Università degli Studi del Piemonte Orientale

"Amedeo Avogadro"

Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche

Dottorato di Ricerca in Biotecnologie Farmaceutiche ed Alimentari

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"Exploring carbohydrates antigens conjugated to pathogen-related protein carriers"

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Alberto Nilo

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Contents

Introduction

General principles of vaccination

The immune responses of mammals can be divided into innate and adaptive immunity. Innate immunity fights intruding pathogens in a fast, yet non-specific way and can be considered a first line of defense. Onset of adaptive immunity instead is delayed, but all responses are directed specifically against the respective intruder. A multitude of pathogen-specific cells are created by clonal expansion of few, "fitting" pre-cursor cells. And, once infection is resolved, some of these cells survive in the body to form immunologic memory ready to fight the same pathogen faster and more efficiently, should a second encounter occur. Vaccination takes advantage of this potential of adaptive immunity. The principle of vaccination is that exposure to a small sample of a disease-causing microorganism - or to a part or portion of it - teaches the immune system to rapidly recognize the menace and to create memory that enables the body to fight the real pathogen efficiently during a later encounter. Vaccination basically mimics a natural infection without causing disease. Several are the components of host immune responses that are set in motion following vaccination. Important vaccine-induced immune effectors are antibodies produced by B lymphocytes. These antibodies are capable to recognize and specifically bind to a toxin or a pathogen (or to a portion representative of it)[1]. Antibody binding interferes with pathogen entry into host cells or other important functions. Moreover bound antibodies facilitate uptake and elimination of the pathogen by other immune cells. Other important immune effectors are cytotoxic CD8+ T lymphocytes (CTL) that may limit the spread of infectious agents by recognizing and killing infected cells or by secreting specific antiviral cytokines. The generation and maintenance of both B and CD8+ T cell responses is supported by growth factors and signals provided by CD4+ T helper (Th) lymphocytes, which are historically subdivided into T helper 1 (Th1) and T helper 2 (Th2) subtypes, and more recently also in T helper 17 (Th17). These effector cells are controlled by regulatory T cells (Treg) that are involved in maintaining immune tolerance.[2] Most of the antigens and vaccines trigger both B and T cell responses, and the two responses benefit of their interconnection: CD4+ T cells are required for most antibody responses, while antibodies exert significant influences on T cell responses to intracellular pathogens[3]

Early protective efficacy of a vaccine is often conferred by microorganism-specific antibodies. But just triggering a specific antibody response is not sufficient. The quality of such antibody responses, e.g., antibody avidity, has been identified as a determining factor of efficacy. Although B lymphocytes

represent the specialized lineage in antibody production once differentiated in plasma cells, T cells can largely contribute to effective and long-lasting immune responses. In fact, these cells are essential to immune memory, and novel vaccine targets have been identified against which T cells are likely to be the prime effectors.

The stimulation of antigen-specific T cell responses requires their activation by specific antigen presenting cells (APCs), essentially dendritic cells (DCs), which reside in tissues or patrol through the body and are recruited by inflammatory signals to the site of infection. When exposed to pathogens, immature DCs will phagocytose and process them proteolytically, so that some pathogen components can be loaded on MHC class II molecules. DCs then undergo maturation, modulate specific surface receptors and migrate towards secondary lymph nodes, where the trigger of T and B cell responses occurs.

The central role for mature DCs in the activation of vaccine responses reflects their unique ability to provide both antigen-specific and co-stimulation signals to T cells. In fact, T cells require at least two signals to become fully activated: i) a first antigen-specific signal, is provided through the T cell receptor which interacts with the antigen peptide-loaded MHC II molecules on the surface of the APCs; ii) a second antigen-unspecific signal, is a co-stimulatory incentive and is provided by the interaction between co-stimulatory molecules expressed on the membrane of the APC upon maturation with its counterparts on the T cell. APCs can also secrete cytokines that are sensed by the interacting T cell and determine its further phenotype (Th1, Th2, Th17).

The very first requirement to elicit vaccine responses is thus to provide sufficient stimuli through vaccine antigens and eventually adjuvants, to trigger an inflammatory reaction that is mediated by cells of the innate immune system[4]. In addition, the antibody response to bacterial polysaccharides is poorly affected by adjuvants, IgM represents the major class of antibodies induced and since their immune response does not induce memory, it is not boosted by subsequent immunizations.

These characteristics are due to the fact that, unlike proteins that are T-cell dependent (TD) antigens, polysaccharides are T-cell independent (TI) antigens in that they do not require T-cell activation for the induction of specific B-cell (antibody) responses. Polysaccharide antigens directly activate polysaccharide- specific B cells which differentiate then into plasma cells to produce antibodies, but memory B cells are not formed; moreover a pre-existing memory B-cell pool can be depleted by immunization with unconjugated polysaccharide, with risk of hypo-responsiveness on subsequent immunizations[5].

Differently from polysaccharides, proteins are TD antigens; following interaction with antigen-presenting cells (APC) like dendritic cells, macrophages and B-cells, protein antigens are internalized and processed into small peptides which are then re-exposed and presented to T lymphocytes in association with the major histocompatibility complex (MHC) class II molecules. Interaction with T cells induces B cells to differentiate into plasma cells and memory B cells, thus initiating downstream adaptive immune responses. Unlike TI antigens, TD antigens are immunogenic early in infancy, the immune response induced can be boosted, enhanced by adjuvants, and is characterized by antibody class switch and production of antigen-specific IgG.

Polysaccharide Vaccines

Polysaccharides are important virulence factor especially for encapsulated bacteria that present on their surface complex carbohydrate structures. Surface polysaccharides have several functions: a) in some cases they protect microorganisms from desiccation when they are exposed to the external environment -an example is the hyaluronic capsule of group A *Streptococcus*, whose adhesive properties help the pathogen to invade the host; b) other capsular polysaccharides prevent the activation of the alternate complement pathway; c) sometimes they mimic molecules produced by human cells so that the pathogen is not recognized as foreign by our immune system (serogroup B meningococcal capsular polysaccharide, hyaluronic acid).

Around 1930s the protective role of antibodies (Abs) induced by pneumococcal polysaccharide started to be investigated and in 1945 the first vaccine composed by purified polysaccharide from selected pneumococcal serotypes was tested in humans[6].

The research on vaccines development was subsequently slowed down by the introduction of antibiotics, however with the emergence of drug resistant strains the development of polysaccharide vaccines started again and a number of them have been studied in large clinical studies. Polysaccharide vaccines against meningococcus serogroup ACWY, *Streptococcus pneumoniae* and *Haemophilus influenzae* type be were licensed between the seventies and the eighties.[7,8] These bacteria possess polysaccharides which are polymers formed by one monosaccharide unit (homopolymers) or more complex oligosaccharide repeats (heteropolymers), that can be charged or neutral.

Polysaccharide vaccines however did not completely solve the problem of bacterial diseases caused by encapsulated microorganisms. Being T-cell independent antigens, one of their main features which emerged from clinical trials, was their scarce immunogenicity in children less than two years of age.[9,10] As a consequence, polysaccharide vaccines can be used in adults, but not in infancy and elderly which are the most sensitive target populations.

Glycoconjugate Vaccines

Glycoconjugate vaccines are among the safest and most efficacious vaccines developed during the last 30 years. They are a potent tool for prevention of life-threating bacterial infectious disease like meningitis and pneumonia.

The limitation of polysaccharides vaccines has been overcome by covalent conjugation to a carrier protein as source of T-cell epitopes. Since 1929 it has been demonstrated by Avery and Goebel that non immunogenic sugars after conjugation to a carrier protein become able to induce antibodies in animal model.[11] However the first application of this concept to a vaccine for human use started only in 1980 with the development of the first conjugate vaccine against *Haemophilus influenzae* type b (Hib) that was later on licensed vaccine between 1987 and 1990.[12,13]

Many other glycoconjugate vaccines have been developed against bacterial pathogens such as *Neisseriae meningitidis*, *Streptococcus pneumoniae* and group B *Streptococcus*. Today glycoconjugate vaccines are among the safest and most efficacious vaccines developed during the last 30 years and they are currently used in the immunization schedules of different countries like for example the UniteStates (US).[14-20]

Vaccine indication	Type of conjugate	Manufacturer
Haemophilus influenzae type b	PRP-TT	Sanofi-Pasteur
	PRP-OMPC	Merck
	PRP-CRM197	Pfizer
	Hib-CRM197	Novartis Vaccines
Haemophilus influenzae type b/Neisseria meningitidis group C	MenC/Hib-TT	GSK
Neisseria meningitidis serogroups $ACW_{135}Y$	MenA-TT	Serum Institute India
	MenC-CRM197	Pfizer, Novartis Vaccines
	MenC-TT	Baxter
	MenACWY-DT	Sanofi-Pasteur
	MenACWY-CRM197	Novartis Vaccines
	MenACWY-TT	GSK
Streptococcus pneumoniae serotypes	7 valent-CRM197 (4, 6B, 9V, 14, 18C, 19F, 23F)	Pfizer
	13 valent-CRM197 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F)	Pfizer
	10 valent-DT/TT Protein D (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F)	GSK

Table 1: Licensed glycoconjugate vaccines.

Chemical conjugation of polysaccharides to protein carriers allows processing of the protein carrier by polysaccharide-specific B cells and presentation, on their surface, of the resulting peptides or in association with MHC class II. Further interaction with carrier-specific cells then induces a TD response already early in life which leads to immunological memory and boosting of the response by further doses of the vaccine (Fig. 1). Recently it has been proposed a novel mechanism according to which internalization of glycoconjugate and following proteolytic digestions generate glycopeptides which are re-exposed by MHCII (Fig. 2) . This model was confirmed by isolation of specific T-cell

clones directed to the sugar. This observation proves that although glycoconjugates are safe and effective, their mechanism of function is still not totally understood.

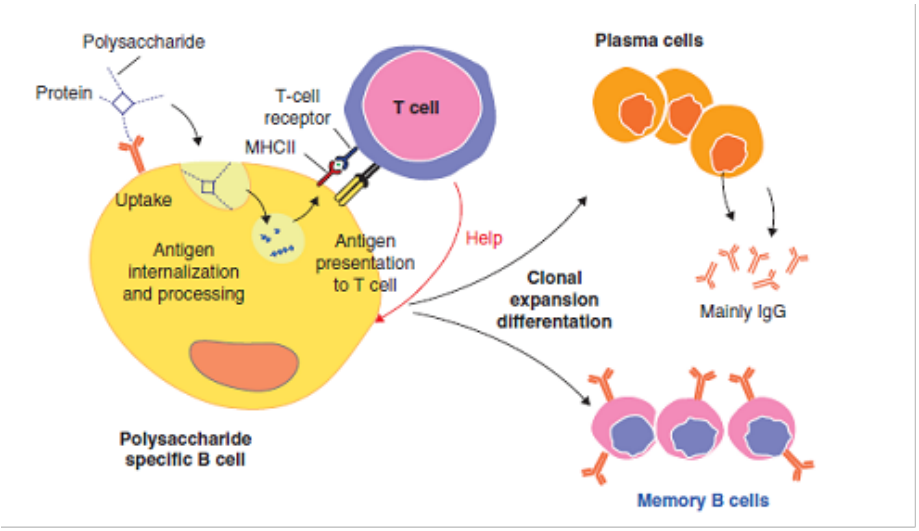

Fig. 1 Mechanism of action of a glycoconjugate vaccine

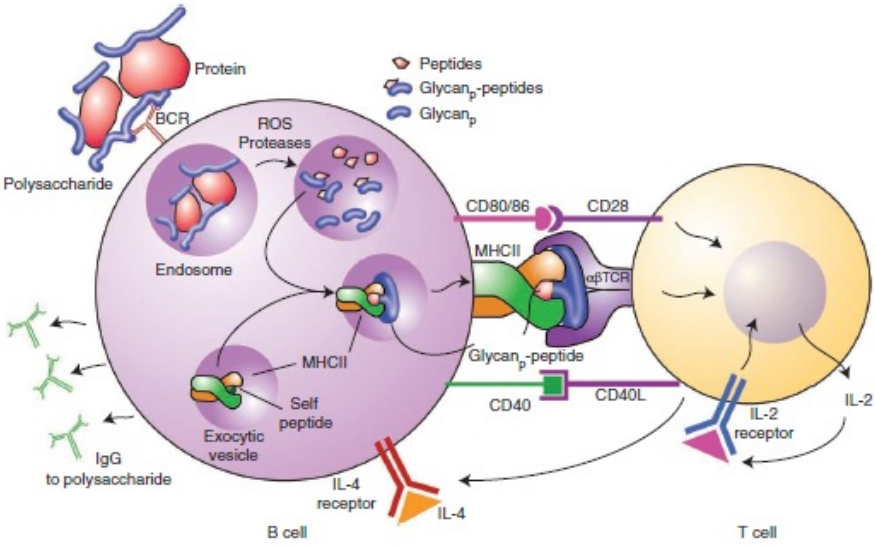

Fig. 2 Recent working model of action of a glycoconjugate vaccine

Chemistry of glycoconjugate vaccines

In glycoconjugate vaccines, a covalent linkage between the carbohydrate moiety and the carrier protein has to be installed. Approaches based on different chemistry of conjugation, have been so far used. One is based on the random chemical activation of the hydroxyl or carboxyl groups of saccharide chain followed by covalent binding with Lys residues, onto glutamic or aspartic residues of the carrier protein. These are usually the most abundant amino acid residues on protein surface, and the regioselectivity of conjugation is hard to achieve. As a consequence these vaccines are effective, but two limitations can be found: a) certain degree of batch-to-batch variability in the immunological properties can be present. b) it has not been possible to apply precise structure-immunogenicity relationships and use the classic medicinal chemistry approach to investigate vaccine candidates.

Recently different methods to target specifically some residues for bioconjugation are emerging. In general, three main approaches for site-selective incorporation of carbohydrates onto proteins can be described[21] (1) chemical ligation-based strategies, which rely on the reaction of two unprotected peptide moieties bearing a C-terminal thioester and a N-terminal cysteine; (2) chemoenzymatic transformations ("remodeling") of glycoproteins with endoglycosidases or other glycan processing enzymes; (3) site selective conjugation to natural and unnatural amino acid residues, such as homoallylglycine, S-allylcysteine or Se-allylselenocysteine, azidoalanine presenting chemical groups, which can react under the mild conditions present in biological systems (aqueous media, mild pH, and temperature). Combination of oligosaccharide synthesis and novel site specific methods may enable an accurate antigen design and a more precise correlation between the glycoconjugate structure and the immunological properties.[22]

Among these methods, targeting tyrosine residues by chemical manipulations has appeared very attractive. Proteins usually present much less tyrosine than lysine, glutamate or aspartate residues, and they are also often buried. The accessible tyrosine residues on protein surface are usually few, thus the regioselectivity of conjugation can be expected. Recently a reliable tyrosine-selective conjugation method via triazolidinone derivatives in tris buffer has been developed.[22] The method enabled the

insertion of alkyne-containing bifunctional linkers onto the tyrosine residues of the genetically detoxified diphtheria toxin CRM_{197}. The subsequent condensation of a synthetic β-glucan by Cu(I) catalyzed azide-alkyne [3+2] cycloaddition (CuAAC)[23,24] enabled the creation of an anti-candidiasis vaccine.[25]

This strategy was proven a powerful method to obtain a robust structure-immunogenicity relationship from glycoconjugates with defined sugars at predetermined sites. In addition, it has been shown that the triazole generated by the cycloaddition is either moderately or non-immunogenic and does not affect the anti-carbohydrate response. Therefore, this conjugation approach appears a robust approach for site-selective incorporation of sugars at predetermined sites of the protein.

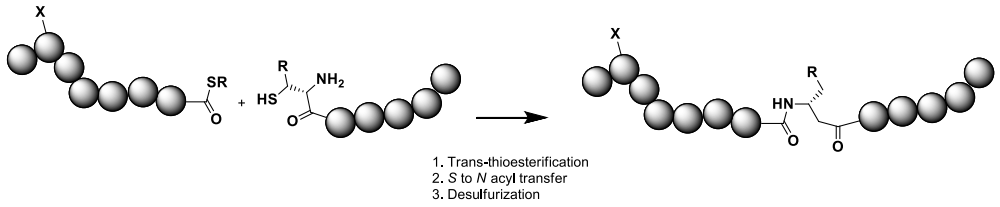

Fig. 3 Chemical ligation based preparation of glycoconjugates

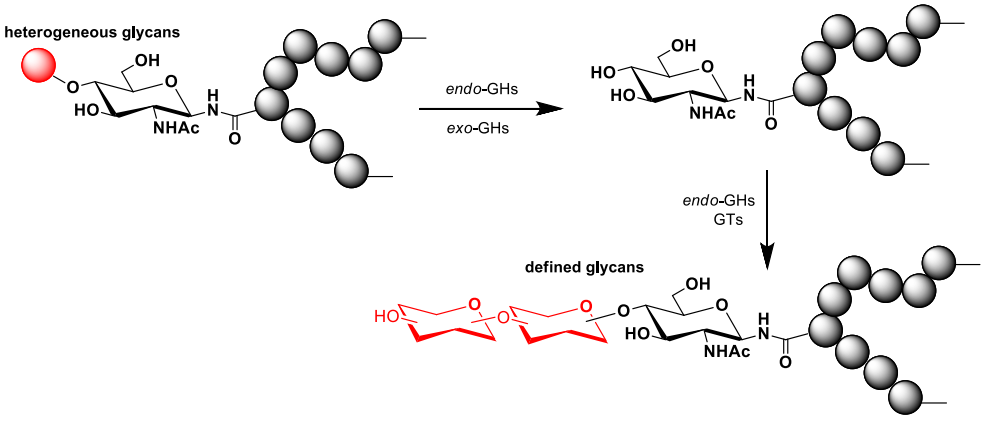

Fig. 4 Chemoenzymatic remodeling of glycoproteins

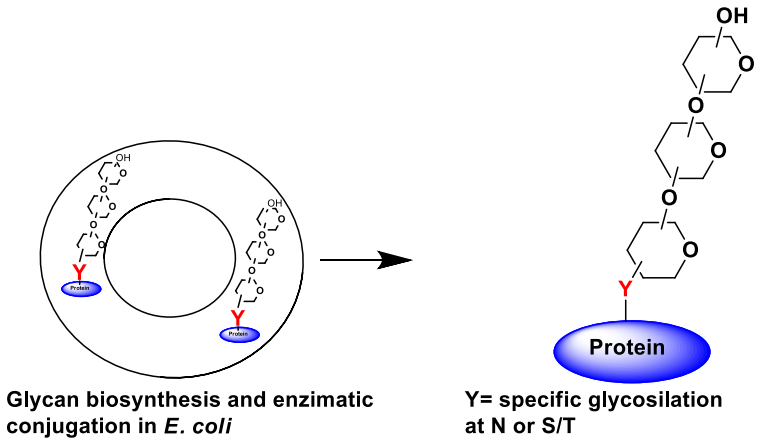

Fig. 5 Glycan biosynthesis and enzymatic conjugation in E.coli and site selective ligation

GBS

Streptococcus agalactiae, also known as group B Streptococcus (GBS), is one of the major causes of sepsis, pneumonia, and meningitis in neonatal and infants in the first three months after birth.[26] It is a Gram positive beta-hemolytic coccus which appears in pairs (Fig.6) or chains[27], that colonizes the urogenital and gastrointestinal tracts of more than 30% of the healthy population and, in particular, the vagina of 25-40% of healthy women.[28-30]

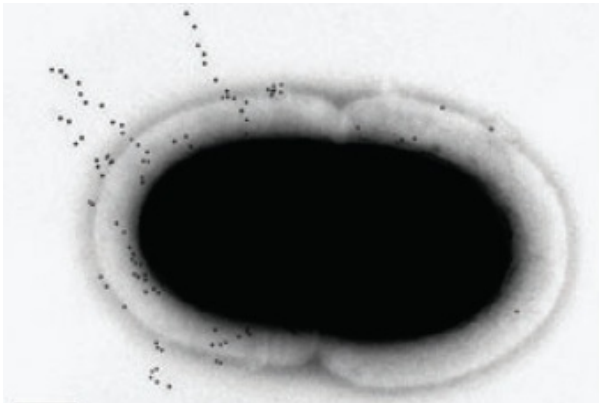

Fig.6 Immunogold Electron Microscopy of GBS strain CJB111*(Rosini, Rinaudo et al. 2006)*.

It was classified for the first time in 1930 by R. Lancefield and R. Hare during a serological differentiation study on human isolates and other groups of hemolytic streptococci.[31] It is included in

the group B of "Lancefield System", where Streptococci are identified with alphabetical letters from A to O, based on capsular polysaccharide antigens and groups A, B and D are the most dangerous[32]. GBS clinical isolates are also classified into ten serotypes, according to the chemical nature of capsular polysaccharides (PS): Ia, Ib, II, III, IV, V, VI, VII, VIII and IX.[33-35] However around 8-14% of the clinical isolates in the Europe and USA are non-typeable strains because cannot be distinguished on the basis of PS antigenicity. All GBS serotypes contain, in different combinations, these carbohydrates: galactose, glucose, rhamnose, N-acetylglucosammine and sialic acid (N-acetylneuraminic acid) (Fig. 7)
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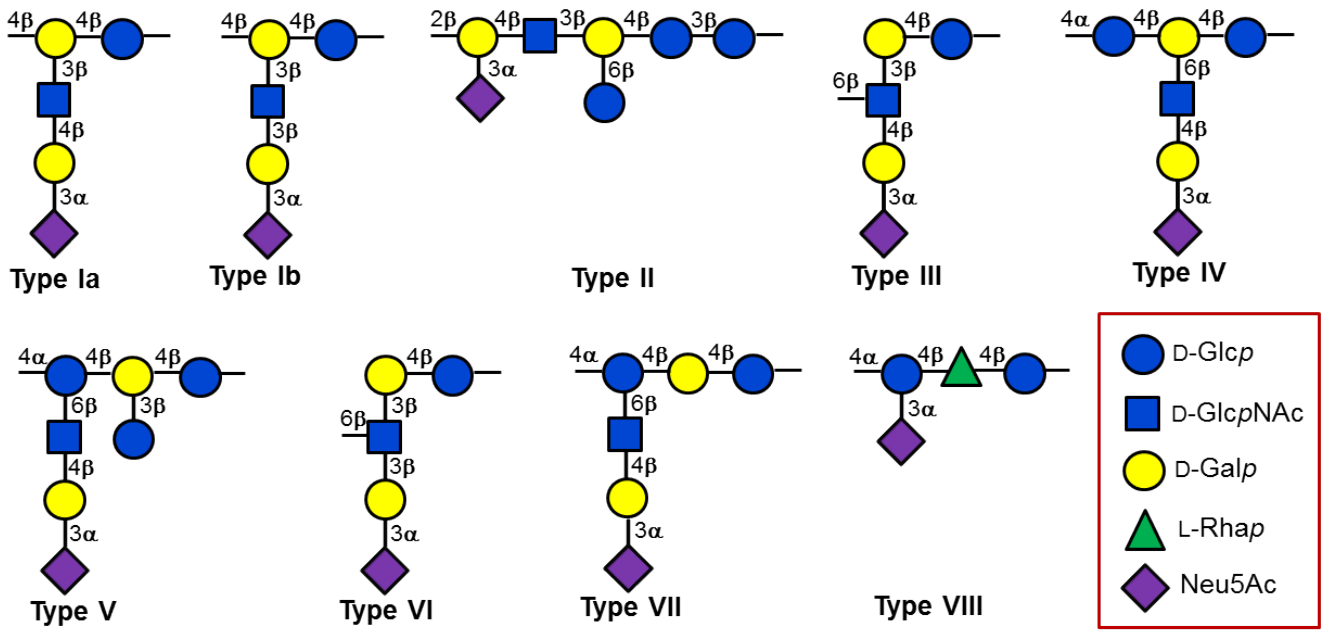

Fig. 7 Chemical structures of isolated GBS serotypes

Streptococcus agalactiae is known as the most frequent cause of sepsis, pneumonia and meningitis in neonates within first months from birth.[36, 37] It is an etiologic agent also in adults, in particular elderly persons, immunocompromised, diabetics, and patients affected by others chronic pathology of liver and kidney. In the cellular hyper-trophism of vaginal epithelium, where it can adheres despite low values of pH[38], it forms a permanent colonization in more than 40% of women. During pregnancy, the uro-genital tract colonization is cause of severe infections/year, leading to chorioamnionitis, endometrial tenderness, tachycardia, cystitis and fever which make necessary antibiotic therapies also after delivery. GBS can also be cause of intrauterine death, aborts, prelabor rupture of the membranes, with preterm labor/delivery, causing the exposure of 90% of premature neonates to infection. In most cases infection

is transferred vertically from asymptomatic mothers and, every year, only for US, 8000-12000 infected babies and 2000 deaths are recorded. Infection appears in uterus or at delivery for contact of throat, ear, nostril, umbilicus and respiratory ways with amniotic liquid or infected vaginal fluids. After the aspiration/ingestion of bacteria, neonatal lungs become the starting focus of infection. From here it can rapidly arrive to flow of blood, continue towards others tissues and organs like blood-brain barrier (Fig.8).

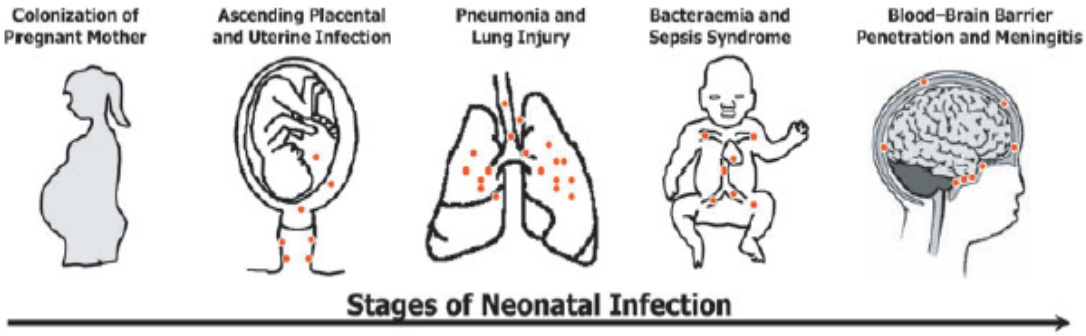

Fig.8 Stages of neonatal group B streptococcal (GBS) infection *(Doran and Nizet 2004)*.

Neonatal disease can occur in two different forms: the **Early-Onset Disease** (EOD) and the **Late-Onset Disease** (LOD).

EOD occurs during the first seven days of life, with the vast majority of cases (approximately 90%) present during the first 24 hours of life.[39] Neonates with EOD present respiratory disease (54%), sepsis without focus (27%) and meningitis (15%).[40] Risk factors include: GBS bacteriuria during pregnancy; gestational age less than 37 weeks; previous infant with invasive GBS disease; preterm labor/delivery; prelabor rupture of membranes; birth weight less than 2500g; black race and teenage mothers.

LOD occurs beyond seven days of life and can develop up to three months of age. In 50% of the cases it has a maternal origin. Neonates with LOD present sepsis (46%), meningitis (37%), urinary infection (7%), osteoarthritis (6%), respiratory disease (4%) and cellulitis (4%). Over 20% of survivors of GBS meningitis have permanent sequelae, including hearing loss, mental retardation, cortical blindness and seizures (Tab. 2).

Tab. 2 Neonatal manifestation of group B streptococcal disease *(Shet and Ferrieri 2004)*.

	Early-onset disease	Late-onset disease
Onset	First week of life (usually within the first 24 h)	One week to 3 months of age
Clinical presentation	Respiratory distress Pneumonia Sepsis	Sepsis Meningitis Osteoarthritis
Incidence of prematury	Increased	No change
Maternal obstetrical complication	Frequent (70%)	Uncommon
Transmission	Vertical: acquired *in utero* or intrapartum	Usually horizontal transmission: can also be intrapartum
Predominant serotypes*	Ia, III, V	III, Ia, V

*In descending order of prevalence

Risk factors include non-white race and preterm birth, but most of time it is an horizontal transmission, after contact with sanitary staff or colonized mothers. LOD is basically due to serotype III strain, with an incidence until 60% depending on the geographic area. Studies performed on non-pregnant adults with GBS associated invasive disease revealed that GBS serotypes Ia, III and V accounted for more than two-third of cases. More than 25% of the subjects had invasive GBS disease caused by type V strains (Tab. 3).

Table 3 Serotype distribution of group B streptococcal isolates from non-pregnant adults with invasive GBS infection, 1992-1999 *(Edwards and Baker 2005)*.

GBS serotype	N° (%) of subjects (n=589)
Ia	143 (24.3)
Ib	72 (12.2)
II	70 (11.9)
III	97 (16.5)
IV	2 (0.3)
V	162 (27.5)
VI	0
VII	0
VIII	1 (0.2)
Non-typeable	42 (7.1)

The clinical manifestations of GBS infection in elderly adults are: skin and soft-tissue infections and in these cases, cellulites is the most frequent clinical manifestation, urinary tract infections (bacteremic urinary tract infections account for 33.4% of the cases in adults >70 years of age), pneumoniae (only in older debilitated adults), bacteremia with no identified focus (in the 15% of non-pregnant adults affected by GBS invasive diseases), arthritis, osteomyelitis, meningitis (in only 2% of non-pregnant adults with GBS invasive disease) and endocarditis.

GBS Vaccine

Early in 1930s Rebecca Lancefield *et al.* demonstrated that polyclonal antibodies from rabbit sera, able to recognize PS epitopes, conferred protection against GBS infection in animal models. During the last two decades, plain GBS polysaccharides have been extensively studied as vaccines in preclinical and human clinical studies. However, the first human clinical trials conducted in the 1980s showed that the purified native PS from serotype III was not sufficient to induce an robust IgG response in adults and insignificant in neonates.

Subsequently, conjugation of PSs to immunogenic proteins, such as tetanus toxoid (TT) and mutated diphtheria toxoid (CRM_{197}),[41,42] was shown to dramatically increase the immune response in children,

eliciting the differentiation of memory cells associated to a long term protection. Following these findings, PS-TT conjugate vaccines based on nine GBS serotypes were produced and tested pre-clinically.[41] These studies, carried out in animal models, showed that conjugate antigens were able to induce functional PS-specific IgG that, in presence of complement, stimulate *in vitro* the opsonization and killing of GBS by human peripheral blood leukocytes. This success constituted the rationale to proceed with the clinical studies in human. Further studies demonstrated that glycoconjugate vaccines constituted by serotypes Ia, Ib, II, III, and V PS linked to TT were safe, well-tolerated and highly immunogenic in human adults,[42] but cross-protection between serotypes was still lacking. Therefore a multivalent vaccine was required in order to obtain a broad coverage of the vaccine against the prevalent circulating GBS serotypes. A tetravalent combination of PS –TT conjugates (serotypes Ia, Ib, II, and III) was successfully tested in a mouse model and further human trials were performed using the combination of two PS TT-conjugates (serotypes II and III). Results showed that the combination had the same immunogenicity and reactogenicity of each monovalent PS vaccine.[42]

However, although recent epidemiological studies[43] suggest that a tetravalent combination of serotypes Ia, Ib, III and V would be sufficient to achieve a coverage against the majority of GBS strains circulating in Europe and North America, there are other geographical areas where such combination would be not be effective owing to a different serotypes distribution (i.e. VI and VIII, predominant in Japan). Moreover, the PS-conjugate vaccine would not protect against all the non-typeable isolates.

An additional obstacle to the licensure of vaccine against GBS is the difficulty of conducting clinical efficacy trials in human: large sample size would be required, but the use of IAP reduces the incidence of neonatal disease. A possible solution to overcome this difficulty came from the studies of Feng-Ying C. Lin and coworkers who carried out a prospective way to estimate the maternal GBS-PSs antibody levels needed to give protection to neonates from EOD. The amount of maternal antibodies against GBS-PSIa was measured by ELISA in 45 case patients (mothers whose neonates developed EOD) and 319 controls (mothers of neonates colonized by GBS-PSIa but without EOD). Distribution of maternal antibody concentrations showed that the probability of developing EOD declined with increasing maternal levels of anti-PSIa IgG.[44] This work demonstrated that it is possible to define thresholds of anti-GBS PSIa specific IgG levels in the mothers which are predictive for the protection of newborns.

More recently, thresholds have also been set for the levels of maternal anti GBS-PSIII IgGs required to protect newborns from EOD caused by GBS serotype III.[45] The results of Feng-Ying C. Lin suggested that complex clinical trials required for a GBS vaccine registration, might be replaced with an *in vitro* correlate of protection based on the quantification of vaccine induced antibodies.

Pilus protein-based vaccine

The protection induced by a polysaccharide based is limited to strains expressing the same CPSs included in the vaccine formulation. For this reason the development of a cross-protective protein vaccine is another relevant option. In order to develop a serotype independent universal GBS vaccine, several efforts have been focused on the identification of highly protective protein antigens. Unlike CPS antigens, proteins antigens are able to induce protective T-cell-dependent antibody responses and long-lasting immunity and conjugation to carrier molecules (i.e. TT or CRM_{197}) is not necessary. Since antibodies directed against surface antigens can interfere with bacterial virulence factors and can promote complement dependent opsonophagocytosis, they are considered excellent vaccine candidates. Before 2005, only a few GBS protein antigens have been identified as potential vaccine candidates; these include Rib, the alpha and beta subunits of the C protein, Sip and the C5a peptidase proteins. However, these proteins, with the exception of C5a peptidase and Sip, are either not expressed on all strains or are highly variable in different isolates.[46] The C-protein complex was able to induce passive protection against GBS infections in an animal model.[47] Further studies also showed that the C-protein complex could be one of the factors that confer resistance to opsonization.[48] Rib is a surface protein with a similar structure and sequence to the alpha subunit of the C protein and it is also able to induce protective immunity.[49] Unfortunately, the alpha subunit of the C protein is present in the genome of only approximately 50% of clinical, while Rib is present in the genome of all serotype III strains, but not in other serotypes. Moreover, both these proteins (the alpha subunit of the C protein and Rib) contain repeated sequences that show strain-to-strain variations and that can affect their immunogenicity. Sip (surface immunogenic protein) is a surface GBS protein that was identified after immunological screening of a genomic library. Sip has been identified in GBS strains of every serotype and the sip gene is highly conserved among GBS isolates.[50] The protection conferred by the Sip protein was determined by a mouse neonatal infection model. Newborn mice were protected against infections of GBS strains of serotypes Ia, Ib, II, III, and V.[50] Furthermore, it has been observed that sera collected from pregnant women and their healthy newborns have Sip antibodies. The C5a peptidase is a serine-protease localized on the surface that inactivates the human C5a a factor produced during complement activation. C5a is highly conserved surface-bound protein that is expressed on the surface of all serotypes of both group A (GAS) and group B streptococcus.[51] The GBS C5a peptidase (ScpB) is 98% identical in sequence to that expressed by GAS. It has been shown that C5a peptidase is a protective

antigen, and could be also used as a carrier protein for type III polysaccharide vaccine. Up to 1995, when the first complete microbial genome sequences became available, vaccines has been classically developed by isolating, inactivating and injecting the cause of the infection. These traditional approaches are time-consuming and expensive. In 1995, a new era named "the genomic era", began chancing completely the approach for vaccine development. The microbial genome sequencing,[52] provided a new impulse to the vaccinology field. A new approach named Reverse Vaccinology, based on integration of several techniques such as genomics, bioinformatics, and molecular biology.[53]

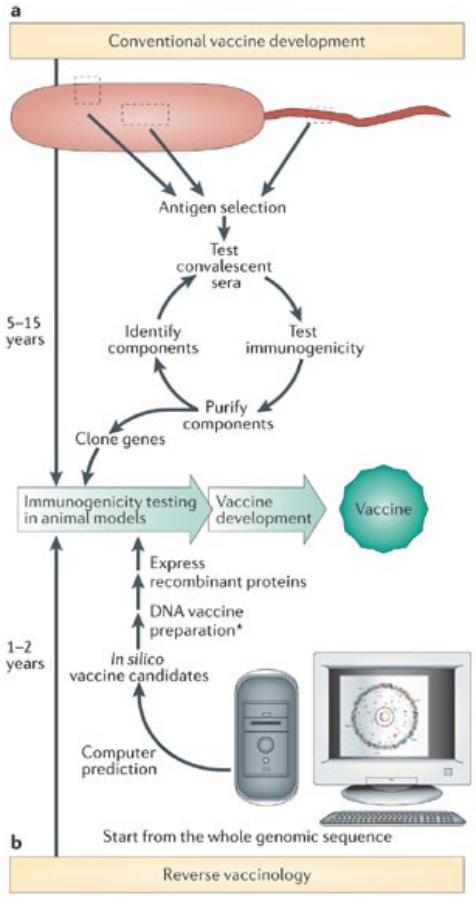

Fig. 9 Comparison between conventional approach and Reverse Vaccinology *(Johri, Paoletti et al. 2006).*

Unlike the conventional vaccine approaches, the reverse vaccinology, permits the identification of less common, low expressed and/or not expressed in vitro antigens and can be applied also on non-cultivable microorganisms. On the other hand, it can be applied only on the discovery of proteins antigens but not to others antigens such as lipopolysaccharides and glycolipids.[54] Application of the reverse vaccinology approach to the development of vaccine against GBS commenced from the

sequencing of the complete genome of a virulent GBS strain (2603v/r, serotype V). However, a Comparative Genome Hybridization (CGH) analysis showed that the genetic variability within the GBS isolates was too high and represented a limit for the identification of vaccine candidates. From this analysis it was evident the need to include genome sequences of more serotypes for the selection of protein antigens. In order to study the genome variability in GBS, Tettelin and coworkers sequenced the genome of 6 GBS strains which represent the most disease-causing serotypes (serotype Ia strains A909 and 515, type Ib strain H36B, type II strain 18RS21, type III strain COH1 and type V strain CJB111). By a comparative analysis of all available genomes, the new sequenced genomes plus the two already published genomes, it was possible to identify two subgenomes: the "core genome" and the "variable genome", together defined as "pan genome".[55] The "core genome" includes genes present in all the strains and is around the 80% of each genome and contains all genes necessary for the basic biology of the bacteria, on the other hand, the "variable genome" is responsible for strain diversity and represents the part of genes that is dispensable and unique to each strain.

The surface availability of the antigens to antibody recognition is a prerequisite for a good protective immune response. Maione and coworkers searched within the GBS pangenome for the genes coding for putative surface-associated and secreted proteins. Using this approach around 589 putative surface proteins were selected, among them 396 belonged to the core genome and 193 were variable genes. The proteins containing more than three trans-membrane domains were excluded because of the difficulties predicted for their production in *E. Coli*. By using a high-throughput cloning and expression approach, 312 of the selected GBS genes were successfully expressed in *E. coli*. Each of the genes was cloned with sequences coding for either an N-terminal 6XHistidine Tag or for a C-terminal glutathione S-transferasetag and the expressed proteins were purified by affinity chromatography. All the 312 purified recombinant GBS antigens were tested by an active maternal immunization/neonatal pup challenge mouse model of GBS infection. Briefly, the antigens were used to immunize female mice with a three dose immunization schedule. After the last immunization, mice were mated and their pups were challenged, within 48 h after birth, with a lethal dose of GBS. The survival of the neonates was monitored for 3 days and immune sera were collected for in vitro analysis. Immunoblot assays were used for the identification of the natural protein in GBS total protein extracts, while flow cytometry assays were carried out to confirm the surface exposure of the antigens. From this first systematic screening four antigens were identified as capable of significantly increasing the survival rate among challenged infant mice. When the four antigens were mixed and administered simultaneously, an almost universal protection was achieved against challenge model using a panel of

strains comprehensive of the most pathogenic GBS serotypes. In particular, the levels of protection reached were similar to those achieved using the polysaccharides-based vaccines. Only one (SAG0032) of these four antigens was part of the "core genome", and this protein was the already described Sip protein. The other three antigens named GBS67 (SAG1408), GBS80 (SAG0645) and GBS104 (SAG0649) were present in the variable portion of the subgenome. The major outcome of this study was that the combination of these antigens, each effective against overlapping populations of isolates, was able to confer broad serotype-independent protection. Characterization studies revealed that three of these antigens (GBS67, GBS80 and GBS104) were component of pilus-like structure (Fig. 10).[56]

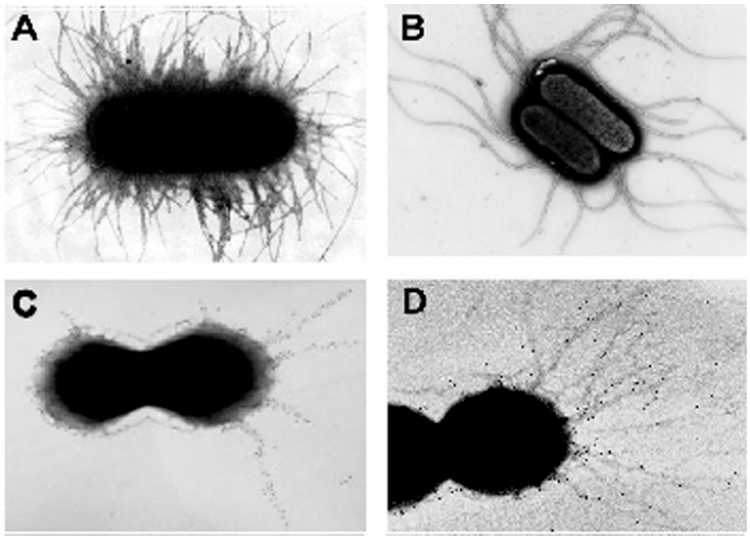

Fig.10 Examples of pilus-like structures in Gram-negative and Gram-positive bacteria. Electron micrographs of pili in Gram-negative organisms: E. coli (A) and Salmonella enterica (B). Electron microscopy of two different types of pili in Gram-positive bacteria: fimbrie in Streptococcus salivarius (C) and pili in Streptococcus agalactiae (D) *(Rosini, Rinaudo et al. 2006)*.

In the 1970s Brinton *et al* had already showed that pilus-based vaccines induced protection in humans against gonococcus and against enterotoxigenic E.*coli*.[57] Genome sequence analysis of GBS revealed three independent loci named Pilus Island 1 (PI-1) and Pilus Island 2a and 2b (PI-2a, PI-2b) which encode structurally distinct pilus types. In fact, Pilus Island 2b (PI-2b) is an allelic variant of PI-2a that, has a similar genetic organization to PI-1 and PI-2a, but varies substantially in gene sequence (Fig. 11).

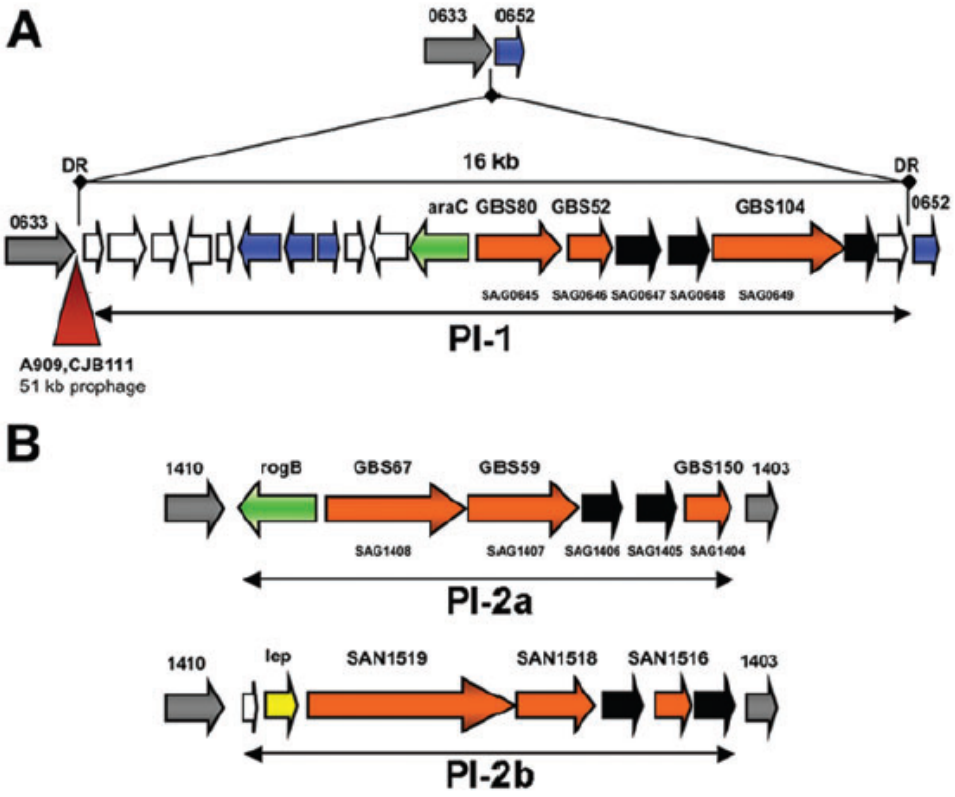

Fig.11 Schematic representation of GBS pilus island regions (A. PilusIsland 1; B. PilusIsland 2) *(Rosini, Rinaudo et al. 2006).*

The overall gene corresponds to the major pilus subunit (backbone protein [BP]) and the two ancillary proteins (AP1 and AP2). BP and AP1 were shown to elicit opsonophagocytic antibodies able to protect mice in the active maternal immunization model. From the studies of Maione *et al*. and later of Rosini *et al*., it was demonstrated that at least 2 of the 3 pilus structural components, the BP and the AP1, encoded by the PI-1 (respectively GBS80 and GBS104) and by the PI-2a (respectively GBS59 and GBS67) were able to induce protective immunity against GBS infection in mice. More recently, Margarit and coworkers showed that all GBS strains carry at least one of the three Pilus Islands, thus a vaccine containing one component of each pilus is capable of providing a high level of protection against different strains (Fig.12).[58]

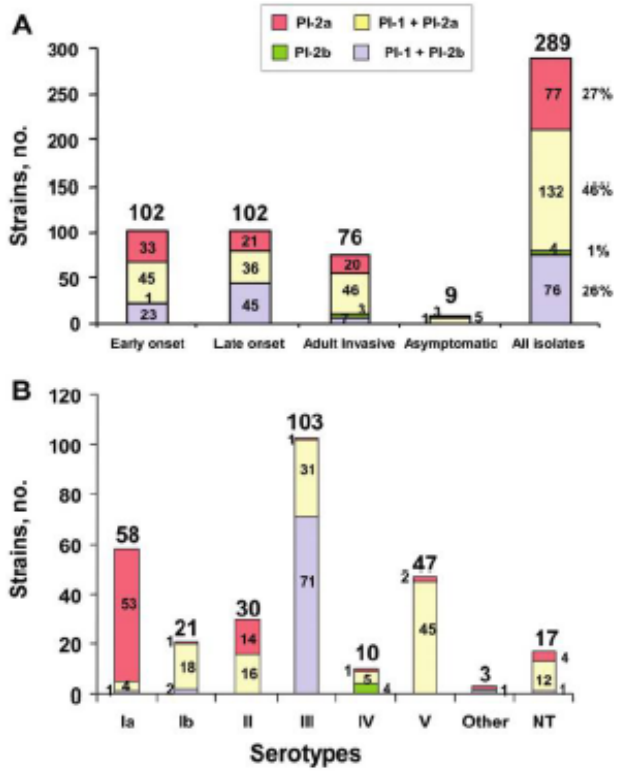

Fig.12 Distribution of pilus islands among 289 GBS isolates grouped by disease type (A) and capsular polysaccharide serotype (B). NT = non-typeable*(Margarit, Rinaudo et al. 2009)*.

Two backbone proteins of PI-1 (GBS80) and PI-2b (GBS1523) and the ancillary protein of PI-2a (GBS67) were identified as potential antigens to include in a final GBS vaccine formulation, in order to increase the coverage of a polysaccharide-based vaccine.[58]

Clostridium difficile

Clostridium difficile is a gram-positive anaerobic bacterium able to infect either humans or animals and commonly found in the environment. It was isolated for the first time in 1935 from the intestinal flora of neonates and was initially considered a normal non-pathogenic resident of the gut. Only in 1970s was *C. difficile* identified as one of the agents responsible for antibiotic- related diarrhea and pseudomembranous colitis.[59-63]

C. difficile infection (CDI) has grown tremendously since 1978, and over the last decade, the incidence and severity of CDI has increased significantly and affected new patient groups. Today, the disease represents a major social and economic burden. Since 2005, CDI has been increasingly reported among young, healthy individuals residing in the community. An estimated 20% to 28% of CDI is community

associated with an incidence of 20 to 50 cases per 100,000 populations in the United States, Sweden and England. At the moment, there is no vaccine against *C. difficile*, despite the increase in the incidence of the disease observed in the last decades. The efficacy of anti-CDI vaccines based on the administration of formalin inactivated toxoids A and B has been described for over 3 decades. This vaccine consists of formalin-detoxified toxins A and B obtained by purification from culture of VPI 10463, which is a hyper-productive strain for both toxins. The use of toxoid-based vaccines in humans has been limited for a long time, despite several studies in animal models having demonstrated the importance of toxin immunity in preventing the lethal outcome of CDI. To overcome the safety issues associated with the large-scale production of toxoids, such as exposure to toxins and spores, Donald and colleagues have recently proposed a novel recombinant toxoid-based candidate vaccine consisting of genetically modified TcdA and B produced in a non-sporulating strain of *C. difficile* lacking the genes for the native toxins. Although site-directed mutations abrogate cytotoxicity linked to the glucosyl- transferase activity of the toxins, a residual toxicity was observed which has been prevented by formalin treatment. This genetically and chemically detoxified recombinant vaccine induced functional antibodies in the hamster model and conferred a partial protection to lethality. A phase I clinical trial of this vaccine is currently ongoing. Polysaccharides coating the surface of bacterial pathogens represent an optimal target for eliciting carbohydrate specific antibodies. Glycans are T cell independent antigens, but they can be turned into molecules able to evoke a T cell memory response following conjugation to a carrier protein. This strategy has found application in the prevention of many deadly infectious diseases. Consequently, great attention has been directed in the recent years to the structural analysis of polysaccharides on the surface of *C. difficile* with the result of identifying 3 glycan structures, named PSI, PSII, and PSIII. Following the discovery of PSII, it was not clear whether PSII was part of a capsule or a surface glycoprotein, or released to the external surface of the bacterium. Antibodies against the conjugated PSII detected the polysaccharide at the surface of the bacterial vegetative cells, thus confirming this molecule as a target for a carbohydrate based vaccine. However the sugar coating was not as thick and uniformly distributed as expected for a capsule. Therefore, it can be hypothesized that PSII is expressed by the bacterium either as cell wall-linked polysaccharide not bound to peptidoglycan or as a conjugate with lipoteichoic acids.

Outline of the thesis

In the last ten years a considerable effort in developing more effective vaccines for the prevention of bacterial infectious diseases has been made. Conjugation to protein carrier is a well-established approach to trigger a T-cell-dependent response against poorly immunogenic microbial carbohydrates. During my PhD I investigated the use of proteins, which are antigens *per se*, as carrier for carbohydrate antigens. We have explored the possibilities of using protein which are 1) bacterial surface antigens or 2) toxins.

In this first case, we have employed GBS pili proteins, which by the "reverse vaccinology" approach have been demonstrated important structures for bacterial adhesion and invasion, and also powerful antigen against GBS infections. In this context we exploited GBS80 pilus protein as antigen and carrier for GBS type II polysaccharide (PSII) in order to broaden the vaccine coverage. Site-selective bioconjugation methods represent potent tools to generate novel therapeutic proteins, chemical biology probes, or to engineer targeted delivery systems and bionanomaterials. While classic procedures for conjugation relies on the random reaction of the with the carrier protein, new efficient methods for site selective conjugation of glycans are emerging. These methods may represent important tools to investigate the effect of the conjugation sites on the immunological properties of glycoconjugate vaccines. Hence, the impact of coupling site on the protective epitopes of GBS80 has been evaluated. Accordingly, as first step we developed a method for efficient tyrosine-directed coupling of small glycans and large polysaccharides to proteins via copper-free click chemistry. Then glycoconjugates from GBS pili proteins and polysaccharides were prepared using a classic random conjugation strategy and the novel method for tyrosine-directed ligation. The capability to elicit anti-carbohydrate and anti-protein antibody titers that induce opsonophagocytic killing of strains expressing exclusively PSII, or GBS80 was evaluated. In addition, survival of new born mice against GBS infection following vaccination of the mothers with the different glycoconjugates was compared.

In another study of my PhD we evaluated the efficacy, in naive mice model, of PSII glycoconjugates where recombinant toxins A and B fragments (TcdA_B2 and TcdB_GT respectively) were used as carriers. Both glycoconjugates were evaluated for the proficiency at inducing anti-PSII IgG titers and retain the functionality of TcdA_B2 and TcdB_GT toxin fragments.

References

1. Cooper NR, Nemerow GR. The role of antibody and complement in the control of viral infections**.** *J Invest Dermatol 83:*121s–127s*,* **1984**
2. Bacchetta R, Gregori S, Roncarolo MG. CD4+ regulatory T cells: mechanisms of induction and effector function. *Autoimmun Rev 4:*491–496*,* **2005**
3. Igietseme JU, Eko FO, He Q, et al. Antibody regulation of T cell immunity: implications for vaccine strategies against intracellular pathogens. *Expert Rev Vaccines 3:*23–34*, **2004.***
4. Hoebe K, Janssen E, Beutler B. The interface between innate and adaptive immunity. *Nat Immunol 5:*971–974*,* **2004**
5. Costantino,P.; Rappuoli, R.; Berti, F. The design of semi-synthetic and synthetic glycoconjugate vaccines *Expert Opin. Drug Discov.2011, 6 (10),* 1045-1066.
6. Macleod, C.M.; Hodges, R.G.; Heidelberg, M.; Bernhard, W.G. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides, *J. Exp. Med,* **1945***, 82,* 445-465.
7. Artenstein, M.S.; Gold, R.; Zimmerly, J.G. Prevention of meningococcal disease by group C polysaccharide vaccine. *N Engl J Med,* **1970***, 282,* 417-20.
8. Gold R.; Artenstein, M.S.; Meningococcal infections. 2. Field trial of group C meningococcal polysaccharide vaccine in 1969-70. *Bull World Health Organ* **1971***, 45,* 279-82.
9. Peltola, H.; Makela, H.; Kayhty, H.; Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age. *N Engl J Med,* **1977***,297,* 686-91.
10. Peltola, H.; Kayhty, H.; Sivonen, A.; Makela, H. Haemophilus influenzae type b capsular polysaccharide vaccine in children: a double-blind field study of 100,000 vaccinees 3 months to 5 years of age in Finland. *Pediatrics,* **1977***, 60,* 730-7
11. Avery,O.T.; Goebel, W.F. Chemo-immunological studies on conjugated carbohydrate-proteins : ii. Immunological specificity of synthetic sugar-protein antigens. *J Exp Med,* **1929***, 50,* 533-50.
12. Schneerson, R.; Barrera, O.; Sutton, A.; Robbins, J.B. Preparation, characterization, and immunogenicity of Haemophilus influenzae type b polysaccharide-protein conjugates. *J Exp Med,* **1980***, 152,* 361-76.

13. Anderson, P.W.; Pichichero, M.E.; Insel, R.A.; Vaccines consisting of periodate-cleaved oligosaccharides from the capsule of Haemophilus influenzae type b coupled to a protein carrier:structural and temporal requirements for priming in the human infant. *J Immunol,* **1986***, 137,* 1181-6.
14. Costantino, P.; Viti, S.; Podda, A.; Development and phase 1 clinical testing of a conjugate vaccine against meningococcus A and C. *Vaccine,* **1992***, 10,* 691-814.
15. Eby, R. Pneumococcal conjugate vaccines. *Pharm Biotechnol,* **1995***, 6,* 695-718.
16. Ravenscroft, N.; Averani, G.; Bartoloni, A. Size determination of bacterial capsular oligosaccharides used to prepare conjugate vaccines. *Vaccine* **1999***, 17,* 2802-16.
17. Lakshman, R.; Finn, A. Meningococcal serogroup C conjugate vaccine. *Expert Opin Biol Ther ,* **2002***, 2,* 87-96.
18. Paoletti, L.C.; Kasper, D.L. Glycoconjugate vaccines to prevent group B streptococcal infections. *Expert Opin Biol Ther,* **2003***, 3,* 975-84.
19. Snape, M.D.; Perrett, K.P.; Ford, K.J. Immunogenicity of a tetravalent meningococcal glycoconjugate vaccine in infants: a randomized controlled trial. *JAMA,* **2008***, 299,*173-84.
20. Bardotti, A.; Averani, G.; Berti, F. Physicochemical characterisation of glycoconjugate vaccines for prevention of meningococcal diseases. *Vaccine,* **2008***, 26,* 2284-96.
21. Adamo, R., Nilo, A., Castagner, B., Boutureira, O., Berti, F., and Bernardes, G. J. L.; **2013** Synthetically defined glycoprotein vaccines: Current status and future directions. *Chem. Sci. 4,* 2995−3008.
22. Hu, Q.-Y., Allan, M., Adamo, R., Quinn, D., Zhai, H., Wu, G., Clark, K., Zhou, J., Ortiz, S., Wang, B., Danieli, E., Crotti, S., Tontini, M., Brogioni, G., and Berti, F. Synthesis of a well-defined glycoconjugate vaccine by a tyrosine-selective conjugation strategy. *Chem. Sci., DOI: 10.1039/C3SC51694F*
23. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B., A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective Ligation of Azides and Terminal Alkynes. *Angew. Chem., Int. Ed.* **2002***, 41,* 2596-2599.
24. Tørnoe, C. W.; Christensen, C.; Meldal, M., Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *J. Org. Chem* **2002***, 67,* 3057-3064

25. Adamo, R.; Martin, A.; Berti, B.; Danieli, E.; Hu, Q.-Y., Tyrosine ligation process and crosslinking agents used to form tyrosine-contg. polypeptide conjugates for raising an immune response in a mammal 2013, *PCT Int. Appl. (2013), WO 2013009564 A1 20130117.*
26. Lauer P, Rinaudo CD, Soriani M, Margarit I, Maione D, Rosini R, Taddei AR, Mora M, Rappuoli R, Grandi G, Telford JL: Genome analysis reveals pili in Group B Streptococcus. *Science.* **2005** *Jul 1; 309(5731):105.*
27. Facklam, R: What Happened to Streptococci: Overview of Taxonomic and Nomenclature Changes ;*CLINICAL MICROBIOLOGY REVIEWS, Oct.* **2002***,* p. 613–630 *Vol. 15, No. 4*
28. Dillon, HC; Gray, E; Pass, MA and Gray, BM Anorectal and Vaginal Carriage of Group B Streptococci During Pregnancy; *THE JOURNAL OF INFECTIOUS DISEASES; VOL. 145, NO.6.* **1982**
29. Schuchat, A ;Epidemiology of Group B Streptococcal Disease in the United States: Shifting Paradigms; *Clinical Microbiology Reviews,July* **1998***;* 497-513
30. Hansen,S ; Uldbjerg, N ; Kilians, M ; Sorensen,UBS ;Dynamics of Streptococcus agalactiae Colonization in Women during and after Pregnancy and in Their Infants; *JOURNAL OF CLINICAL MICROBIOLOGY,* **2004***,* p. 83–89
31. Lancefield, R. C. **1934** A serological differentiation of specific types of Bovine Hemolytic Streptococci (Group B), *J. Exp. Med. 59,* 441-458
32. Lancefield, R.C. and Hare , R.:The Serological Differentiation of Pathogenic and Non-Pathogenic Strains of Hemolytic Streptococci from Parturient Women
33. Wagner, M; Wagner, B; Kubin, V:Immunoelectron Microscopic Study of the Location of Group-specific and Type-specificPolysaccharide Antigens on Isolated Walls of Group B Streptococci; *Journal of General Microbiology* **1980***, 120,* 369-376.
34. Kong, Gowan et al. **2002**: Serotype Identification of Group B Streptococci by PCR and Sequencing; *Journal Of Clinical MIrobiology;* p.216-226
35. Slotved, Kong et al. **2007** : Serotype IX, a Proposed New Streptococcus agalactiae Serotype ; *JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2007,* p. 2929–2936
36. McCracken 1973: Group B streptococci: The new challenge in neonatal infections
37. Schuchat, A.:Epidemiology of Group B Streptococcal Disease in the United States: Shifting Paradigms; *Clinical microbiology reviews July* **1998** p.497-513

38. Tamura, Kuypers et al. 1994: Adherence of Group B Streptococci to CulturedEpithelial Cells: Roles of Environmental Factors and Bacterial Surface Components; *INFECTION AND IMMUNITY, June* **1994**, p. 2450-2458
39. Garland, S. 1991:Early Onset Neonatal Group B Streptococcus Infection: Associated Obstetric Risk Factors
40. Yagupsky, Menegus et al. **1991**; The changing spectrum of Group B Streptococcal disease in infants : an eleven-year experience in a tertiary care hospital
41. Paoletti, L. C., Wessels, M. R., Michon, F., Difabio, J., Jennings, H. J., and Kasper, D. L. **1992** Group-B Streptococcus Type-II polysaccharide-Tetanus Toxoid conjugate vaccine, *Infect. Immun. 60,* 4009-4014.
42. Paoletti, L. C., Peterson, D. L., Legmann, R., and Collier, R. J. **2001** Preclinical evaluation of group B streptococcal polysaccharide conjugate vaccines prepared with a modified diphtheria toxin and a recombinant duck hepatitis B core antigen; *Vaccine 20,* 370-376.
43. Harrison, Elliott et al. **1998**; Serotype Distribution of Invasive Group B Streptococcal Isolates in Maryland: Implications for Vaccine Formulation; *The Journal of Infectious Diseases 1998;177:*998–1002
44. Lin FY, Philips JB 3rd, Azimi PH, Weisman LE, Clark P, Rhoads GG, Regan J, Concepcion NF, Frasch CE, Troendle J, Brenner RA, Gray BM, Bhushan R, Fitzgerald G, Moyer P, Clemens JD; Level of maternal antibody required to protect neonates against early-onset disease caused by group B Streptococcus type Ia: a multicenter, seroepidemiology study.
45. Lin FY1, Weisman LE, Azimi PH, Philips JB 3rd, Clark P, Regan J, Rhoads GG, Frasch CE, Gray BM, Troendle J, Brenner RA, Moyer P, Clemens JD; Level of maternal IgG anti-group B streptococcus type III antibody correlated with protection of neonates against early-onset disease caused by this pathogen.
46. Madoff LC1, Michel JL, Gong EW, Rodewald AK, Kasper DL: Protection of neonatal mice from group B streptococcal infection by maternal immunization with beta C protein. *Infect Immun.* **1992** *Dec ;60 (12):*4989-94.
47. Yang HH1, Mascuch SJ, Madoff LC, Paoletti LC: Recombinant group B Streptococcus alpha-like protein 3 is an effective immunogen and carrier protein. *Clin Vaccine Immunol. 2008 Jul; 15(7):1035-41. doi: 10.1128/CVI.00030-08. Epub 2008 May 7.*
48. Payne NR, Ferrieri P: The relation of the Ibc protein antigen to the opsonization differences between strains of type II group B streptococci. *J Infect Dis.* **1985** *Apr; 151(4):*672-81.

49. Musser JM1, Mattingly SJ, Quentin R, Goudeau A, Selander RK : Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease. *Proc Natl Acad Sci U S A.* **1989** *Jun;86(12):*4731-5
50. Brodeur BR1, Boyer M, Charlebois I, Hamel J, Couture F, Rioux CR, Martin D: Identification of group B streptococcal Sip protein, which elicits cross-protective immunity. *Infect Immun.* **2000** *Oct; 68(10):*5610-8.
51. Wexler DE, Chenoweth DE, Cleary PP: Mechanism of action of the group A streptococcal C5a inactivator; *Proc Natl Acad Sci U S A.* **1985** *Dec;82(23):*8144-8.
52. Pizza M, Scarlato V, Masignani V, Giuliani MM, Aricò B, Comanducci M, Jennings GT, Baldi L, Bartolini E, Capecchi B, Galeotti CL, Luzzi E, Manetti R, Marchetti E, Mora M, Nuti S, Ratti G, Santini L, Savino S, Scarselli M, Storni E, Zuo P, Broeker M, Hundt E, Knapp B, Blair E, Mason T, Tettelin H, Hood DW, Jeffries AC, Saunders NJ, Granoff DM, Venter JC, Moxon ER, Grandi G, Rappuoli R: Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing; *Science.* **2000** *Mar 10; 287(5459):*1816-20.
53. Capecchi B, Serruto D, Adu-Bobie J, Rappuoli R, Pizza M: The genome revolution in vaccine research; *Curr Issues Mol Biol.* **2004** *Jan;6(1):*17-27.
54. Serruto D, Rappuoli R: Post-genomic vaccine development; *FEBS Lett.* **2006** *May 22; 580 (12):*2985-92.
55. Maione, D., Margarit, I., Rinaudo, C. D., Masignani, V., Mora, M., Scarselli, M., Tettelin, H., Brettoni, C., Iacobini, E. T., Rosini, R., D'Agostino, N., Miorin, L., Buccato, S., Mariani, M., Galli, G., Nogarotto, R., Nardi Dei, V., Vegni, F., Fraser, C., Mancuso, G., Teti, G., Madoff, L. C., Paoletti, L. C., Rappuoli, R., Kasper, D. L., Telford, J. L., and Grandi, G. **2005** Identification of a universal Group B streptococcus vaccine by multiple genome screen, *Science 309,* 148-150.
56. Lauer P, Rinaudo CD, Soriani M, Margarit I, Maione D, Rosini R, Taddei AR, Mora M, Rappuoli R, Grandi G, Telford JL: Genome analysis reveals pili in Group B Streptococcus; *Science.* **2005** *Jul 1; 309 (5731):105.*
57. Morgan RL, Isaacson RE, Moon HW, Brinton CC, To CC: Immunization of suckling pigs against enterotoxigenic Escherichia coli-induced diarrheal disease by vaccinating dams with purified 987 or K99 pili: protection correlates with pilus homology of vaccine and challenge; *Infect Immun.* **1978** *Dec;22(3):*771-7.

58. Margarit, I., Rinaudo, C. D., Galeotti, C. L., Maione, D., Ghezzo, C., Buttazzoni, E., Rosini, R., Runci, Y., Mora, M., Buccato, S., Pagani, M., Tresoldi, E., Berardi, A., Creti, R., Baker, C. J., Telford, J. L., and Grandi, G. **2009** Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm, *J. Infect. Dis. 199,* 108-115.
59. Keessen EC, Gaastra W, Lipman LJ. Clostridium difficile infection in humans and animals, differences and similarities. *Vet Microbiol 2011; 153:205- 17; PMID:21530110;*
60. Al Saif N, Brazier J S. The distribution of Clostridium difficile in the environment of South Wales. *J Med Microbiol 1996; 45:133-7; PMID: 8683549; http:// dx.doi.org/10.1099/00222615-45-2-133).*
61. Zidaric V, Beigot S, Lapajne S, Rupnik M. The occurrence and high diversity of Clostridium difficile genotypes in rivers. *Anaerobe* **2010***; 16:*371-5*; PMID: 20541023; http://dx.doi.org/10.1016/j.*
62. Hall IC, O'Toole E. Intestinal flora in new-born infants: with a description of anew pathogenic anaerobe, Bacillus difficilis. *Am J Dis Child* **1935***; 49:*390-402*; http://dx.doi.org/10.1001/ archpedi.1935.019700201050105.*
63. Bartlett JGMN, Moon N, Chang TW, Taylor N, Onderdonk AB. Role of Clostridium difficile in antibiotic-associated pseudomembranous colitis. *Gastroenterology* **1978***; 75:*778-82*; PMID:700321*

Recombinant *Clostridium difficile* toxin fragments as carrier protein for PSII surface polysaccharide preserve their neutralizing activity

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1. Introduction

Clostridium difficile is a Gram-positive, spore-forming and toxin-producing anaerobic gastrointestinal pathogen that is the major cause of antibiotic-associated colitis. *C. difficile* has been isolated from several domestic and nondomestic animal species, and has been associated with diarrhea in horses, pigs, dogs and cats. In humans, *C. difficile* associated diarrhea (CDAD) is the most commonly diagnosed cause of hospital-associated and antimicrobial-associated diarrhea.[1]

C. difficile infection (CDI) has grown tremendously since 1978, and over the last decade, the incidence and severity of CDI has increased significantly and affected new patient groups. Today, the disease represents a major social and economic burden.[2] Since 2005, CDI has been increasingly reported among young, healthy individuals residing in the community. An estimated 20% to 28% of CDI is community associated with an incidence of 20 to 50 cases per 100 000 population in the United States, Sweden and England.[3]

At the moment, there is no vaccine against *C. difficile*, despite the increase in the incidence of the disease observed in the last decades.[4-5]

The virulence of *C. difficile* is conferred primarily by 2 large exotoxins, toxins A and B, and there is evidence that protection against severe CDI is mediated by systemic antibodies to TcdA and TcdB.[6-8] Both toxins present three distinct functional domains: an N-terminal enzymatic domain consisting of glucosyl-transferase (GT) and cysteine protease (CP) moieties, a central translocation (T) domain that mediates import into host cells and a C-terminal receptor binding domain (RBD) with 38 tandem repeats.[9]

Although a number of studies have demonstrated that anti-toxin circulating antibodies are effective in the treatment of severe CDI,[10-11] supporting the key role of toxin immunity in preventing the lethal outcome of this infection, the use of toxoid-based vaccines in humans has been limited for a long time. Recently, preparations of formaldehyde-inactivated toxoid from *C. difficile* culture supernatants have been able to confer protective immunity in clinical trials.[11-14]

To overcome the safety issues potentially associated to the large-scale production of toxoids, such as exposure to toxins and spores, the use of recombinant proteins has been proposed as an attractive alternative for development of vaccines against CDAD.[15] Several studies have demonstrated the ability of recombinant toxin fragments to induce robust immunity against lethal challenge with *C. difficile*. In particular, TcdA and TcdB RBDs, cloned and purified from a variety of hosts, have been proven to induce both systemic and mucosal neutralizing antibodies in animal models.[16-18] Our group has recently shown that co-administration of a cell binding domain fragment of TcdA (TcdA_B1) and the glucosyltransferase moiety of TcdB (TcdB_GT) can induce systemic IgGs, neutralizing both toxins and protecting vaccinated animals from death in hamster animal model of lethal infection. The presence of anti-TcdA and TcdB antibodies was assessed in gut contents, suggesting that systemic vaccination with this pair of recombinant polypeptides can limit the disease caused by toxin production during *C. difficile* infection.[19] However, anti-toxins antibodies elicited by toxin fragments are not able to limit the level of bacterial load in the gut.[20]

Recently it has been shown that *C. difficile* vegetative cells express three highly complex polysaccharides on their cell surface, named PSI, PSII and PSIII. Among those three carbohydrates, PSII has been found to be the more abundantly expressed by the hypervirulent rybotype O27.[21] The PSII is a polysaccharide composed of a hexaglycosyl phosphate repeating unit [-6)-β-D-Glcp-(1-3)-β-D-GalpNAc-(1-4)-α-D-Glcp-(1-4)-[β-D-Glcp-(1-3)]-β-D-GalpNAc-(1-3)-α-D-Manp-(1-P].[22]

We have previously employed the high-resolution magic angle spinning (HR-MAS) NMR on vegetative whole cells from a collection of clinical isolates and have detected PSII on the surface of different rybotypes, such as 001, 018, 027, 078 and 126.[23] The list of isolates analyzed by this technique has been further updated, detecting PSII in a number of clinical and environmental isolates, including strain 630.[24] Therefore, PSII is as a surface antigen conserved among the most common strains and can represent a relevant target for the development of a carbohydrate-based vaccine.

In confocal microscopy, examination of vegetative cells using anti-PSII antibodies revealed that PSII does not appear as a typical thick and even bacterial capsule; then it can be hypothesized that the polysaccharide is present either as cell wall-linked polysaccharide not bound to peptidoglycan or as a conjugate with lipoteichoic acids.[21, 24]

Interestingly, strain 630 and the hypervirulent strain R20291 can form *in vitro* structured biofilms, where the presence of PSII could be detected by antibodies against the phosphorylated hexaglycosyl

repeating unit.[25] This suggests that extracellular PSII could play a role in determining the biofilm's architecture of *C. difficile* as component of extracellular matrix.

Glycans are T cell independent antigens, but they can be turned into molecules able to evoke a T cell memory response following conjugation to a carrier protein.[26] Anti unconjugated PSII IgM antibodies have been generated in pregnant pigs vaccinated with a non-adjuvanted PSII containing an average of 6 repeating units.[27] PSII, after conjugation to CRM_{197} (non-toxic mutant of diphtheria toxin),[28] a carrier protein widely used for the manufacturing of glycoconjugate vaccines,[29] was formulated with the adjuvant MF59 and tested in Balb/C mice, inducing high levels of specific anti carbohydrate IgG, a class of antibodies which is generally relevant to induce protection against the sugar coated pathogens.[23] Therefore, conjugation of PSII to the carrier protein could ensure, as expected, the IgM-to-IgG switch. Noteworthy, glycoarray analysis has demonstrated that specific IgA antibodies in the stool of patients infected with *C. difficile* can recognize the nonphosphorylated PSII hexasaccharide hapten, suggesting that under exposure to PSII the human immune system may furnish a mucosal response against carbohydrate epitopes from PSII.[30]

The co-administration of multiple *C. difficile* antigens, by using recombinant toxin fragments conjugated to PSII could have the potential to prevent colonization and protect against *C. difficile* disease. We envisaged in conjugation of PSII to toxins as a strategy to ensure co-delivery of the two antigens, while using the toxin as carrier protein for the polysaccharide. With this aim, we have evaluated the immunological response of PSII-toxin based glycoconjugates in mouse, investigating the possible double role of the two TcdA_B2 and TcdB_GT fragments, as carrier protein for the PSII polysaccharide and antigens able to elicit antibodies with toxin neutralizing activity.

2. Results and Discussion

2.1. PSII-toxins conjugate

PSII is composed of hexaglycosyl repeating units hold together by phosphodiester bonds,[22] and the assigned structure has been confirmed by synthesis of the non-reducing end terminal phosphorylated repeating unit.[31]

Pure PSII with an average degree of polymerization (avDP) of 15, obtained from fermentation of the R20291 strain (Stoke Mandeville -ribotype 027) as previously reported,[23] was conjugated to the two *C. difficile* recombinant fragments derived from TcdA and TcdB after chemical modification of the

mannose sugar of the repeating unit at the reducing end. PSII was first reduced with $NaBH_4$ and then oxidized with sodium periodate to introduce an aldehyde group useful for the coupling to the lysine residues of the protein by reductive amination[23] (Fig. 1). The occurrence of complete conjugation was assessed by SDS-PAGE, and confirmed by the formation of a broad smear and the concomitant disappearance of the narrow band of the proteins (Fig. 2). Subsequently, the glycoconjugates were purified by size exclusion chromatography to remove unbound saccharide and analyzed for their protein content and in terms of total and free saccharide by HPAEC-PAD HPLC as described in literature.[23]

Table 1 summarizes the physico-chemical characterization of the purified glycoconjugates in term of total and free saccharide and protein content, compared to the PSII-CRM_{197} conjugate of which the preparation and the characterization were previously reported.[23] Notably, the degrees of glycosylation, ranging from 0.2 to 0.3 (w/w), was comparable for both the products.

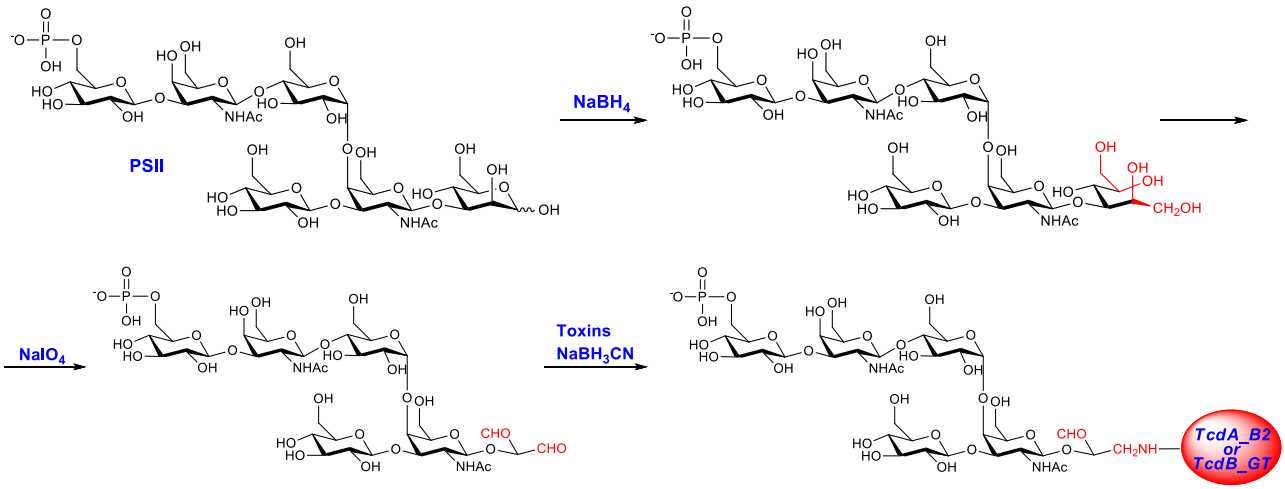

Figure 1. Conjugation Scheme of PSII-Toxins.

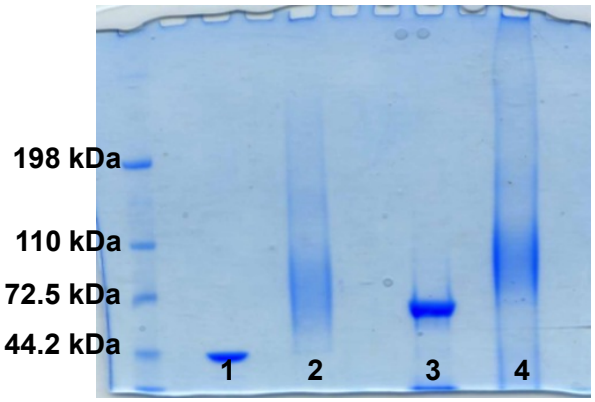

Figure 2. SDS-Page of PSII-Toxin Conjugates 1. TcdA_B2 fragment protein; 2. PSII-TcdA_B2 conjugate, 3. TcdB_GT fragment protein; PSII-TcdB_GT conjugate.

Table 1. Characteristics of PSII Conjugates.

Conjugates	***PSII avDP***	***Free Saccharide %***	***Saccharide/Protein w/w***
PSII-TcdA_B2	15	7.7	0.28
PSII-TcdB_GT	15	22.7	0.33
PSII-CRM$_{197}$*	21	11.2	0.24

**characterization previously described*[23]

2.2. Immunological Evaluation of PSII-toxins conjugates

To assess the ability of conjugates to induce anti-PSII antibodies, groups of 8 female BALB/c mice were intraperitoneally immunized three times with 2.5 μg carbohydrate based doses of conjugates, at three week-interval between the first and the second dose and two week-interval from the second and the third dose. The conjugates were formulated with the adjuvant MF59, an oil in water emulsion frequently used for seasonal flu vaccination.[32] Adjuvant alone in phosphate buffer (PBS) was used as a negative control, while the PSII-CRM$_{197}$ conjugate previously shown capable of inducing a robust anti-

polysaccharide immune response,[23] and the TcdA_B2 and TcdB_GT fragment proteins already shown to be highly immunogenic,[19] were used as a positive control.

Sera obtained after two weeks from the third dose (*post 3 sera*) were analyzed by ELISA for their content of anti-PSII and anti-Toxin IgGs. Additionally, the functionality of the anti-Toxin antibodies was investigated *in vitro* to assess the capacity to neutralize the cytotoxicity of TcdA and TcdB.

The PSII-TcdB_GT conjugate was highly immunogenic, eliciting anti-PSII IgG titres comparable to those obtained with the PSII-CRM_{197} conjugate. Conversely, PSII-TcdA_B2 conjugate induced an anti-polysaccharide response significantly lower than the CRM_{197} conjugate (p 0.006), where IgGs against PSII were induced in three mice only (Fig. 3). These results evidenced a better capability of the TcdB_GT fragment protein to function as carrier for the PSII moiety in comparison to the TcdA_B2 peptide.

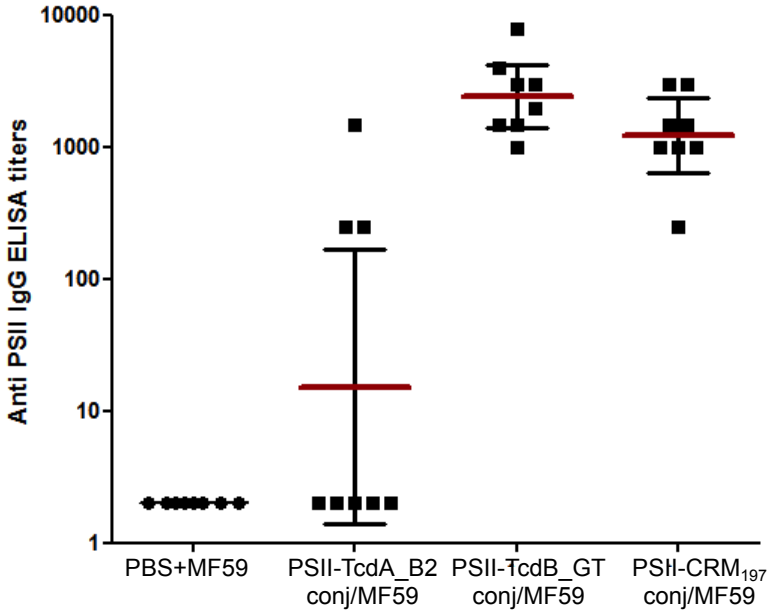

Figure 3. Anti-PSII IgG levels detected in individual post 3 sera of BALB/c mice; each dot represents single mouse sera; vertical bars indicate geometric mean titers of each group with 95% statistical confidence intervals as red bars.

The antibodies response to the TcdA_B2 and TcdB_GT fragment proteins as carrier was measured and compared with the IgG titers obtained with unconjugated TcdA_B2 and TcdB_GT fragment proteins.

As shown in Tab. 2, both conjugated toxin fragments evoked a robust anti protein response. However, unconjugated TcdA_B2 elicited significantly higher anti toxin fragment antibodies in comparison to the corresponding PSII conjugate (p 0.002), while TcdB_GT induced IgGs levels comparable to the conjugated form.

Table 2. IgG levels detected in individual post 3 sera of BALB/c mice against **TcdA_B2** and **TcdB_GT** coating.

	IgG Titer (GMT95% CI)
PBS	2
TcdA_B2 fragment protein	1,024,000 (374,868-4,023,000)
PSII-TcdA_B2 conjugate	51,499 (28,721-109,279)
TcdB_GT fragment protein	206,317 (47,610-400,390)
PSII-TcdB_GT conjugate	106,936 (69,489-174,511)
PSII-CRM$_{197}$	2

Next, the functionality of the antibodies was verified by the *in vitro* toxin neutralization assay of pooled sera from mice immunized with the conjugates. Remarkably, as shown in Fig. 4, both PSII conjugates elicited toxin neutralizing activity comparable to that induced by their respective protein carrier in unconjugated form, indicating that chemical conjugation did not alter the critical epitopes of recombinant toxin A and B fragments. As previously demonstrated,[19] TcdA_B2 and TcdB_GT induce neutralizing antibodies only against the cognate toxin (data not shown), suggesting that vaccination

should include combinations of fragments derived from each toxin to achieve the concurrent neutralization of TcdA and TcdB.

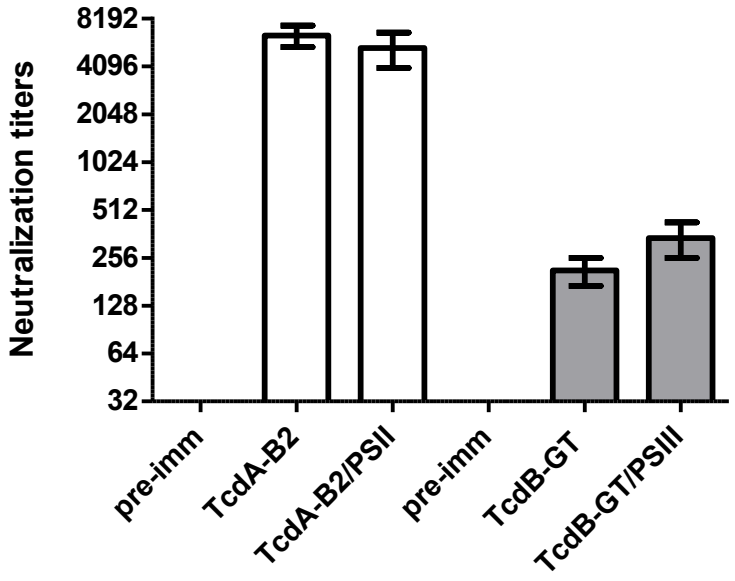

Figure 4. Neutralization titers of sera from mice immunized with toxin A and B fragments, either alone or conjugated to PSII, in the presence of MF59 adjuvant. Titers were defined as the reciprocal of the highest dilution able to inhibit 100% rounding in IMR-90 human fibroblasts treated with 1 CTU_{100} of toxin A (white column) or B (grey column). Pre-immune sera were used as negative controls. Values reported in the table represent the mean dilution from 3-5 independent experiments.

3. Experimental Section

Preparation and purification of PSII glycoconjugates

PSII at avDP 15 was obtained as previously reported in literature. Purified PSII[23] was reduced at the mannose sugar with 50 mM $NaBH_4$ (Sigma) in 10 mM sodium phosphate buffer pH 9.0 at room temperature for 2 hours; the reduced PSII was purified by Sephadex G25 chromatography (G&E Healthcare) in water and then oxidized with 15 equivalent of $NaIO_4$ (Sigma) in 10 mM sodium

phosphate buffer pH 7.2 at room temperature for 2 hours at the dark. The oxidized PSII was then purified by Sephadex G25 chromatography (G&E Healthcare) in water. The oxidized PSII (10 mg/ml) was then conjugated to carrier proteins using a stoichiometry of 4:1 (weight PSII per weight Protein) in 200 mM sodium phosphate/1 M NaCl buffer pH 8.0, and in presence of $NaBH_3CN$ (2:1, weight PSII per weight $NaBH_3CN$). The mixture was incubated for 48-72 hours at 37°C, mixing very gently with a magnetic stirrer. Conjugates were purified from excess of unconjugated PSII using size exclusion Superdex 75 chromatography (G&E Healthcare) in 10 mM sodium phosphate/10 mM NaCl buffer pH7.2.

Characterization of PSII glycoconjugates

Conjugates were characterized by SDS-PAGE using 7% Tris-Acetate gels (NuPAGE, Invitrogen). The samples (5 µg in term of protein) were added of 0.5 M dithiothreitol (1/5 v/v) and NuPAGE LDS sample buffer (1/5 v/v). The mixtures were heated at 100°C for 1 min. The gel containing loaded samples was electrophoresed at 45 mA in in NuPAGE Tris-Acetate SDS running buffer (20x, Invitrogen) and stained with Simply Blue Safe Stain (Invitrogen).

Protein concentration was determined by MicroBCA protein assay kit (Thermo Scientific). Total saccharide concentration was determined by High Performance Anionic Exchange Chromatography-Pulsed Amperometric Detection (HPAEC-PAD) analysis. Unconjugated saccharide was separated by SPE C4 hydrophobic interaction column (0.5 mL resin, Bioselect, Grace Vydac) and subsequently estimated by HPAEC-PAD analysis.

Cloning, expression and purification of recombinant fragments

TcdA_B2 and TcdB_GT were cloned, expressed and purified as previously described.[19] Briefly, fragments were cloned in pet15b+ vector (N-term-His tag) using the Polymerase Incomplete Primer Extension (PIPE) method. The sequences coding for TcdA_B2 (residues 2303-2710 in TcdA) and TcdB_GT (residues 1-543 in TcdB) were amplified by PCR from the *C. difficile* strain 630 genomic DNA and the vector was amplified from the pet15b+ vector; *E.coli* HK100 cells were then transformed with vector/insert hybrids.

PIPE method was employed to generate TcdB_GT (D270A, R273A, Y284A, D286A and D288A) mutant with abrogated enzymatic activity.

The proteins were expressed in *E.coli* BL21 (DE3) cells (Novagen) grown in LB. The expression of the protein was induced by addition of 1 mM IPTG to the culture at exponential growth phase and incubation for 4 hours at 25°C.

The protein expression was checked by SDS- PAGE.[19]

Recombinant TcdA_B2 and TcdB_GT were purified by Immobilized Metal Ion Affinity Chromatography (IMAC) and buffer exchange was performed by PD-10 desalting column (GE Healthcare) or by dialysis. Protein quantification was performed by BCA assay (Thermo Scientific).

Immunization protocol

Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in the conduct of all animal studies.

Groups of 8 female BALB/c mice (5-6 week old) were immunized on days 1, 21 and 35 with 2.5 µg of conjugated carbohydrate antigen formulated with MF59 (mixing equal volume of conjugate and MF59 suspension) as adjuvants. All immunizations were performed by administering a 200 µl of vaccine via intraperitoneal route. Adjuvant alone was used for negative control groups. Sera were collected on days 0 (before the first immunization), 34 and 49 (two weeks after the third immunization).

ELISA assay

Specific antibodies titers were determined 2 weeks after the third immunization by ELISA assay. For that purpose 96-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with 100 µl/well of 2 µg/ml (protein content) PSII-HSA conjugate (prepared as previously reported[23]) or TcdA_B2 protein or TcdB_GT protein in PBS pH 7.2. Plates were incubated over night at 4°C, then washed three times with TPBS (0.05% Tween 20 in PBS, pH 7.4) and blocked with 100 µl/well of 3% BSA (Sigma-Aldrich) for 1 hour at 37°C. Each incubation step was always followed by triple TPBS wash. Serum samples were initially diluted 1:1000 in TPBS, transferred into coated-blocked plates (200 µL) and serially two-fold diluted followed by 2 hours incubation at 37°C. Then 100 µl/well of 1:10000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) were added and left for 1 hour at 37°C. Visualization of bound alkaline phosphatase was performed by adding 100 µl/well of 1 mg/ml para-nitrophenyl-phosphate (pNPP) disodium hexahydrate (Sigma-Aldrich) in 0.5 M diethanolamine buffer pH 9.6. After 30 minutes of development at room temperature, the OD of each sample was measured at 405 nm with a microplate spectrophotometer (Biorad). Antibody titres were expressed as

the reciprocal of sera dilution corresponding to a cut off OD = 1.0. Each group of immunization was represented as the geometrical mean (GMT) of the single mouse titers.

The statistical and graphical analysis was performed using GraphPad Prism 5 software by applying the Mann Whitney test for statistical analysis.

In vitro neutralization assay

Toxin A and Toxin B were purified from *C. difficile* VPI10463 strain as previously described.[19] IMR-90 human fibroblasts were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown to 80-90% confluence in 96-well plates in EMEM (Eagle's Minimum Essential Medium) with 10% fetal calf serum. The minimal doses of Toxin A and Toxin B needed to cause 100% rounding in 24 hours (1 CTU_{100}) were defined as 20 ng/ml and 10 pg/ml, respectively. For neutralization assay two-fold dilutions of mouse sera from 1:8 to 1:32000 were pre-incubated with 1 CTU_{100} of each toxin in cell medium for 90 minutes at 37°C. Sera and toxins mixtures were then added to the cells and incubated for 16-18 hours before analysis. Pre-immune sera were used as negative controls. The endpoint titers were defined as the reciprocal of the highest dilution able to inhibit cell rounding.

4. Conclusions

C. difficile is considered the most important identifiable cause of healthcare-acquired diarrhea. The tendency of CDI to relapse and lack of efficacious preventative therapies render a vaccine highly recommendable. Typically, the two toxins A and B have been targeted for vaccine development. The surface polysaccharide PSII conjugated to CRM_{197} has also been proven an optimal target for a carbohydrate-based vaccine. In the present study we explored the feasibility of a glycoconjugate vaccine where the PSII saccharide was conjugated to the two protein fragments TcdA_B2 and TcdB_GT from toxin A and B, respectively.

We demonstrated that TcdB_GT is a very efficient carrier for PSII, since PSII-TcdB_GT was highly immunogenic and induced high titers of anti-polysaccharide IgG antibodies, in a comparable manner to the PSII-CRM_{197} conjugate. On the other hand the PSII-TcdA_B2 conjugate was less efficient in inducing anti-PSII IgGs. Since the chemical characteristics of the two conjugates are comparable, the reason for this different behavior might reside in the different intrinsic ability of the two carriers to drive the antibody response toward the carbohydrate moiety.

It is important to note that conjugation to the polysaccharide does not impact the neutralizing activity of both TcdA_B2 and TcdB_GT. Therefore, we conclude that conjugation of *C. difficile* carbohydrate antigens to toxin fragments is a promising approach for the design of a conjugate vaccine which targets both surface exposed carbohydrate as well as secreted toxins. Further evaluation in suitable animal models is needed to fully understand the capacity of such constructs to prevent colonization and neutralize toxin activity.

References and Notes

1. Kelly, C.P.; LaMont, J.T. *Clostridium difficile*--more difficult than ever. *N. Engl. J. Med.* **2008**, *359(18)*, 1932-1940.
2. Rebeaud, F.; Bachmann, M.F. Immunization strategies for *Clostridium difficile* infections. *Expert Rev. Vaccines* **2012**, *11(4)*, 469-479.
3. Chitnis, A.S.; Holzbauer, S.M.; Belflower, R.M.; Winston, L.G.; Bamberg, W.M.; Lyons, C.; Farley, M.M.; Dumyati, G.K.; Wilson, L.E.; Beldavs, Z.G.; Dunn, J.R.; Gould, L.H.; MacCannell, D.R.; Gerding, D.N.; McDonald, L.C.; Lessa, F.C. Epidemiology of Community-Associated *Clostridium difficile* Infection, 2009 through 2011. *JAMA Internal Medicine* **2013**, *173(14)*, 1359-1367.
4. Kyne, L.; Hamel, M.B.; Polavaram, R.; Kelly, C.P. Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile*. *Clin. Infect. Dis.* **2002**, *34(3)*, 346-353.
5. Petrella, L.A.; Sambol, S.P.; Cheknis, A.; Nagaro, K.; Kean, Y.; Sears, P.S.; Babakhani, F.; Johnson, S.; Gerding, D.N. Decreased cure and increased recurrence rates for *Clostridium difficile* infection caused by the epidemic *C. difficile* BI strain. *Clin. Infect. Dis.* **2012**, *55(3)*, 351-357.
6. Kyne, L.; Warny, M.; Qamar, A.; Kelly, C.P. Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. *Lancet* **2001**, *357(9251)*, 189-193.
7. Leav, B.A.; Blair B, Leney, M.; Knauber, M.; Reilly, C.; Lowy, I.; Gerding, D.N.; Kelly, C.P.; Katchar, K.; Baxter, R.; Ambrosino, D.; Molrine, D. Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI). *Vaccine* **2010**, *28(49)*, 965-969.
8. Steele, J.; Mukherjee, J.; Parry, N.; Tzipori, S. Antibody against TcdB, but not TcdA, prevents development of gastrointestinal and systemic *Clostridium difficile* disease. J. Infect. Dis. **2013**, *207*(2), 323-330.
9. Jank, T.; Giesemann, T.; Aktories, K. Rho-glucosylating *Clostridium difficile* toxins A and B: new insights into structure and function. *Glycobiology* **2007**, *17(4)*, 15R-22R.

10. Lowy, I.; Molrine, D.C.; Leav, B.A.; Blair, B.M.; Baxter, R.; Gerding, D.N.; Nichol, G.; Thomas, W.D.Jr.; Leney, M.; Sloan, S.; Hay, C.A.; Ambrosino, D.M. Treatment with monoclonal antibodies against *Clostridium difficile toxins*. *N. Engl. J. Med.* **2010**, *362(3)*, 197-205.

11. Marozsan, A.J.; Ma, D.; Nagashima, K.A.; Kennedy, B.J.; Kang, Y.K.; Arrigale, R.R.; Donovan, G.P.; Magargal, W.W.; Maddon, P.J.; Olson, W.C. Protection against *Clostridium difficile* infection with broadly neutralizing antitoxin monoclonal antibodies. *J. Infect. Dis.* **2012**, 206(5), 706-713.

12. Kotloff, K.L.; Wasserman, S.S.; Losonsky, G.A.; Thomas, W.Jr.; Nichols, R.; Edelman, R.; Bridwell, M.; Monath, T.P. Safety and immunogenicity of increasing doses of a *Clostridium difficile* toxoid vaccine administered to healthy adults. *Infect. Immun.* **2001**, *69(2)*, 988-995.

13. Aboudola, S.; Kotloff, K.L.; Kyne, L.; Warny, M.; Kelly, E.C.; Sougioultzis, S.; Giannasca, P.J.; Monath, T.P.; Kelly, C.P. *Clostridium difficile* vaccine and serum immunoglobulin G antibody response to toxin A. *Infect. Immun.* **2003**, *71(3)*, 1608-1610.

14. Sougioultzis, S.; Kyne, L.; Drudy, D.; Keates, S.; Maroo, S.; Pothoulakis, C.; Giannasca, P.J.; Lee, C.K.; Warny, M.; Monath, T.P.; Kelly, C.P. *Clostridium difficile* toxoid vaccine in recurrent *C. difficile*-associated diarrhea. *Gastroenterology* **2005**, 128(3), 764-770.

15. Donald, R.G.; Flint, M.; Kalyan, N.; Johnson, E.; Witko, S.E.; Kotash, C.; Zhao, P.; Megati, S.; Yurgelonis, I.; Lee, P.K.; Matsuka, Y.V.; Severina, E.; Deatly, A.; Sidhu, M.; Jansen, K.U.; Minton, N.P.; Anderson, A.S. A novel approach to generate a recombinant toxoid vaccine against *Clostridium difficile*. *Microbiology* **2013**, 159(Pt7), 1254-1266.

16. Ryan, E.T.; Butterton, J.R.; Smith, R.N.; Carroll, P.A.; Crean, T.I.; Calderwood, S.B. Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated Vibrio cholerae vector strain. *Infect. Immun.* **1997**, *65(7)*, 2941-2949.

17. Ward, S.J.; Douce, G.; Figueiredo, D.; Dougan, G.; Wren, B.W. Immunogenicity of a Salmonella typhimurium aroA aroD vaccine expressing a nontoxic domain of *Clostridium difficile* toxin A. *Infect. Immun.* **1999**, *67(5)*, 2145-2152.

18. Tian, J.H.; Fuhrmann, S.R.; Kluepfel-Stahl, S.; Carman, R.J.; Ellingsworth, L.; Flyer, D.C. A novel fusion protein containing the receptor binding domains of *C. difficile* toxin A and toxin B elicits protective immunity against lethal toxin and spore challenge in preclinical efficacy models. *Vaccine* **2012**, 30(28), 4249-4258).

19. Leuzzi, R.; Spencer, J.; Buckley, A.; Brettoni, C.; Martinelli, M.; Tulli, L.; Marchi, S.; Luzzi, E.; Irvine, J.; Candlish, D.; Veggi, D.; Pansegrau, W.; Fiaschi, L.; Savino, S.; Swennen, E.; Cakici, O.; Oviedo-Orta, E.; Giraldi, M.; Baudner, B.; D'Urzo, N.; Maione, D.; Soriani, M.; Rappuoli, R.; Pizza, M.; Douce, G.R.; Scarselli, M. Protective efficacy induced by recombinant *Clostridium difficile* toxin fragments. *Infect. Immun.* **2013**, *81(8)*, 2851-2860.

20. Spencer, J.; Leuzzi, R.; Buckley, A.; Irvine, J.; Candlish, D.; Scarselli, M.; Douce, G.R. Vaccination against *Clostridium difficile* using toxin fragments. Observations and analysis in animal models. *Gut Microbes*, **2014**, *5(2)*.

21. Monteiro, M.A.; Ma, Z.; Bertolo, L.; Jiao, Y.; Arroyo, L.; Hodgins, D.; Mallozzi, M.; Vedantam, G.; Sagermann, M.; Sundsmo, J.; Chow, H. Carbohydrate-based *Clostridium difficile* vaccines. *Expert Rev. Vaccines* **2013**, *12(4)*, 421-431.

22. Ganeshapillai, J.; Vinogradov, E.; Rousseau, J.; Weese, J.S.; Monteiro, M,A. *Clostridium difficile* cell-surface polysaccharides composed of pentaglycosyl and hexaglycosyl phosphate repeating units. *Carbohydr. Res.* **2008**, 343(4), 703–710.

23. Adamo, R.; Romano, M.R.; Berti, F.; Leuzzi, R.; Tontini, M.; Danieli, E.; Cappelletti, E.; Cakici, O.S.; Swennen, E.; Pinto, V.; Brogioni, B.; Proietti, D.; Galeotti, C.L.; Lay, L.; Monteiro, M.A, Scarselli, M.; Costantino, P. Phosphorylation of the synthetic hexasaccharide repeating unit is essential for the induction of antibodies to *Clostridium difficile* PSII cell wall polysaccharide. ACS Chem. Biol. **2012**, *7(8)*, 1420–1428.

24. Reid, C.W.; Vinogradov, E.; Li, J.; Jarrell, H.C.; Logan, S.M.; Brisson, J.R. Structural characterization of surface glycans from *Clostridium difficile*. *Carbohydr. Res.* **2012**, 354, 65–73.

25. Đapa, T.; Leuzzi, R.; Ng, Y.K.; Baban, S.T.; Adamo, R.; Kuehne, S.A.; Scarsell,i M.; Minton, N.P.; Serruto, D.; Unnikrishnan, M. Multiple factors modulate biofilm formation by the anaerobic pathogen *Clostridium difficile*. *J. Bacteriol.* **2013**, *195(3)*, 545-555.

26. Berti, F.; Adamo, R. Recent mechanistic insights on glycoconjugate vaccines and future perspectives. *ACS Chem. Biol.* **2013**, *8(8)*, 1653-1663.

27. Bertolo, L.; Boncheff, A.G.; Ma, Z.; Chen, Y.H., Wakeford, T.; Friendship, R.M; Rosseau, J.; weese, J.S.; Chu, M.; Mallozzi, M.; Vedantam, G.; Monteiro, M.A. *Clostridium difficile* carbohydrates: glucan in spores, PSII common antigen in cells, immunogenicity of PSII in swine and synthesis of a dual *C. difficile*-ETEC conjugate vaccine. *Carbohydr. Res.* **2012**, *354*, 79–86.

28. Bröker, M.; Costantino, P.; DeTora, L.; McIntosh, E.D.; Rappuoli, R. Biochemical and biological characteristics of cross-reacting material 197 CRM197, a non-toxic mutant of diphtheria toxin: Use as a conjugation protein in vaccines and other potential clinical applications. *Biologicals* **2011**, *39(4)*, 195–204.

29. Costantino, P.; Rappuoli, R.: Berti, F. The design of semi-synthetic and synthetic glycoconjugate vaccines. *Expert Opin. Drug Discovery* **2011**, *6 (10)*, 1045−1067.

30. Oberli, M.A.; Hecht, M.L.; Bindschädler, P.; Adibekian, A.; Adam. T.; Seeberger, P.H. A possible oligosaccharide-conjugate vaccine candidate for *Clostridium difficile* is antigenic and immunogenic. *Chem. Biol.* **2011**, *18(5)*, 580–588.

31. Danieli, E.; Lay, L.; Proietti, D.; Berti, F.; Costantino. P.; Adamo, R. First synthesis of *C. difficile* PS-II cell wall polysaccharide repeating unit. *Org. Lett.* **2011**, *13(3)*, 378–381.

32. Galli, G.; Medini, D.; Borgogni, E.; Zedda, L.; Bardelli, M.; Malzone, C.; Nuti, S.; Tavarini, S.; Sammicheli, C.; Hilbert, A.K.; Brauer, V.; Banzhoff, A.; Rappuoli, R.; Del Giudice, G.;

Tyrosine-directed conjugation of large glycans to proteins via copper-free click chemistry

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Introduction

Site-selective bioconjugation methods represent powerful tools to generate novel therapeutic proteins, molecular probes, targeted delivery systems and bionanomaterials.[1,2] Glycosylation of proteins can profoundly affect the pharmacokinetics and activity of protein-based medicines.[3] Glycans are poorly immunogenic, however conjugation to protein carriers ensures elicitation of carbohydrate specific T-cell-dependent memory response.[4] Carbohydrate-based vaccines are often produced by coupling the saccharides to the surface abundant lysine residues of the protein carrier, but the regioselectivity of conjugation is hard to achieve. Thus, batch-to-batch variability in the immunological properties can be introduced. In contrast, tyrosine residues are usually much less present and accessible than lysine residues, hence regioselective conjugation can be expected. We have recently developed a robust tyrosine-selective amination via a triazolidinone-ene reaction in tris buffer.[5] The method enabled the creation of an anti-candidiasis vaccine through Cu(I) catalyzed azide-alkyne [3+2] cycloaddition (CuAAC)[6,7] of a synthetic β-glucan onto specific tyrosine residues of the genetically detoxified diphtheria toxin CRM_{197}. By this strategy we obtained a detailed structure-immunogenicity relationship of glycoconjugates with defined sugars at predetermined sites, and deciphered the contribution of the glycan, the linker and the attachment site on their immune properties.[8]

A few carrier proteins, such as CRM_{197}, diphtheria (DT) and tetanus toxoid (TT), are generally employed for licensed vaccines or candidates under clinical development.[9] The use the same carriers for multivalent vaccines or subsequent vaccinations can in some cases interfere with the anti-polysaccharide immune response.[10-12] Therefore, the identification of novel carrier protein is a relevant topic for the development of the future vaccines.

Group B *Streptococcus* (*Streptococcus agalactiae* - GBS) is a Gram positive pathogen that causes severe invasive neonatal infections, such as pneumonia, septicemia and meningitis.[13] Of the 10 serotypes identified on the basis of the capsular polysaccharide (CPS) structure,[14,15] five (Ia, Ib, II, III, and V) are commonly associated with human diseases. A glycoconjugate vaccine comprising conjugates of PS Ia, Ib and III, which covers the majority of the infections, is currently under phase II

clinical trial.[16, 17] After genome sequencing of GBS strains, and high-throughput expression and testing of a number of surface proteins by the *reverse vaccinology* approach,[18] pilus proteins have been found to protect immunized mice against a lethal challenge with GBS.[19,20] The conjugation of the capsular polysaccharides to proteins of the same antigen could be a general strategy to broaden the strain coverage of glycoconjugate vaccines.

In the newly developed tyrosine-ligation we envisaged a powerful method to direct polysaccharide conjugation at predetermined sites of the protein, and ensure higher batch-to-batch consistency in comparison to classic nonspecific conjugation procedures, particularly when the protein is used with the dual role of antigen and carrier.

To our surprise, preliminary attempts to conjugate high molecular weight negatively charged GBS polysaccharides to the tyrosine residues of CRM_{197} by CuAAC provided the desired glycoconjugates at poor yield. Conversely, condensation of 4-pentinyl lactose **4**[21] (Figure 2) was successful, indicating that our method,[5] which was proven very efficient which small-medium size oligosaccharides, was not suitable for larger polysaccharides, such as the ones from GBS. We reasoned that the CuAAC could be hampered by the reduced accessibility of the catalyst to these large coupling partners.[22]

We, therefore, hypothesized that strain-promoted azide-alkyne [3+2] cycloaddition (SPAAC),[23-25] which benefits of high rate of the cycloaddition bypassing the need of a catalyst[26] would represent an optimal approach for the conjugation of this type of polysaccharides. Herein, we report a two-step strategy for copper-free conjugation of defined glycans equipped with a cyclooctyne arm to predetermined tyrosine residues of CRM_{197} and GBS pilus proteins modified with a novel azide-linker, and its utilization for the coupling of streptococcal polysaccharides (Figure 1). Integrity of protein epitopes in the modified proteins was ascertained by competitive ELISA, and covalent attachment of protein and polysaccharide in the glycoconjugates was confirmed by immunoblot assays. The conjugated polysaccharide was quantified by HPAEC-PAD.

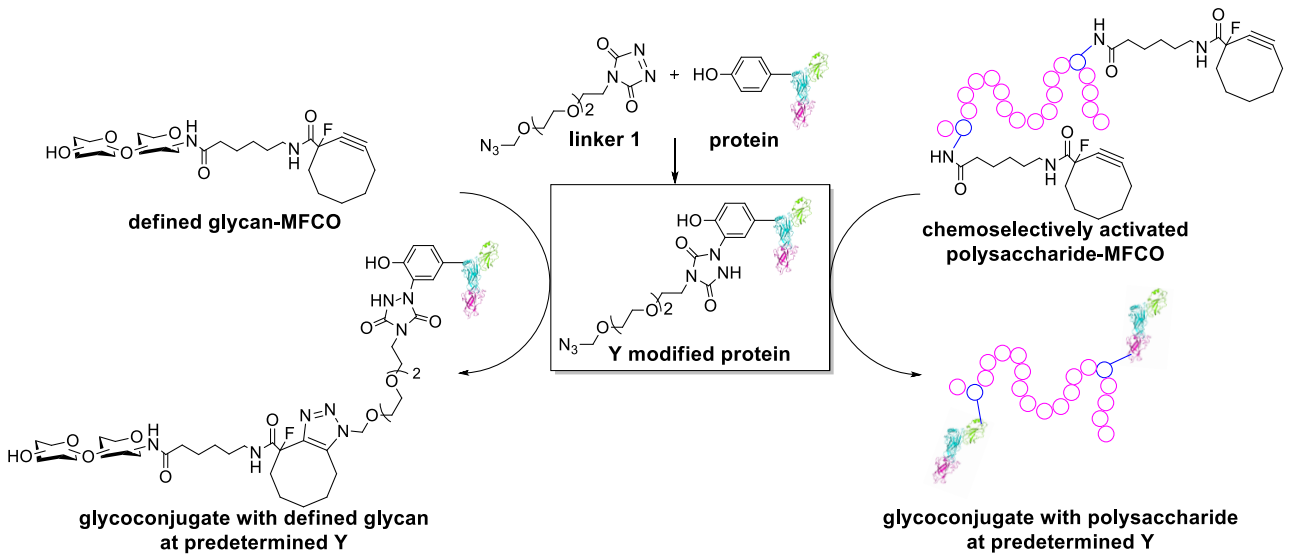


Figure 1. Synthesis of glycoconjugates by tyrosine-ligation copper-free azide-alkyne [3+2] cycloaddition.

Results and discussion

To develop a general strategy suitable for tyrosine directed conjugation of both well-defined glycans and complex polysaccharides through SPAAC (Figure 1), we initially designed and synthesized a set of different length sugars bearing an amine function for coupling to N-hydroxysuccinimyl ester of the monofluoro-cyclooctyne (MFCO)[23] linker (Figure 2).

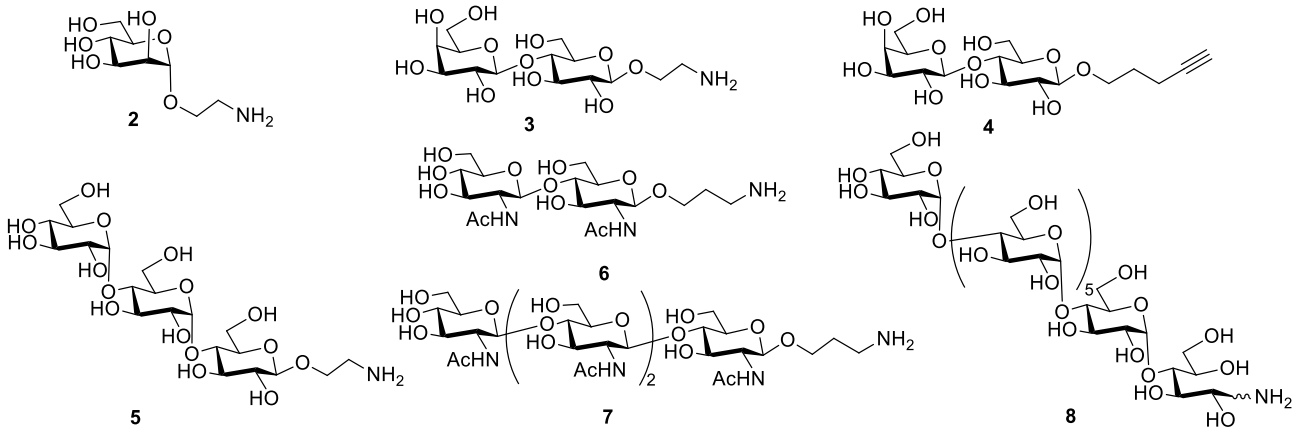
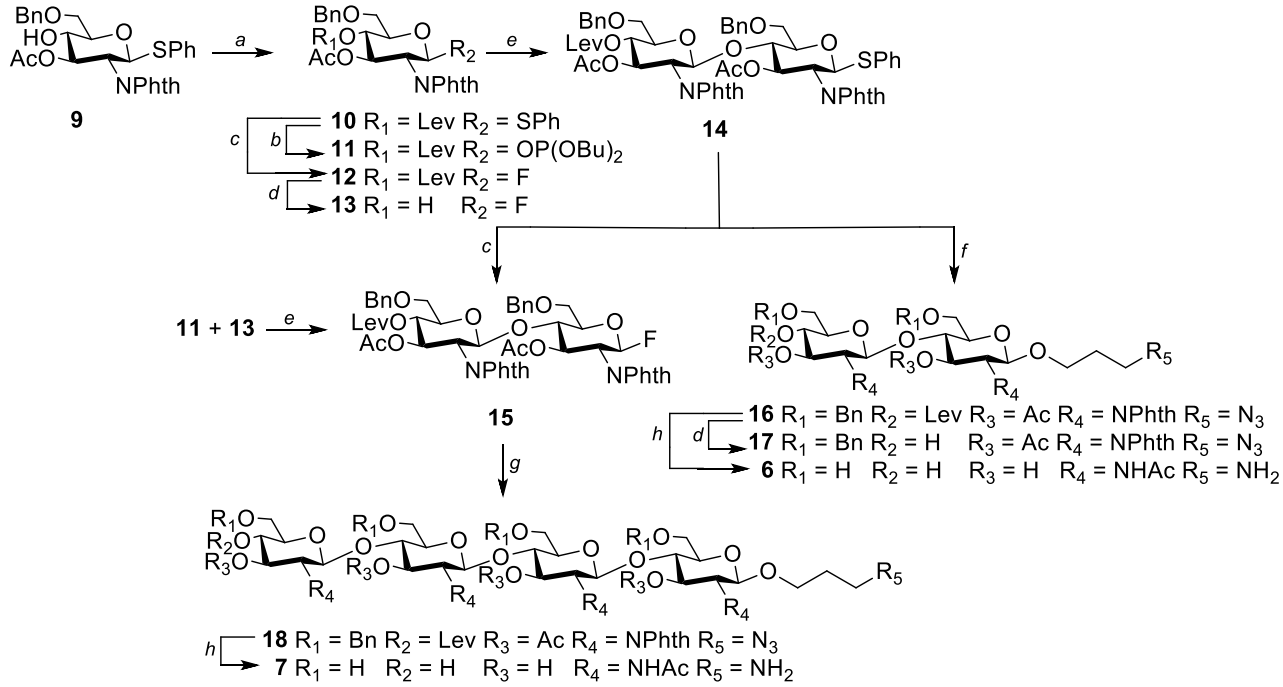


Figure 2. Structures of the defined glycans used for the tyrosine ligation via copper-free azide-alkyne [3+2] cycloaddition.

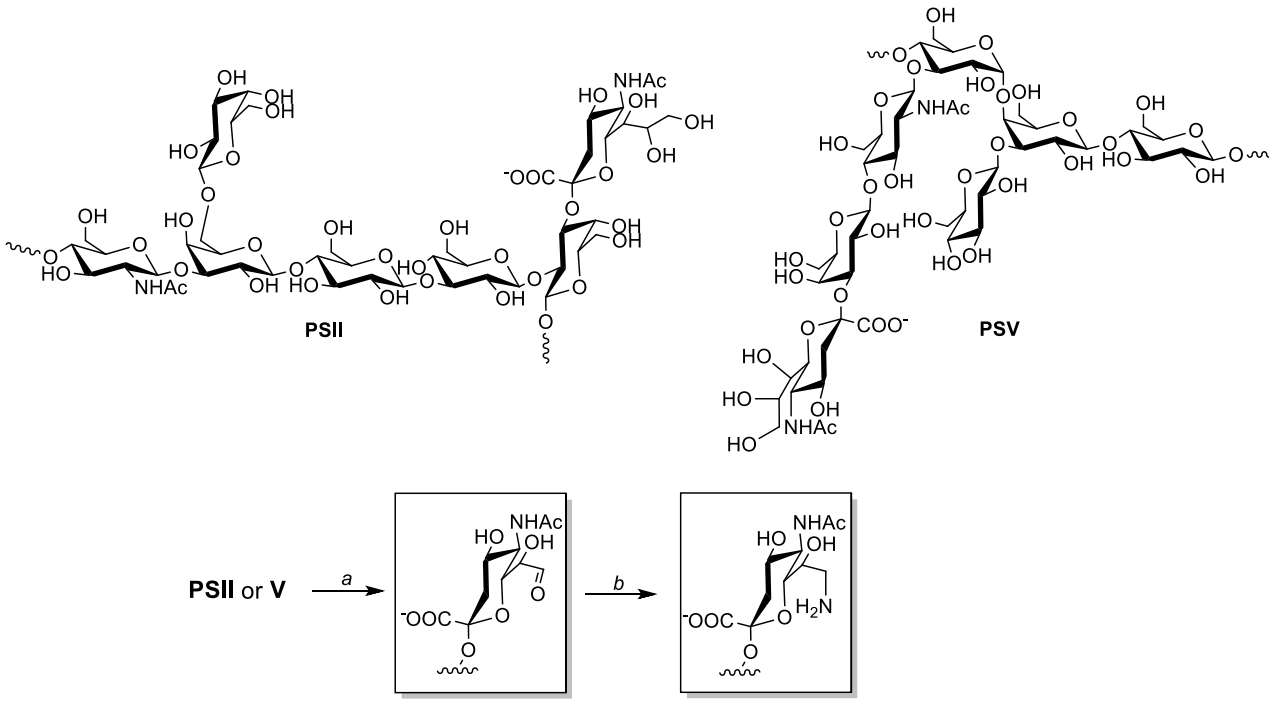
Compounds **2-5** and **8** were prepared by standard manipulations of commercially available sugars (as described in SI).[27] To further test our approach in the context of a convergent total synthesis of glycoconjugates, the β-(1→4)-chitin dimer **6** and tetramer **7** were assembled as depicted in Scheme 1. These structures are biologically relevant, since chitins have been found in the inner core of fungal cell wall[28] and as part of bacterial peptidoglycan.[29] The assembly of the β-(1→4)-oligoglucosamines is particularly challenging due to the presence of the deactivating acetamido group at the C-2 position of each repeating unit.[30,31] Our synthesis commenced from the known thioglycoside **9**,[31] which was used as acceptor for trimethylsilyl trifluoromethanesulfonate (TMSOTf) promoted glycosylation with **11,** obtained by levulinoylation at C-3 hydroxyl group and following conversion to phosphate donor, to give disaccharide **14**.[32]

The dimer **6,** derivatized at the end terminus with an arm for conjugation, was prepared by reaction of **14** with 3-azidepropanol in the presence of N-iodosuccinimide **(**NIS)/Trifluoromethanesulfonic acid (TfOH) and a subsequent series of deprotecting steps that include removal of N-phthalamido groups, reacetylation, methanolysis of the acetyl esters and final debenzylation. The assembly of the tetramer **6** was achieved from the disaccharide acceptor **17**, readily available by 3-*O*-develvulinoylation of **16,** through a [2+2] convergent approach. For this purpose, the disaccharide fluoride donor **15** was prepared from thioglycoside **14** by treatment with diethylaminosulfur trifluoride (DAST)/N-bromosuccinimide (NBS)[33] or, alternatively, by chemoselective TMSOTf promoted glycosylation with phosphate donor **11** of fluoride acceptor **13,** obtained from **10** by reaction with DAST/NBS succeeded by orthogonal removal of the 3-*O*-levulinoyl ester. Bis(cyclopentandienyl) Hafnium(IV) dichloride (Cp_2HfCl_2)/silver trifluoromethanesulfonate (AgOTf) promoted glycosylation of **16** with **15** yielded the protected tetramer **18.** To our best knowledge, this represents the first example of selective glycosylation using phosphate and fluoride partners. Deprotection of **18**, similarly to dimer **6**, afforded the target tetrasaccharide **7.**



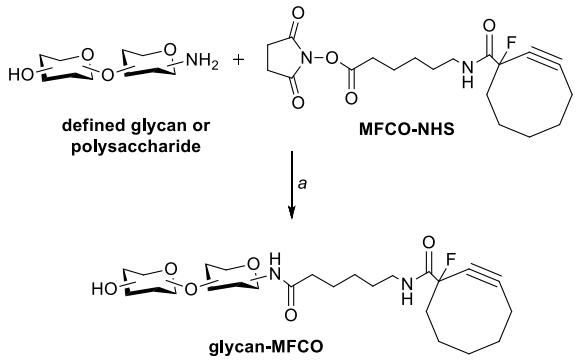
Scheme 1. Reactions leading to chitin dimer **6** and tetramer **7.** *Reagents and conditions:* a. Levulinic acid, DMAP, DCC, CH_2Cl_2, 91%; b. $HOPO(OBu)_2$, NIS-TfOH, CH_2Cl_2, -40°C to rt, 87%; c. DAST-NBS, CH_2Cl_2, -10°C to rt, 80% from **10**, 65% from **14**; d. $H_2NNH_2 \cdot AcOH$, CH_2Cl_2, 85% from **12**, 94% from **16**; e. TMSOTf, **9**, CH_2Cl_2, -30°C to rt, 93%; f. $HO(CH_2)_2N_3$, CH_2Cl_2, -40°C to rt, 69%; g. Cp_2HfCl_2-AgOTf, CH_2Cl_2, -10°C to rt, 55%; h. $NH_2CH_2CH_2NH_2$, EtOH, 60°C; Ac_2O, MeOH; NH_3, MeOH; H_2, 5% Pd-C, MeOH, 64% from **16**, 53% from **18**.

Insertion of the amine groups in GBS PSII and V for coupling with MFCO-NHS was accomplished by targeting 20% chemoselective periodate oxidation of the NeuAc 9,8-diol, and subsequent reductive amination of the generated aldehyde intermediate (Scheme 2).[34]



Scheme 2. Modification of PSII or V. *Reagents and conditions:* a. $NaIO_4$, 10 mM NaPi; b. NH_4OAc, $NaCNHB_3$, 5 mM NaOAc, 60-65%.

Both defined glycans and polysaccharide were then coupled to MFCO active ester to deliver the molecules ready for strain-promoted coupling to the labeled proteins (Scheme 3).



Scheme 3. Insertion of the MFCO linker on the carbohydrates.
Reagents and conditions: MFCO-NHS, DMSO or 9:1 DMSO-H_2O, TEA, 80-90%.

For the tyrosine-directed modification of the proteins, linker **1** was designed without the phenyl group present in our first generation of spacers, which we have shown to induce unwanted antibodies.[8]

Incubation of CRM_{197} with linker **1** (prepared as described in SI), under the conditions previously reported,[5] gave the modified protein with an average incorporation of 3.5 azides, as determined by LC-MS analysis. As expected, MS/MS analysis of proteolytic digests showed, in agreement with our previous findings, that Y27, Y46, Y358 and Y380 were modified among the 18 tyrosine residues totally available in CRM_{197}.[5,8]

Having demonstrated that insertion of linker **1** proceeded uneventfully as reported for similar tyrosine modifications, we directed our attention to the GBS pilus proteins, GBS80 and GBS67.

GBS assembles two types of pili on its surface that mediate bacterial adherence to host cells.[35] Two pilus island (PI) variants, PI-1 and PI-2, have been described, with the latter differentiated into components 2a and 2b.[36] GBS80 is a three domains protein, which constitutes the major component of GBS PI-1 pilus,[37] while GBS67 is an ancillary highly conserved four domains protein of 2a pilus.[38]

GBS80 and GB67 were modified with **1** in a fashion similar to CRM_{197}, and an average labelling of 2 and 3.5 tyrosine residues, respectively, was ascertained by LC-MS. Semi-quantitative MS analysis of peptide digests enabled to cover 17 of the 18 tyrosine residues of GBS80 and identification of Y16, Y23, Y44 and Y135 as the modified sites (Table S1, SI). Interestingly, in the case of GBS67 36 of the 39 tyrosine residues were covered, and Y744 resulted in a higher level of modification, while Y282/283, Y336/337 and Y403 were modified at a lower extent (Table S2, SI).

By competitive ELISA we ascertained that the modification of the tyrosine residues did not impair the exposition of peptide epitopes onto GBS80 or GBS67, as unmodified and modified proteins were comparably strong as inhibitors of the binding of polyclonal anti-protein murine sera to the immobilized proteins (Figure 3).

Following our main purpose, we first developed a procedure for conjugation of the defined glycans **2**-**8** with the MFCO linker installed. In spite of the moderate constant rate reported for the MFCO in SPAAC,[23] a good progression of the reaction was observed already in 1 h by mixing the labelled protein at 300 μM azide concentration (~5 mg/mL protein concentration) with a 10-fold excess of MFCO-glycan (Figure S1). In general, overnight incubation of the labelled proteins at 200-300 μM azide concentration with a 2-fold excess of MFCO-glycan was sufficient to achieve complete reaction regardless of the sugar length (Table 1; Figures S2-S6 for SDS page electrophoresis and MALDI TOF MS).[39] Although cycloalkynes enabling more expeditious click reactions have been recently described,[25] we deemed these conditions satisfying for the preparation of the target GBS PS conjugates.

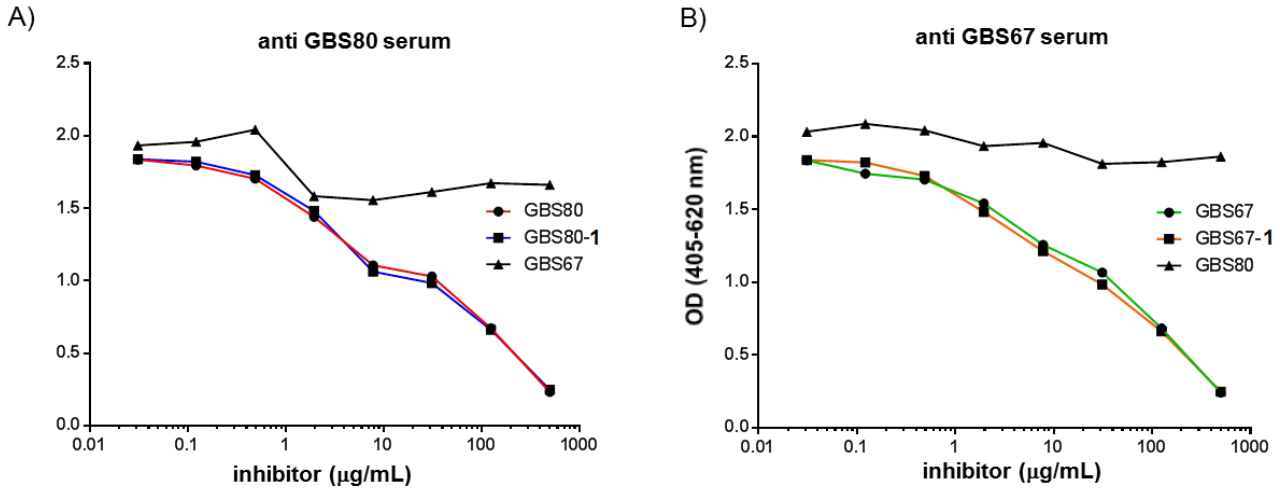


Figure 3. Competitive ELISA of the binding between specific anti-protein polyclonal sera and the protein as coating reagent, using unmodified and labeled proteins as inhibitors. A) GBS80 and GBS80-**1** are equipotent inhibitors of the binding (IC_{50} = 15 and 10 µg/mL, respectively); GBS67 is the negative control. B) GBS67 and GBS67-**1** are equipotent inhibitors of the binding (IC_{50} = 33 and 18 µg/mL, respectively); GBS80 is the negative control.

Table 1. Conjugation of different length oligosaccharides to the proteins

Glycan[a]	**Glycan loading (Coupling efficiency %)**		
	CRM_{197}	**GBS80**	**GBS67**
2	3.5 (>95%)	2.0 (90%)	3.3 (94%)
3	3.5 (>95%)	2.0 (>95%)	3.5 (>95%)
4	nd[b]	2.0 (>95%)	nd[b]
5	3.5 (>95%)	2.0 (>95%)	3.5 (>95%)
6	3.5 (>95%)	2.0 (95%)	3.5 (>95%)
7	3.5 (>95%)	2.0 (>95%)	3.1 (89%)
8	3.5 (>95%)	2.0 (>95%)	2.8 (85%)

a. All glycans were coupled to MFCO-NHS prior to conjugation as described in SI, with the exception of **4**,

which was directly coupled via CuAAC;
b. not determined.

Next, we tested the optimized protocol in the conjugation of GBS PSII and V to the different proteins. Modified proteins (~5 mg/mL) and MFCO-activated polysaccharides were gently shacked overnight at different w/w ratios, as summarized in Table 2. After chromatography of the produced glycoconjugates on hydroxyapatite column, the amount of conjugated and unconjugated sugar in the final products was estimated by HPAEC-PAD (Table 2).[40] Notably, when the same stoichiometry of polysaccharide and protein was applied, conjugation by SPAAC gave the GBS-PSII construct at higher yield in comparison to CuACC (36 vs 9%; Table 2, entry 2 and 3). The amount of condensed polysaccharide was proportional to that used in the conjugation step (Table 2, entry 3 and 4).

Table 2. Characteristics of the synthesized GBS glycoconjugates

entry	Glycoconjugate	Carbohydrate:protein stoichiometry (w/w)[a]	Glycosylation ratio (w/w)[b,c]	Free saccharide (%)[b]	Yield (%)[d]
1	PSII-Y-CRM_{197}	3:1	1.8	< 2.8	41
2	PSII-Y-GBS80	6:1	2.7	< 1.8	36
3	PSII-Y-GBS80[e]	6:1	1.9	13.5	9
4	PSII-Y-GBS80	1:1	1.1	< 4.5	32
5	PSII-Y-GBS67	4:1	1.3	< 3.8	32
6	PSV-Y-GBS80	4:1	2.2	< 5.5	37
7	PSV-Y-GBS67	4:1	2.3	< 5.1	24

a. Ratio of reagents used in the conjugation reaction; b. carbohydrate:protein ratio in the purified glycoconjugate; c. conjugated and unconjugated PS in the purified products were estimated by HPAEC-PAD quantification of Gal for PSII adducts or GlcNAc for PSV adducts; d. determined by micro-BCA quantification of purified glycoconjugate vs the starting protein; e. this conjugate was prepared by CuAAC.

For instance, a 1:1 carbohydrate-protein ratio (w/w) during the coupling step (~4:1 mol MFCO/mol azide) was sufficient to attain the glycoconjugate PSII-GBS80 with a final glycosylation degree of 1.1 (w/w). Incubation of polysaccharide and protein in 3-4:1 ratio (w/w) (12-16:1 mol MFCO/mol azide) guaranteed a higher glycosylation degree (1.8-2.3:1, w/w).

The covalent linkage between the protein and polysaccharide was confirmed by Western blot (WB) analysis (Figure 4), demonstrating that only glycoconjugates were stained concomitantly by specific anti-protein and anti-polysaccharide murine sera, when compared to the protein, the polysaccharide and their physical mixture as controls.

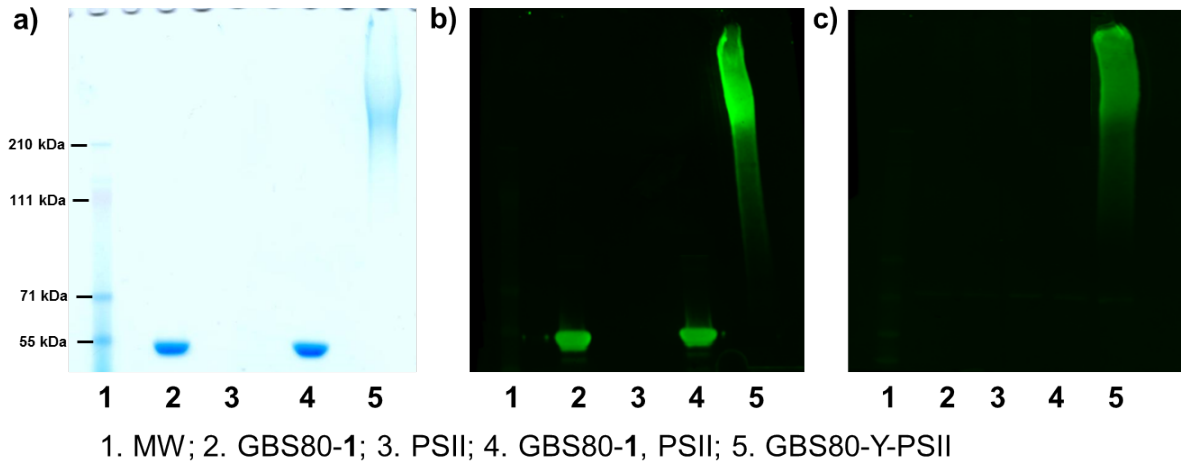


Figure 4. Example of immunoblot characterization of GBS glycoconjugates. a) SDS page of GBS80-Y-PSII (entry 4; Table 1), using the labeled protein, PS and their mixture as controls; b) WB analysis with anti-protein murine sera shows staining for the starting protein, its mixture with the sugar and the glycoconjugate; c) WB analysis with anti-PS murine sera highlights staining only for the glycoconjugate, clearly confirming the covalent linkage of PS with the protein.

Conclusions

We described the development of a two-step conjugation strategy based on copper-free [3+2] cycloaddition of sugars modified with a cyclooctyne and proteins derivatized at the tyrosine residues with the novel azido-linker **1**. This method was first proven efficient in the preparation of glycoconjugates with defined attachment point from minimal amounts of synthetic carbohydrates, whose preparation generally requires multistep syntheses and substantial effort. Next, the novel approach was successfully applied to the preparation of glycoconjugates from GBS PSII and PSV and the pilus proteins GBS80 and GBS67, previously selected as vaccine antigens through the *reverse vaccinology* approach. By competitive ELISA we verified that the proteins are not affected by the selective ligation of the linker onto the tyrosine residues. The covalent linkage of the saccharides and the protein was ascertained by immunoblot analysis. This technology appears very appealing for the conjugation of carbohydrate haptens to protein antigens,[41] as it ensures high consistency in the conjugation step and easy in process MS analysis of the modified protein whose functionality needs to be preserved. This approach will be useful to extend the coverage of glycoconjugate vaccines. Ongoing immunological evaluation of the prepared candidates will be presented in due course.

References

1. Spicer, C. D., and Davis, B. G. **2014** Selective chemical protein modification. *Nat. Commun. 5*, doi:10.1038/ncomms5740.

2. Shimanovich, U., Bernardes, G. J. L., Knowlesa, T. P. J., and Cavaco-Paulo, A. **2014** Protein micro- and nano-capsules for biomedical applications. *Chem. Soc. Rev. 43,* 1361-1371.

3. Wang, L.-X., and Amin, M. N. **2014** Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chem. Biol. 21*, 51-66.

4. Avci, F. Y., and Kasper, D. L. **2010** How bacterial carbohydrates influence the adaptive immune system. *Annu. Rev. Immunol. 28*, 107-110.

5. Hu, Q.-Y., Allan, M., Adamo, R., Quinn, D., Zhai, H., Wu, G., Clark, K., Zhou, J., Ortiz, S., Wang, B., Danieli, E., Crotti, S., Tontini, M., Brogioni, G., and Berti, F. **2013** Synthesis of a well-defined glycoconjugate vaccine by a tyrosine-selective conjugation strategy. Chem. Sci. 4, 3827-3832; Adamo, R., Martin, A., Berti, B., Danieli, E., and Hu, Q.-Y. **2013** Tyrosine ligation process and crosslinking agents used to form tyrosine-contg. polypeptide conjugates for raising an immune response in a mammal, From PCT Int. Appl., *WO 2013009564 A1 20130117.*

6. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. **2002** A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective ligation of azides and terminal alkynes. *Angew. Chem. Int. Ed. 41*, 2596-2599.

7. Tørnoe, C. W., Christensen, C., and Meldal, M. **2002** Peptidotriazoles on solid phase: [1,2,3]-Triazoles by regiospecific Copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057-3064.

8. Adamo, R., Hu, Q.-Y., Torosantucci, A., Crotti, S., Brogioni, G., Allan, M., Chiani, P., Bromuro, C., Quinn, D., Tontini, M., and Berti, B. **2014** Deciphering the structure–immunogenicity relationship of anti-Candida glycoconjugate vaccines. *Chem. Sci. 5*, 4302-4311.

9. Costantino, P., Rappuoli, R., and Berti, F. **2011** The design of semi-synthetic and synthetic glycoconjugate vaccines. *Expert Opin. Drug Disc. 6,* 1045-1067.

10. Pollabauer, E. M., Petermann, R., and Ehrlich, H. J. **2009** The influence of carrier protein on the immunogenicity of simultaneously administered conjugate vaccines in infants. *Vaccine 27*, 1674-1679.

11. Barington, T., Skettrup, M., Juul, L., and Heilmann, C. **1991** Non-epitope specific suppression of the antibody response to Haemophilus influenzae type b conjugate vaccine by preimmunization with vaccine components. *Infect. Imm. 61*, 432-438.

12. Granoff, D. M., Holmes, S. J., Belshe, R. B., Osterholm, M. T., McHugh, J. E., and Anderson, E. L. **1994** Effect of carrier protein priming on antibody responses to Haemophilus influenzae Type b conjugate vaccines in infants. *J. Am. Med. Assoc. 272*, 1116-1121.

13. Gibbs, R. S., Schrag, S., and Schuchat, A. **2004** Perinatal infections due to Group B streptococci. *Obstet. Gynecol. 104*, 1062-1076.

14. Madoff, L. C., Paoletti, L. C., and Kasper, D. L. **2006** Surface structures of Group B streptococci important in human immunity. Gram-Positive Pathogens Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A., and Rood, J. I., Eds pp 169-185, *Am. Soc. Microbiol.*, Washington, DC,.

15. Berti, F., Campisi, E., Toniolo, C., Morelli, L., Crotti, S., Rosini, R., Romano, M. R., Pinto, V., Brogioni, B., Torricelli, G., Janulczyk, R., Grandi, G., and Margarit, I. **2014** Structure of the type IX Group B Streptococcus capsular polysaccharide and its evolutionary relationship with types V and VII. *J. Biol. Chem. 289*, 23437-2348.

16. Heath, P. T., and Feldman, R. G. **2005** Vaccination against Group B Streptococcus. *Expert Rev. Vaccines 4,* 207-218.

17. Adamo, R., Nilo, A., Castagner, B., Boutureira, O., Berti, F., and Bernardes, G. J. L. **2013** Synthetically defined glycoprotein vaccines: Current status & future directions. *Chem. Sci. 4*, 2995-3008.

18. Rappuoli, R. **2000** Reverse vaccinology. *Curr. Opin. Microbiol. 3*, 445-450.

19. Maione, D., Margarit, I., Rinaudo, C. R., Masignani, V., Mora, M., Scarselli, M., Tettelin, H., Brettoni, C., Iacobini, E. T., Rosini, R., D'Agostino, N., Miorin, L., Buccato, S., Mariani, M., Galli, G., Nogarotto, R., Nardi Dei, V., Vegni, F., Fraser, C., Mancuso, G., Teti, G., Madoff, L. C., Paoletti , L. C., Rappuoli, R., Kasper, D. L., Telford, J. L., and Grandi, G. **2005** Identification of a universal Group B Streptococcus vaccine by multiple genome screen. *Science 109*, 148-150.

20. Margarit, I., Rinaudo, C. R., Galeotti, C. L., Maione, D., Ghezzo, C., Buttazzoni, E., Rosini, R., Runci, Y., Mora, M., Buccato, S., Pagani, M., Tresoldi, E., Berardi, A., Creti, R., Baker, C. J., Telford, J. L., and Grandi, G. **2009** Preventing bacterial infections with pilus-based vaccines: the Group B Streptococcus paradigm. *J. Infect. Dis. 199*, 108-115.

21. Crotti, S., Zhai, H., Zhou, J., Allan, M., Proietti, D., Pansegrau, W., Hu, Q.-Y., Berti, F., and Adamo, R. **2014** Defined conjugation of glycans to the lysines of CRM197 guided by their reactivity mapping. *ChemBioChem 14,* 836-843.

22. Meldal, M., and Tornøe, C. W. **2008** Cu-catalyzed azide-alkyne cycloaddition. *Chem. Rev. 108,* 2952–3015.

23. Agard, N. J., Baskin, J. M., Prescher, J. A., Lo, A., and Carolyn R. Bertozzi (2006) A comparative study of bioorthogonal reactions with azides **2006** *ACS Chem. Biol. 1*, 644-648.

24. Hudak, J. E., Barfield, R. M., de Hart, G. W., Grob, P., Nogales, E., Bertozzi, C. R., and Rabuka, D. **2012** Synthesis of heterobifunctional protein fusions using copper-free click chemistry and the aldehyde tag. Angew. *Chem. Int. Ed. 51*, 4161-4165.

25. Jewett, J. C., and Bertozzi, C. R. **2010** Cu-free click cycloaddition reactions in chemical biology. *Chem. Soc. Rev. 39*, 1272–1279.

26. Kurra, Y., Odoi, K. A., Lee, Y.-J., Yang, Y., Lu, T., Wheeler, S. E., Torres-Kolbus, J., Deiters, A., and Liu, W. R. **2014** Two rapid catalyst-free click reactions for in vivo protein labeling of genetically encoded strained alkene/alkyne functionalities. Bioconj. Chem., DOI: 10.1021/bc500361d.

27. Šardzík, R., Noble, G. T., Weissenborn, M. J., Martin, A., Webb, S. J., and Flitsch, S. L. **2010** Preparation of aminoethyl glycosides for glycoconjugation. Beilstein *J. Org. Chem. 6*, 699-703.

28. Mora-Montes, H. M., Netea, M. G., Ferwerda, G., Lenardon, M. D., Brown, G. D., Mistry, A. R., Kullberg, B. J., O'Callaghan, C. A., Sheth, C. C., Odds, F. C., Brown, A. J. P., Munro, C. A., and Gow, N. A. R. **2011** Recognition and blocking of innate immunity cells by Candida albicans chitin. *Infect. Immun.,* 1961–1970.

29. Vollmer, W., Blanot, D., and de Pedro, M. A. **2008** Peptidoglycan structure and architecture. *FEMS Microbiol. Rev. 32*, 149-167.

30. Kanie, O., Ito, Y., and Ogawa, T. **1994** Orthogonal glycosylation strategy in oligosaccharide synthesis. *J. Am. Chem. Soc. 116*, 12073-12074.

31. Yamago, S., Yamada, T., Maruyama, T., and Yoshida, J. **2004** Iterative glycosylation of 2-deoxy-2-aminothioglycosides and its application to the combinatorial synthesis of linear oligoglucosamines. *Angew. Chem., Int. Ed. 43*, 2145 -2148.

32. Plante, O. J., Palmacci, E. R., Andrade, R. B., and Seeberger, P. H. **2001** Oligosaccharide synthesis with glycosyl phosphate and dithiophosphate triesters as glycosylating agents. *J. Am. Chem. Soc. 123*, 9545-9554.

33. Toshima, K. **2000** Glycosyl fluorides in glycosidations. *Carbohydr. Res. 327*, 15–26.

34. Wessels, M. R., Paoletti, L. C., Kasper, D. L., Di Fabio, J. L., Michon, F., Holme, K., and Jennings, H. J. **1990** Immunogenicity in Animals of a Polysaccharide-protein conjugate vaccine against type III Group B Streptococcus. *J. Clin. Invest. 86*, 1428-1433.

35. Lauer, P., Rinaudo, C. D.,Soriani, M., Margarit, I., Maione, D., Rosini, R., Taddei, A. R., Mora, M., Rappuoli, R., Grandi, G., and Telford, J. L. **2005** Genome analysis reveals pili in Group B Streptococcus. *Science 309*, 105.

36. Rosini, R., Rinaudo, C. D., Soriani, M., Lauer, P., Mora, M., Maione, D., Taddei, A., Santi, I., Ghezzo, C., Brettoni, C., Buccato, S., Margarit, I., Grandi, G., and Telford, J. L. **2006**

Identification of novel genomic islands coding for antigenic pilus-like structures in Streptococcus agalactiae. *Mol. Microbiol. 61*, 126–141.

37. Vengadesan, K.,Ma, X., Dwivedi, P., Ton-That, H., and Narayanan, S. V. L. **2010** Purification, crystallization and halide phasing of a Streptococcus agalactiae backbone pilin GBS80 fragment. *Acta Cryst. F66*, 1666–1669.

38. Hendrickx, A. P. A., Budzik, J. M., Oh, S.-Y., and Schneewind, O. **2011** Architects at the bacterial surface - sortases and the assembly of pili with isopeptide bonds. *Nature Rev. 9,* 167-176.

39. Dougherty, C. A., Furgal, Mallory, J. C., van Dongen, A., Goodson, T. III, Banaszak Holl, M. M., Manono, J., and Di Maggio, S. **2014** Isolation and characterization of precise dye/dendrimer ratios. *Chem. Eur. J. 20,* 4638 – 4645

40. Van der Puta, R. M. F., de Haana, A., van den IJssela, J. G. M., Hamidia, A., and Beurret, M. HPAEC-PAD quantification of Haemophilus influenzae type b polysaccharide in upstream and downstream samples. *Vaccine, DOI: 10.1016/j.vaccine.2014.07.028.*

41. Adamo, R., Berti, F., and Hu, Q.-Y. **2014** Cycloalkyne derivatized saccharides. From PCT Int. Appl., *WO 2014111344 A1 20140724.*

Anti-Group B *Streptococcus* carbohydrate-based vaccines using pilus proteins as carriers: Comparing lysine and tyrosine-directed conjugation

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Introduction

Gram-positive *Streptococcus agalactiae* or Group B *Streptococcus* (GBS) is a leading cause of invasive infections in pregnant women, newborns and the elderly people.[1] Most GBS strains possess a capsular polysaccharide (PS) on their surface which is a major virulence factor. Ten different PS serotypes have been characterized, five of which are responsible for the vast majority of the disease in North America and Europe. GBS infection can be contracted by neonates from the maternal genital tract, and can result in prematurity of the newborn or bacteremia - generally pneumonia or menigitidis.[2] Therefore vaccination of pregnant women represents the best strategy for prevention of GBS infection in newborns.[3, 4] Early in 1930s Rebecca Lancefield *et al.* demonstrated that polyclonal antibodies from rabbit sera, able to recognize PS epitopes, conferred protection against GBS infection in animal models.[5-7] During the last two decades, plain GBS polysaccharides have been extensively studied as vaccines in preclinical and clinical studies.[1, 2, 8] However, the first human clinical trials conducted in the 1980s showed that the purified native PS from serotype III was not sufficient to induce a robust IgG response in adults.[1, 9] Conjugation of poorly immunogenic microbial polysaccharides to protein carriers, such as the chemically detoxified diphtheria and tetanus toxin (DT and TT, respectively) or the genetically detoxified diphtheria toxin (CRM_{197}), is a well-known mode to trigger an anti-carbohydrate T-cell-dependent memory response early in life, susceptible of boost effect.[10, 11] The conjugation of GBS PSs to TT and CRM_{197} has been shown to elicit long term protection in mice.[12-15] A trivalent GBS polysaccharide-protein conjugate vaccine composed of capsular epitopes from serotypes Ia, Ib and III is undergoing phase-II evaluation among pregnant women in Europe, North America and Africa.[16] In the future it may be necessary to broaden coverage against others strains that are emerging, primarily GBS II and V.[17] In this instance, several efforts have been focused on the identification of highly protective protein antigens that are present in most strains, including the non-capsulated ones. Unlike the conventional vaccine approaches, genome based "reverse vaccinology" has enabled the rapid identification of protective protein antigens.[18, 19] By this approach, it has been demonstrated that a

combination of GBS pilus proteins, besides being important structures in bacterial adhesion and invasion, confers wide protection against GBS. Genome sequence analysis of GBS revealed three independent loci, named Pilus Island 1 (PI-1) and Pilus Island 2a and 2b (PI-2a, PI-2b) that encode structurally distinct pilus types, and the backbone protein of PI-1 (GBS80) was identified among the potential protein antigens.[20, 21]

It has been observed that pre-exposure to the carrier can in some cases lead to reduction of the anti-carbohydrate immune response against glycoconjugate vaccines (*carrier epitope suppression*).[22] Use of carrier proteins alternative to DT, TT and CRM_{197}, that are usually employed in the manufacturing of most the glycoconjugate vaccines currently in the market, could be a way to overcome this effect.[23] Conjugation of streptococcal polysaccharides to GBS80 pilus protein could, therefore, be an attractive approach to extend the vaccine coverage.

Usually glycoconjugate vaccines are prepared by non-selective covalent coupling of the carbohydrate antigen to carrier protein through □-amine group or carboxyl group of K or D/E residues respectively. Over the recent years, methods for site selective bioconjugation are emerging.[24, 25] Our group has recently developed a robust method for insertion of alkyne-containing bifunctional linkers onto the tyrosine residues of the carrier protein and subsequent condensation of glycans via copper mediated azide-alkyne [3+2] cycloaddition.[26-28] This strategy was proven a powerful technology to establish a robust structure-immunogenicity relationship from anti-*Candida albicans* glycoconjugate prepared with defined sugars at predetermined Y residues.[29] This two-step conjugation to tyrosine residues allows fast determination by LC ESI MS of the sites where the glycan coupling will be directed, thus ensuring a higher batch-to-batch consistency in vaccine production in comparison to the classic random conjugation, and to control preservation of protective protein epitopes. We have also shown that conjugation of high molecular weight charged GBS polysaccharides can be achieved with high efficiency by using a copper-free based conjugation strategy.[30] Following that work, in the present study we explored the possibility to obtain conjugates of GBS PSII to GBS80, where the protein is used with the dual role of carrier and antigen. In this context, the effect on *in vivo* efficacy of vaccines obtained using Y-directed conjugation and non-selective K conjugation were compared.

Results and Discussion

Preparation of glycoconjugates. To investigate the potential use of streptococcal pili proteins as both, protective antigens and carriers for GBS polysaccharides, glycoconjugates of GBS80 and PSII were prepared. PSII is a complex negatively charged high molecular weight polysaccharide, whose repeating unit is depicted in Figure 1.

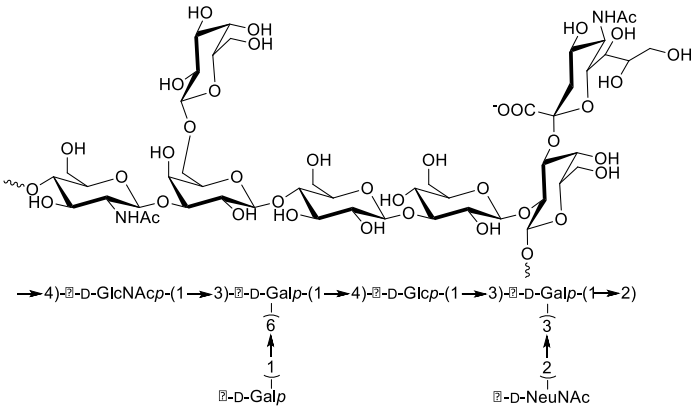


Figure 1. GBS CPSII repeating unit.

Pilus protein GBS80 is composed by three domains, namely N1, N2 and N3, and constitutes the major component of GBS PI-1 pilus.[31] The N2N3 domain, consisting of the 35 kDa C-terminal fragment, and its structure has been recently resolved at atomic level. As we have reported, the insertion of the monofluoro-cyclooctyne (MFCO) arm enables copper-free azide-alkyne [3+2] cycloaddition of the protein labelled with an azide linker (Scheme 1).[30] This approach provided the desired conjugate with excellent coupling efficiency when compared to the direct reductive amination (Table 1).

Table 1. Characteristics of the synthesized glycoconjugates.

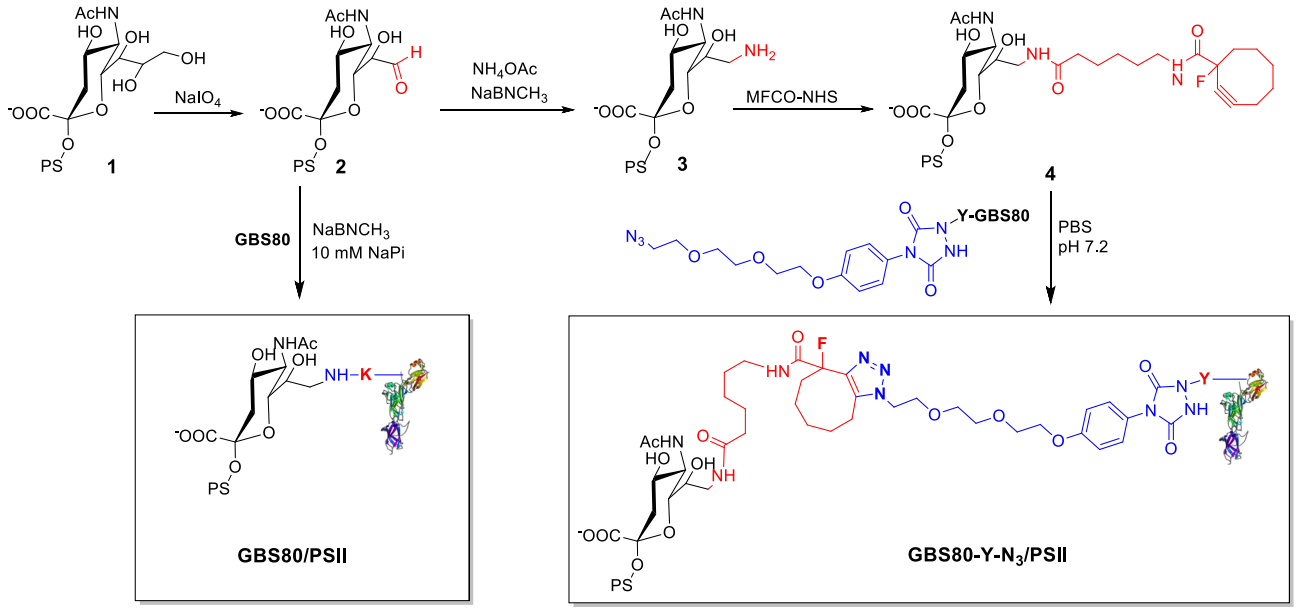
Glycoconjugate	Saccharide:protein stoichiometry (w/w)[a]	Glycosylation ratio (w/w)[b]	Free saccharide[c]	Conjugation efficiency[d]
GBS80/PSII	2:1	1.8	14.7%	86%
GBS80-Y-N_3/PSII	1:1	1.1	< 4.5%	>95%

a. Ratio of reagents used in the conjugation reaction; b. carbohydrate:protein ratio in the purified glycoconjugate; c. conjugated and unconjugated PS in the purified products were estimated by HPAEC-PAD quantification of Gal; d. percentage of carbohydrate attached to protein in respect to the amount used in conjugation.

Interestingly, labelling of GBS80 with the triazolidinone linker occurred at the residues Y16, Y23, Y44 and Y135 of the N1 protein domain, as determined by LC MS of the digested peptides. This data

indicated that the N-terminal domain is the more flexible and accessible portion of the protein. Preliminary data showed that the insertion of the linker onto the N1 domain did not impair the recognition of the epitopes from sera generated against full GBS80.[30] Therefore, we could predict that the protein immunogenicity was being preserved by the tyrosine-directed conjugation.

For random polysaccharide II conjugation, regioselective $NaIO_4$ oxidation targeting the glycerol chain of 20% sialic acid residues was used to generate aldehyde groups on the polymer. This material was used for direct conjugation of GBS80 through random reductive amination with sodium cyanoborohydride (Scheme 1). Alternatively, reductive amination of the foregoing aldehydes in presence of ammonium acetate provided the amine groups for the insertion of the N-hydroxysuccinimide ester of the MFCO linker. This modification permitted to introduce the cyclooctyne enabling subsequent high efficiency coupling to the protein by copper-free click chemistry. Purification of the final glycoconjugates was performed by chromatography on hydroxyapatite, and tuning of the phosphate buffer concentration used for elution enabled to separate the glycoconjugate from the unconjugated polysaccharide and protein. The characteristics of the synthesized glycoconjugates are described in Table 1. Both Y-directed conjugation via copper-free click chemistry and classic reductive amination showed very good efficiency in terms of incorporated saccharide.



Scheme 1. Conjugation of PSII to GBS80.

Immunogenicity of the glycoconjugates. The synthesized glycoconjugates were tested in mice for their capability to elicit specific anti-PSII and anti-GBS80 antibodies, which can meditate opsonophagocytic killing (OPKA) of strains expressing the capsular polysaccharide and the protein, respectively, and confer protection to the offspring from GBS infection (Tables 2 and 3).

To this end, two groups of sixteen CD-1 female mice were immunized with three subcutaneous injections of the prepared conjugates, with a dose of 1 μg in terms of polysaccharide and protein content, respectively. The animals received booster doses of the same vaccine at three and six weeks after the initial vaccination. The responses induced by the glycoconjugates were compared to the unconjugated PSII, when the protein was evaluated as carrier for the sugar, and to GBS80, when it was tested as antigen. The adjuvant alone was the negative control.

Following ELISA analysis of the elicited antibodies, the functionality was assessed by *in vitro* killing-based opsonophagocytosis assay (OPKA).[32] This is a well-established analysis that mimics the *in vivo* process of bacterial killing by host effector cells, following opsonization by specific antibodies.[32] In addition, it has been demonstrated that passive protection of mice by sera from individuals immunized with GBS polysaccharide-based vaccines correlate with high functional antibody titers measured by OPKA, suggesting that this assay can constitute a viable surrogate of the effectiveness of a GBS vaccine.[33] By using the strain 5401, which is heavily coated by PSII, while Pl-1 proteins are very poorly expressed, the functional activity of the antibodies against bacteria producing the polysaccharide was measured. *Vice versa*, the strain CHO1 was used to assess the functionality against strains which do not express PSII but GBS80 only.

The capability to transfer functional antibodies from the vaccinated female mice to the offspring was finally tested by survival model.[20, 21] To distinguish the effectiveness of the conjugated PSII and the protein as vaccine antigens, pups were challenged 35 days after the third immunization with a killing dose of either GBS strain 5401 or CHO1, respectively. The results of these *in vivo* experiments in correlation with the measured IgG and OPKA titers are shown in Tables 2 and 3.

Table 2. Evaluation of GBS80 as carrier for PSII at 1 μg polysaccharide dose.

Glycoconjugate	Anti PSII IgG GMT (95% CI)	OPKA titer (strain 5401)	Protected/Treated (challenge strain type 5401)	Survival (%)
PBS	<10	<30	6/59	10
GBS80/PSII	4956 (1273 ; 19299)	2443	36/60	60
GBS80-Y-N_3/PSII	384 (97 ; 1523)	2327	68/69	98
GBS80, PSII	<10	<30	42/79	53

ELISA analysis of sera generated at equal dose of carbohydrate hapten indicated that, while PSII non-covalently linked to GBS80 did not produce any anti-carbohydrate antibody response, both the glycoconjugates prepared by random conjugation and Y-directed ligation induced specific anti-PSII IgGs (Table 2). The antibody response elicited by GBS80/PSII was approximatively 10 fold higher than that achieved by vaccination with GBS80-Y-N_3/PSII. This effect could be due to the presence of non-responder mice in the group immunized with the latter vaccine. Importantly, the sera from mice vaccinated with the two conjugates showed comparable killing of the 5401 strain, which translated into an effective protection of the pups from a lethal dose of 5401 strain. These findings, therefore, highlighted that the protein GBS80 can efficiently function as carrier for PSII, regardless of the conjugation chemistry. A higher protection was observed in this model by vaccination with GBS80-Y-N_3/PSII in comparison to GBS80/PSII.

Table 3. Evaluation of GBS80 as antigen at 1 μg protein dose.

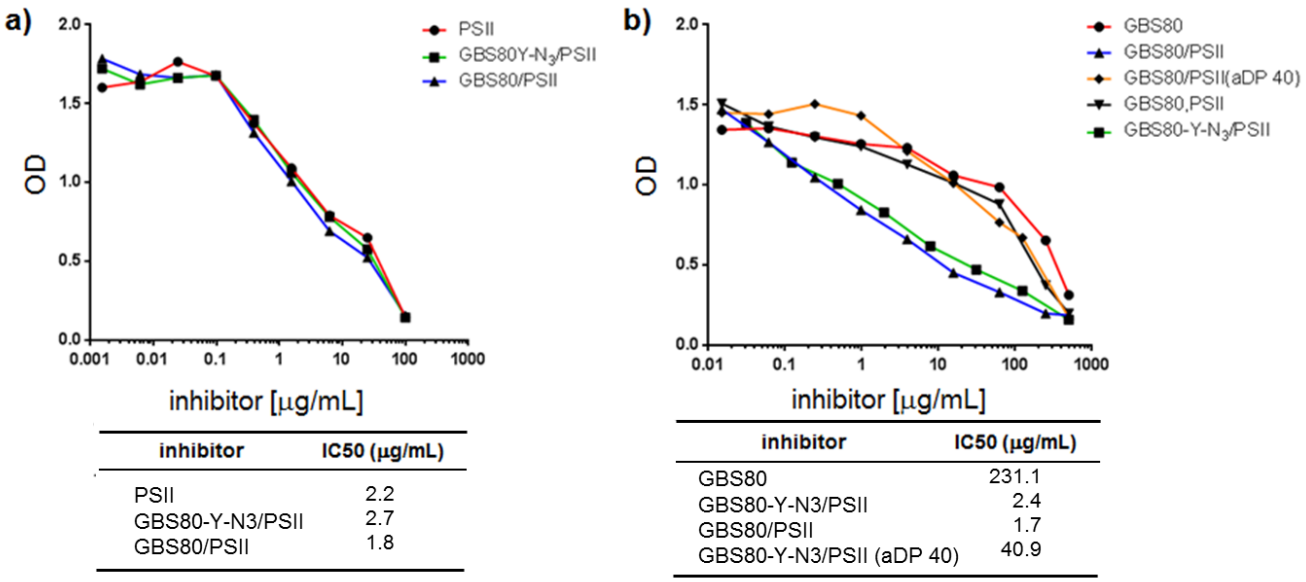
Glycoconjugate	Anti GBS80 IgG GMT (95% CI)	OPKA titer (strain COH1)[a]	Protected/Treated (challenge strain type COH1)	Survival (%)
PBS	10	<30	28/80	35
GBS80	3099 (69 ; 137225)	276	35/60	58
GBS80/PSII	775 (83 ; 7240)	676	30/60	50
GBS80-Y-N_3/PSII	3111 (426 ; 22705)	282	49/70	70

When the glycoconjugates were compared at the same protein dose (Table 3), we measured by ELISA analysis anti-protein IgG antibodies at statistically comparable levels to those elicited by the unconjugated GBS80 in the sera obtained from both the constructs. Interestingly, the conjugate synthesized by Y-selective coupling raised four fold higher anti-protein titers than the counterpart prepared by reductive amination at K. This result could be explained with the more homogenous conjugation of the polysaccharide achieved through the Y-directed ligation or, alternatively, with the slightly lower glycosylation degree of this conjugate. In accordance with the IgG levels, the OPKA titers induced by the two conjugates against the CHO1 strain were comparable each other and to the GBS80 alone. The generated antibodies were also effective in inducing good protection of the offspring from a lethal infection with COH1 strain, with again a moderately higher protection for the vaccination with GBS80-Y-N_3/PSII.

Overall, these results clearly demonstrated that GBS80 can be conjugated to PSII and function as carrier while maintaining its properties of antigen. The two constructs prepared by diverse conjugation

approaches are both effective in raising functional antibodies against PSII and GBS80 expressing strains. Despite the relatively high variability of the animal model, a moderately higher percentage of survival seems to be conferred by vaccination with GBS80-Y-N3/PSII in respect to GBS80/PSII (98 vs. 60% for 5401; 70 vs. 50% for CHO1).

Analysis of epitope recognition. The recognition of polysaccharide and protein epitopes in the two conjugates was further analyzed by competitive ELISA (Figure 3). When the binding of specific anti-PSII polyclonal sera to HSA-PSII conjugate was inhibited by GBS80-Y-N3/PSII and GBS80/PSII, both the constructs exhibited a half maximal inhibitory concentration (IC50) comparable to the unconjugated PSII (Figure 3A). Intriguingly, inhibition of the protein recognition from anti-GBS80 sera showed that the conjugates were better inhibitors than the protein itself (Figure 3B).



inhibitor	IC50 (μg/mL)
PSII	2.2
GBS80-Y-N3/PSII	2.7
GBS80/PSII	1.8

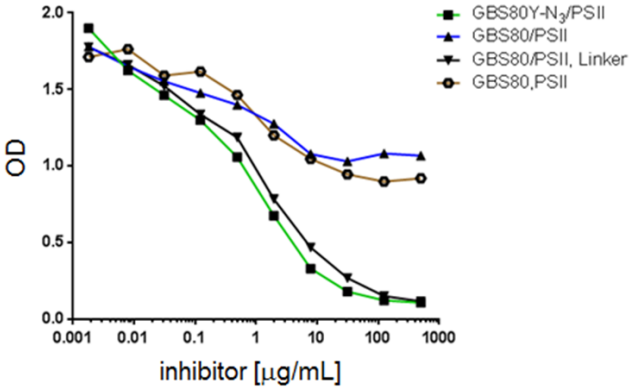
inhibitor	IC50 (μg/mL)
GBS80	231.1
GBS80-Y-N3/PSII	2.4
GBS80/PSII	1.7
GBS80-Y-N3/PSII (aDP 40)	40.9

Figure 3. a) Competitive ELISA of anti-PSII polyclonal sera generated from mice immunized with PSII conjugated to a non-pilus protein, using HSA-PSII as coating reagent. GBS80-Y-N3/PSII and GBS80/PSII are compared PSII as inhibitors. b) Competitive ELISA of anti-GBS80 polyclonal sera using the protein as coating reagent. GBS80-Y-N3/PSII and GBS80/PSII are compared to the protein, its mixture with PSII, and a conjugate prepared with shorter PSII fragments conjugated to GBS80.

We reasoned that this behavior could be due to the multivalent presentation of different GBS80 units along one polysaccharide chain. To test this hypothesis we reduced PSII into fragments with an average degree of polymerization (aDP) of 40 units by controlled partial N-deacetylation followed by nitrous acid deamination.[34] The aldehyde of the generated 2,5-anhydro-D-mannose residue was coupled to

GBS80 by reductive amination (Scheme S1, SI). The resulting glycoconjugate, presenting shorter PSII fragments around one protein core, induced inhibition very similar to the plain GBS80. This clearly proved that the stronger inhibition of GBS80-Y-N3/PSII and GBS80/PSII conjugates in respect to GBS80 was remarkably affected by the protein presentation. Overall, these findings corroborated the observation that polysaccharide and protein epitopes are well-preserved in both the constructs.

Analysis of anti-linker antibodies. Rigid rings have been found to generate unwanted anti-linker. We have previously shown that in our first generation of linkers where a phenyl and a triazole ring were concomitantly present, these antibodies were predominantly addressed to the first one. In any case, the anti-glycan response was not affected by these antibodies.[35] Hence, we were interested to evaluate the presence of antibodies directed to the neoepitope generated by condensation of the monofluoro-cyclooctyne and the azide linker during the copper-free click chemistry reaction. To this end, a competitive ELISA, using the conjugate GBS80-Y-N3/PSII as coating reagent and the corresponding anti-serum was carried out (Figure 4).



inhibitor	IC50 (µg/mL)
GBS80-Y-N3/PSII	0.8
GBS80/PSII	0.5
GBS80/PSII, linker	1.3
GBS80, PSII	1.4

Figure 4. Competitive ELISA of anti-GBS80-Y-N_3/PSII sera using GBS80-Y-N3/PSII as coating reagent. GBS80/PSII and its mixture with a conjugate CRM_{197}-Y-N_3/MenY, containing the MFCO linker, were compared as inhibitors. GBS80, PSII and their physical mixture were used as controls.

While GBS80-Y-N3/PSII efficiently inhibited the binding of the sera, conjugate GBS80/PSII did not fully inhibit the binding to the conjugate made by the copper-free click chemistry. In contrast, the mixture of GBS80/PSII with a CRM_{197}-Y-N_3/MenY conjugate, presenting in common with GBS80-Y-N_3/PSII the linker portion included between the modified Y residue of the protein and the oxidized sialic acid of the oligosaccharide (Figure 2), showed complete inhibition. This suggested that anti-linker antibodies were present in serum from construct GBS80-Y-N_3/PSII.

The level of antibodies directed to neoepitopes generated during the coupling reactions was further determined by ELISA analysis of the anti-glycoconjugate sera against a series of constructs presenting the oxidized sialyl residue produced during the random conjugation of PSII to GBS80 (Figure 5A), and the condensed cyclooctene-triazole system originated by the copper-free click chemistry (Figure 5C). For the preparation of the coating reagents, a tetrasaccharide [α-D-Glcp(1□4)-α-D-NeuNAc-(2□6)]$_2$ fragment from meningococcal serogroup Y (MenY) capsular polysaccharide was employed. This polymer has only the terminal end sialyl moiety in common with GBS polysaccharide. By doing so, we could roll out in the analysis of the sera any interference from the carrier and the other sugar residues. An additional reagent was used to measure antibodies directed to the triazole ring generated by the azide-alkyne [3+2] cycloaddition (Figure 5B). This comparative analysis evidenced that the presence of relatively low level of anti-linker IgGs which were primarily directed against the cyclooctene, rather than the triazole ring. Most importantly, the anti-linker antibodies do not impair the anti-carbohydrate and anti-protein immune responses (Figure 5).

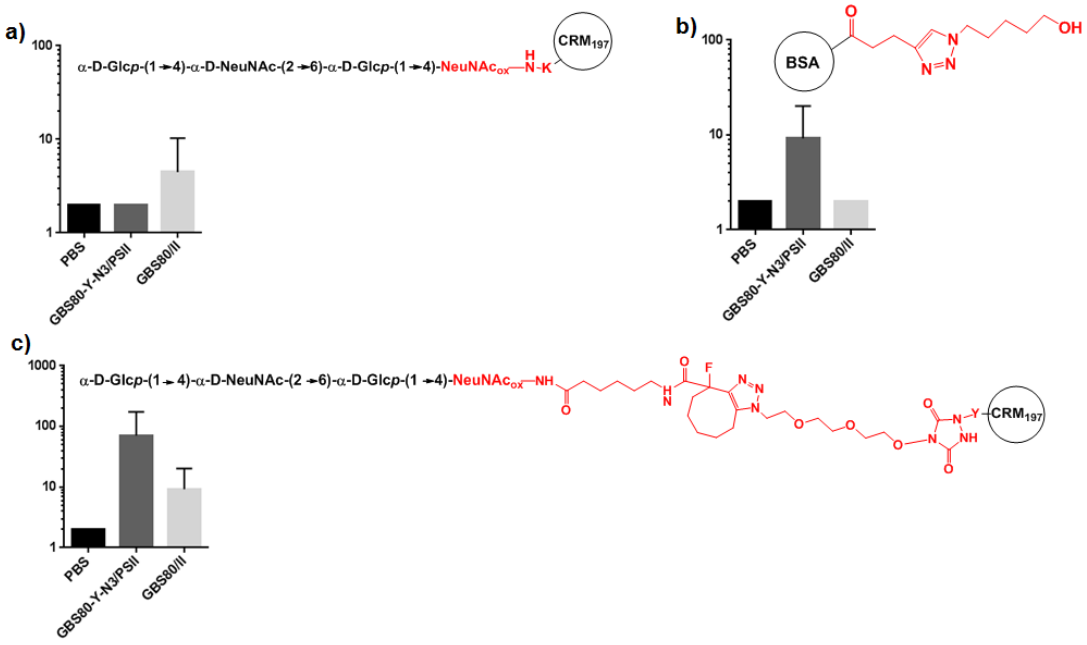


Figure 5. ELISA analysis of anti-linker antibodies using pooled polyclonal sera from mice vaccinated with GBS conjugates using as coating reagents: a) MenY tetramer directly coupled to CRM_{197}; b) the triazole ring generated on BSA; c) MenY tetramer modified with the MFCO linker and condensed to CRM_{197} labeled with the azide linker.

In conclusions, we have shown that the tyrosine-directed conjugation via copper-free click chemistry represents an excellent strategy to attach large polysaccharides to protein antigens, enabling to target predetermined site and to ensure high reproducibility in the conjugate formation.*(30)* This approach permits easy and fast assessment of preservation of the protein epitopes prior to polysaccharide conjugation.*(30)* In the present study we compared two conjugates prepared from GBS type II polysaccharide and GBS80 pilus protein through a classic random conjugation and the recently developed Y-directed ligation, respectively. We found that both the constructs showed a good capability to elicit *in vivo* anti-carbohydrate and anti-protein functional antibodies that induce opsonophagocitosis in strains expressing exclusively PSII or GBS80. In addition, the two glycoconjugates were both effective in protecting new born mice against GBS infection following vaccination of the mothers. The conjugate made by the tyrosine ligation via copper free chemistry generated relatively low levels of anti-linker antibodies that, however, do not affect the anti-polysaccharide and anti-protein immune responses. By contrast it conferred moderately higher protection against strains expressing PSII or GBS80 than the counterpart prepared by random conjugation at lysine residues. These results clearly demonstrated that GBS80 pilus protein can be used as carrier for PSII polysaccharide while maintaining its properties of protein antigen. Furthermore, these findings highlight that protein antigens selected by "reverse vaccinology" can be combined with capsular polysaccharides through appropriate conjugation chemistries that preserve the protein epitopes to create vaccines with broader coverage. This approach is expected to lead to significant advancements towards the next generation of rationally designed glycoconjugate vaccines.

Methods

Bacterial strains. GBS serotype II strain 18RS21 was obtained from Dennis Kasper (Harvard Medical School, Boston, MA). The purification process was based on previously described procedures.[36] Briefly, the bacterial pellet was recovered by centrifugation at 4,000 rpm for 20 min and incubated with 0.8 N NaOH at 37 °C for 36 h. After centrifugation at 4,000 rpm for 20 min, 1 M Tris buffer (1:9, v/v) was added to the supernatant and diluted with 1:1 (v/v) HCl to reach a neutral pH.

GBS80 expression. Pilus protein was expressed in *E. coli* by a plasmid vector derived from pET24b+ allowing the expression of the protein GBS 80 from *S. agalactiae*. The clone has been obtained by recombinant DNA techniques. The DNA fragment coding for amino acids 38-520 of GBS 80 from *S. agalactiae* strain 2603/VR was introduced in the plasmid pET24b+ for inducible expression.

Protein modification. Similarly as we reported in literature,[30] to the protein (0.001 μmol) in Tris 0.5 M pH 7.4 (67 μL) at 4°C was added four portions of 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-3H-1,2,4-triazole-3,5(4H)-dione (0.25 μL, 0.005 μmol) in acetonitrile every minute (total of 20 equivalents). The mixture was agitated at 4°C for 30 minutes. The mixture was desalted and buffer exchanged to PBS pH 7.4 two times using Zeba 7K MW cut off spin columns.

Polysaccharide modification

Periodate oxidation of PSII. Oxidation was targeted to 20% of sialic acid residues.(*35)* GBS type II PS (100 mg) was stirred with 151 μL 0.1M of sodium periodate in 9.8 mL of 10 mM sodium phosphate buffer in the dark, for 2 h at RT. The mixture was purified by tangential flow filtration (Sartocon 10kD filter).

For the preparation of GBS80-PSII glycoconjugate through random chemistry at lysine, the oxidized polysaccharide (20 mg) was dissolved in a 200 mM/NaCl 2M Sodium Phosphate buffer pH 7.2 and mixed to the protein (10 mg) with a final concentration of PSII of 4 mg/mL and for GBS80 2 mg/mL. For conjugation of PSII to tyrosine modified GBS80, the following procedure was followed. The oxidized PS (50 mg) dissolved in H_2O milli-Q at a concentration of 5 mg/mL, was subjected to reductive amination by treatment with ammonium acetate (5.5 g) and sodium cyanoborohydride (1 g). After incubation for 5 days at 37°C, the crude mixture was filtered by Tangential Flow Filtration (TFF,Sartocon 10kDa filter). Quantification of polysaccharide and amino groups were performed by colorimetric assays. Partially aminated polysaccharide (30 mg, corresponding to 4.5×10^{-3}mmol of oxidized sialic acid) was dissolved in 3 mL of DMSO, MFCO-N-hydroxysuccinimide (Berry&Associates, 10 equiv) and 50 μL of triehtylamine were added. Reaction was stirred for 3 h at RT. The crude was precipitated and washed (8 × 30 mL) in EtOAc at 4°C in order to remove the excess of unreacted linker. Then the PS was lyophilized and sialic acid was quantified by colorimetric assay.

Modified GBS80 (3.5 mg) and activated polysaccharide (3.5 mg) were mixed in PBS at pH 7.2 and incubated overnight at RT. Conjugation was monitored by SDS-PAGE 3-8% of polyacrylamide in Tris Acetate. The conjugates were purified by a CHT™ hydroxyapatite column (Biorad laboratories). In the

first step of purification, unreacted protein was removed by elution with 2 mM sodium phosphate pH 7.2 (90 mL, 1 mL/min) followed by 400 mM sodium phosphate pH 7.2 (90 mL, 1 mL/min). Then the conjugate was purified from free polysaccharide by another run on the same column with 2 mM sodium phosphate /550 mM NaCl pH 7.2 (50 mL, 0.5 mL/min), 10 mM sodium phosphate pH 7.2 (50 mL, 1 mL/min), 35 mM sodium phosphate pH 7.2 (40 mL, 1 mL/min), 400 mM sodium phosphate pH 7.2 (40 mL, 1 mL/min). The conjugate is detected by measuring UV absorption at 215 nm, 254 nm and 280 nm. Protein content in the purified glycoconjugates was determined by micro-BCA (Thermo-scientific).

Total saccharide was quantified by HPAEC-PAD. A standard sample with five increasing concentrations of Gal ranging between 0.2 and 4.0 ug/mL (as saccharide powder/mL), was prepared for building a calibration curve. Two GBS PSII samples were prepared targeting final concentrations in the calibration curve range. The reference and analytical samples for Gal were prepared in 4 M trifluoroacetic acid, incubated at 100 °C for 2 h, dried under vacuum (SpeedVac Thermo), and suspended in water. All analytical samples were filtered with 0.45 μm Acrodisc (Pall) filters before analysis. HPAEC-PAD analysis was performed with a Dionex ICS3000 equipped with a CarboPac PA1 column (4 x 250 mm; Dionex) coupled with PA1 guard to column (4 x 50 mm; Dionex). Samples (50 μL injection volume) were run at 1 mL/min, using isocratic elution with 24 mM NaOH, followed by a washing step with 0.5 M NaOH. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was applied. Unconjugated saccharide was separated by SPE C4 hydrophobic interaction column (0.5 mL resin, Bioselect, Grace Vydac, Columbia, MD, USA) and subsequently estimated by HPAEC-PAD analysis. The resulting chromatographic data were processed using Chromeleon software 6.8 (Dionex).

Preparation of PSII-HSA for ELISA coating. To HSA (Sigma), at a concentration of 10 mg/mL in 100 mM MES (Sigma) buffer pH 5, EDAC (1.5 equiv relative to HSA carboxylic groups) was added, and the mixture was gently mixed to allow complete solubilization; then, ADH (13.4 equivrelative to HSA carboxylic groups) was added to the solution. The reaction was kept under gently stirring for 1 h at RT. Next, the reaction was quenched by adding 1/6 of the reaction volume of 400mM sodium phosphate buffer, pH 7.2.

The conjugation reaction was performed at GBS PSII concentration of 5 mg/mL in 100mM MES/250 mM NaCl buffer pH 5.EDAC (1:1, mol PS/mol EDAC) and the SulfoNHS (1:1, mol PS/mol SulfoNHS) were added to GBS PSII solution.

After the reageents were dissolved, HSA-ADH (2:1 protein/polysaccharide w/w) was added and the reaction was incubated at RT under gently stirring for 12-15 h, at which time the reaction was quenched by adding 1/5 of the reaction volume of 400mM NaPi buffer pH 7.2. The crude reaction was purified by size-exclusion chromatography (SEC) on a Sephacryl S400 resin (G&E Healthcare). The chromatographic step was performed on Akta ™ system (G&E Healthcare) at 2 mL/min flow rate, detecting the conjugate by measuring UV absorption at 215 nm, 254 nm and 280 nm.

Preparation of conjugate GBS80/PSII (aDP40). For the depolymerization of PSII, 10 mg of polysaccharide were dissolved in 5 mL of 0.5 M of NaOH and stirred at 70° C. After 6 h the solution was cooled, 0.5 mL of acetic acid and 1 mL of a solution 5% of nitrous acid were added and stirred at 4°C for 2 h. Then the mixture was desalted on a G-25 column (Sephadex G-25 GE Healthcare). For the conjugation reaction to GBS80, 8.3 mg of depolymerized PSII were dissolved in 10 mM sodium phosphate buffer pH 7.2 were added to 2.5 mg of GBS80 in a solution with a final concentration of 10mg/mL in protein. Then 8.3 mg of $NaBCNH_3$ were added to the solution and incubated O.N. at R.T. Conjugation was monitored by SDS-PAGE 4-8% of polyacrylamide in MOPS. Unreacted saccharide was removed by 10 cycles of filtration with Vivaspin (cut-off 30 KDa)

Periodate oxidation of MenY DP2. MenY DP2 was obtained by controlled acidic hydrolysis of MenY polysaccharide and purifications of different size oligosaccharide by anionic exchange chromatography on Mono Q column as described in literature.[37] 50% of sialic acid residues were targeted for oxidation. MenY DP2 (100 mg) was stirred with 491 µL 0.1M of sodium periodate in 9.5 mL of 10mM sodium phosphate buffer in the dark, for 2 h at RT. The mixture was purified by gel filtration chromatography on a Sephadex G-10 column (G&E Healthcare) using 10 mM NaCl pH 7.2 as elution buffer (190 mL, 1mL/min). Subsequently reductive amination and linker insertion was performed with the same conditions reported for GBS PSII. CRM_{197} Modified at tyrosine residues (CRM_{197}-Y-N_3) was prepared as described above.

Conjugation of CRM_{197}-Y-N_3 to modified MenY DP2. to 1.5 mg of protein (2.5×10^{-5} mmol) in 10 mM of sodium phosphate buffer pH 7.2, the saccharide (2.1 mg, 60 eq) was added at a final concentration of 2 mg/mL in term of protein. The mixture was incubated overnight at RT. Conjugation

was monitored by SDS-PAGE 4-8% of polyacrylamide in MOPS. Unreacted saccharide was removed by 10 cycles of filtration with Vivaspin (30 KDa MW cut off). The loading of saccharide was determined by MALDI-TOF MS analysis (Bruker Daltonics).

Immunogenicity of conjugates in mice. Protocols were approved by the Italian Ministry of Health. Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in the conduct of all animal studies. Two groups of 8 CD-1 mice were immunized by intraperitoneal injection of 1 μg in polysaccharide or in protein content of each glycoconjugate using alum hydroxide as adjuvant. Alum hydroxide, GBS80 and the mixture of GBS80 and PSII were used as controls. Mice received the vaccines at days 1, 21 and 35. Sera were bled at days 1, 35 and 49.

Challenge and survival protocol. The protective efficacy of GBS conjugates was measured by the maternal immunization-neonatal mouse challenge model of GBS infection. At day 35 a male mouse was added to the female mice groups. Infants were challenged with a lethal dose 90% of GBS within 48 h of life. Newborns were monitored for 4 days and after they were sacrificed.

ELISA. Direct enzyme-linked immunosorbent assay titers of PS-specific and protein antibody were determined using as coating reagents PSII-HAS, GBS80 or constructs with different linkers. Microtiter plates (NUNC Polysorp; Nalge Nunc International Corp., Rochester, NY) were coated by adding 100 μL per well of coating reagent (1-1.5 μg/mL) in PBS 1x pH 7.2. The plates were incubated overnight at 4°C and were washed with PBS containing 0.05% Tween 20 (PBS-T) then blocked with 0.5% bovine serum albumin in PBS for 1.5 h at 37°. The wells were then filled with 100 μL of serum at dilution 1:400 in PBS and incubated at 37°C for 1h. After 3 washes, 100 μL for well of peroxidase-labeled goat anti-mouse was added (Sigma-Aldrich) and plates incubated for 1.5 h at 37°C. The plates were again washed 3 times with PBS-T and finally 100 μL of peroxidase substrate (1 μg/mL in diethanolamine pH 9.8) was added to each well, and following incubation of the plates for 30 min at room temperature. The reaction was stopped by the addition of 100 μL of a solution of EDTA 7% and the plates were read immediately at 405 nm.

Competitive ELISA. 96-well Microtiter plates (NUNC Polysorp; Nalge Nunc International Corp., Rochester, NY) were coated by adding 100 μL per well of GBS80 protein (1.5 μg/mL) in PBS 1x pH 7.2. The plates were incubated overnight at 4°C and were washed three times with PBS containing

0.05% Tween 20 (PBS-T) then blocked with 0.5% bovine serum albumin in PBS for 1.5 h at 37°. A pool of antiserum versus GBS80 (dilution 1:1500 in PBS 1x pH 7.2) were incubated 15 minutes at RT with different concentrations of the protein used as inhibitor in a final volume of 100 µL and then transferred into coated plates. After 2 h of incubation at 37°C, plates were washed three times and of peroxidase-labeled goat anti-mouse diluted 1:10000 (100 μL for well, Sigma-Aldrich) was added and incubated for 1.5 h at 37°. The plates were again washed 3 times with PBS-T and finally peroxidase substrate (100 μL of a 1 μg/mL solution in diethanolamine pH 9.8) was added to each well, and following incubation of the plates for 30 min at room temperature. Then the plates were read immediately at 405 nm. The statistical and graphical analysis was performed using GraphPad Prism 6 software.

Opsonophagocytosis killing assay (OPKA). The functional activity of the sera was determined in OPKA assay. HL-60 cells were grown in RPMI 1640 with 20% fetal calf serum. Incubation was at 37 °C with 5% CO_2. HL-60 cells were differentiated to neutrophils with 0.78% dimethylformamide (DMF) and after 4-5 days are used as source of phagocytes.

Serum antibodies serially diluted in HBSS red were mixed with 6 $\times 10^4$ CFU per well of GBS type II strain 5401 cells or type III strain COH1. HL-60 cells (2 $\times$ 10^6 cell/well) and rabbit complement (diluted at 2-10% in water) were added and incubated at 37°C for 1 h under shaking. Before (T0) and after (T60) the incubation, the mixtures were diluted and plated in blood agar plates. (NUNC Polysorp; Nalge Nunc International Corp., Rochester, NY) Each plate was then incubated overnight at 37°C with 5% of CO_2 counting CFUs the next day. OPA titer was expressed as the reciprocal serum dilution leading to 50% killing of bacteria and the % of killing is calculated as follow: Killing% : (T0-T60)\T0

Where T0 is the mean of the CFU counted at T0 and T1 is the average of the CFU counted at T60 for the two replicates of each serum dilution.

References

1 Baker, C. J., Rench, M. A., Edwards, M. S., Carpenter, R. J., Hays, B. M., and Kasper, D. L. **1988** Immunization of pregnant women with a polysaccharide vaccine of group B streptococcus, *N. Engl. J. Med. 319*, 1180-1185.

2 Zalenik, D. F., Rench, M. A., Hillier, S., Krohn, M. A., Platt, R., Lee, M. T., Flores, A. E., Ferrieri, P., and Baker, C. J. **2000** Invasive disease due to Group B Streptococcus in pregnant women and neonates from diverse population groups, *Clin. Infect. Dis. 30*, 276-282.

3 Chen, V. L., Fikri, Y. A., and Kasper, D. L. **2013** A maternal vaccine against group B *Streptococcus*: Past, present, and future, *Vaccine 31*, 13-19.

4 Munoz, F. M., and Ferrieri, P. **2013** Group B *Streptococcus* vaccination in pregnancy: Moving toward a global maternal immunization program, *Vaccine*, 46-52.

5 Lancefield, R. C. **1934** A serological differentiation of specific types of Bovine Hemolytic Streptococci (Group B), *J. Exp. Med. 59*, 441-458.

6 Lancefield, R. C. **1938** Two serological types of Group B Hemolytic Streptococci with related, but not identical, type-specific substances, *J. Exp. Med*. *67*, 25-40.

7 Lancefield, R. C., McCarty, M., and Everly, W. N. **1975** Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens, *J. Exp. Med. 142*, 165-179.

8 Lancaster, L., Saydam, M., Markey, K., Ho, M. M., and Mawas, F. **2011** Immunogenicity and physico-chemical characterisation of a candidate conjugate vaccine against group B streptococcus serotypes Ia, Ib and III, *Vaccine 29*, 3213-3242.

9 Baker, C. J., Edwards, M. S., and Kasper, D. L. **1981** Role of antibody to native type-III polysaccharide of Group-B Streptococcus in infant infection, *Pediatrics 68*, 544-549.

10 Avci, F. Y., and Kasper, D. L. **2010** How bacterial carbohydrates influence the adaptive Immune system, *Annu. Rev. Immunol. 28,* 107-130.

11 Avci, F. Y., Li, X., Tsuji, M., and Kasper, D. L. **2012** A novel mechanism for glycoconjugate vaccine activation of the adaptive immune system, *Nat. Med. 17*, 1602-1609.

12 Paoletti, L. C., Wessels, M. R., Michon, F., Difabio, J., Jennings, H. J., and Kasper, D. L. **1992** Group-B Streptococcus Type-II polysaccharide-Tetanus Toxoid conjugate vaccine, *Infect. Immun. 60*, 4009-4014.

13 Wessels, M. R., Paoletti, L. C., Rodewald, A. K., Michon, F., DiFabio, J., Jennings, H. J., and Kasper, D. L. **1993** Stimulation of protective antibodies against type Ia and Ib group B streptococci by a type Ia polysaccharide-tetanus toxoid conjugate vaccine, *Infect. Immun. 61*, 4760-4766.

14 Wessels, M. R., Paoletti, L. C., Pinel, J., and Kasper, D. L. **1995** Immunogenicity and Protective Activity in animals of a Type-V Group-B Streptococcal polysaccharide Tetanus Toxoid conjugate vaccine, *J. Infect. Dis. 171*, 879-884.

15 Paoletti, L. C., Peterson, D. L., Legmann, R., and Collier, R. J. **2001** Preclinical evaluation of group B streptococcal polysaccharide conjugate vaccines prepared with a modified diphtheria toxin and a recombinant duck hepatitis B core antigen *Vaccine 20*, 370-376.

16 Madhi, S. A., Dangor, Z., Heath, P. T., Schrag, S., Izu, A., Sobanjo-ter Meulen, A., and Dull, P. M. **2013** Considerations for a phase-III trial to evaluate a group B *Streptococcus* polysaccharide-protein conjugate vaccine in pregnant women for the prevention of early- and late-onset invasive disease in young-infants, *Vaccine 31*, 52-57.

17 Le Doare, K., and Heath, P. T. **2013** An overview of global GBS epidemiology, *Vaccine 31*, 7-12.

18 Rappuoli, R. **2000** Reverse vaccinology, *Curr. Opin. Microbiol. 3*, 445-450.

19 Rappuoli, R., and Serruto, D. **2006** Post-genomic vaccine development, *FEBS Letters 580*, 2985-2992.

20 Maione, D., Margarit, I., Rinaudo, C. D., Masignani, V., Mora, M., Scarselli, M., Tettelin, H., Brettoni, C., Iacobini, E. T., Rosini, R., D'Agostino, N., Miorin, L., Buccato, S., Mariani, M., Galli, G., Nogarotto, R., Nardi Dei, V., Vegni, F., Fraser, C., Mancuso, G., Teti, G., Madoff, L. C., Paoletti, L. C., Rappuoli, R., Kasper, D. L., Telford, J. L., and Grandi, G. **2005** Identification of a universal Group B streptococcus vaccine by multiple genome screen, *Science 309*, 148-150.

21 Margarit, I., Rinaudo, C. D., Galeotti, C. L., Maione, D., Ghezzo, C., Buttazzoni, E., Rosini, R., Runci, Y., Mora, M., Buccato, S., Pagani, M., Tresoldi, E., Berardi, A., Creti, R., Baker, C. J., Telford, J. L., and Grandi, G. **2009** Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm, *J. Infect. Dis. 199*, 108-115.

22 Dagan, R., Poolman, J., and Siegrist, C. **2010** Glycoconjugate vaccines and immune interference: A review, *Vaccine 28*, 5513-5524.

23)Santamaria, R., Goulart, C., Perciani, C. T., Barazzone, G. C., Carvalho, R., Goncalves, V. M., Leite, L. C. C., and Tanizaki, M. M. **2011** Humoral immune response of a pneumococcal conjugate vaccine: Capsular polysaccharide serotype 14-Lysine modified PspA, *Vaccine 29*, 8689-8695.

24 Spicer, C. D., and Davis, B. G. **2013** Selective chemical protein modification, *Nat. Commun.5,* DOI: 10.1038/ncomms5740.

25 Adamo, R., Nilo, A., Castagner, B., Boutureira, O., Berti, F., and Bernardes, G. J. L. **2013** Synthetically defined glycoprotein vaccines: current status and future directions, *Chem. Sci. 4*, 2995-3008.

26 Hu, Q., Allan, M., Adamo, R., Quinn, D., Zhai, H., Wu, G., Clark, K., Zhou, J., Ortiz, S., Wang, B., Danieli, E., Crotti, S., Tontini, M., Brogioni, G., and Berti, F. **2013** Synthesis of a well-defined glycoconjugate vaccine by a tyrosine-selective conjugation strategy, *Chem. Sci. 4*, 3827-3832.

27 Baskin, J. M., Prescher, J. A., Laughlin, S. T., Agard, N. J., Chang, P. V., Miller, I. A., Lo, A., Codelli, J. A., and Bertozzi, C. R. **2007** Copper-free click chemistry for dynamic in vivo imaging, *Proc. Natl. Acad. Sci. USA 104*, 16793-16797.

28 Jewett, J. C., and Bertozzi, C. R. **2010** Cu-free click cycloaddition reactions in chemical biology, *Chem. Soc. Rev. 39*, 1272-1279.

29 Adamo, R., Allan, M., Berti, F., Danieli, E., and Hu, Q. **2013** Tyrosine ligation process and crosslinking agents used to form tyrosine-contg. polypeptide conjugates for raising an immune response in a mammal, *PCT Int. Appl. (2013), WO 2013009564 A1 20130117.*

30 Nilo, A., Allan, M., Brogioni, B., Proietti, D., Cattaneo, V., Crotti, S., Sokup, S., Zhai, H., Margarit, I., Berti, F., Hu, Q., and Adamo, R. **2014** Tyrosine-directed conjugation of large glycans to proteins via copper-free click chemistry, *Submitted*.

31 Vengadesan, K., Ma, X., Dwivedi, P., Ton-That, H., and Narayanan, S. V. L. **2010** Purification, crystallization and halide phasing of a Streptococcus agalactiae backbone pilin GBS80 fragment, *Acta Cryst. F66*, 1666–1669.

32Guttormsen, H., Liu, Y., and Paoletti, L. C. **2008** Functional activity of antisera to group B streptococcal conjugate vaccines measured with an opsonophagocytosis assay and HL-60 effector cells, *Hum. Vaccin. 4*, 370-374.

33 Brigtsen, A. K., Kasper, D. L., Baker, C. J., Jennings, H., J., and Guttormsen, H. **2002** Induction of Cross-reactive antibodies by immunization of healthy adults with Types Ia and Ib Group B Streptococcal polysaccharide–Tetanus Toxoid conjugate vaccines, *185*, 1277-1284.

34 Michon, F., Uitz, C., Sarkar, A., D'Ambra, A. J., Laude-Sharp, M., and Fusco, P. C. **2006** Group B Streptococcal Type II and III conjugate vaccines: Physicochemical properties that influence immunogenicity, *Clin. Vacc. Immunol. 13*, 936-943.

35 Adamo, R., Hu, Q., Torosantucci, A., Crotti, S., Brogioni, G., Allan, M., Chiani, P., Bromuro, C., Quinn, D., Tontini, M., and Berti, F. **2014** Deciphering the structure–immunogenicity relationship of anti-Candida glycoconjugate vaccines, *Chem. Sci. 5,* 4302-4311

36 Wessels, M. R., Paoletti, L. C., Kasper, D. L., DiFabio, J., Michon, F., Holme, K., and Jennings, H., J. **1990** Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against Type III Group B Streptococcus, *J. Clin. Invest. 86*, 1428-1433.

37 Costantino, P., Norelli, F., Giannozzi, A., D'Ascenzi, S., Bartoloni, A., Kaur, S., Tang, D., Seid, R., Viti, S., Paffetti, R., Bigio, M., Pennatini, C., Averani, G., Guarnieri, V., Gallo, E., Ravenscroft, N.,

Lazzeroni, C., Rappuoli, R., and Ceccarini, C. **1999** Size fractionation of bacterial capsular polysaccharides for their use in conjugate vaccines, *Vaccine 17*, 1251-1263.

Conclusions

Glycoconjugate vaccines are among the safest and most efficacious vaccines developed during the last 30 years. They are a potent tool for prevention of life-threating bacterial infectious disease like meningitis and pneumonia[1].

The weak immunogenicity of polysaccharides, T-cell independent antigens, can be increased after their conjugation to protein carrier; with this strategy a glycoconjugate induced a T-cell response (TD) already early in life which leads to immunological memory and boosting of the response by further doses of the vaccine[2].

In the context of glycoconjugate vaccines, with saccharide antigen (B-cell epitope) coupled to a protein carrier (T-cell epitope), the development of more defined vaccines to improve glycoconjugate design has been investigated.

A first study was directed to develop a two-step conjugation strategy based on copper-free [3+2] cycloaddition of sugars modified with a cyclooctyne and proteins derivatized at the tyrosine residues with the novel azido-linker. This method was first proven fast and highly efficient in the preparation of glycoconjugates with defined attachment point from minimal amounts of synthetic carbohydrates, whose preparation generally requires multistep syntheses and substantial effort. Next, the novel approach was successfully applied to the preparation of glycoconjugates from GBS PSII and the pilus protein GBS80. By competitive ELISA we verified that the protein is not affected by the selective ligation of the linker onto the tyrosine residues. The covalent linkage of the saccharides and the protein was ascertained by immunoblot analysis. This technology appears very appealing for the conjugation of carbohydrate haptens to protein antigens[3], as it ensures high consistency in the conjugation step and easy in process MS analysis of the modified protein whose functionality needs to be preserved.

In the second study, by comparing two conjugates prepared from GBS type II polysaccharide and GBS80 pilus protein through a classic random conjugation and the recently developed Tyr-directed

ligation, respectively, we found that both the constructs showed comparable capability to elicit *in vivo* anti-carbohydrate and anti-protein functional antibodies that induce opsonophagocitosis in strains expressing exclusively PSII or GBS80 proteins. In addition, the two glycoconjugates were both effective in protecting new born mice against GBS infection following vaccination of the mothers. The conjugate made by the tyrosine ligation via copper free chemistry generated relatively low levels of anti-linker antibodies that do not impair the anti-polysaccharide immune response. The protection conferred to the dams was higher than that achieved by random conjugation. These results clearly demonstrated that GBS80 pilus protein can be used as carrier for PSII polysaccharide while maintaining its properties of protein antigen. These findings highlight, for the first time, that GBS polysaccharide haptens and protein antigens selected by "reverse vaccinology" can be combined to extend the current trivalent GBS vaccine under clinical trial. This approach is expected to lead to significant advancements towards the next generation of glycoconjugate vaccines. The Tyr-directed conjugation via copper-free click chemistry represents an excellent strategy to attach large polysaccharides to protein antigens, enabling fine tuning of the conjugation chemistry. This approach permits easy and fast assessment of preservation of the protein epitopes prior to polysaccharide conjugation.

In conclusion, the PhD work has given a contribution to improve the glycoconjugate vaccine design, by preparing more defined structures able to maintain their immunogenicity and using pili proteins in a double role of antigens and carrier

In another study the surface polysaccharide PSII of *C. difficile* conjugated to CRM_{197} has also been proven an optimal target for a carbohydrate-based vaccine. We explored the feasibility of a glycoconjugate vaccine where the PSII saccharide was conjugated to the two protein fragments TcdA_B2 and TcdB_GT from toxin A and B, respectively. We demonstrated that TcdB_GT is a very efficient carrier for PSII, since PSII-TcdB_GT was highly immunogenic and induced high titers of anti-polysaccharide IgG antibodies, in a comparable manner to the PSII-CRM_{197} conjugate. On the other hand, the PSII-TcdA_B2 conjugate was less efficient in inducing anti-PSII IgGs. Since the chemical characteristics of the two conjugates are comparable, the reason for this different behavior might reside in the different intrinsic ability of the two carriers to drive the antibody response toward the carbohydrate moiety. It is important to note that conjugation to the polysaccharide does not impact the neutralizing activity of both TcdA_B2 and TcdB_GT.

References

1. Costantino,P.; Rappuoli, R.; Berti, F. The design of semi-synthetic and synthetic glycoconjugate vaccines , *Expert Opin. Drug Discov.**2011**, 6 (10),* 1045-1066

2. Avery, O.T.; Goebel, W.F. Chemo-immunological studies on conjugated carbohydrate-proteins : Immunological specificity of synthetic sugar-protein antigens. *J Exp Med,* **1929***, 50,* 533-50.

3. Adamo, R.; Berti, F.; Hu, Q.-Y., Cycloalkyne derivatized saccharides. *From PCT Int. Appl. (2014), WO 2014111344 A1 20140724.)*

List of Publications

Nilo A, Allan M, Brogioni B, Proietti D, Cattaneo V, Crotti S, Sokup S, Zhai H, Margarit I, Berti F, Hu QY, Adamo R: Tyrosine-Directed Conjugation of Large Glycans to Proteins via Copper-Free Click Chemistry; *Bioconjug Chem.* **2014** *Nov 21*

Morelli L, Cancogni D, Tontini M, **Nilo A**, Filippini S, Costantino P, Romano MR, Berti F, Adamo R, Lay L: Synthesis and immunological evaluation of protein conjugates of Neisseria meningitidis X capsular polysaccharide fragments; *Beilstein J Org Chem.* **2014** *Oct 13;10:*2367-76

Adamo R, **Nilo A**, Harfouche C, Brogioni B, Pecetta S, Brogioni G, Balducci E, Pinto V, Filippini S, Mori E, Tontini M, Romano MR, Costantino P, Berti F: Investigating the immunodominance of carbohydrate antigens in a bivalent unimolecular glycoconjugate vaccine against serogroup A and C meningococcal disease*; Glycoconj J.* **2014** *Dec;31(9):*637-47

Romano MR, Leuzzi R, Cappelletti E, Tontini M, **Nilo A**, Proietti D, Berti F, Costantino P, Adamo R, Scarselli M; Recombinant Clostridium difficile toxin fragments as carrier protein for PSII surface polysaccharide preserve their neutralizing activity; *Toxins (Basel). **2014** Apr 22;6(4):*1385-96

Gao Q, Tontini M, Brogioni G, **Nilo A**, Filippini S, Harfouche C, Polito L, Romano MR, Costantino P, Berti F, Adamo R, Lay L: Immunoactivity of protein conjugates of carba analogues from Neisseria meningitidis a capsular polysaccharide; *ACS Chem Biol.* **2013** *Nov 15;8(11)*

Adamo R, **Nilo A**, Castagner B, Boutureira O, Berti F and Bernardes G J L: Synthetically defined glycoprotein vaccines: current status and future directions; *Chem. Sci.,* **2013***, 4, 2995*

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