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Antibacterial Carbohydrate Vaccines

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1.1 Introduction

Despite the impressive advances of modern medicine and microbiological research, infectious and parasitic diseases are still a significant burden with a profound socio-economic impact worldwide, particularly in low- and middle-income countries (LMIC). Indeed, infectious diseases increase healthcare expenditures and decrease the country's economic growth, thereby representing a vital concern of the global economy to cope with outbreaks of novel pathogens, such as the SARS-CoV-2 pandemic. It should be noted that the impact of infectious diseases is not only confined to the healthcare system, but different cost categories in various sectors of the economy are involved. The production losses due to illness and premature death from the disease, or even broader economic effects such as those due to reduced trade and tourism are, indeed, additional factors that need to be considered.

In 1992, the World Bank and the World Health Organization (WHO) commissioned the first Global Burden of Disease (GBD) study. One of the main aims of this study was to quantify the burden of disease with a measure that could be used for cost-effectiveness analysis. The GBD study used a novel and single metric, the disability-adjusted life year (DALY), corresponding to the sum of the years of life lost to premature death (YLL) and the years lived with disability (YLD) for varying degrees of severity. DALY, therefore, represents a common metric for death and disability, disaggregating the contribution of comorbidity. The first GBD report covered eight geographic areas and five age groups, with estimates through 1990. Subsequent GBD studies progressively expanded the diseases and injuries number, the risk factors, and the geographic areas [1]. Besides injuries, causes of death and disability can be distinguished into communicable (infectious diseases, along with maternal, perinatal, and nutritional conditions) and noncommunicable (chronic) diseases. In

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the GBD study 2001, the vast majority of DALYs caused worldwide by communicable diseases were due to infectious and parasitic diseases (21% out of 37%) [1]. The latest GBD report (2019) [2] reveals a substantial decline in the burden of communicable diseases and an improvement of the health of the world's population. For instance, global life expectancy at birth increased from 67.2 years in 2000 to 73.5 years in 2019 [3], even if the disability is becoming an increasingly large component of disease burden. However, the 2019 GBD study shows that the global burden of infectious and parasitic diseases still has a prominent place in terms of disability-adjusted life year's percentage (17% out of 26% total DALYs due to communicable diseases). In addition, another factor contributing to worsening the situation is the growing phenomenon of antimicrobial resistance (AMR), due to the constant emergence of antibiotic-resistant bacterial strains. It was in the 1990s when the first examples of AMR were documented [4, 5]. To ensure their survival, pathogens started to develop evolutionary defense mechanisms which progressively impaired the effectiveness of the antimicrobial agents, thus making the continuous development of new and more sophisticated antibiotics necessary. To date, AMR is one of the major global healthcare challenges, undermining the capacity to prevent and cure a number of infectious diseases that were once treatable. It has been estimated that AMR kills about 700 000 people each year worldwide, a number that, without urgent action, is expected to increase dramatically in the next decades, until reaching 10 million deaths per year by 2050 [6, 7].

The adoption of preventive strategies appears to be the most promising means to efficiently tackle both known and emerging infectious diseases. More than two centuries after the seminal experiments of Edward Jenner, vaccines have become one of the most powerful weapons of modern medicine in the fight against life-threatening infectious diseases, and capable of saving millions of lives, until being recognized by the WHO as "one of the most cost-effective ways to prevent disease" [8]. Indeed, records collected by the Centers for Disease Control and Prevention (CDC) since 1912 have shown a significant decrease in the number of reported cases of infectious diseases before and after the availability of a vaccine [9]. Particularly impressive is the impact of vaccination on life expectancy at birth: according to WHO, current immunization programs contributed to the substantial reduction in mortality of children under 5 years of age from 93 deaths per 1000 live births in 1990 to 39 deaths per 1000 live births in 2018 [10]. In addition, the vaccination practice is also a powerful tool to fight AMR [11]. Besides directly preventing the transmission of bacteria that are already resistant to antibiotic therapies, vaccination helps to reduce transmission of all types of infections, including viral infections, thus avoiding unnecessary or excessive antibiotic use that is a major cause of AMR.

1.1.1 A Brief History of Vaccines

Although the mass vaccination became a routine practice only in the twentieth century [12], it has had a tremendous impact on the health of the world's population. With the exception of safe water, no other intervention, not even antibiotics, has had such a major effect on mortality reduction and population growth. Vaccination, indeed, allowed to control 14 major diseases (smallpox, diphtheria, tetanus, yellow

fever, pertussis, *Haemophilus influenzae* type b (Hib) disease, poliomyelitis, measles, mumps, rubella, typhoid fever, rabies, rotavirus, and hepatitis B), at least in parts of the world.

The word "vaccine" originates from the Latin term "variolae vaccinae" (smallpox), when Edward Jenner in 1798 observed that cowpox (a less virulent version of smallpox) could be used to prevent smallpox in humans. Contrary to the general belief, however, the first known real vaccination practice is owed not to a physician nor a scientist, but to a cattle breeder named Benjamin Jesty. Based on the observation that dairymaids seemed to be protected from smallpox after they had contracted cowpox, in 1774, he deliberately inoculated his wife and two children with cowpox to avoid a smallpox epidemic. His experiment was successful, as they were unaffected by the outbreak. Jesty never attempted to publicize his experiment or vaccinate anyone else. He nevertheless proved for the first time the key vaccination principle: inoculation with one moderately harmless disease (cowpox) could provide protection against another far more dangerous disease (smallpox). More than 20 years later, Edward Jenner demonstrated that cowpox could be passed directly from one person to another and that the stimulation of the immune system with a weaker version of the pathogen of interest can confer protection against the related disease [13, 14]. For this reason, despite Jesty's successful vaccination of his family, Jenner is considered the "father of modern human vaccination."

The second key milestone after Jenner's findings was the work of Louis Pasteur on the attenuation of the chicken cholera bacterium in the late 1870s, shortly thereafter followed by his research on anthrax bacillus. After intensive laboratory research, in 1880, Pasteur announced to the scientific community the vaccination against chicken cholera, while the first public controlled experiment of anthrax vaccination took place in 1881. Although both vaccines were not a success, Pasteur introduced the modern concept of vaccination, involving the creation of vaccines in the laboratory using the same agent that caused the disease. Pasteur's methodology paved the way for subsequent epochal developments. In the last decade of the nineteenth century, the novel advances in the newly born discipline called bacteriology provided a rationale for vaccine development based on techniques to inactivate whole bacteria, the discovery of bacterial toxins, and the production of antitoxins. It was realized that the immune system produced soluble substances (antibodies) capable of neutralizing toxins and stopping bacterial growth. Antitoxins and vaccines against rabies, diphtheria, tetanus, anthrax, cholera, plague, typhoid, tuberculosis, and more were developed through the 1930s, taking advantage of these new pieces of knowledge [15]. Likewise, the middle of the twentieth century was a very active time for vaccine research, fueled by the development of methods for growing viruses in the laboratory, which led to rapid discoveries and innovations, including the creation of vaccines for polio. Researchers targeted other common childhood diseases such as measles, mumps, and rubella, and vaccines for these diseases reduced the disease burden greatly. In the second half of the twentieth century, new and more sophisticated techniques were introduced and employed for vaccine development. Examples include the formulation of the first recombinant vaccine, licensed in 1986 against hepatitis B [16–19], and the recombinant, quadrivalent human papillomavirus (HPV) vaccine, licensed in the United States in 2006 and

followed in 2014 by a nine-valent version [20]. In 1995, the first complete genome sequence of *H. influenzae* Rd was published [21], leading to a new breakthrough in vaccine research. The availability of bacterial strain genome sequences was successfully applied for the first time to identify vaccine candidates for Meningococcus serogroup B (MenB), thanks to the bioinformatic technique known as *reverse vaccinology* [22]. The sequencing of a MenB strain allowed to predict hundreds of specific protein antigens as possible vaccine candidates [23] and ultimately led to the licensure of the first vaccine against the MenB disease [24].

Today, vaccines can be classified into four different types:

- **Whole-cell killed or inactivated**: based on virulent microorganisms, no longer able to cause the disease, that have been killed by exposure to chemicals, heat, or radiation. Examples include the polio vaccine, hepatitis A vaccine, rabies vaccine, and some influenza vaccines.
- **Whole-cell attenuated**: contain live-attenuated microorganisms. Many of them are active viruses cultivated under conditions that disable their virulent properties, or closely related but less dangerous mutant strains that induce a broad immune response. Examples include the viral diseases yellow fever, measles, mumps, rubella, and the bacterial disease typhoid fever. Attenuated vaccines have some pros and cons. They typically provoke more durable immunological responses and are the preferred type for healthy adults, but they may not be safe in immunocompromised individuals and, on rare occasions, mutate to a virulent and disease-causing form.
- **Toxoid vaccines**: based on inactivated pathogenic toxins, but still retaining their immunizing capacity. Examples of toxoid-based vaccines include tetanus and diphtheria.
- **Subunit vaccines**: different from whole-cell-based vaccines, subunit vaccines contain only a specific and conserved microbial component of the microorganism. They can be obtained either by isolation and purification of the key antigens from the natural source (the pathogen) or by production of the antigen(s) by genetic engineering or chemical synthesis. Subunit vaccines do not contain "live" components of the pathogen; they cause only minor side-effects and are safer and more stable than other kinds of vaccines containing whole pathogens. Subunit vaccines can be divided into two groups:
  - *Protein-based vaccines* contain proteins present on the surface of the pathogen. An example is the subunit vaccine against Hepatitis B virus, composed of only the surface viral proteins, which were previously extracted from the blood serum of chronically infected patients and are now produced by recombination of the viral genes in yeast [19].
  - *PS-based vaccines* contain PSs either extracted from encapsulated bacteria (capsular polysaccharides (CPSs)) or portions of lipopolysaccharides (LPS, usually the O-antigen) of Gram-negative bacteria. However, as explained in more detail in Section 1.2, plain PSs are not able *per se* to induce B-cell-mediated immunological memory. The breakthrough in this field has been the introduction of *glycoconjugate vaccines*, obtained by chemical conjugation of pathogen-associated saccharide antigens to immunogenic proteins (e.g. toxins). The administration of glycoconjugate vaccines induces stronger activation of the immune system, resulting in persistent immunological memory and durable protection of the host.

An additional type of vaccine that has gained considerable attention in recent years, particularly following the COVID-19 pandemic, is mRNA vaccines [25]. Strictly speaking, they cannot be classified in any of the above-mentioned categories because they combine features of subunit vaccines and live-attenuated vectors. They are based on synthetic mRNA molecules that direct the production of the antigen that will generate an immune response and are able to mimic a viral infection eliciting both humoral and cellular immune responses. The interest in mRNA-based technology for the development of prophylactic vaccines against infectious disease stems from the potential to expedite vaccine development, to have improved safety and efficacy, have flexible production, to address maternal vaccination, and to tackle diseases that have not been possible to prevent with other approaches. However, this technology has been used to target mainly viral infections. There is only one study in the field of bacterial infections describing a self-amplifying mRNA vaccine to elicit immune responses against group A and group B streptococci [26], thus it is out of the scope of this chapter.

1.2 Carbohydrate-Based Vaccines

Carbohydrates are by far the most abundant organic molecules found in nature and are fundamental constituents of all living organisms. Besides their well-established structural and energetic (storage and production) functions, carbohydrates play a vital role in a great deal of biological and biochemical processes and are involved in cellular adhesion and differentiation, signal transmission, fetal development, fertilization, and all kinds of cellular recognition events. Both eukaryotic and prokaryotic cells expose on their surfaces a dense array of complex carbohydrates, mainly as components of glycoproteins, proteoglycans, and cell surface glycolipids, which are collectively referred to as *glycocalyx*. In mammalian cells, glycans can be attached not only to proteins and lipids on the cell surface, but also in the cytoplasm or even in the nucleus. The glycocalyx exerts a protective function from ionic and mechanical stress, preserving the integrity of the membrane and acting as a barrier from invading microorganisms. Furthermore, the glycocalyx is critical in determining the development of innate and adaptive immunity in response to pathogens or the growth and spread of cancer [27–29]. On the other hand, the glycocalyx of bacterial pathogens may include either CPSs of encapsulated bacteria, LPS of the outer membrane of Gram-negative microorganisms, or other lipid-linked or peptidoglycan-attached glycan chains. Bacterial surface PSs are key virulence factors. They can trigger bacterial adhesion and host cells infection, and interfere with innate immunity by preventing the activation of the alternative complement pathway. Overall, the bacterial glycocalyx plays many functions, regulating the interactions of the organism with its environment and allowing the bacterium to establish and maintain an infection. Additionally, since carbohydrates derive from posttranslational modifications, they remained structurally highly conserved throughout evolution and, in most cases, are uniquely associated to microbial species, even if some examples of bacterial surface carbohydrates mimicking host self-antigens have been reported [24]. It is, therefore, anything but surprising that these pathogenic glycans have proven to be attractive targets for vaccine development.

The first PS-based vaccine stemmed from the seminal findings of Avery and Heidelberger, who demonstrated the immunoreactivity of CPSs from the encapsulated bacterium *Streptococcus pneumoniae* (Sp) [30, 31]. Accordingly, a CPS-based vaccine targeting four relevant Sp serotypes was licensed in 1945 [32]. The advent of chemotherapeutics and antibiotics in later years, however, dampened the enthusiasm toward vaccines. It was a general belief that these new drugs could represent the ultimate solution to heal any kind of infectious disease, but the emergence and the constant increase of multidrug resistance phenomenon revealed the intrinsic limitations of antibiotic therapies and awakened a renewed interest in preventive strategies. In the following years, a large body of literature data highlighted the role of carbohydrate-specific antibodies in preventing microbial infections and provided a new impetus to the development of carbohydrate-based vaccines. These endeavors led to the approval of numerous CPS-based monovalent and multivalent vaccines against *Neisseria meningitidis* (MPSV4, introduced in 1978) [33], Sp (the current version including 23 out of approximately 100 known serotypes was launched by Merck and Co. in 1983 with the trade name PneumoVax) [34], Hib, and *Salmonella typhi* [15]. These PS-based vaccines were proven effective in preventing disease in healthy adults and older children. They appeared, however, to be poorly immunogenic in infants and young children (under two years of age), in the elderly, and in immunocompromised individuals. Even in adults, they induce only short-lasting antibody responses and fail to generate conventional B-cell-mediated immunological memory [35–38].

The limited clinical efficacy of PS vaccines is largely attributed to the T-cell-independent immune response they induce, which is typically triggered by repetitive polymeric antigens (see Section 1.2.1) [39, 40]. Once again, an old discovery was the key. In 1921, Landsteiner introduced the *hapten* concept, meaning any small organic molecule that alone does not elicit an immune response but is able to produce specific antibody responses when conjugated to an immunogenic protein [41]. A few years later, Avery and Goebel put in place this concept and chemically conjugated pneumococcal CPSs to proteins with the aim to enhance their immunogenicity [42]. This seminal experiment paved the way for the development of glycoconjugate vaccines, a revolutionary breakthrough in the field of vaccinology, although the first glycoconjugate vaccine, targeting Hib, was authorized only in 1987 [43, 44]. Subsequently, at least 12 more monovalent Hib conjugate vaccines have been licensed. Likewise, several formulations of glycoconjugate vaccines were licensed in the following years, targeting meningococcal [45] and pneumococcal disease (PCVs, pneumococcal conjugate vaccines). The first version of the latter was introduced in 2000 and contained seven serotypes (PCV7) [46]. Higher valent formulations (PCV15 and PCV20), however, have been authorized by the Food and Drug Administration (FDA) in 2021 to achieve broader serotype coverage [47, 48]. In the last few years, two *S. typhi* conjugate vaccines were also licensed [49, 50]. An updated picture of current glycoconjugate vaccines licensed by FDA and/or prequalified by WHO is given in Table 1.1. Table 1.2 reports the chemical structures of the repeating units (RUs) of the CPSs used in commercially available glycoconjugate vaccines.

The advent of glycoconjugate vaccines, capable of conferring long-term protective immunity even in high-risk groups, opened a new era of vaccinology. Glycoconjugate antigens raise an immune response improved in quality and quantity compared to plain PSs, as outlined in Section 1.3.1.

Table 1.1 List of glycoconjugate vaccines licensed by FDA[a] for use in the USA and/or prequalified by WHO[b] (content current as of 04 April 2022).

Pathogen	Serogroup *or* serotype	Pharmaceutical company[c] (country of manufacture)	Tradename (*if any*) (FDA Licensed, Prequalified by WHO)	Formulation type[d] (valency) (vaccine components)	Carrier protein[e]	Adjuvant	Saccharide antigen size[f]
Haemophilus influenzae	Type b	Merck Sharp & Dohme Corp. (USA)	Liquid PedvaxHIB (1990)	Monovalent	OMPC	Amorphous aluminum hydroxyphosphate sulfate	MEDIUM
	Type b	Sanofi Pasteur (France)	Act-HIB (1993, 1998) *used as vaccine component in Hexaxim® and Pentacel®*	Monovalent	TT	—	LONG
	Type b	Serum Institute of India Pvt. Ltd. (India)	*Haemophilus influenzae* type b conjugate vaccine (2008)	Monovalent	TT	*Information not available* (N/A)	N/A
	Type b	GlaxoSmithKline Biologicals SA (Belgium)	HIBERIX (2009)	Monovalent	TT	—	MEDIUM-LONG
	Type b	Centro de Ingenieria Genetica y Biotecnologia (Cuba)	Quimi-Hib (2010)	Monovalent	TT	Aluminum phosphate	SHORT
	Type b	Biological E. Limited (India)	ComBE Five (2011)	Combination (DTPw–HBV–Hib)	TT	Aluminum phosphate	N/A

(Continued)

Table 1.1 Continued

Pathogen	Serogroup *or* serotype	Pharmaceutical company[c] (country of manufacture)	Tradename (*if any*) (FDA Licensed, Prequalified by WHO)	Formulation type[d] (valency) (vaccine components)	Carrier protein[e]	Adjuvant	Saccharide antigen size[f]
	Type b	Panacea Biotec Ltd. (India)	Easyfive-TT (2013)	Combination (DTPw–HBV–Hib)	TT	Aluminum phosphate Gel	N/A
	Type b	Sanofi Pasteur (France)	Hexaxim (also known as Hexyon *or* Hexacima) (2014)	Combination (DTPa–HBV–IPV-Hib)	TT	Aluminum hydroxide	LONG
	Type b	Sanofi Healthcare India Private Limited (India)	Shan-5 (2014)	Combination (DTPw–HBV–Hib)	TT	N/A	N/A
	Type b	PT Bio Farma (Persero) (Indonesia)	Pentabio (2014)	Combination (DTPw–HBV–Hib)	TT	Aluminum phosphate	N/A
	Type b	LG Chem Ltd (Republic of Korea)	Eupenta (2016)	Combination (DTPw–HBV–Hib)	TT	Aluminum hydroxide	N/A
	Type b	MSP Vaccine Company (USA)	VAXELIS (2020)	Combination (DTPa–HBV–IPV–Hib)	OMPC	Aluminum salts	MEDIUM
	Type b	Sanofi Pasteur (Canada)	Pentacel (known in the UK and Canada as Pediacel) (2021)	Combination (DTPa–HBV–IPV–Hib)	TT	Aluminum phosphate	LONG

Neisseria meningitidis	A	Serum Institute of India Pvt. Ltd. (India)	MenAfriVac (2010)	Monovalent	TT	Aluminum phosphate	MEDIUM
	$ACYW_{135}$	Sanofi Pasteur Inc. (USA)	Menactra (2005, 2014)	Polyvalent (4-valent)	DT	—	MEDIUM-LONG
	$ACYW_{135}$	GlaxoSmithKline Biologicals SA (Belgium)	Menveo (2010, 2013)	Polyvalent (4-valent)	CRM_{197}	—	MEDIUM-LONG
	$ACYW_{135}$	Pfizer Europe MA EEIG (Belgium)	Nimerix (2016)	Polyvalent (4-valent)	TT	—	MEDIUM
	$ACYW_{135}$	Sanofi Pasteur Inc. (USA)	MenQuadfi (2020, 2022)	Polyvalent (4-valent)	TT	—	MEDIUM-LONG
Salmonella enterica	typhi Ty2 (Vi antigen)	Bharat Biotech International Ltd. (India)	Typbar-TCV (2017)	Monovalent	TT	—	N/A
	typhi Ty2 (Vi antigen)	Biological E. Limited (India)	TYPHIBEV (2020)	Monovalent	CRM_{197}	—	N/A
Streptococcus pneumoniae	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	GlaxoSmithKline Biologicals SA (Belgium)	Synflorix (2009)	Polyvalent (10-valent)	NTHi PD, TT, and DT	Aluminum phosphate	MEDIUM
	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	Pfizer (USA)	Prevnar 13 (2010, 2010)	Polyvalent (13-valent)	CRM_{197}	Aluminum phosphate	LONG

(Continued)

Table 1.1 Continued

Pathogen	Serogroup *or* serotype	Pharmaceutical company[c] (country of manufacture)	Tradename (*if any*) (FDA Licensed, Prequalified by WHO)	Formulation type[d] (valency) (vaccine components)	Carrier protein[e]	Adjuvant	Saccharide antigen size[f]
	1, 5, 6A, 6B, 7F, 9V, 14, 19A, 19F and 23F	Serum Institute of India Pvt. Ltd. (India)	Pneumosil (2019)	Polyvalent (10-valent)	CRM_{197}	Aluminum phosphate	LONG
	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, 33F	Merck Sharp & Dohme Corp. (USA)	Vaxneuvance (2021)	Polyvalent (15-valent)	CRM_{197}	Aluminum phosphate	LONG
	1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, 33F	Pfizer (USA)	Prevnar 20/ Apexxnar (2021)	Polyvalent (20-valent)	CRM_{197}	Aluminum phosphate	LONG

a) https://www.fda.gov/vaccines-blood-biologics/vaccines/vaccines-licensed-use-united-states.
b) https://extranet.who.int/pqweb/vaccines/prequalified-vaccines.
c) MSP Vaccine Company is a U.S.-based joint-partnership between Merck and Sanofi Pasteur, Merck Sharp & Dohme Corp. is a U.S.-based subsidiary of Merck & Co.
d) DTPw–HBV–Hib = Diphtheria–Tetanus–Pertussis (whole cell), Hepatitis B Virus (rDNA), and *Haemophilus influenzae* type b conjugate vaccine (absorbed); DTPa–HBV–IPV–Hib = Diphtheria–Tetanus–Pertussis (acellular), Hepatitis B Virus, Poliovirus (Inactivated), and Haemophilus influenzae type b conjugate vaccine (absorbed); DTPa–IPV–Hib = Diphtheria–Tetanus–Pertussis (acellular), Poliovirus (Inactivated), and Haemophilus influenzae type b conjugate vaccine (absorbed).
e) OMPC = meningococcal outer membrane protein complex; TT = Tetanus Toxoid; DT = Diphtheria Toxoid; CRM_{197} = Cross Reacting Material 197; NTHi PD = Nontypeable *H. influenzae* Protein.
f) LONG = native PS; MEDIUM = depolymerized and/or sized-fractionated PS; SHORT = synthetic PS.

Table 1.2 Chemical structure of CPS repeating units within serogroups *or* types in licensed vaccines.

Pathogen	Serogroup *or* type	Repeating unit
Haemophilus influenzae	Type b	→3)-β-D-Rib*f*-(1→1)-D-Rib-ol-(5-OPO_3→
Neisseria meningitidis	A	→6)-α-D-Man*p*NAc(3/4OAc)-(1-OPO_3→
	C	→9)-α-D-Neu*p*5Ac(7/8OAc)-(2→
	W	→6)-α-D-Gal*p*-(1→4)-α-D-Neu*p*5Ac(7/9OAc)-(2→
	Y	→6)-α-D-Glc*p*-(1→4)-α-D-Neu*p*5Ac(7/9OAc)-(2→
Salmonella enterica	typhi Vi	→4)-α-D-Gal*p*NAcA(3OAc)-(1→
Streptococcus pneumoniae	1	→3)-α-D-AATGal*p*-(1→4)-α-D-Gal*p*A(2/3OAc)-(1→3)-α-D-Gal*p*A-(1→
	3	→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→
	4	→3)-β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*2,3(S)Pyr-(1→
	5	→4)-β-D-Glc*p*-(1→4)-[α-L-Pne*p*NAc-(1→2)-β-D-Glc*p*A-(1→3)]-α-L-Fuc*p*NAc-(1→3)-β-D-Sug*p*-(1→
	6A	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Rib-ol-(5→OPO_3→
	6B	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→OPO_3→
	7F	→6)-[β-D-Gal*p*-(1→2)]-α-D-Gal*p*-(1→3)-β-L-Rha*p*(2OAc)-(1→4)-β-D-Glc*p*-(1→3)-[α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*(1→4)]-β-D-Gal*p*NAc-(1→
	8	→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→
	9V	→4)-α-D-Glc*p*(2/3OAc)-(1→4)-α-D-Glc*p*A(2/3OAc)-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc(4/6OAc)-(1→4)-β-D-Glc*p*-(1→
	10A	→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→OPO_3→5)-β-D-Gal*f*-(1→3)-β-D-Gal*p*(1→
	11A	→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[Gro-(1→OPO_3→4)]-α-D-Glc*p*(3OAc)-(1→4)-α-D-Gal*p*(2OAc)-(1→
	12F	→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→
	14	→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→
	15B	→6)-[α-D-Gal*p*(2/3/4/6OAc)-(1→2)-[Gro-(2→OPO_3→3)]-β-D-Gal*p*-(1→2)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→

(Continued)

Table 1.2 (Continued)

Pathogen	Serogroup *or* type	Repeating unit
	18C	→4)-β-D-Glc*p*-(1→4)-[α-D-Glc*p*(6OAc)-(1→2)] [Gro-(1→OPO_3→3)]-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→
	19A	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→3)-α-L-Rha*p* -(1→OPO_3→
	19F	→4)-β-D-ManpNAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p* -(1→OPO_3→
	22F	→4)-β-D-Glc*p*A-(1→4)-[α-D-Glc*p*-(1→3)]-β-L-Rha*p*(2OAc)-(1→4)-α-D-Glc*p*-(1→3)-α-D-Gal*f*-(1→2)-α-L-Rha*p*-(1→
	23F	→4)-β-D-Glc*p*-(1→4)-[α-L-Rha*p*-(1→2)]-[Gro-(2→OPO_3→3)]-β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→
	33F	→3)-β-D-Gal*p*-(1→3)-[α-D-Gal*p*-(1→2)]-α-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→3)-β-D-Glc*p*(1→5)-β-D-Gal*f*(2OAc)-(1→

Abbreviations: Glc, glucose; Gal, galactose; Neu5Ac, *N*-acetylneuraminic acid (sialic acid); Rha, rhamnose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; FucNAc, *N*-acetylfucosamine; ManNAcA, *N*-acetylmannuronic acid; PneNAc, *N*-acetylpneumosamine (2-acetamido-2,6-dideoxytalose); GlcA, glucuronic acid; Gro, glycerol; Rib-ol, ribitol; Sug, 2-acetamido-2,6-deoxyhexose-4-ulose; AATGal, 2-acetamido-4-amino-2,4,6-trideoxy-galactose; 2,3(S)Pyr, *trans*-2,3-(S) cyclic pyruvate ketal modification at galactose; *p*, pyranose form; *f*, furanose form; OPO_3, phosphate group in phosphodiester linkages.

1.2.1 Mechanism of the Immune Response to Carbohydrate-Based Vaccines

Bacterial CPSs have been recognized as key virulence factors for more than a century and have been explored as potential vaccine antigens from the outset [51, 52]. The licensure of the previously mentioned CPS-based vaccines against *N. meningitidis*, *S. pneumoniae*, *H. influenzae* b, and *S. typhi* allowed an efficient control of the disease in adults and older children, especially for short-lasting exposures (travelers and soldiers in military campaigns). Plain PS-based vaccines, however, are unable to mount a protective immune response in the immature immune systems of newborns and young children (under two years of age), who are the major group at risk for these infections. Even in adults and adolescents, PS vaccines are not able to induce memory B cells (MBCs), avidity maturation, and antibody isotype switching from immunoglobulin M (IgM) to immunoglobulin G (IgG). Most of the antibodies produced, indeed, are low-affinity IgM, which are only poor activators of the complement system, a key arm of the innate immune system that enhances the humoral responses [53, 54]. Furthermore, immunization of adults and older children with PS vaccines leads to apoptosis of MBCs, thus reducing the response to subsequent administrations, a phenomenon usually referred to as hyporesponsiveness [55]. PSs are T-cell-independent type 2 (TI-2) immunogens, which are typically high-mass polymers with repeating structures of 5–10 nm. Due to their polymeric structure,

PSs activate B cells by cross-linking approximately 15–20 B-cell receptors (BCRs), triggering a series of protein phosphorylation steps which leads to an increase in free intracellular calcium (Figure 1.1a) [56]. The need to crosslink multiple BCRs to achieve B-cell activation is noteworthy, as it explains the reason why the immunogenicity of PS vaccines is size-dependent, with only high-molecular-weight antigens capable of inducing an effective immune response. Following stimulatory cytokines or co-stimulatory signals expressed by other cells of the immune system [57–59], B cells are finally activated. They mature into plasma cells and secrete antibodies

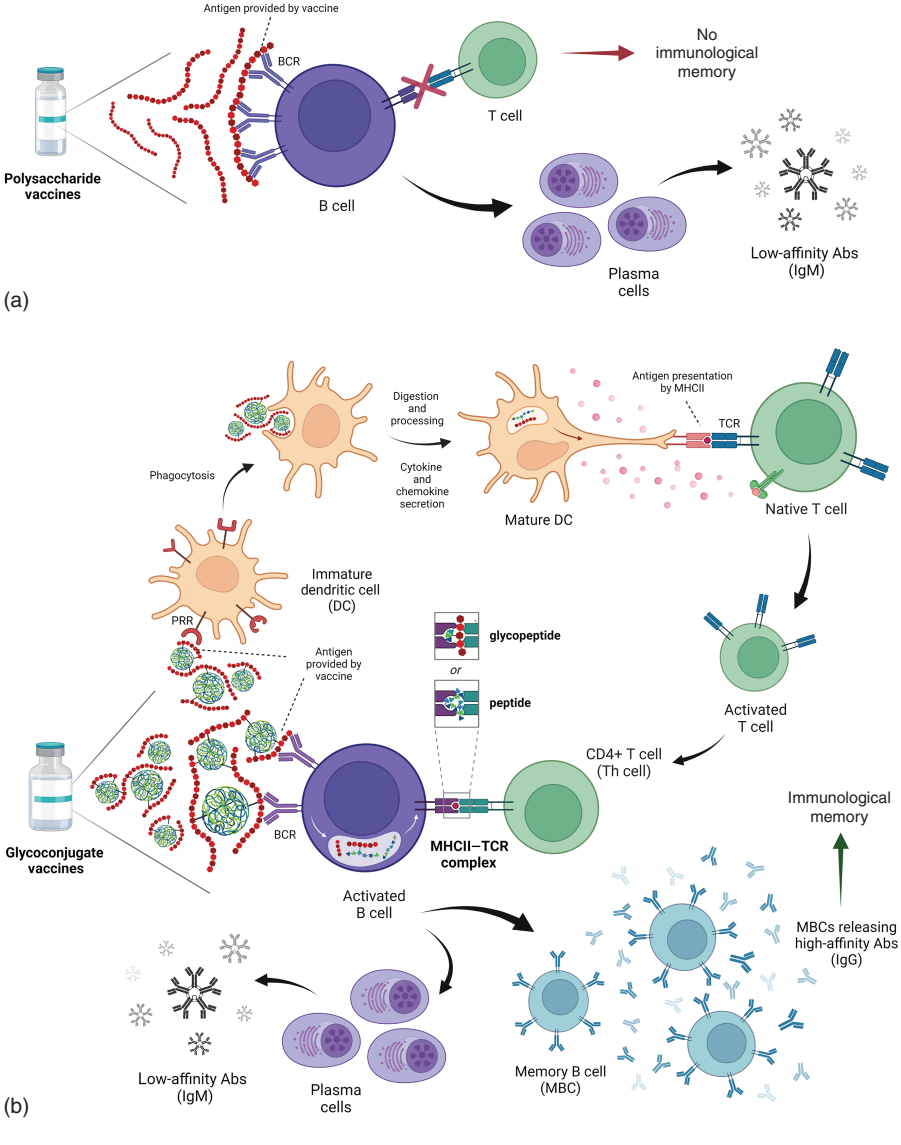

Figure 1.1 Immune response following immunization with polysaccharides (a) and glycoconjugates (b).

(mainly IgM) but since the entire process takes place without the direct involvement of T cells, the development of MBCs and the induction of immunological memory do not occur.

Unlike PSs, proteins and peptides are instead T-cell-dependent (TD) antigens, as they stimulate helper T lymphocytes to elicit an immune response leading to specific antibody response. TD antigens are immunogenic even in early childhood, the immune response induced can be boosted and enhanced by adjuvants, and it is characterized by an antibody class switch with the production of high-affinity and antigen-specific IgG. The hapten-carrier concept [60] underpinning the development of glycoconjugate vaccines is based on the fact that the covalently linked protein carrier confers T-cell-dependent properties to the glycoconjugate, which is eventually able to produce carbohydrate-specific MBCs. The molecular mechanism of the immune response to glycoconjugate vaccines has been thoroughly investigated [61, 62], albeit some steps are still unclear. After administration, the conjugate is taken up by antigen-presenting cells (APCs), mainly dendritic cells (DCs), but also macrophages and B cells can play the same role. Engulfment by APCs is promoted by stimulation of pathogen recognition receptors (PRRs), a large family of receptors expressed on APCs surface able to respond to a huge variety of pathogen-associated molecular patterns (PAMPs), structurally and chemically diverse compounds highly conserved in pathogens but absent in their multicellular host. PRRs stimulation creates the necessary pro-inflammatory context (expression of costimulatory molecules and secretion of soluble cytokines and chemokines), leading to full maturation of DCs, antigen uptake, and intracellular processing (Figure 1.1b). In particular, the glycoconjugate antigen is chopped through the intracellular endosomal compartments. While the saccharide portion is depolymerized by oxidative agents (reactive oxygen species, ROS, and reactive nitrogen species, RNS) [63, 64], the protein portion is processed by proteases into small peptides. Within a few days, mature DCs reach the draining lymph nodes, where peptide antigens are conveyed to the cell surface in association with the major histocompatibility complex class II (MHC-II) protein to be presented to T lymphocytes via interaction with the T-cell receptor (TCR). T lymphocytes are then activated as helper T cells (Th or $CD4^+$ T cells), which provide appropriate stimulatory signals to elicit a conventional TD immune response. Th cells prime the maturation process of resting B cells, driving their proliferation and differentiation into antibody-secreting (mainly IgM) plasma cells and MBCs, ensuring the establishment of the immunological memory. Contrary to plasma cells, MBCs survive for a long time in the body and respond rapidly to subsequent exposures of the same antigen by secreting high-affinity IgG antibodies.

Recent findings [65] provided more details on the mechanism of action of glycoconjugate vaccines, highlighting the crucial role of the formation of germinal centers (GCs) to elicit an immune response. GCs are sites in lymph nodes where mature B-cell proliferation and differentiation occur. The GC formation requires the presence of PS-specific B cells, which display the antibody on their surface as a BCR, the follicular helper T (Tfh) cells, which are able to recognize the protein carrier antigen, and the follicular dendritic cells (FDCs), highly specialized APCs, which

contain and present the antigen to the B cells. The formation of GCs is an essential step during the immune response to an infection or after vaccination, since the affinity maturation process and the class switch from IgM to IgG occur in the GCs, specifically in two distinct regions called the light and dark zones.

Also, Kasper and coworkers suggested that not only peptides but also glycopeptide fragments are generated by glycoconjugate processing in APCs and presented to TCR in the context of MHC-II [66, 67]. According to this model, the lipophilic peptide portion of the glycopeptide antigen binds to MHC-II, whereas the hydrophilic carbohydrate portion is exposed to the TCR, leading to the generation of carbohydrate-specific Th, called T carb cells.

It should be emphasized that the different mechanisms of the immune response toward PS and glycoconjugate antigens also have practical consequences in vaccine design. Since crosslinking of surface immunoglobulin molecules on B cells is not required, glycoconjugate vaccines can also be produced from small saccharide chains obtained by size fractionation of native PSs or by chemical synthesis.

The development of glycoconjugate vaccines has been one of the greatest success stories of modern medicine [68, 69], as demonstrated by the drastic reduction of *S. pneumoniae*, *H. influenzae* b, and *N. meningitidis* infections in those countries where the corresponding conjugate vaccines have been introduced in routine vaccination programs. Overall, these vaccines have had a huge impact on global infant mortality and morbidity, saving millions of lives.

1.3 Components of Glycoconjugate Vaccines

A vaccine is a biological product able to induce an immune response that confers protection against an infection upon successive exposures to a pathogen [10]. The efficacy of protection is based on preexisting antibodies in the serum, which prevent the disease but not the infection [70]. Although most vaccines are based on TD protein antigens, PSs can also be used to induce protective immune responses, at least in an immunocompetent host. However, as explained in Section 1.2.1, the T-cell independence of PSs prevents the proliferation of MBCs and the occurrence of immunological memory, making PS-based vaccines ineffective for infants and young children, as well as for immunocompromised individuals. Protein–PS conjugate vaccines provided the solution to overcome these limitations, enabling the use of bacterial surface PSs as antigens to induce long-lasting protection from infectious diseases. Saccharide antigens employed for the construction of glycoconjugate vaccines are either derived from the pathogen or produced synthetically to mimic the components of the microorganism. They can be present in the form of microbial poly- or oligosaccharides (OSs) (the latter obtained by size-fractionation of the native polymer) or as synthetic low-molecular-weight compounds, covalently linked to a carrier protein. In addition, subunit glycoconjugate vaccines can be formulated with adjuvants to enhance the low immunogenicity of carbohydrate antigens.

Impressive progress has been made in the field of antibacterial vaccine development over the past two decades. Surprisingly, the important milestones that have outlined the history of antibacterial vaccines have been reached with an empirical approach [12]. The main reason is that only in recent times has immunology started to give a useful contribution to vaccine design. Recent advances in basic immunology are now unveiling the immunological principles that govern susceptibility to infections and protection of the host, ushering in the era of rational vaccine design. Indeed, progresses in vaccinology permitted us to realize that different, often interconnected, parameters can affect the immunogenicity of the glycoconjugate. Besides the choice of the T-cell helper protein, factors more related to the design of the saccharide antigen and its conjugation to the carrier protein have been recognized as important variables to determine immunogenicity. The elements to consider for the development of an efficient, immunogenic, and protective vaccine setting are now much clearer, and vaccinology is moving to a new era where the "vaccine" should be regarded as a pharmaceutical rather than as a biological product [71]. The following paragraphs of Section 1.3 report a more detailed analysis of each key component of a glycoconjugate vaccine, highlighting how they affect the efficacy of the construct and the robustness of the elicited immune response.

1.3.1 The Carbohydrate Antigen

Antibacterial glycoconjugate vaccines may contain full-length or size-reduced PSs, generally derived from CPS or the O-antigen portion of LPS. The PS portion of the construct is the key player in the glycoconjugate; indeed, the efficacy of the vaccine is measured by the magnitude and the quality of the immune response against the carbohydrate antigen. The size, and therefore the length, of the PS fragment is the principal determinant of its immunogenicity [72]. The glycan chain of a PS contains epitopes or antigenic determinants, also called glycoepitopes, that are the specific portions of the PS recognized by antibodies and responsible for inducing the antigen-specific immune response of the host. An epitope is commonly identified as a sequence of an average of six/seven contiguous residues (linear or branched), but for long saccharide chains, conformational epitopes are also formed, where discontinuous monosaccharide units closely organized in space are simultaneously engaged by the BCRs [73].

The lesson from traditional plain PS vaccines is that the large size of the PSs ensures cross-linking of multiple BCRs to elicit the TI immune response. The presence of repetitive antigenic determinants increases the avidity of antibody–antigen binding, thus facilitating the activation of B cells even when they express low-affinity antibodies [74]. On the other hand, the immunological mechanism of glycoconjugate vaccines does not depend exclusively on cross-linking of BCRs. In glycoconjugates, the simultaneous exposition of multiple copies of shorter PS fragments covalently attached to the carrier protein triggers the activation of the immune response. Yet, also in this case, a relationship between the length of the protein-conjugated PS fragment and the immunogenicity of the vaccine persists.

Consequently, significant efforts are aimed at determining the minimal OS length capable of maintaining the epitope determinants required to engage the antibody and elicit a T-cell-dependent protective immune response. However, the correlation between these two parameters depends on the type of glycoconjugate produced [72]. Typically, size-reduced PSs are obtained through chemical or mechanical fragmentation of the natural PSs isolated from cultures of pathogenic bacteria, followed by multiple purification and characterization steps to reduce glycan heterogeneity [75]. The use of more defined saccharide fragments facilitates the conjugation with the protein carrier and allows for improved batch-to-batch consistency of the final glycoconjugate. PS fragments are then conjugated to the protein either randomly, through the hydroxyl or carboxyl groups occurring along the saccharide chain, or via functional groups localized at the glycan end of the sugar chain (typically the anomeric carbon of the reducing end monosaccharide) [37]. The latter methodology generally includes the use of a linker to facilitate the glycan–protein coupling and to alleviate the steric hindrance between protein and saccharide (see Section 1.3.2). The two different strategies lead to heterogeneous cross-linked structures, usually involving multiple saccharide chains and protein molecules, and more defined structures with a radial exposition of saccharide chains end-terminal conjugated to a single protein molecule, respectively. The manufacture of random cross-linked conjugates uses PS fragments of about 100–300 kDa, while the selective attachment at the reducing end of the sugar is carried out on shorter fragments of 5–20 kDa, generally referred to as OSs [76]. Studies in the literature support the observation that higher immunogenicity of end-linked glycoconjugates correlates with the use of medium-size OSs, while longer PS fragments are preferable in obtaining randomly cross-linked effective glycoconjugates. As an example, a study in mice using end-linked conjugate preparation of *Salmonella enterica sv typhi* Vi antigen coupled to cross-reacting material 197 (CRM197) showed that shorter chain constructs (glycan size about 9.5 kDa) induced a more prolonged proliferation of Vi-specific B cells and a slower decline of Vi-specific IgG antibodies compared to their longer chain counterparts (glycan size about 165 kDa) [77]. On the other hand, a study on cross-linked glycoconjugates of *Francisella tularensis* OAgs coupled to tetanus toxoid (TT) evidenced that a genetically induced, very-high-molecular-size OAg (around 220 kDa) provided a marked increase in protection. The LPS-specific antibodies induced by this larger-sized O-antigen exhibit significantly enhanced relative affinity compared to low and native molecular weight preparations (25 and 80 kDa, respectively), albeit with comparable antibody titers [78]. This study supports the importance of conformational OAg epitopes in the protection induced by the vaccine.

The shape of the glycoconjugate and the way the glycoconjugates are presented to the immune system are critical and interconnected aspects of vaccine efficacy. In this regard, the carbohydrate-to-protein ratio is considered a significant parameter. Hib PS fragments with different chain lengths were coupled to diphtheria toxin (DT) carrier protein. The average degree of polymerization (avDP) was 8 or 20 monosaccharide RUs, and a maximum of three sugar moieties per protein molecule were conjugated [79]. The shorter avDP8 oligomer stimulated a poorer anticarbohydrate

response than the avDP20, but immunogenicity comparable to the avDP20 oligomer was then obtained in a later study where four/five sugar fragments with avDP8 were coupled to TT, thus supporting the argument that a higher level of protein glycosylation could compensate the shorter chain length [80].

The evidence that even a short OS chain can induce the production of specific antibodies that can protect the host from a pathogen and current advances in OSs synthesis have opened a new era of vaccine research that aims to prepare the carbohydrate antigen by chemical synthesis [81]. Structurally defined synthetic OSs, designed based on molecular understanding of antigen–antibody interactions, offer a promising alternative for developing semisynthetic (containing fully synthetic carbohydrates conjugated to an immunogenic carrier protein) and fully synthetic glycoconjugate vaccines based on synthetic OS antigens conjugated to synthetic carrier molecules, such as glycolipids or peptides [82–84]. The access to the active components of the vaccines by chemical synthesis, made possible by the tremendous progresses and recent technological innovations in glycan assembly [85–95], would result in more reproducible and homogeneous vaccine preparations, characterized by robust biological properties and better safety profile. Accordingly, the manufacturing process of the glycoconjugates thus obtained would be facilitated and accelerated, enabling timely combat of emerging or antimicrobial-resistant infectious diseases and/or addressing currently unmet medical needs [71, 96].

One additional aspect to consider when investigating carbohydrate antigens for immunological applications is that the size and structure of the PS can be affected by the manufacturing process. The production of the PS is designed to improve the yields and consistency of the final product. For example, native CPSs are commonly isolated by bacterial culture. The yield (expressed in x mgl^{-1}, where x are mg of pure CPSs extracted from 1 l of culture broth) and the molecular weight of bacterial PS produced during fermentation are influenced by the genetic nature of the microorganism and the fermentation conditions (i.e. the carbohydrate source and the carbon/nitrogen ratio in the culture medium, pH, and temperature) [78, 97–99]. Other factors influence the consistency of the final products: the type of PS fragmentation (i.e. chemical or mechanical), the size-fractionation and purification processes, and the activation chemistry before conjugation. Due to the highly specific mechanism for immunological activation, it is important to reduce extensive chemical modifications of the native PS structure during the production of the glycoconjugates. For example, the loss of labile *O*-acetyl groups, which are present (often in random order) in different PSs, might impair the immunogenicity of a PS, leading to a less effective vaccine. In this regard, the meningococcal NeisVac-C vaccine, which uses de-O-acetylated MenC OSs [100], shows less efficacy than other licensed MenC glycoconjugate vaccines, like Menjugate, that are derived from acetylated OS. Also, the random periodate oxidation of the vicinal hydroxy groups of the PS units to generate multiple aldehyde groups to be reacted with the protein by reductive amination should be tightly controlled to affect only some monosaccharides in order to avoid extensive PS chemical modification and, consequently, the loss of immunogenicity.

1.3.2 Linkers for Carbohydrate–Protein Conjugation

In glycoconjugate vaccines, the covalent conjugation of the saccharide antigen to the carrier protein can be achieved either directly or through short linear spacer molecules, called linkers. The introduction of a linker facilitates the coupling of the glycan to the protein because the steric hindrance that may reduce the reactivity between two relatively large molecules is reduced. In addition, the presence of a linker affects the spatial orientation of the glycan epitopes around the protein and the way they are presented to the immune system. Indeed, the nature of the linker and the type of conjugation chemistry are other key factors in the development of a vaccine candidate. They are interconnected and have to be considered jointly because the conjugation chemistry should be based on a highly efficient chemoselective coupling step between two specific functional groups on the carbohydrate and the protein [61]. On one side, there are the chemically reactive sites of the carbohydrate moiety, which are mostly (i) aldehyde groups obtained by periodate oxidation of *cis*-diols in the sugar ring or at the glycerol moiety of sialic acid residues, (ii) carboxyl groups of sialic or uronic acid residues, (iii) cyanate esters generated by random functionalization of the sugar hydroxyls with cyanilating agents, and (iv) primary amino groups, generally obtained by an end terminal reductive amination of sized OSs. Bacterial OS fragments obtained by chemical synthesis are generally designed with short linkers at their reducing end bearing a terminal reactive group suitable for protein conjugation, such as an amine, a thiol, an alkyne, or an azide. On the other side, the common protein functional groups that are more suitable for chemical linkage to sugars are the carboxylic groups of aspartic and glutamic acid residues and the ε-amino group of lysines. These amino acid residues are generally well exposed on the surface of the protein, thanks to their hydrophilic character. Based on the functional groups that are present on the two coupling partners, the protein and the glycan, different types of linkers have been developed over the last 30 years [37].

Homobifunctional linkers contain the same reactive group at the two ends. The glycoconjugate construct is generally obtained through a two-step protocol, which involves the initial coupling of one of the two partners in the presence of an excess of the linker, followed by the conjugation of the activated intermediate to the second partner moiety. The Di-(*N*-succinimidyl)-adipate (DSA) and the dithio-propionates Dithio bis(succinimidylpropionate) (DSP) and Dithiobis(sulfosuccinimidylpropionate) (DTSSP) are examples of linkers containing two terminal carboxyls activated as *N*-hydroxysuccinimidyl (NHS) esters, which react with the amino groups of the lysine residues on the protein and of the sugar to give stable nonhydrolyzable amide bonds (Figure 1.2) . Chemical conjugation through stable chemical bonds can also be obtained by exploiting the cystamine or ADH homobifunctional linkers that contain two amino or hydrazide functional groups at both ends, respectively (Figure 1.2). These are crosslinking reagents able to engage aspartic and glutamic residues on the protein in an EDC-mediated coupling, while they can give reductive amination with the formyl groups on the sugar or be employed in nucleophilic additions to cyanide groups to form

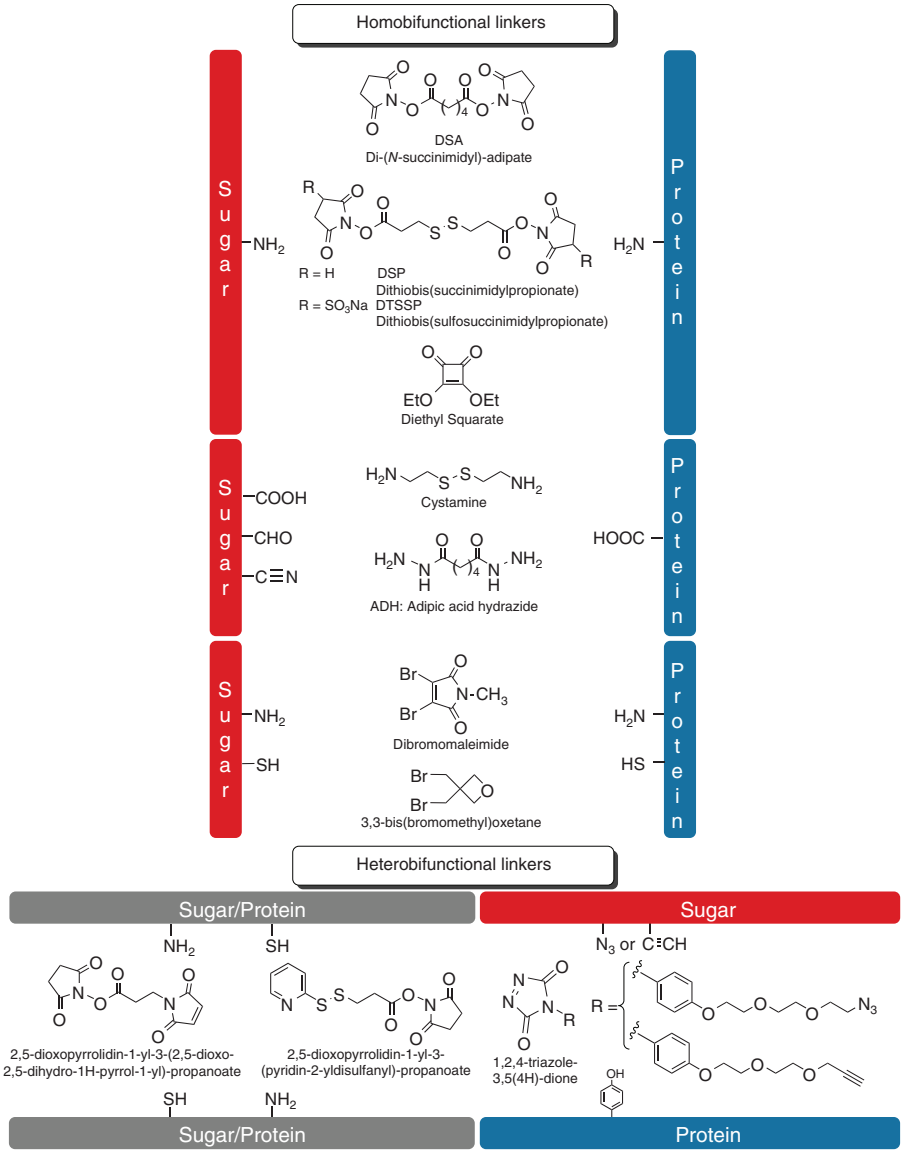

Figure 1.2 Representative examples of homo- and heterobifunctional linkers for glycoconjugation.

amidine bonds. All these homobifunctional linkers are soluble in water and ensure highly specific and easy conjugation protocols that have been exploited in the preparation of different glycoconjugate vaccines [101-103]. Diethyl squarate also reacts with amino groups on the sugar and the protein to give cross-linked coupling products [104]. Halogen-bearing homofunctional spacers, such as dibromomaleimide or 3,3-bis(bromomethyl)oxetane, are also available (Figure 1.2). They are characterized by a cyclic scaffold and by the presence of two halogens that can be displaced in a two-step

protocol by nucleophiles such as amines or thiols either on the sugar or on the protein. While the protein conjugate with dibromomaleimide suffers from intrinsic instability, due to the reversible nature of the nucleophilic substitution reaction in a reducing environment, the cross-linked product with 3,3-bis(bromomethyl)oxetane gives a chemically defined and irreversible conjugate [105, 106].

The other class of spacer reagents is represented by heterobifunctional linkers that contain two different reactive end-groups connected by an organic spacer. The two ends of the linker target different functional groups in the reaction partners and are activated orthogonally for site-selective conjugations that require a two-step protocol. The family of heterobifunctional linkers is quite large due to the different combinations of functional groups that can be targeted in the protein and/or the sugar [37]. Among the most representative examples, it is worth mentioning the propanoate derivatives reported in Figure 1.2 that are designed to react with an amine group on one end and with a thiol on the other. Indeed, one of the two extremities of the spacer contains an activated ester, which is the first to be conjugated to the sugar/protein to give an amide, while at the other extremity is a specific group that can react with thiol-containing molecules to form a disulfide linkage or a thioether by Michael addition. In addition, heterobifunctional linkers that contain one functional group suitable for azide–alkyne Huisgen 1,3-dipolar cycloaddition (AAC) are gaining increasing prominence [107–109]. In this regard, triazole-3, 5-dione scaffolds can be easily substituted with alkylated phenols to obtain alkyne or azido-ending linkers suitable for AAC on one side and for ene-like reactions on the other.

An exhaustive picture of the type and chemistry of the linkers is out of the scope of the present account due to the great number of alternative options that have been recently developed. However, a general requirement in this field is that the linker should be easily introduced under mild conditions and should allow an efficient and selective coupling step that is easy to control and scale up. The use of a linker should allow the sugar and the protein to maintain the correct conformational arrangement to be recognized by the immune system, preserving sugar epitopes. In addition, several studies have demonstrated that a kind of intrinsic immunogenicity of the linker could be present, and any type of nonspecific response against the linker that drives the immune response away from the targeted hapten epitopes should be avoided [110, 111]. Data from the literature support that rigid and constrained spacers could induce a significantly high amount of undesirable antibodies, while these unwanted effects are reduced by the use of flexible alkyl linkers [112]. On the other hand, a study in mice with a GBS PS-based conjugate vaccine, where a GBS type II polysaccharide (PSII) was conjugated to a pilus protein, showed that the presence of moderate levels of antibodies against rigid triazole rings generated by click chemistry did not affect the anti-PS immune response [113]. In addition, it was reported that in some cases, the presence of the linker might divert the antibody response of the conjugate from the target sugar epitopes, or conversely, it can enhance the immunogenicity of carbohydrate antigens [101, 111, 114, 115]. Overall, the choice of the linker is case-specific and should be tailored to the particular glycoconjugate construct under development.

1.3.3 The Carrier Protein

The early discovery of Avery and Goebel that conjugation of PSs with proteins stimulates T-cell help and the production of IgG against encapsulated bacteria [42] set the stage for the development of the first glycoconjugate vaccine against *H. influenzae* [116], followed by all the other highly effective antibacterial vaccines based on the same technology. The protein antigen, linked to the saccharide, is the key agent responsible for the thymus-dependent (elicited by TD antigens) immune response and is the so-called T helper protein/peptide antigen. It generally contains multiple sites for saccharide attachment. Five carrier proteins have been developed and successfully used as T helper antigens in licensed glycoconjugate vaccines. TT and DT were the first TD antigens used for this scope. They were discovered at the beginning of the twentieth century by Gustav Ramon, who developed a method for inactivating the respective toxins using formaldehyde. TT and DT were initially selected as carriers for Hib conjugate vaccines due to their high safety profiles established over decades of vaccination against tetanus and diphtheria. Their use was then extended to other vaccines, such as Menactra®, the first licensed quadrivalent meningococcal vaccine [117, 118]. Later on, during mutagenesis studies of the phage containing the gene encoding DT [119], the CRM_{197} was identified. CRM_{197} and DT are structurally very similar [120] and differ only by a single amino acid substitution from glycine to glutamate in position 52 [121]. While they are immunochemically indistinguishable, the big advancement is that CRM_{197} is naturally nontoxic. For this reason, CRM_{197} was considered an ideal carrier for conjugate vaccines, and it is still currently used as a component of meningococcal and pneumococcal glycoconjugate vaccines [122]. It can be isolated from lysogens of *Corynebacterium diphtheriae* or produced by recombinant DNA techniques in heterologous organisms. The other two commonly used T helper antigens are the outer membrane protein complex (OMPC) of *N. meningitidis* serogroup B strain B11 and the outer membrane protein D (PD) derived from non-typeable *H. influenzae* (NTHi). They are components of vaccines against *H. influenzae* and pneumococcus [123–125].

The nature of the carrier protein is an important parameter that strongly affects the efficacy of glycoconjugate vaccines. Comparative studies on the clinical impact of the different carrier proteins used in licensed vaccines were not conclusive in proving the superiority of one protein over the other, due to the high number of variations in the formulation of different vaccines. However, some general hints for the selection and/or development of new carrier proteins can be defined. A good protein carrier should be safe and devoid of any toxic or enzymatic activity. In this regard, the production of carrier proteins with recombinant techniques is preferred over chemical detoxification, which can also result in extensive structure modification and heterogeneity. The maintenance of the protein effective T cell help should be balanced with eventual immune interferences caused by the carrier that diverts the immune system from the relevant saccharide target. Previous exposure to the carrier or carrier-related protein (i.e. coadministration of different vaccines) has been correlated with the carrier-induced epitope suppression, i.e. attenuation or even suppression of the antibody response against the glycoepitope. A high ratio by

weight of the carrier protein with respect to the saccharide hapten in the vaccine formulation has also been associated to antigen hyporesponsiveness [126, 127]. In addition, a deep knowledge of the immunological and physicochemical characteristics of the protein is necessary to ensure a reproducible production of the glycoconjugate vaccine. The protein carrier should be soluble, stable, and contain a sufficient number of accessible amino acids for a controlled glycoconjugation. Lastly, the protein should be produced in high yield at a large scale and according to the cGMP protocols required by regulatory agencies.

Many other proteins have been tested in clinical and preclinical studies or are under development as alternatives to the currently used carriers. A larger range of options could reduce the problem of carrier-induced epitope suppression associated to the extensive use of current T helpers in vaccine manufacturing. A detailed picture of the research in this field has been recently reviewed [128, 129]. New carriers are derived from bacteria, like a recombinant nontoxic form of *Pseudomonas aeruginosa* exotoxin A (rEPA), which has been conjugated to PSs of *Staphylococcus aureus* types 5 and 8 [130], and the Vi antigen of *S. typhi* [131, 132]. Furthermore, most of the potential new carriers are produced through recombinant techniques with the purpose of reducing sophisticated culture conditions and developing solid protocols. For example, the tetanus toxin's native C-fragment (Hc) is a safe, low-cost, and highly immunogenic peptide with easy purification. It has been tested as a carrier for pneumococcal PSs (PS14 and PS23F) and has shown immunogenicity levels comparable to CRM_{197} and TT glycoconjugates [133].

The carrier protein may also provide protection against the pathogen from which it is derived. Protein D is one of the examples of proteins with dual role of carriers and antigens. It is expressed by NTHi and was selected as the carrier of 8 PSs in the 10-valent antipneumococcal protein D conjugate vaccine (PHiD-CV), in which the other two serotypes are conjugated to TT and DT, respectively, with the aim to provide protection against NTHi acute otitis media [134]. Other examples are *S. aureus* α toxin Hla and the *S. pneumoniae* protein PiuA, which were effective in animal models in inducing protection against *S. aureus* and pneumococcal infections [135, 136]. Another interesting approach regards a truncated version of the rotavirus spike protein VP8* that was covalently conjugated to Vi capsular PS of *S. typhi* to develop a bivalent vaccine. The Vi-ΔVP8* conjugate vaccine elicited high antibody titers and functional antibodies against *S. typhi* and rotavirus. This study showed that the conjugation of the antigenic peptide to the saccharide epitope enhanced the intrinsically low immunogenicity of the short ΔVP8 truncated peptide and allowed the switching to a specific TD response against the saccharide [137]. Due to their role as antigens eliciting functional antibodies, it is important to preserve the correct topography of the protein during the process of conjugation to avoid the masking of B-cell epitopes. In this regard, a significant example is the conjugate of the GBS type II (GBSII) PS with the antigenic GBS80 pilus protein, selected by genome-based reverse vaccinology. The GBSII PS was conjugated to the GBS80 protein by catalyst-free strain-promoted azide–alkyne cycloaddition (SPAAC) for glycan–protein coupling, exploiting a tyrosine-selective protein derivatization that enables targeting of predetermined less abundant and less exposed sites of the protein (tyrosine vs.

lysines), ensuring to preserve crucial protein epitopes. The GBSII-GBS80 construct was very effective in eliciting antibodies that recognized the glycan and the protein epitopes individually [113].

1.3.4 The Adjuvant

Adjuvants are immunostimulatory substances generally added to nonliving vaccines to enhance the immunogenicity of antigens. The goal of using adjuvants is to endow potentially weak antigens with the immunostimulatory capabilities of microbial pathogens. Adjuvants are in fact immune potentiators able to boost the immune system through a broad variety of molecular and cellular mechanisms of innate and adaptive immunity to provide a strong response even in less-responding individuals [138]. In addition, they are able to increase the stability and efficacy of the vaccine formulation [139]. Alum, one of the few adjuvants approved for human use, is the only adjuvant authorized for carbohydrate-based vaccines, but its beneficial effect in improving or amplifying the specific response to carbohydrate antigens cannot be generalized. Overall, it is well established that Alum is almost ineffective in plain PS vaccines, while it could act as an immune stimulator in glycoconjugate vaccines. Nevertheless, it is contained only in a few licensed antibacterial glycoconjugate vaccines, such as pneumococcal and anti-*H. influenzae* formulations. Alum is the adjuvant of choice for novel vaccines, thanks to its strong safety profile in all age groups [140]. It is a crystalline substance consisting of phosphate and/or hydroxide salts of aluminum. It induces an immune response by improving the attraction and uptake of the antigen from APCs and triggering innate immunity pathways. Glycoconjugates are often formulated with Alum in the form of Alhydrogel®, an aluminum hydroxide suspension. The vaccine formulation contains the glycoconjugate that is adsorbed at pH 5–7 onto the aluminum salt. Alhydrogel® particles possess a net positive charge at this pH and are well suited for the absorption of negatively charged antigens, like many saccharide epitopes that contain carboxylate or phosphate groups. The final product is stored as a lyophilized powder or as a suspension.

Although explored alternatives to the use of alum in vaccine formulation have been mostly unsuccessful so far, the last great advances in the understanding of the signaling pathways of innate immunity spurred and revitalized the search for new adjuvants [141]. New immune activators, including MF59 and AS03, the monophosphoryl lipid A (MPLA), and the CpG oligonucleotides, have been approved for human use and are active components of licensed vaccines for influenzae, herpes, and hepatitis B. MF59 and AS03 are two similar oil-in-water emulsions based on squalene [142, 143]. MPLA and CpG oligonucleotides are recognized by Toll-like receptors (TLRs), a group of PRRs expressed APCs surface. In particular, MPLA is a Toll-like receptor-4 (TLR4) agonist obtained by detoxification of LPS, while CpG oligonucleotides are characteristic nonmethylated oligonucleotide sequences recognized by TLR9 [144, 145]. Unfortunately, none of them were completely successful in enhancing the immune response in antibacterial conjugate vaccines. A human clinical trial in which the TLR9 agonist CpG 7909 has been co-administered with

antipneumococcal PCV-7 vaccine to HIV-positive patients has shown a positive effect of the adjuvant in improving the titers and the persistence of the vaccine-specific IgG antibody response, but an increased number of mild side-reactions to PCV-7 vaccine have occurred [146]. In another clinical trial, the use of MPLA in co-administration with PCV-9 conjugate vaccine has not enhanced the production of IgG pneumococcal capsular PS antibodies with respect to the use of Alum in the control group [147]. The overall picture says that despite promising results in animal models, it is hard to translate preclinical data to clinic. The most probable explanation lies in the fact that the immunomodulatory effect of adjuvants is better in priming than in boosting the immune response. It is hence very difficult to enhance the immune response in humans that have already been primed or preexposed to the pathogen. Nevertheless, novel adjuvants capable of initiating and enhancing immune responses are under evaluation. The general strategy is to develop/identify agents able to target specific signaling activators of innate immunity (i.e. TLRs) with the purpose of delivering the adjuvant specifically to APCs and/or lymph nodes, thus avoiding unnecessary stimulation of other tissues. In this regard, a novel Alum-TLR7 adjuvant in which the TLR7 agonist SMIP7.10 is adsorbed to aluminum hydroxide has been proven in a mouse model to potentiate, right after the first immunization, the response to glycoconjugate antigens of *N. meningitidis* strains with respect to the addition of the sole aluminum hydroxide [148]. On the other side, a phase I randomized study in healthy adults who had received a MenC conjugate vaccine adjuvanted with a TLR7 agonist adsorbed on aluminum hydroxide (AS37) only highlighted a good safety profile of the new vaccine formulation. A comparison with the control group, vaccinated with a licensed MenC conjugate alum-adjuvanted vaccine, did not reveal any enhancement of the immune response [149]. Alternatively, the adjuvant can be directly covalently linked to the antigen to allow the simultaneous uptake of the two species by the specifically targeted APCs. This is the so-called self-adjuvanting approach, which is mainly explored in antitumor vaccines, even if some studies in animal models about the development of MPLA glycoconjugates have been reported [150]. An overall picture of the research in this context has been recently reviewed [38].

1.4 Technologies Employed for Production of Glycoconjugate Vaccines

As explained in Section 1.2, the T-cell independence of PS antigens calls for the development of glycoconjugate vaccines to improve the immunogenicity of saccharide haptens and ensure protective efficacy in infants and young children. However, despite the huge impact that conjugate vaccines have had on global health over the past 30 years, there are still some limitations to the use of this vaccination approach. For example, some immunogenicity issues still persist for certain groups at high risk, such as the elderly or immunocompromised individuals. Furthermore, continuous variations in global serotype distributions, as well as serotype replacement events, are reported [151, 152], and a constant check of the vaccine serotype

coverage is required, often followed by the need to include new serogroups in already licensed vaccines [47, 48]. Additionally, the massive use of a limited number of protein carriers (TT, DT, and CRM_{197}) may cause the already-mentioned "carrier-induced epitope suppression," hence leading to reduced immunogenicity against the PS hapten [153].

Other relevant issues concern the complexity and manufacturing cost of the traditional, currently licensed glycoconjugate vaccines [154]. They are based on bacterial PSs originated by bacterial growth with an avDP; therefore, they have a heterogeneous composition with inevitable microbial contamination and batch-to-batch variability. Additionally, although the traditional technique is effective and safe, there are significant drawbacks related to the use of PSs derived from bacterial biomass, such as the production cost – mainly due to the need for multiple isolation and purification steps, quality control tests, and arduous physicochemical characterization of the final constructs – and the limited availability of B-cell antigen due to the generally low yields of isolated bacterial PSs. In this regard, innovative strategies are needed to avoid the hazardous handling of pathogens. Thus, there is an urgent demand for new solutions apt to obtain homogeneous and pure bacterial PSs in a safe and cost-effective manner to be used for the manufacture of improved vaccine constructs.

Various methodologies, based on advanced chemical and biochemical approaches, have been thoroughly investigated to overcome the intrinsic limitations of traditional glycoconjugate vaccines.

Chemical and (chemo-)enzymatic synthesis of carbohydrate antigens, glycoprotein bioengineering, use of glycoengineered vesicles, and glyconanoparticles are some of the innovative technologies aimed at optimizing traditional constructs and streamlining the manufacturing processes of glycoconjugate vaccines. In this section, we will describe the state of the art of the diverse technologies employed for the preparation of glycoconjugate vaccines (Figure 1.3), with a focus on the novel approaches explored for a faster production of vaccine constructs with a safer and more efficient profile.

1.4.1 Traditional Glycoconjugates

The traditional approach mainly consists of the conjugation of a PS or OS derived from cultivated pathogens to a carrier protein. As mentioned above, random conjugation leading to cross-linked architectures is usually employed for long sugar chains (PSs, more than 15 RUs), while end-linked chemistry is the preferred approach with shorter sugar chains (OSs, <20 RUs). Currently licensed vaccines are based on random conjugation chemistry (Figure 1.3a), and PSs are usually activated along the biopolymer chain mainly by oxidation of vicinal diols followed by reductive amination or by cyanylation followed by condensation. Periodate oxidation generates carbonyl groups along the chain (e.g. aldehydes from 1,2-diols of sialic acid residues). Oxidated PSs undergo either a direct reductive amination with ε-amino groups of protein lysines, or they are further converted with a bifunctional spacer (see Section 1.3.2) [155–158]. The cyanylation chemistry makes use of cyanogen bromide, recently replaced with the milder and safer 1-cyano-4-dimethylamminopyridinium

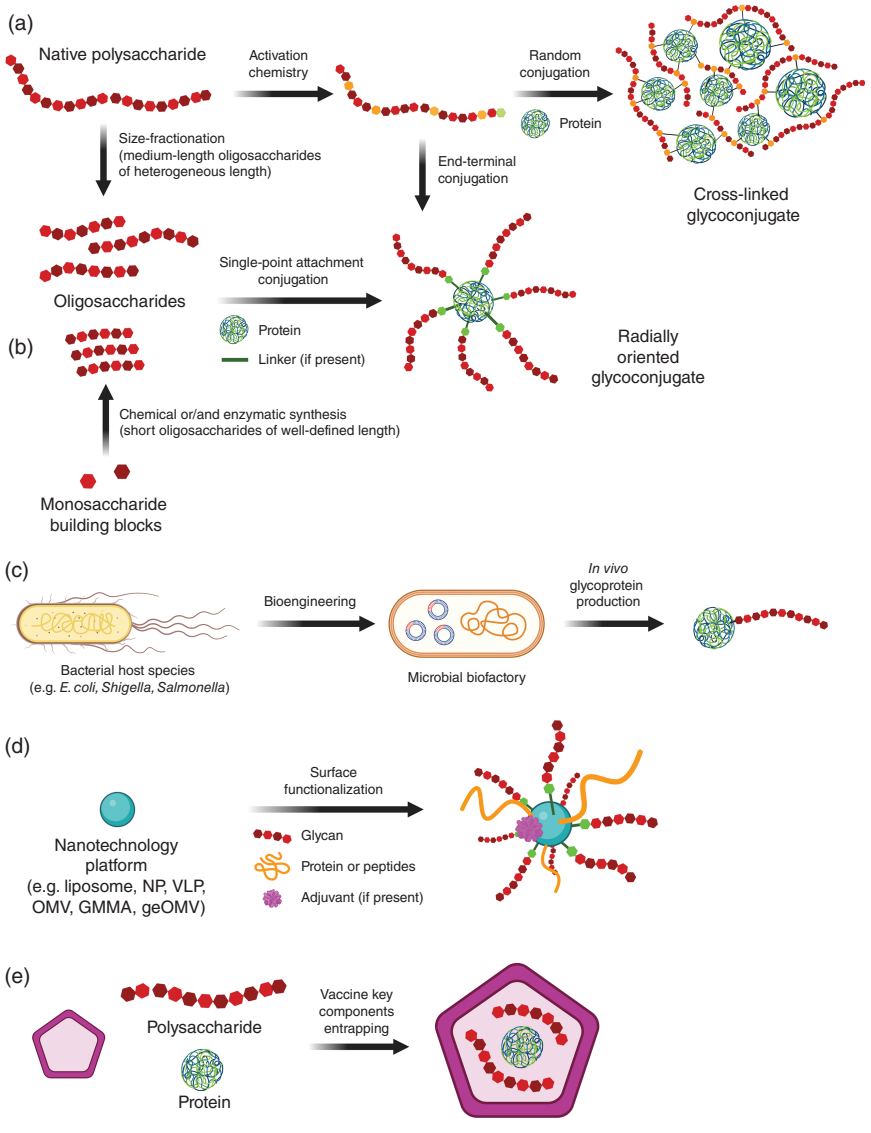

Figure 1.3 Summary of the technologies employed for glycoconjugate production: (a) traditional approaches, (b) chemical and/or enzymatic approach, (c) bioengineered synthesis, (d) nanotechnology-based approach, and (e) noncovalent constructs.

tetrafluoroborate (CDAP), to convert the carbohydrate hydroxyl groups into the corresponding cyanate esters, which are then conjugated with amine or hydrazide groups [159–161]. In selective end-terminal conjugation (Figure 1.3a), native CPSs should be fragmented via hydrolysis (acid or peroxide) and then size-modulated by ultrafiltration or chromatography techniques into shorter and better defined OSs. Shorter saccharides are selectively conjugated by direct reductive amination with ε-amino groups of protein lysine residues or further functionalized with a spacer

prior to protein conjugation. For more details on conjugation techniques, we refer the reader to more specialized accounts [162].

Overall, traditional glycoconjugates prepared by random conjugation are obtained as rather heterogeneous mixtures of compounds with a variety of glycoforms containing a variable number of glycoepitopes and multiple glycosylation sites, making it very challenging to decipher their structure–immunogenicity relationship.

1.4.2 Glycoconjugates Based on Synthetic Carbohydrate Antigens

The use of chemical synthesis to obtain large amounts of saccharide antigens in a reproducible manner, often combined with site-selective protein conjugation chemistry, is considered a cutting-edge strategy for vaccine design [163]. In recent decades, improvements in various methodologies (e.g. automated solid-phase [164], one-pot [165, 166], or chemoenzymatic synthesis [167-169]) have fostered the use of synthetic procedures to attain OS fragments unachievable by microbial fermentation. In addition, chemical synthesis may provide analogs with improved stability [170, 171] or structural hybrids conceived to confer enhanced serotype coverage [172] [173]. Furthermore, synthetic saccharide fragments can be activated more efficiently to maximize the yield of protein glycoconjugation and achieve a controlled and reproducible carbohydrate-to-protein ratio. Compared to structures isolated from natural sources, synthetic OSs are highly pure, homogeneous, and well-defined, hence easier to characterize (Figure 1.3b). In addition, synthetic antigens may be helpful tools to detect the glycoepitopes within a PS chain [174–176].

The first and, so far, only licensed glycoconjugate vaccine based on a fully synthetic OS antigen was developed in 2004 by Vicente Verez-Bencomo and coworkers with the trade name Quimi Hib and is addressed to Hib [177]. The synthetic nature of the Quimi Hib vaccine was a revolutionary breakthrough, demonstrating that it is possible to produce a protective vaccine for human use by large-scale chemical synthesis under Good Manufacturing Practice (GMP) conditions.

Very recently, a semisynthetic glycoconjugate vaccine candidate targeting *Shigella flexneri* 2a has successfully completed preclinical studies and entered the first-in-human clinical trial in healthy adults [178–180]. *S. flexneri* 2a belongs to the *Shigella* species, and it is a major causative agent of shigellosis, a severe intestinal infectious disease considered a serious threat to human health and affecting children below five years of age.

Despite the great potential of synthetic carbohydrate antigens, there are crucial factors that still pose significant hurdles to their use in the construction of glycoconjugate vaccines. First, low-molecular-weight OSs, such as those typically achieved by chemical synthesis, are inherently poorly immunogenic. Moreover, the synthesis of complex carbohydrates is still a challenging process, which needs multistep procedures, including problematic and time-consuming purification of intermediates, leading to relatively low overall yield of the final product. The stereochemical control of the glycosylation steps may be difficult, and in particular the stereoselective formation of certain types of glycosidic linkages is still tricky (e.g. formation of 1,2-*cis*-glycosidic bonds). Optimization procedures are still needed to improve and

simplify the large-scale chemical synthesis of complex PS fragments and to make synthetic carbohydrate antigens appealing enough for manufacture and industrial development.

1.4.2.1 Site-Selective Protein Conjugation

Over the last few years, numerous studies have highlighted that the type of chemistry used for conjugation may influence the efficacy and the immunogenicity of glycoconjugates. Accordingly, site-selective protein conjugation, intended as the preferential glycosylation of a specific set of amino acids over others of the same type present in the protein, has raised the interest of many researchers. In comparison to random approaches, glycoconjugates obtained by site-selective protein glycosylation should be characterized by higher homogeneity and easier physicochemical characterization, thereby accelerating the manufacture and regulatory processes.

Regioselective conjugation techniques comprise conjugation to specific protein amino acids, as well as unnatural residues artificially introduced by protein premodification, and *in vivo* production of glycoconjugates (glycoengineering, described in Section 1.4.4).

Site-selective conjugation of natural amino acids targets predominantly the ε-amino group of lysines, which are common amino acids highly exposed at the surface of the protein and easily accessible in water. Although Lys glycosylation is generally achieved with a random approach, a recent study shows that preferential conjugation of defined Lys residues over others can be attained [181]. In another example, the most solvent-exposed lysine residues of the protein flagellin (FliC) from *P. aeruginosa* were modified by the introduction of azide groups using a diazo-transfer reaction, allowing the glycosylation of the chemically modified protein at predetermined sites [182]. On the other side, cysteine residues can also be reacted regioselectively with a variety of electrophiles and thiophilic agents with very high regioselectivity, thanks to the high nucleophilicity of the sulfhydryl group and to their relatively low abundancy within proteins [37]. In addition, cysteines can be delivered by reductive cleavage of disulfide bridges followed by stapling of the resulting thiol groups by alkylation with electrophilic agents [114]. Recently, a useful protein modification method targeting tyrosine residues has been developed and applied to the construction of glycoconjugate vaccine candidates. In particular, tyrosine residues can be reacted with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) through an ene-like reaction. Using a properly modified PTAD, the protein is provided with a chemical handle regioselectively installed on a few Tyr residues and suitable for coupling with carbohydrate antigens [183].

On the other hand, unnatural amino acids (uAAs, i.e. not naturally encoded) suitable for site-selective conjugation can be installed into proteins by using molecular biology methods [184, 185]. Alternatively, proteins incorporating uAA can be expressed from *E. coli*-derived cell-free extracts [186, 187]. All these methods allow the introduction of diverse uAA at predetermined positions to enable regioselective protein glycosylation [188, 189]. Relevant examples include proteins modified with para-azido-L-phenylalanine [190], para-azidomethyl-L-phenylalanine [191], selenocysteine [192], and an uAA containing the SeH group instead of the SH of cysteine.

Incorporation of uAA-bearing bicyclononyne (BCN) and trans-cyclooctene (TCO) is also a very useful methodology to allow site-specific protein glycoconjugation by Huisgen cycloaddition and inverse electronic demand Diels–Alder [109, 193], respectively.

Many other protocols have been developed for chemical protein modifications, targeting both natural and uAAs, with the aim of obtaining homogeneous glycoproteins with a defined glycosylation pattern [194–198]. A comprehensive account of these methodologies is, however, beyond the scope of this chapter.

We believe that glycoconjugates obtained by site-selective protein modification with fully synthetic saccharide antigens represent the most promising candidates for a new generation of innovative, safer, and more effective carbohydrate-based vaccines.

1.4.3 Enzymatic and ChemoEnzymatic Approach

Enzymatic formation of glycosidic bonds is an attractive method to accomplish regio- and stereoselective linkages in high purity without applying a demanding protecting group strategy. Enzymatic synthesis occurs in aqueous solutions and can be considered a green approach since no toxic contaminants derive from experimental procedures. However, the exquisite substrate specificity still poses a limitation to the use of fully enzymatic synthesis of glycoproteins. Conversely, the combination of chemical and enzymatic methods has been recently developed for faster vaccine manufacture, taking advantage of the *in vitro* production of long PS chains by recombinant capsule polymerases. In principle, chemoenzymatic methods allow to reduce both synthetic and purification steps.

The chemoenzymatic synthesis of *N. meningitidis* serogroup C (MenC), W, and Y PSs was investigated [199, 200], and the specific enzymes needed for these processes have been identified. In particular, Vann and coworkers produced size-controlled MenC antigens using bacterial sialyl transferases [201]. They employed a recombinant sialyltransferase similar to the enzyme CSTII from *Campylobacter jejuni*, which recognizes lactosides as acceptors and is able to transfer multiple sialic acid residues. Lactosides functionalized at the reducing end with a short azido-spacer were sequentially sialylated using CMP-sialic acid as the donor. Polymer elongation was controlled using a CMP-9-deoxy-NeuNAc as process inhibitor. The OSs were then linked to TT protein, and the resulting glycoconjugates were able to elicit specific and protective anti-MenC antibody response [201, 202].

Recently, modern automated synthesis techniques have been employed in this area. In general, two approaches to immobilization are used to investigate the automated synthesis of PSs mediated by enzymes, where either the enzyme or the saccharide substrate is immobilized on the resin/support. The first example of chemical synthesis and enzymatic elongation combined by using an immobilized enzyme was addressed to the production of *N. meningitidis* serogroup X (MenX) OSs [203]. A mixture of the fully synthetic MenX trisaccharide acceptor [204] and the UDP-GlcNAc donor was eluted through a metal affinity column (HisTrap column, Ni Sepharose High-Performance column chromatography) and derivatized with a truncated MenX capsular polymerase (ΔN58ΔC99-CsxA), providing size-controlled

MenX fragments with an avDP of 12 units. Chemoenzymatic MenX-CRM_{197} conjugates were able to raise anti-MenX-protective antibodies. The enzyme is immobilized on the column and can only be removed at the end of the elongation process. A limitation of on-column chemoenzymatic synthesis is that pure OSs can be obtained only after careful purification of the eluates released by the column, which contain a mixture of PS fragments and other reagents. A recent methodology has been developed to overcome this issue. The new protocol has been applied to mammalian glycans and is based on the functionalization of sugar primers with sulfonate tags in order to facilitate the purification process by solid-phase extraction [205].

1.4.4 Bioengineered Glycoconjugates

In recent years, a new approach termed Protein Glycan Coupling Technology (PGCT) was developed, based on the use of a bacterial host species (in particular *E. coli*, *Shigella*, *or Salmonella*) as a biofactory for glycoprotein production (Figure 1.3c) [206]. In the last few years, PGCT has been finding application in the preparation of glycoconjugates for vaccine use. In PGCT, both the saccharide antigen and the carrier protein are biosynthesized and covalently coupled into the bacterial host species. This technique was first reported in the early 2000s, based on the remarkable discovery that the N-linked glycosylation system of *C. jejuni* can be functionally transferred into *E. coli* cells by the N-linking oligosaccharyltransferase (OTase) PglB [207]. In the following years, PGCT techniques employing both N-linking and O-linking OTases were developed and applied to the production of bacterial glycoconjugate vaccines. In particular, the *in vivo* production of glycoconjugates consists of three stages (Figure 1.4a): *glycan expression*, with a genetic locus (or loci) able to encode for glycan

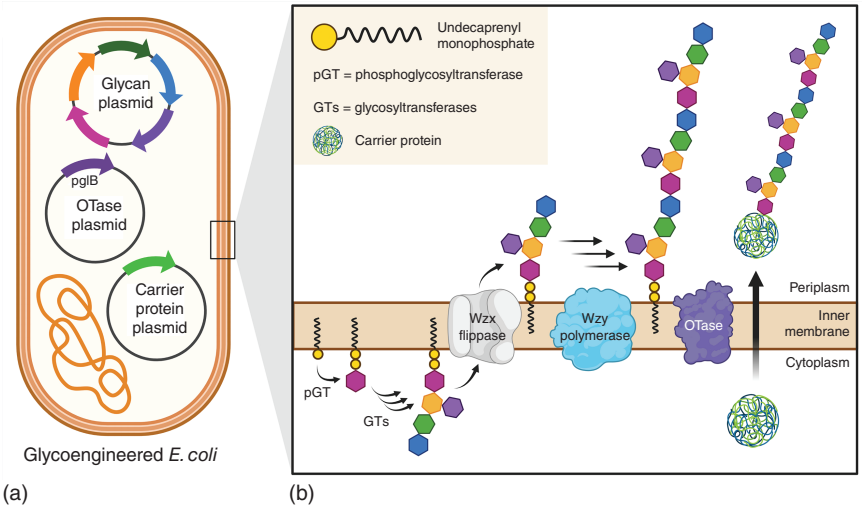

Figure 1.4 The protein glycan coupling technology (PGCT): schematic illustrations of (a) a glycoengineered *E. coli*, the preferred host for vaccine bioproduction, and (b) the biosynthetic Wzy-dependent pathway by which pure glycoconjugates are produced.

biosynthesis; *protein design and expression*, with a plasmid encoding a carrier protein; and *coupling* or *bioconjugation*, provided by an active OTase (N- or O-linking). In the cytosol (Figure 1.4b), a monosaccharide is firstly assembled on the undecaprenyl pyrophosphate (Und-PP) lipid carrier at the level of the inner membrane, and then the Und-PP-monosaccharide is sequentially elongated by glycosyltransferases (GTs).

Once glycan assembly is complete, the Und-PP-linked OS is flipped to the periplasm by a Wzx flippase, polymerized by a Wzy polymerase, and then transferred for final conjugation by the bacterial OTase complex to the acceptor protein. Protein glycosylation usually occurs in two ways, depending on the site for the attachment of the glycan: the Und-PP-linked OS is coupled to a side-chain amide nitrogen atom of asparagines within the Asp/Glu-X1-Asn-X2-Ser/Thr consensus sequence (N-linked, where X1 and X2 can be any amino acid except proline) or to a side-chain hydroxyl of a serine or threonine residue (O-linked).

The N-linking PglB system has been the most employed OTase to produce different bacterial glycoconjugates, such as *S. enterica*, *Shigella* species, *S. aureus* serotypes 5 and 8, *Francisella tularensis*, and extraintestinal pathogenic *E. coli* [208–211], but neither PglB nor PglL systems are able to transfer glycans with glucose at their reducing ends. On the contrary, the newly discovered PglS system possesses this ability, and it has been used for producing Group B *Streptococcus* glycoconjugates [212]. OTases can recognize various lipid-linked OSs and their target carrier proteins. CRM_{197} is a suitable carrier for bioconjugation, although recent PGCT-based vaccine candidates contain engineered *P. aeruginosa* exotoxin A (rEPA) carrier proteins [212] or other antigenic proteins derived from the same pathogen of the glycan hapten. The use of homologous proteins is optimal to confer protection against pathogens with multiple serotypes featuring many different PS structures. A significant example includes the PGCT-mediated conjugation of a *S. pneumoniae* protein antigen (PiuA) with different *S. pneumoniae* CPSs in *E. coli*. The obtained glycoconjugates were proven to stimulate an antibody response comparable to that generated by the commercial vaccine Prevnar-13 [136].

A limitation of the PglB-based PGCT platform is the requirement of a GlcNAc unit at the reducing end of the glycan hapten. The presence of a 2-acetamido group directly linked to the lipid carrier enables GTs and enzymes involved in sugar biosynthesis to transfer the glycan hapten onto the carrier protein [213]. A second structural limitation seems to be related to the sugar residues nearby the lipid carrier, since PglB OTase is unable to transfer an Und-PP-linked OS with a β-(1→4) linkage between the two terminal GlcNAc residues of the saccharide sequence [214]. However, considering the high potential of the technique, the mentioned shortcomings of the PglB-based PGCT platform are under optimization. Recent studies deal with the use of PglB orthologs as alternatives to cover a broader range of glycan structures and the search for other OTases able to recognize different protein sequons for site-selective conjugation. The OTase PglS, capable of transferring OSs containing glucose as the first residue of the growing sugar chain, has been used to develop the first vaccine candidate against hypervirulent *Klebsiella pneumoniae*, where glucose is the residue at the reducing end of K1 and K2 Klebsiella CPSs, the most virulent serotypes responsible for the majority of Klebsiella infections [215].

PGCT is a versatile platform and a promising alternative for the manufacture of a low-cost new generation of glycoconjugate vaccines, and many bioconjugated vaccine candidates are currently in different phases of clinical trials [216].

1.4.5 Nanotechnology-Based Glycoconjugate Vaccines

Various nanotechnology-based strategies have been explored to improve traditional glycoconjugate vaccines. The major advantage of nanotechnology tools is the ability to display carbohydrate antigens in a polyvalent fashion, thereby improving the instauration of multiple and simultaneous protein–glycan interactions, resulting in an increased binding affinity (Figure 1.3d) [217]. Multivalent presentation is particularly relevant for conjugates composed of low-molecular-weight OSs, typically characterized by low binding affinity. Nanotechnology-based vaccines have attracted much interest due to the high versatility of nanocarriers that can be easily functionalized with multicopies of ligands and, in addition, with more than one antigen (e.g. biantigenic NPs). For example, liposomes and gold nanoparticles (AuNPs) are biocompatible delivery systems used as multivalent scaffolds in recent novel antibacterial vaccine prototypes. The saccharide antigen is generally displayed on the surface of the nanoparticles but can also be encapsulated or associated with the nanomaterial.

In this regard, different nanostructured materials, allowing for modular functionalization with B- and T-cell antigens, have been explored and evaluated as carriers for a more efficient delivery of carbohydrate vaccine components, and they are briefly discussed in Sections 1.4.5.1 and 1.4.5.2.

1.4.5.1 Outer Membrane Vesicles (OMVs) and Generalized Modules for Membrane Antigens (GMMA)

Bacteria naturally release membrane vesicles (MVs) during growth through a "membrane blebbing" process in response to a specific external chemical stimulus. MVs are generally spherical lipid membrane nanoparticles with a diameter of 20–400 nm, and they are composed of microbial biomolecules (such as proteins, lipids, and nucleic acids) derived from the parental bacterial species, either Gram-negative [218] or Gram-positive [219]. MVs play different roles both in microbe–microbe interactions (e.g. biofilm formation, antibiotic resistance, nutrient acquisition, microbial defense) and microbe–host interactions (i.e. pathogenesis, communication) [220].

MVs were ignored and defined as insignificant for decades, but in the mid-1990s, it was found that outer membrane vesicles (OMVs) produced by Gram-negative bacteria, especially *P. aeruginosa*, mimic the bacterial surface, displaying various antigens (e.g. LPS, periplasmic proteins, outer membrane proteins, and lipoproteins). Since then, researchers have started to study OMVs in terms of their physiological and antigenic roles, and as delivery systems. Bacteria were genetically modified to increase blebbing and reduce the intrinsic toxicity of the lipid A portion [221], leading to Generalized Modules for Membrane Antigens (GMMAs) secretion [222]. GMMA combines the multivalent display of carbohydrates, favoring B-cell activation, with optimal size for immune stimulation. GMMA also works as self-adjuvants due to the presence of structures acting as agonists of TLRs 2 and 4.

GMMA were tested as novel tools for antibacterial vaccine design since their surface is naturally decorated with *O*-polysaccharide antigens. From a manufacturing point of view, only two steps of tangential flow filtration are needed to obtain GMMA of high purity. The simple and low-cost fabrication process makes these novel nanocarriers attractive for the production of vaccines addressed to developing countries [223]. In recent years, various GMMA-based vaccine candidates have been investigated and, in some cases, they have demonstrated greater serum bactericidal activity compared to classical protein-based glycoconjugates [224–227]. A novel GMMA-based vaccine candidate against *Shigella sonnei*, known as 1790GAHB, was evaluated in Kenya, a *Shigella*-endemic country, and tested in Phase 1 and 2 clinical trials. 1790GAHB was found to be highly immunogenic and well tolerated [228, 229]. Recently, OMVs were engineered in *E. coli* generating glycoengineered outer membrane vesicles (geOMVs). With this technique, both recombinant *O*-polysaccharide biosynthesis and vesciculation occur in the *E. coli* biofactory, and the surface of geOMVs can be decorated with heterologous polysaccharide antigens [230, 231].

Although OMV-based platforms are intriguing tools since they offer a simple and versatile method for low-cost production of antibacterial vaccines, further studies are still needed before they can be translated into viable and well-established vaccine settings approved for human use.

1.4.5.2 Gold Nanoparticles, Liposomes, and Virus-Like Particles

Vaccine protection is restricted to those serotypes or serogroups included in the formulations. However, the emergence of new epidemics requires novel vaccines with flexible composition that are easy to design and manufacture and available at affordable prices for a broader distribution worldwide. Carbohydrate-based vaccine candidates, which may meet these requirements, are based on single-point attachment chemistry, allowing short synthetic OSs to be displayed in a multivalent fashion on nanoparticles of differing conceptions such as AuNPs, liposomes, and virus-like particles (VLPs) [232] [6]. Peptides, oligonucleotides, and lipids are the proper immunogenic carriers for these types of nanoconstructs. Nanotechnology-based approaches exploit preferentially short synthetic glycans instead of long-chain native PSs because, in principle, there is no need for a high number of RUs to efficiently trigger TCR recognition. As mentioned above, the RU of PSs consists of one to a maximum of five (with a few cases extending beyond) monosaccharides, and the TCR-binding site can accommodate short sugar sequences (no more than four/six monosaccharides). The surface of the nanoconstructs allows for the presentation of the short synthetic sugar chains in a multivalent fashion. Another key advantage is that the number and type of ligand(s) loaded on the nanoconstruct can be controlled and modulated, so that NPs can be functionalized with more than one antigen (multiantigenic loading) with fine control of their stoichiometry.

AuNPs were the first nanosystems to be used as scaffolds for a fully synthetic pneumococcal vaccine candidate by Pènades and coworkers [233]. In this pioneering study, mice immunization showed that AuNPs bearing the synthetic RU of

S. pneumoniae serotype 14 (Sp14), together with $OVA_{323-339}$ peptide as T helper antigen, were able to elicit IgG titers even if in a lower amount than the positive control (Sp14-CRM_{197} conjugate). Despite this, AuNPs were proven to be very promising platforms for innovative vaccine development, and this study set the stage for further advancements. More recently, bi-antigenic AuNPs displaying synthetic Sp14 and Sp19F RUs combined on the same nanoparticle with $OVA_{323-339}$ peptide were able to trigger an anti-Sp14 immune response in mice comparable to licensed 13-valent pneumococcal vaccine [234].

Liposomes are nontoxic and attractive nanocarriers for multivalent antigen presentation. These phospholipid vesicles are safe and biocompatible nanotools for the co-delivery of the saccharide antigen(s) (encapsulated into the liposomal carrier) and a T helper entity (protein or antigenic peptide or natural killer T-cell adjuvant), which is exposed on the liposomal surface. In the last decade, liposomal encapsulation of polysaccharides (LEPS) has been widely investigated as an effective alternative strategy targeting pneumococcal diseases. Deng et al. demonstrated that a liposomal anti-Sp14 construct, made by a combination of saccharide and NKT cell antigens, is able to elicit a robust immune response comparable to the one obtained with the licensed vaccine [235]. This strategy was expanded to include multiple pneumococcal strains [236, 237, 238]. Jones and coworkers used the LEPS technique to successfully codeliver two pathogen-related proteins, GlpO (an α-glycerophosphate oxidase) and PncO (a bacteriocin ABC transporter transmembrane protein), together with 20 pneumococcal CPSs [237, 239]. This formulation was re-designed, increasing the CPS valency (up to 24 serotypes) and raising fourfold the dose of PncO as a result of the removal of GlpO protein antigen due to off-target immunogenicity associated with GlpO [238]. Indeed, a nonnegligible number of bacterial proteins in the human microbiome were found to share 50% or higher sequence identity to GlpO.

Mulard and co-workers have developed liposomes coated at their surface with two fully synthetic fragment mimics of *S. flexneri* 2a O-antigen (B-cell epitope) and a universal T helper peptide derived from influenza virus hemagglutinin ($HA_{307-319}$, known as PKY). The lipopeptide Pam_3CAG was used as adjuvant and anchor for the covalent linking of both sugar and peptide antigens to the liposomal surface [240]. These vesicles were able to induce a protective immune response in mice, demonstrating that liposomes are a promising and attractive alternative for the design of novel antibacterial vaccines.

VLPs are supramolecular constructs that mimic the architecture of a virus, preserving its conformational epitope but lacking the viral genome. VLPs are like empty shells able to induce a strong B-cell response by themselves, and they are, therefore, interesting platforms for glycoconjugate-based vaccine development. Bacteriophage Qβ VLPs, as an example, have been functionalized with short synthetic CPS fragments of *S. pneumoniae* serotypes 3 and 14. Qβ can be synthesized in *E. coli* as a self-assembled icosahedral particle and consists of 180 copies of a 132-amino acid monomeric protein [241]. VLP-pneumococcal prototypes were able to elicit protective, long-lived, and serotype-specific IgG antibodies with nM affinity in mice.

1.4.6 Nonprotein-Based Glycoconjugates

It is now a well-established principle that the intrinsically poor immunogenicity of saccharide haptens can be strongly enhanced by their covalent linking to an immunogenic carrier protein. Despite this, novel platforms based on the use of nonprotein carriers have been explored and proven useful for the development of semisynthetic carbohydrate-based vaccines.

The synthetic glycolipid antigen α-galactosyl ceramide (α-GalCer, KRN7000). α-GalCer is an activator of natural killer iNKT cells, which are a very abundant subpopulation of T cells, restricted by the nonpolymorphic CD1d molecule that is expressed by B cells. Recent immunization studies in mice reported that the coupling of *S. pneumoniae* PSs or OSs to α-GalCer gives conjugates able to induce high titers of class switched antibodies with high affinity and specificity for the PS used in the vaccination [235, 242, 243]. Indeed, the use of α-GalCer can offer an alternative approach to the development of a new type of self-adjuvanting glycovaccines where the TD response is evoked by the presence of the glycolipid.

The 1-*O*-dephosphorylated derivative of lipid A, known as MPLA, is a nontoxic glycolipid mimic of the hydrophobic anchor of bacterial LPS and a safe and effective vaccine adjuvant [144, 244]. Notably, Guo and coworkers showed that MPLA derivatives can be chemically linked to OS antigens and used as carrier molecules for the development of nonprotein-based semisynthetic antibacterial vaccines [150, 245, 246]. Short synthetic fragments of α-(2-9)-polysialic acid (the meningococcus C capsula) were conjugated to MPLA and investigated as antimeningococcal vaccine candidates [245]. Tri- and tetra-oligosialic acids linked to the glycolipid through a short spacer elicited a protective immune response comparable to the one induced by licensed protein-conjugate anti-MenC vaccine. In addition, in a recent work, an OS–MPLA conjugate was explored as an antituberculosis vaccine candidate. The authors extensively evaluated the adjuvant properties of MPLA carriers and the importance of the position of antigen conjugation to MPLA (1-MPLA vs. 6′-MPLA conjugates were compared) [246]. In this study, a 6′-amino-MPLA derivative was synthesized and coupled with the upstream tetrasaccharide fragment of lipoarabinomannan (LAM, a major virulence factor of *Mycobacterium tuberculosis*). LAM tetrasaccharide was also linked to 1-OH of MPLA, and the immunological activity of both prototypes was assessed. This study showed that the 6′-N-MPLA–LAM tetrasaccharide conjugate elicited higher IgG titers and is a better immunogen as compared to 1-O-MPLA–LAM conjugate.

1.4.7 Noncovalent Vaccines

In recent years, other noteworthy technologies have emerged for the development of innovative carbohydrate-based vaccines against infectious diseases. Although there are no examples reported in the literature supporting that the co-administration of both key antigenic components is effective [65, 66, 247], novel “covalent linkage free” macromolecular complexes have been investigated (Figure 1.3e). They include

the Multiple Antigen Presenting System (MAPS) and the Protein Capsular Matrix Vaccines (PCMVs), with the aim of further simplifying vaccine manufacturing while preserving the ability to induce durable protection of the host.

Richard Malley and coworkers at Boston Children's Hospital were the first to develop the MAPS platform, where the covalent binding is replaced by an affinity-based coupling between a pathogen-specific protein genetically fused with rhizavidin and a biotinylated sugar antigen [248]. This strategy was first used to develop a pneumococcal vaccine candidate, ASP3772, including 24 pneumococcal CPSs and two pneumococcal proteins. The Phase 2 clinical trial recently conducted to assess the efficacy of the novel formulation in the elderly demonstrated a protective antibody response against both carbohydrate (all 24 PSs) and protein antigens [249]. These intriguing results open up the prospect of possible competitiveness with the well-established Prevnar multicomponent vaccine series (see Table 1.1), since MAPS platforms can optimize pneumococcal vaccine production, reducing production costs. Several MAPS platforms, conceived for including the most challenging bacterial pathogens, are currently under development by Affinivax Inc., a biopharmaceutical company in Cambridge, Massachusetts [250].

In 2015, Matrivax (a biotechnology company in Boston) [251] developed a cross-linked polymer matrix embedding the sugar antigen with the carrier protein in collaboration with John J. Mekalanos (Harvard Medical School) [252]. More recently, this "virtual conjugate" approach was found to be a viable alternative to classical conjugate vaccines to confer protection from typhoid fever [253]. Immunological data from a Phase 1 clinical study demonstrated that Typhax vaccine, made of Vi sugar antigen entrapped in a cross-linked CRM_{197} matrix, was able to induce IgG levels comparable to, and even better than, the commercial vaccine currently in use (Typhim Vi) [254].

1.5 Conclusion

Vaccination is a simple, safe, and effective way of protecting individuals against harmful, often life-threatening, infectious diseases, and there is no doubt that it has been one of the greatest revolutions of modern medicine. After the provision of adequate sanitation facilities and safe drinking water, the development of vaccines provided one of the most significant contributions to human health, leading to an increase in life expectancy from around 40 years up to almost double. Over the past century, vaccines were able to defeat devastating infectious diseases, in some cases until their complete eradication (smallpox), and for this reason, vaccination is considered by the WHO to be the most cost effective of all potential prevention strategies.

In this regard, carbohydrate-based vaccines hold a prominent role. They are classified as subunit vaccines based on the concept that only a component of the pathogen rather than the entire microorganism is sufficient to raise a specific immune response capable of inducing long-term protection of the host. The introduction of glycoconjugate vaccines represented a further fundamental advancement. They are

based on CPSs or LPS OAg (for bacterial pathogens), as well as any other glycan uniquely expressed on the surface of the microorganism (viruses, fungi, parasites, or even tumor cells) and covalently linked to an immunogenic protein carrier. Glycoconjugate vaccines allow to overcome the intrinsic limitations of vaccines based on plain PSs that are poorly immunogenic in infants and young children (under two years of age), in the elderly, and in immunocompromised individuals due to the T-cell independence of unconjugated carbohydrates. Conversely, protein conjugation ensures a T-cell memory response and boosts effect of the vaccine, conferring persistent immunological memory and durable immune protection even in high-risk groups.

Interestingly, the fundamental milestones that marked the history and development of carbohydrate-based vaccines have been reached with an empirical approach. The tremendous advances in immunology and glycobiology of the last few decades, however, provided new insights to steer the immune response and to design innovative vaccine settings devoid of the side effects and drawbacks which typically distinguish the traditional, currently licensed glycoconjugates.

New technologies, including emerging techniques for the analysis of surface PSs structures and to decipher carbohydrate–protein interactions, became available and have been explored to produce in high yields more homogeneous glycoconjugates based on well-defined carbohydrate structures in order to simplify and accelerate the manufacture and regulatory processes. Notably, the use of synthetic chemistry is gaining an increasingly prominent role, fueled by the recent great progresses and achievements in the chemical synthesis of complex carbohydrates. The synthetic approach has indeed demonstrated that even short, but well-designed OSs can act as protective antigens. Accordingly, glycoconjugates obtained by site-selective protein modification with synthetic saccharide antigens are among the most promising candidates for a new generation of innovative, safer, and more effective carbohydrate-based vaccines. In addition, chemoenzymatic approaches, nanotechnology-based platforms, and glycoengineering techniques are highly promising outlooks to conceive novel vaccine candidates able to confer enhanced protection and broader serotype coverage.

Finally, a challenge for the near future is the development of novel glycoconjugates intended for infant vaccination to prevent future pandemic diseases. In particular, an additional effort is needed to develop economically viable technologies which will enable to introduce novel glycoconjugate vaccines (safer, easily scalable, flexible, and thermostable) at affordable prices into the market of LMIC.

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