteriae type 1 O-polysaccharide antibodies, three employ V-genes that differ extensively from those of the fourth. Mol Immunol. 1996;33:1217–22. doi:10.1016/S0161-5890(96)00105-8
19. Carter JA, Blondel CJ, Zaldivar M, Alvarez SA, Marolda CL, Valvano MA, Contreras I. O-antigen modal chain length in *Shigella flexneri* 2a is growth-regulated through RfaH-mediated transcriptional control of the wzy gene. Microbiol. 2007;153:3499–507. doi:10.1099/mic.0.2007/010066-0.
20. Ferreccio C, Prado V, Ojeda A, Cayyazo M, Abrego P, Guers L, Levine MM. Epidemiologic patterns of acute diarrhea and endemic *Shigella* infections in children in a poor periurban setting in Santiago, Chile. Am J Epidemiol. 1991;134:614–27.
21. Cohen D, Green MS, Block C, Rouach T, Ofek I. Serum antibodies to lipopolysaccharide and natural immunity to shigellosis in an Israeli military population. J Infect Dis. 1988;157:1068–71.
22. Cohen D, Green MS, Block C, Slepon R, Ofek I. Prospective study of the association between serum antibodies to lipopolysaccharide O antigen and the attack rate of shigellosis. J Clin Microbiol. 1991;29:386–89.
23. Robin G, Cohen D, Orr N, Markus I, Slepon R, Ashkenazi S, Keisari Y. Characterization and quantitative analysis of serum IgG class and subclass response to *Shigella sonnei* and *Shigella flexneri* 2a lipopolysaccharide following natural *Shigella* infection. J Infect Dis. 1997;175:1128–33.
24. Cohen D, Block C, Green MS, Lowell G, Ofek I. Immunoglobulin M, A, and G antibody response to lipopolysaccharide O antigen in symptomatic and asymptomatic *Shigella* infections. J Clin Microbiol. 1989;27:162–67.
25. Orr N, Robin G, Lowell G, Cohen D. Presence of specific immunoglobulin A-secreting cells in peripheral blood after natural infection with *Shigella sonnei*. J Clin Microbiol. 1992;30:2165–68.
26. Cohen D, Orr N, Robin G, Slepon R, Ashkenazi S, Ashkenazi I, et al. Detection of antibodies to *Shigella* lipopolysaccharide in urine after natural *Shigella* infection or vaccination. Clin Diagn Lab Immunol. 1996;3:451–55.
27. Robbins JB, Chu C, Schneerson R. Hypothesis for vaccine development: protective immunity to enteric diseases caused by nontyphoidal salmonellae and shigellae may be conferred by serum IgG antibodies to the O-specific polysaccharide of their lipopolysaccharides. Clin Infect Dis. 1992;15:346–61.
28. Robbins JB, Schneerson R, Szu SC. Perspective: hypothesis: serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. J Infect Dis. 1995;171:1387–98.
29. Turbyfill KR, Clarkson KA, Vortherms AR, Oaks EV, Kaminski RW. Assembly, Biochemical Characterization, Immunogenicity. Adjuvanticity, and efficacy of *Shigella* artificial invaplex. mSphere. 2018;3. doi:10.1128/mSphere.00583-17
30. Obiero CW, Ndiaye AGW, Scire AS, Kaunyangi BM, Marchetti E, Gone AM, Schütte LD, Riccucci D, Auerbach J, Saul A, et al. A phase 2a randomized study to evaluate the safety and immunogenicity of the 1790GAHB generalized modules for membrane antigen vaccine against *Shigella sonnei* administered intramuscularly to adults from a shigellosis-endemic country. Front Immunol. 2017;8:1884. doi:10.3389/fimmu.2017.01884.
31. Avery OT, Goebel WF. Chemo-immunological studies on conjugated carbohydrate-proteins: II. Immunological specificity of synthetic sugar-protein antigens.. J Exp Med. 1929;50:533–50.
32. Avery OT, Goebel WF. Chemo-immunological studies on conjugated carbohydrate-proteins: V. The immunological specifity of an antigen prepared by combining the capsular polysaccharide of type III pneumococcus with foreign protein.. J Exp Med. 1931;54:437–47.
33. Paul WE, Katz DH, Benacerraf B. Augmented anti-S 3 antibody responses to an S 3 -protein conjugate. J Immunol. 1971;107:685–88.

34. Schneerson R, Barrera O, Sutton A, Robbins JB. Preparation, characterization, and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J Exp Med. 1980;152:361–76.
35. Vella M, Pace D. Glycoconjugate vaccines: an update. Expert Opin Biol Ther. 2015;15:529–46. doi:10.1517/14712598.2015.993375.
36. Pollard AJ, Perrett KP, Beverley PC. Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. Nat Rev Immunol. 2009;9:213–20. doi:10.1038/nri2494.
37. Rappuoli R. Glycoconjugate vaccines: principles and mechanisms. Sci Transl Med. 2018;10(456):1–6. doi:10.1126/scitranslmed.aat4615
38. Taylor DN, Trofa AC, Sadoff J, Chu CY, Bryla D, Shiloach J, Cohen D, Ashkenazi S, Lerman Y, Egan W, et al. Synthesis, characterization, and clinical-evaluation of conjugate vaccines composed of the O-specific polysaccharides of *Shigella dysenteriae* Type-1, *Shigella flexneri* Type2a, and *Shigella sonnei* (*Plesiomonas shigelloides*) bound to bacterial toxoids. Infect Immun. 1993;61:3678–87.
39. Passwell JH, Harlev E, Ashkenazi S, Chu C, Miron D, Ramon R, Farzan N, Shiloach J, Bryla DA, Majadly F. Safety and immunogenicity of improved *Shigella* O-specific Safety and immunogenicity of improved *Shigella* O-specific polysaccharide-protein conjugate vaccines in adults in Israel. Infect Immun. 2001;69:1351–57. doi:10.1128/IAI.69.3.1351-1357.2001.
40. Passwell JH, Ashkenazi S, Harlev E, Miron D, Ramon R, Farzam N, Lerner-Geva L, Levi Y, Chu C, Shiloach J, et al. Safety and immunogenicity of Shigella sonnei-CRM9 and *Shigella flexneri* type 2a-rEPAsucc conjugate vaccines in one- to four-year-old children. Pediatr Infect Dis J. 2003;22:701–06. doi:10.1097/01.inf.0000078156.03697.a5.
41. Cohen D, Ashkenazi S, Green M, Lerman Y, Slepon R, Robin G, Orr N, Taylor DN, Sadoff JC, Chu C, et al. Safety and immunogenicity of investigational *Shigella* conjugate vaccines in Israeli volunteers. Infect Immun. 1996;64:4074–77.
42. Cohen D, Ashkenazi S, Green MS, Gdalevich M, Robin G, Slepon R, Yavzori M, Orr N, Block C, Ashkenazi I, et al. Double-blind vaccine-controlled randomised efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults. Lancet. 1997;349:155–59. doi:10.1016/S0140-6736(96)06255-1.
43. Ashkenazi S, Passwell JH, Harlev E, Miron D, Dagan R, Farzan N, Ramon R, Majadly F, Bryla DA, Karpas AB, et al. Safety and immunogenicity of *Shigella sonnei* and *Shigella flexneri* 2a O-specific polysaccharide conjugates in children. J Infect Dis. 1999;179:1565–68. doi:10.1086/314759.
44. Passwell JH, Ashkenzi S, Banet-Levi Y, Ramon-Saraf R, Farzam N, Lerner-Geva L, Even-Nir H, Yerushalmi B, Chu C, Shiloach J, et al. Age-related efficacy of *Shigella* O-specific polysaccharide conjugates in 14-year-old Israeli children. Vaccine. 2010;28:2231–35. doi:10.1016/j.vaccine.2009.12.050.
45. Laird RM, Ma Z, Dorabawila N, Pequegnat B, Omari E, Liu Y, Maue AC, Poole ST, Maciel M, Satish K, et al. Evaluation of a conjugate vaccine platform against enterotoxigenic *Escherichia coli* (ETEC), *Campylobacter jejuni* and *Shigella*. Vaccine. 2018. doi:10.1016/j.vaccine.2018.09.052.
46. Robbins JB, Kubler-Kielb J, Vinogradov E, Mocca C, Pozsgay V, Shiloach J, Schneerson R. Synthesis, characterization, and immunogenicity in mice of *Shigella sonnei* O-specific oligosaccharide-core-protein conjugates. Proc Natl Acad Sci U S A. 2009;106:7974–78. doi:10.1073/pnas.0900891106.
47. Kubler-Kielb J, Vinogradov E, Mocca C, Pozsgay V, Coxon B, Robbins JB, Schneerson R. Immunochemical studies of *Shigella flexneri* 2a and 6, and *Shigella dysenteriae* type 1 O-specific polysaccharide-core fragments and their protein conjugates as vaccine candidates. Carbohydr Res. 2010;345:1600–08. doi:10.1016/j.carres.2010.05.006.
48. Hatz CF, Bally B, Rohrer S, Steffen R, Kramme S, Siegrist CA, Wacker M, Alaimo C, Fonck VG. Safety and immunogenicity of a candidate bioconjugate vaccine against *Shigella dysenteriae* type 1 administered to healthy adults: A single blind, partially randomized Phase I study. Vaccine. 2015;33:4594–601. doi:10.1016/j.vaccine.2015.06.102.
49. Riddle MS, Kaminski RW, Di Paolo C, Porter CK, Gutierrez RL, Clarkson KA, Weerts HE, Duplessis C, Castellano A, Alaimo C, et al. Safety and immunogenicity of a candidate bioconjugate vaccine against *Shigella flexneri* 2a Administered to healthy adults: a single-blind, randomized phase I study. Clin Vaccine Immunol. 2016;23:908–17. doi:10.1128/CVI.00224-16.
50. Talaat KR, Alaimo C, Bourgeois AL, Kaminski RW, Dreyer A, Porter CK, Chakraborty S, Clarkson KA, Brubaker J, Elwood D, et al. Flexyn2a, a candidate bioconjugate vaccine against *Shigella flexneri* 2a induces protective immune response in a controlled human infection model. In: Management M, editor. Vaccines for Enteric Diseases. Albufeira (Portugal): NAU Sao Rafael Atlantico Hotel; 2017 Oct 9–11.
51. Pozsgay V, Kubler-Kielb J, Schneerson R, Robbins JB. Effect of the nonreducing end of *Shigella dysenteriae* type 1 O-specific oligosaccharides on their immunogenicity as conjugates in mice. Proc Natl Acad Sci USA. 2007;104:14478–82. doi:10.1073/pnas.0706969104.
52. van der Put RM, Kim TH, Guerreiro C, Thouron F, Hoogerhout P, Sansonetti PJ, Westdijk J, Stork M, Phalipon A, Mulard LA. A synthetic carbohydrate conjugate vaccine candidate against shigellosis: improved bioconjugation and impact of alum on immunogenicity. Bioconjugate Chem. 2016;27:883–92. doi:10.1021/acs.bioconjchem.5b00617.
53. Cohen D, Atsmon J, Artaud C, Meron-Sudai S, Gougeon ML, Bialik A, Goren S, Asato V, Ariel-Cohen O, Reizis A, et al. A phase I dose escalation study to assess the safety and immunogenicity of the SF2a-TT15 conjugate vaccine against *S. flexneri* 2a in healthy adult volunteers (Preliminary Results). In: Management M, editor. Vaccines for enteric diseases. Albufeira (Portugal): NAU Sao Rafael Atlantico Hotel; 2017 October 9–11.
54. Hu Z, Bongat White AF, Mulard LA. Efficient iterative synthesis of O-acetylated tri- to pentadecasaccharides related to the lipopolysaccharide of *Shigella flexneri* type 3a through di- and trisaccharide glycosyl donors. Chem Asian J. 2017;12:419–39. doi:10.1002/asia.201600819.
55. Hu Z, Cornil J, Guerreiro C, Thouron F, Ligeour C, Hoos S, England P, Phalipon A, Mulard LA. Progress toward a bivalent *Shigella flexneri* synthetic carbohydrate-based vaccine candidate. In: Management M, editor. Vaccines for enteric diseases. Albufeira (Portugal): NAU Sao Rafael Atlantico Hotel; 2017 Oct 9–11.
56. Dmitriev BA, Knirel YA, Kochetkov NK. Somatic antigens of *Shigella*. Structural investigation on the O-specific polysaccharide chain of *Shigella dysenteriae* type 1 lipopolysaccharide. Eur J Biochem. 1976;66:559–66.
57. Perepelov AV, Shekht ME, Liu B, Shevelev SD, Ledov VA, Senchenkova SN, L'vov VL, Shashkov AS, Feng L, Aparin PG, et al. *Shigella flexneri* O-antigens revisited: final elucidation of the O-acetylation profiles and a survey of the O-antigen structure diversity. FEMS Immunol Med Microbiol. 2012;66:201–10. doi:10.1111/j.1574-695X.2012.01000.x.
58. Kenne L, Lindberg B, Petersson K, Katzenellenbogen E, Romanowska E. Structural studies of the O-specific side-chains of the *Shigella-sonnei* phase-I lipopolysaccharide. Carbohydr Res. 1980;78:119–26. doi:10.1016/S0008-6215(00)83665-4.
59. Polotsky VY, Robbins JB, Bryla D, Schneerson R. Comparison of conjugates composed of lipopolysaccharide from *Shigella flexneri* type 2a detoxified by two methods and bound to tetanus toxoid. Infect Immun. 1994;62:210–14.
60. Chu CY, Liu BK, Watson D, Szu SS, Bryla D, Shiloach J, Schneerson R, Robbins JB. Preparation, characterization, and immunogenicity of conjugates composed of the O-specific polysaccharide of *Shigella dysenteriae* type 1 (Shiga's bacillus) bound to tetanus toxoid. Infect Immun. 1991;59:4450–58.
61. Pavliakova D, Chu C, Bystricky S, Tolson NW, Shiloach J, Kaufman JB, Bryla DA, Robbins JB, Schneerson R. Treatment with succinic anhydride improves the immunogenicity of

Shigella flexneri type 2a O-specific polysaccharide-protein conjugates in mice. Infect Immun. 1999;67:5526–29.
62. Pavliakova D, Moncrief JS, Lyerly DM, Schiffman G, Bryla DA, Robbins JB, Schneerson R. *Clostridium difficile* recombinant toxin A repeating units as a carrier protein for conjugate vaccines: studies of pneumococcal type 14, *Escherichia coli* K1, and *Shigella flexneri* type 2a polysaccharides in mice. Infect Immun. 2000;68:2161–66.
63. Robin G, Keisari Y, Slepon R, Ashkenazi S, Cohen D. Quantitative analysis of IgG class and subclass and IgA serum response to Shigella sonnei and Shigella flexneri 2a polysaccharides following vaccination with Shigella conjugate vaccines. 1999;17:3109–15. doi:10.1016/S0264-410X(99)00136-X
64. Cobb BA, Kasper DL. Characteristics of carbohydrate antigen binding to the presentation protein HLA-DR. Glycobiol. 2008;18:707–18. doi:10.1093/glycob/cwn050.
65. Farzam N, Ramon-Saraf R, Banet-Levi Y, Lerner-Geva L, Ashkenazi S, Kubler-Kielb J, Vinogradov E, Robbins JB, Schneerson R. Vaccination with *Shigella flexneri* 2a conjugate induces type 2a and cross-reactive type 6 antibodies in humans but not in mice. Vaccine. 2017;35:4990–96. doi:10.1016/j.vaccine.2017.07.070.
66. Pozsgay V, Chu C, Pannell L, Wolfe J, Robbins JB, Schneerson R. Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from *Shigella dysenteriae* type 1. Proc Natl Acad Sci U S A. 1999;96:5194–97. doi:10.1073/pnas.96.9.5194.
67. Kubler-Kielb J, Pozsgay V. A new method for conjugation of carbohydrates to proteins using an aminooxy-thiol heterobifunctional linker. J Org Chem. 2005;70:6987–90. doi:10.1021/jo050934b.
68. Dagan R, Eskola J, Leclerc C, Leroy O. Reduced response to multiple vaccines sharing common protein epitopes that are administered simultaneously to infants. Infect Immun. 1998;66:2093–98.
69. Shepherd JG, Wang L, Reeves PR. Comparison of O-antigen gene clusters of *Escherichia coli* (*Shigella*) *sonnei* and *Plesiomonas shigelloides* O17: sonnei gained its current plasmid-borne O-antigen genes from *P. shigelloides* in a recent event. Infect Immun. 2000;68:6056–61.
70. Ahamad N, Katti DS. A two-step method for extraction of lipopolysaccharide from *Shigella dysenteriae* serotype 1 and *Salmonella typhimurium*: an improved method for enhanced yield and purity. J Microbiol Methods. 2016;127:41–50. doi:10.1016/j.mimet.2016.05.014.
71. Hegerle N, Bose J, Ramachandran G, Galen JE, Levine MM, Simon R, Tennant SM. Overexpression of O-polysaccharide chain length regulators in Gram-negative bacteria using the Wzx-/Wzy-dependent pathway enhances production of defined modal length O-polysaccharide polymers for use as haptens in glycoconjugate vaccines. J Appl Microbiol. 2018;125:575–85. doi:10.1111/jam.13772.
72. Feldman MF, Wacker M, Hernandez M, Hitchen PG, Marolda CL, Kowarik M, Morris HR, Dell A, Valvano MA, Aebi M. Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. Proc Natl Acad Sci U S A. 2005;102:3016–21. doi:10.1073/pnas.0500044102.
73. Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW, et al. N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. Science. 2002;298:1790–93. doi:10.1126/science.298.5599.1790.
74. Vozza NF, Feldman MF. Glyco-engineering O-antigen-based vaccines and diagnostics in *E. coli*. Methods Mol Biol. 2015;1321:57–70. doi:10.1007/978-1-4939-2760-9_5.
75. Kowarik M, Young NM, Numao S, Schulz BL, Hug I, Callewaert N, Mills DC, Watson DC, Hernandez M, Kelly JF, et al. Definition of the bacterial N-glycosylation site consensus sequence. Embo J. 2006;25:1957–66. doi:10.1038/sj.emboj.7601087.
76. Ihssen J, Kowarik M, Dilettoso S, Tanner C, Wacker M, Thony-Meyer L. Production of glycoprotein vaccines in *Escherichia coli*. Microb Cell Fact. 2010;9:61. doi:10.1186/1475-2859-9-61.
77. Ravenscroft N, Haeuptle MA, Kowarik M, Fernandez FS, Carranza P, Brunner A, Steffen M, Wetter M, Keller S, Ruch C, et al. Purification and characterization of a *Shigella* conjugate vaccine, produced by glycoengineering *Escherichia coli*. Glycobiol. 2016;26:51–62. doi:10.1093/glycob/cwv077.
78. Kampf MM, Braun M, Sirena D, Ihssen J, Thony-Meyer L, Ren Q. In vivo production of a novel glycoconjugate vaccine against *Shigella flexneri* 2a in recombinant *Escherichia coli*: identification of stimulating factors for in vivo glycosylation. Microb Cell Fact. 2015;14:12. doi:10.1186/s12934-015-0195-7.
79. Perepelov AV, L'Vov V L, Liu B, Senchenkova SN, Shekht ME, Shashkov AS, Feng L, Aparin PG, Wang L, Knirel YA. A similarity in the O-acetylation pattern of the O-antigens of *Shigella flexneri* types 1a, 1b, and 2a.. Carbohydr Res. 2009;344:687–92. doi:10.1016/j.carres.2009.01.004.
80. Kubler-Kielb J, Vinogradov E, Chu CY, Schneerson R. O-Acetylation in the O-specific polysaccharide isolated from *Shigella flexneri* serotype 2a. Carbohydr Res. 2007;342:643–47. doi:10.1016/j.carres.2006.09.017.
81. Mulard LA. Bacterial polysaccharides as major surface antigens: interest in O-acetyl substitutions. Carbohydr Chem. 2018;43:71–103. doi:10.1039/9781788010641-00071
82. Berti F, De Ricco R, Rappuoli R. Role of O-Acetylation in the immunogenicity of bacterial polysaccharide vaccines. Molecules. 2018;23. doi:10.3390/molecules23061340
83. Ilg K, Zandomeneghi G, Rugarabamu G, Meier BH, Aebi M. HRMAS NMR reveals a pH-dependent LPS alteration by de-O-acetylation at abequose in the O-antigen of *Salmonella enterica* serovar Typhimurium. Carbohydr Res. 2013;382:58–64. doi:10.1016/j.carres.2013.10.002.
84. Porter CK, Lynen A, Riddle MS, Talaat K, Sack D, Gutierrez RL, McKenzie R, DeNearing B, Feijoo B, Kaminski RW, et al. Clinical endpoints in the controlled human challenge model for *Shigella*: A call for standardization and the development of a disease severity score. PLoS One. 2018;13:e0194325. doi:10.1371/journal.pone.0194325.
85. Goebel WF. Chemo-immunological studies on conjugated carbohydrate-proteins: XII. The immunological properties of an artificial antigen containing cellobiuronic acid. J Exp Med. 1938;68:469–84.
86. Kabat EA. The upper limit for the size of the human antidextran combining site. J Immunol. 1960;84:82–85.
87. Mage RG, Kabat EA. Immunochemical studies on dextrans. III. The specificities of rabbit antidextrans. Further findings on antidextrans with 1,2- and 1,6-specificities. J Immunol. 1963;91:633–40.
88. Ekborg G, Eklind K, Garegg PJ, Gotthammar B, Carlsson HE, Lindberg AA, Svenungsson B. Artificial disaccharide-protein conjugates as immunogens for the preparation of specific anti-*Salmonella* O-antisera. Immunochem. 1977;14:153–57.
89. Svenson SB, Nurminen M, Lindberg AA. Artificial *Salmonella* vaccines: O-antigenic oligosaccharide-protein conjugates induce protection against infection with *Salmonella typhimurium*. Infect Immun. 1979;25:863–72.
90. Svenson SB, Lindberg AA. Artificial Salmonella vaccine: *Salmonella typhimurium* O-antigen-specific oligosaccharide-protein conjugates elicit protective antibodies in rabbits and mice. Infect Immun. 1981;32:490–96.
91. Svenson SB, Lindberg AA. Immunochemistry of *Salmonella* O-antigens: preparation of an octasaccharide-bovine serum albumin immunogen representative of *Salmonella* serogroup B O-antigen and characterization of the antibody response. J Immunol. 1978;120:1750–57.
92. Theillet FX, Chassagne P, Delepierre M, Phalipon A, Mulard LA. Multidisciplinary approaches to study O-antigen: antibody recognition in support of the development of synthetic carbohydrate-

based enteric vaccines. In: Kosma P, Müller-Loennies S, editors. Anticarbohydrate Antibodies - from molecular basis to clinical applications. Vienna,Austria: Springer-Verlag GmbH; 2012. p. 1–36.doi:10.1007/978-3-7091-0870-3_1
93. Adamo R, Nilo A, Castagner B, Boutureira O, Berti F, Bernardes GJ. Synthetically defined glycoprotein vaccines: current status and future directions. Chem Sci. 2013;4:2995–3008. doi:10.1039/c3sc50862e.
94. Adamo R. Advancing homogeneous antimicrobial glycoconjugate vaccines. Acc Chem Res. 2017;50:1270–79. doi:10.1021/acs.accounts.7b00106.
95. Pavliak V, Nashed EM, Pozsgay V, Kovac P, Karpas A, Chu C, Schneerson R, Robbins JB, Glaudemans CP. Binding of the O-antigen of *Shigella dysenteriae* type 1 and 26 related synthetic fragments to a monoclonal IgM antibody. J Biol Chem. 1993;268:25797–802.
96. Miller CE, Mulard LA, Padlan EA, Glaudemans CP. Binding of modified fragments of the *Shigella dysenteriae* type 1 O-specific polysaccharide to monoclonal IgM 3707 E9 and docking of the immunodeterminant to its modeled Fv. Carbohydr Res. 1998;309:219–26.
97. Pozsgay V. Synthesis of glycoconjugate vaccines against *Shigella dysenteriae*type1. J Org Chem. 1998;63:5983–99. doi:10.1021/jo980660a
98. Pozsgay V, Coxon B, Glaudemans CPJ, Schneerson R, Robbins JB. Towards an oligosaccharide-based glycoconjugate vaccine against *Shigella dysenteriae* type 1. Synlett. 2003;743–67. doi:10.1002/chin.200329260.
99. Falt IC, Lindberg AA. Epitope mapping of six monoclonal antibodies recognizing the *Shigella dysenteriae* type 1 O-antigenic repeating unit expressed in *Escherichia coli* K-12. Microb Pathog. 1994;16:27–41. doi:10.1006/mpat.1994.1003.
100. Coxon B, Sari N, Batta G, Pozsgay V. NMR spectroscopy, molecular dynamics, and conformation of a synthetic octasaccharide fragment of the O-specific polysaccharide of *Shigella dysenteriae* type 1. Carbohydr Res. 2000;324:53–65. doi:10.1016/S0008-6215(99)00278-5
101. Nyholm PG, Mulard LA, Miller CE, Lew T, Olin R, Glaudemans CP. Conformation of the O-specific polysaccharide of Shigella dysenteriae type 1: molecular modeling shows a helical structure with efficient exposure of the antigenic determinant α-L-Rhap-(1→2)-α-D-Galp. Glycobiol. 2001;11:945–55. doi:10.1093/glycob/11.11.945
102. Pozsgay V, Coxon B, Yeh H. Synthesis of di- to penta-saccharides related to the Ospecific polysaccharide of *Shigella dysenteriae* typeSynthesis of di- to penta-saccharides related to the O-specific polysaccharide of Shigella dysenteriae type 1, and their nuclear magnetic resonance study. Bioorg Med Chem. 1993;1:237–57. doi:10.1016/S0968-0896(00)82129-X
103. Pozsgay V, Ekborg G, Sampathkumar SG. Synthesis of hexa- to tridecasaccharides related to *Shigella dysenteriae* type 1 for incorporation in experimental vaccines. Carbohydr Res. 2006;341:1408–27. doi:10.1016/j.carres.2006.04.006.
104. Pozsgay V. Towards a synthetic carbohydrate vaccine against *Shigella dysenteriae* type 1. In: Nifantiev NE, editor. Progress in the synthesis of complex carbohydrate chains of plant and microbial polysaccharides. Trivandrum (India): Transworld Research Network; 2009. p. 327–44.
105. Coster TS, Hoge CW, VanDeVerg LL, Hartman AB, Oaks EV, Venkatesan MM, Cohen D, Robin G, Fontaine-Thompson A, Sansonetti PJ, et al. Vaccination against shigellosis with attenuated *Shigella flexneri.* 2a strain SC602. 1999;67:3437–43.
106. Phalipon A, Costachel C, Grandjean C, Thuizat A, Guerreiro C, Tanguy M, Nato F, Vulliez-Le Normand B, Bélot F, Wright K, et al. Characterization of functional oligosaccharide mimics of the *Shigella flexneri* serotype 2a O-antigen: implications for the development of a chemically defined glycoconjugate vaccine. J Immunol. 2006;176:1686–94. doi:10.4049/jimmunol.176.3.1686
107. Theillet FX, Frank M, Vulliez-Le Normand B, Simenel C, Hoos S, Chaffotte A, Bélot F, Guerreiro C, Nato F, Phalipon A, et al. Dynamic aspects of antibody: oligosaccharidecomplexes characterized by molecular dynamics simulations and saturation transfer difference nuclear magnetic resonance. Glycobiol. 2011;21:1570–79. doi:10.1093/glycob/cwr059.
108. Vulliez-Le Normand B, Saul FA, Phalipon A, Belot F, Guerreiro C, Mulard LA, Bentley GA. Structures of synthetic O-antigen fragments from serotype 2a *Shigella flexneri* in complex with a protective monoclonal antibody. Proc Natl Acad Sci U S A. 2008;105:9976–81. doi:10.1073/pnas.0801711105.
109. Theillet FX, Simenel C, Guerreiro C, Phalipon A, Mulard LA, Delepierre M. Effects of backbone substitutions on the conformational behavior of *Shigella flexneri* O-antigens: implications for vaccine strategy. Glycobiol. 2011;21:109–21. doi:10.1093/glycob/cwq136.
110. Gauthier C, Chassagne P, Theillet FX, Guerreiro C, Thouron F, Nato F, Delepierre M, Sansonetti PJ, Phalipon A, Mulard LA. Nonstoichiometric O-acetylation of *Shigella flexneri* 2a O-specific polysaccharide: synthesis and antigenicity. Org Biomol Chem. 2014;12:4218–32. doi:10.1039/c3ob42586j.
111. Phalipon A, Tanguy M, Grandjean C, Guerreiro C, Belot F, Cohen D, Sansonetti PJ, Mulard LA. A synthetic carbohydrate-protein conjugate vaccine candidate against *Shigella flexneri* 2a infection. J Immunol. 2009;182:2241–47. doi:10.4049/jimmunol.0803141.
112. Wright K, Guerreiro C, Laurent I, Baleux F, Mulard LA. Preparation of synthetic glycoconjugates as potential vaccine against *Shigella flexneri* serotype 2a disease. Org Biomol Chem. 2004;2:1518–27. doi:10.1039/b400986J.
113. Belot F, Guerreiro C, Baleux F, Mulard LA. Synthesis of two linear PADRE conjugates bearing a deca- or pentadecasaccharide B epitope as potential synthetic vaccines against *Shigella flexneri* serotype 2a infection. Chemistry. 2005;11:1625–35. doi:10.1002/chem.200400903.
114. Hassane FS, Phalipon A, Tanguy M, Guerreiro C, Belot F, Frisch B, Mulard LA, Schuber F. Rational design and immunogenicity of liposome-based diepitope constructs: application to synthetic oligosaccharides mimicking the *Shigella flexneri* 2a O-antigen. Vaccine. 2009;27:5419–26. doi:10.1016/j.vaccine.2009.06.031.
115. Verez-Bencomo V, Fernandez-Santana V, Hardy E, Toledo ME, Rodriguez MC, Heynngnezz L, Rodriguez A, Baly A, Herrera L, Izquierdo M, et al. A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type b. Science. 2004;305:522–25. doi:10.1126/science.1095209.
116. Salamone S, Guerreiro C, Cambon E, Andre I, Remaud-Simeon M, Mulard LA. Programmed chemo-enzymatic synthesis of the oligosaccharide component of a carbohydrate-based antibacterial vaccine candidate. Chem Commun. 2015;51:2581–84. doi:10.1039/C4CC08805K.
117. Pfister HB, Mulard LA. Synthesis of the zwitterionic repeating unit of the O-antigen from *Shigella sonnei* and chain elongation at both ends. Org Lett. 2014;16:4892–95. doi:10.1021/ol502395k.
118. Chassagne P, Fontana C, Guerreiro C, Gauthier C, Phalipon A, Widmalm G, Mulard LA. Structural studies of the O-acetyl-containing O-antigen from a *Shigella flexneri* serotype 6 strain and synthesis of oligosaccharide fragments thereof. Eur J Org Chem. 2013;4085–106. doi:10.1002/ejoc.201300180.
119. Sun X, Stefanetti G, Berti F, Kasper DL. Polysaccharide structure dictates mechanism of adaptive immune response to glycoconjugate vaccines. Proc Natl Acad Sci U S A. 2019;116:193–98. doi:10.1073/pnas.1816401115.
120. Trouilleux P, Potier P, Driguez PA. Kilogram-scale production of synthetic heparin analogs: some chemical considerations. In: Vidal S, editor. Protecting groups: strategies and applications in carbohydrate chemistry first edition. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co; 2019. p. 473–91. doi:10.1002/9783527697014.ch17