UNIVERSITÀ DEGLI STUDI DI TRIESTE

IIIVI CICLO DEL DOTTORATO DI RICERCA IN BIOMEDICINA MOLECOLARE

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Investigation of alternative technologies for the development of polysaccharide-based vaccines

Settore scientifico-disciplinare: **BIO/10**

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Tesi redatta con il contributo finanziario di
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ABSTRACT

Conjugation is a well-established approach for the development of polysaccharide-based vaccines. Chemical linkage to a carrier protein improves polysaccharides immunogenicity and overcomes the limitations of their T-independent nature, including lack of immunological memory and no efficacy in infants. A double-hit approach, meaning that both polysaccharide and carrier protein belong to the same pathogen, may be very useful for bacterial species with large glycan variability. Recently, bacterial protein glycosylation has been exploited to obtain glycosylated proteins in *E. coli* cytoplasm. This system relies on a N-glucosyltransferase enzyme which catalyzes the transfer of a single β-linked glucose onto engineered N-x-S/T sequons on recombinant proteins. In my PhD work, I have used this technology for the development of novel selective glycoconjugates, with the aim of preserving the antigenicity of the carrier protein. *Klebsiella pneumoniae* (Kp) capsular (K-antigens) and subcapsular (O-antigens) polysaccharides have been selected as model antigens for the generation of double-hit vaccines using MrkA as carrier protein and potential Kp protective antigen. Kp has been listed by WHO among the multidrug-resistant pathogens for which development of new interventions is a priority. MrkA, the major component of Kp type 3 fimbriae, possesses a high degree of sequence conservation among different isolates, and thus its use in a glycoconjugate might increase the vaccine coverage, considering the diversity of Kp K- and O-antigens.

Two stabilized constructs of MrkA monomer were designed, recombinantly expressed in *E. coli* and tested in mice. MrkA version A induced significantly higher anti-MrkA IgG response with respect to MrkA version B after two subcutaneous immunizations, so MrkA version A was chosen for the generation of selective glycoconjugates exploiting protein cytoplasmic glycosylation. A glycosylation pathway was successfully established in *E. coli* for MrkA modification with a lactose moiety, after deleting *lacZ* gene to prevent the disaccharide catabolism. Lac-MrkA, as the unmodified one, was obtained with a good purity and yield (~ 60 mg/L). Mass spectrometry analysis confirmed the actual modification and its position in the protein sequence, with an occupancy of 100% as quantified by anion exchange chromatography. In parallel, Kp O-antigens and K-antigens were purified and fully characterized prior conjugation. O1v1 O-antigen (~ 20 kDa) and K2 K-antigen (~ 150 kDa) were randomly derivatized with an aldehyde-reactive linker and selectively conjugated to oxidized Lac-MrkA by reductive amination. Through anion exchange chromatography an average number of 3 MrkA molecules linked to O1v1 and approximately 20 to K2 were

calculated. The immunogenicity of the resulting conjugates was evaluated in mice in comparison to random glycoconjugates as well as to MrkA protein alone. The ability of MrkA to work as carrier for polysaccharides was proportional to the molecular mass of the final glycoconjugate. Interestingly, the mice study highlighted the ability of a long polysaccharide like K2 to work as "carrier" for MrkA increasing the immunogenicity, poor *per se*, of the protein selectively linked along its chain. In agreement with anti-MrkA ELISA results, sera from selective conjugate immunized mice showed a higher binding capacity towards an heterologous Kp strain expressing high levels of MrkA in flow cytometry, compared to sera from random conjugate and protein alone groups. A study in a different animal model (i.e., rabbits) is ongoing and will help to consolidate the results obtained in mice. The development of a biofilm inhibition assay will allow to assess anti-MrkA sera functionality to further distinguish the quality of the immune response elicited by the different constructs.

Cytoplasmic glycosylation allowed reproducible modification of MrkA in *E. coli*. I have also shown that sequons can be easily engineered in different sites of the protein supporting additional studies to understand if the glycosylation position can affect the immunogenicity of resulting selective conjugates.

The new conjugation approach developed in this work can be easily extended to other pathogens, combining polysaccharide and protein antigens in novel effective glycoconjugate vaccines with broader coverage.

Overall, my work represents an innovative example of how the glycoengineering technology can be combined to conjugation chemistry for the development of effective glycoconjugate vaccines.

LIST OF ABBREVIATIONS

AA	Aminoacid
Ab	Antibody
Adipic Acid Dihydrazide	Adipic acid dihydrazide
AMR	Antimicrobial resistance
bp	Base pair
BSA	Bovine serum albumin
CDAP	1-cyano-4-dimethylaminopyridine tetrafluoroborate
CPS	Capsular Polysaccharides
CRM_{197}	Cross Reacting Material 197
DOSY	Diffusion-Ordered Spectroscopy
dRI	Differential Refractive Index
DT	Diphtheria Toxoid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GAS	Group A *Streptococcus*
GMMA	Generalized Modules for Membrane Antigens
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPLC-SEC	High Performance Liquid Chromatography – Size Exclusion Chromatography
hv	Hypervirulent
IMAC	Immobilized-Metal Affinity Chromatography
LB	Luria Bertani
LC-MS/MS	Liquid Chromatography coupled to Tandem Mass Spectrometry
LMICs	Low- and Middle- Income Countries
LPS	Lipopolysaccharide
KDO	3-deoxy-D-manno-oct-2-ulosonic acid
Kp	*Klebsiella pneumoniae*
mAb	Monoclonal antibody
MDR	Multidrug-resistant

MM	Molecular Mass
MFI	Mean Fluorescence Intensity
MS	Mass Spectrometry
NGT	N-glycosyltransferase
NMR	Nuclear Magnetic Resonance
OAc	O-acetyl/acetylation/acetylated
OAg	O-Antigen
OPKA	Opsono Phagocytic Killing Assay
OS	Oligosaccharide
OTase	Oligosaccharyltransferase
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffer Saline
PRR	Pattern Recognition Receptor
PS	Polysaccharide
RT	Retention time
RU	Repeating Unit
SBA	Serum Bactericidal Assay
SEC	Size Exclusion Chromatography
SLO	Streptolysin O
spp	Species
SPR	Surface Plasmon Resonance
TLR	Toll-Like Receptor
TT	Tetanus Toxoid
WHO	World Health Organization

1 INTRODUCTION

1.1 Immune system and general principles of vaccination

The immune system is an interactive network that protects us from external and internal threats. A foreign substance recognized as dangerous and capable of triggering an immune response is known as antigen. The immune response is divided into two parts, constantly interacting with each other, differing in the speed and specificity of the reaction: innate and adaptative immune responses. Innate immunity is able to recognize conserved molecular patterns found on all microorganisms and fights invading pathogens in a fast, yet non-specific way. It can be considered a first line of defense against different classes of microbes. The adaptive immunity is instead directed against a specific pathogenic agent, and it is composed of B cells/antibodies (humoral response) and T cells (cell-mediated response). This response takes longer to occur than the innate response, but in successive exposures to that particular pathogen it will intervene more rapidly, thanks to its memory component (Figure 1.1). Vaccination takes advantage of this feature of the adaptive immunity. The exposure to a live attenuated or inactivated form 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 the disease in the vaccinee, leading to the production of vaccine-induced immune effectors called antibodies.

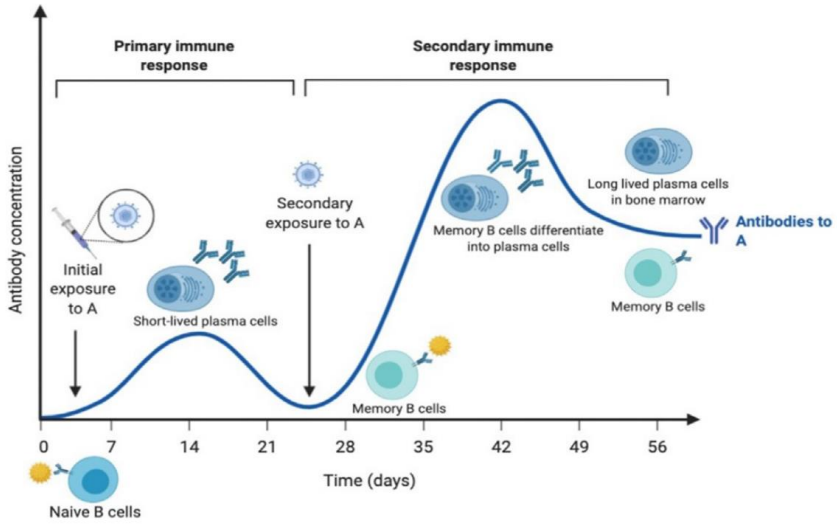


Figure 1.1. Plot showing the effects of immunological memory: upon antigen re-exposure, antibodies from mature B cells that have undergone isotype switching and somatic hypermutation are more rapidly produced. *Reproduced from [1].*

These antibodies, produced by B cells, are a class of proteins called Immunoglobulins (Ig), which are capable to recognize and specifically bind to a pathogen [2], blocking its ability to bind to receptors on host cells. Antibody binding also marks invading pathogens for destruction, mainly by making it easier for phagocytic cells of the innate immune system to ingest them. The quality of such antibody responses, such as antibody subclass and antibody avidity, has been identified as a determining factor for vaccine efficacy. Although B cells represent the specialized lineage in antibody production, once differentiated in plasma cells, T cells, the other arm of adaptive immunity, can largely contribute to effective and long-lasting immune responses. The main actors of cell-mediated response are CD4 and CD8 T cells: CD4 cells (or T-helper cells) play a major role in mediating immune responses through the secretion of specific cytokines, stimulating B-cells to generate antibodies; CD8 cells (or T-cytotoxic cells) are essential for immune defense against intracellular pathogens, including viruses and bacteria, and for tumor surveillance [3]. CD4 cells only recognize the major histocompatibility complex (MHC) class II protein, while CD8 cells the MHC class I protein. Both classes of proteins share the task of presenting peptides on the cell surface for recognition by T-cells [4]. Unlike B cells, T cells can only recognize an antigen that has been processed and presented by antigen-presenting cells (APCs) [5]. In fact, T cells require at least two signals to become fully activated: a first antigen-specific signal provided through the T-cell receptor which interacts with the antigen peptide-loaded MHC class II molecules on the surface of the APCs; a second antigen-unspecific signal provided by the interaction between co-stimulatory molecules expressed on the membrane of the APCs upon maturation with its counterparts on the T cell.

1.2 Bacterial Carbohydrates

Carbohydrates, in the form of capsular polysaccharides (CPS) or lipopolysaccharides (LPS), are the major components found on the surface of bacteria. The CPS may be present in both Gram-negative and in Gram-positive (Figure 1.2), while LPS is only present in Gram-negative bacteria. CPS are a diverse class of high molecular mass polysaccharides (PSs) that protect bacteria from desiccation when they are exposed to the external environment and mimic molecules produced by human cells, preventing bacteria phagocytosis. LPS is part of the outer membrane and contributes to its structural integrity, protecting the membrane from access by many antibacterial agents. It consists of three different components: lipid A, core oligosaccharide and O-polysaccharide (OAg), which confers serotype specificity.

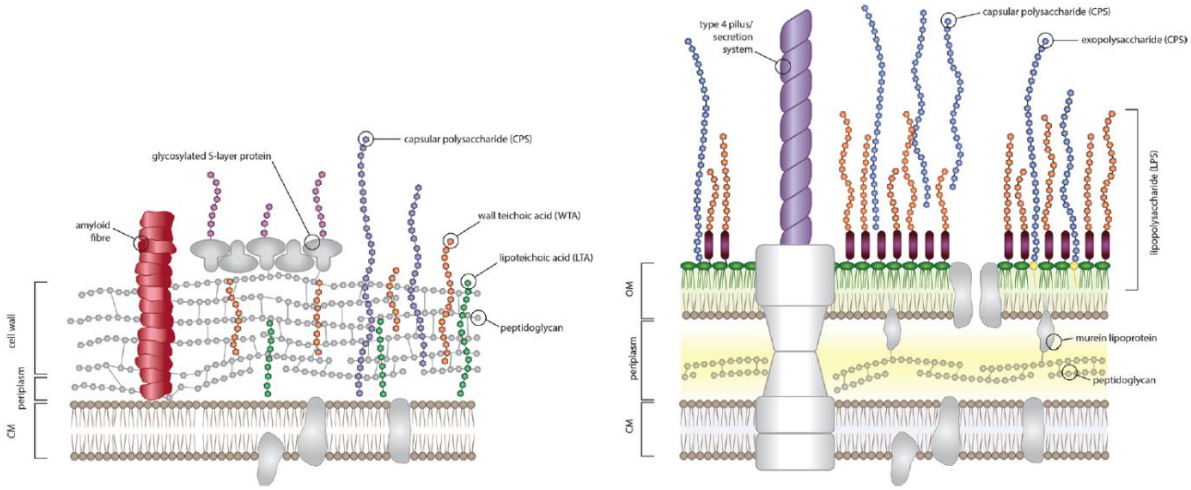


Figure 1.2. Schematic representation of the cell envelope of Gram-positive (left) and Gram-negative (right) bacteria. *Reproduced from* [6]. Gram-positive cells are characterized by a thick membrane layer (200-800 Å) of peptidoglycan whereas Gram-negative ones have a thinner layer (50-100 Å). In Gram-negative bacteria, an intermediate compartment called periplasm is enclosed by the outer membrane (OM) and the cytoplasmic membrane (CM). The CM and the inner leaflet of OM are made of phospholipids, while the outer leaflet of the OM is mainly LPS.

Bacterial PSs are polymers formed by one monosaccharide unit (homopolymers) or more complex oligosaccharide repeats (heteropolymers), that can be charged or neutral. They can covalently bind proteins or lipids, forming glycoproteins and glycolipids, respectively [7]. The LPS features many negatively charged functional groups including phosphoryl groups on the lipid A and heptose sugars and carboxyl groups on the 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) sugars. Alteration of this charge density, through glycan modification, affords resistance to antibiotics and antimicrobial peptides. It is reported that the introduction of positively-charged moieties on lipid A by ArnT, a periplasmic glycosyl transferase, confers resistance to the last-resort polycationic antibiotic polymyxin in *E. coli* and *Salmonella enterica*, as well as in serious pathogens including *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [8]. PSs expressed in pathogenic bacteria play major roles in the virulence of these organisms, protecting them from the host immune response. Upon infection, the innate immune system responds to pathogen-associated molecular patterns (PAMPS), such as LPS and endotoxin, and activate immediate host inflammatory and antimicrobial responses. PSs are T-cell-independent antigens as they do not require T-cell activation for the induction of specific B-cell responses. They directly activate polysaccharide-specific B cells which differentiate into plasma cells producing mainly IgM antibodies, without induction of immunological memory.

1.3 Polysaccharide-based and glycoconjugate vaccines

Playing a crucial role in pathogenesis, bacterial PSs are considered relevant targets for vaccine development. Around the 1930s the protective role of antibodies induced by pneumococcal PSs started to be investigated and in 1945 the first vaccine composed of purified PSs from selected pneumococcal serotypes was tested in humans [9]. The introduction of antibiotics slowed down the research on vaccines development; however, with the emergence of drug resistant strains, the development of vaccines started again and a number of them were studied in large clinical studies. PS vaccines against *Neisseria meningitidis* serogroups ACWY, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) were licensed between the seventies and the eighties [10,11]. Despite their efficacy in adults, PS based vaccines fail to evoke immunological memory or long-lived antibody production being T-cell-independent antigens. Furthermore, PS vaccines can be used in adults but elicit scarce immunogenicity in children less than two years of age and elderly which are the most sensitive target populations [12-14]. Conjugation of bacterial PSs to carrier proteins has allowed to overcome limitations related to the immunization with plain PSs. Differently from PSs, proteins are T-dependent-antigens:

interaction with T cells induces B cells to differentiate into plasma cells and memory B cells, thus initiating downstream adaptive immune responses. The T-cell help provided by protein epitopes present in glycoconjugates imparts the capacity to induce a long lasting and boostable IgG antibody production also in children below the age of two [12,13]. Vaccination with glycoconjugates has been proven more effective than the one with PSs in adult population [15,16]. Finally, conjugate vaccines have been shown to reduce carriage or impact on transmission of meningococci, while there is some evidence that plain PS vaccines cannot [17].

In 1931 Avery and Goebel were the first to demonstrate that protein conjugates of oligosaccharides (OSs) instead of PSs alone could also elicit protective antibodies: in their first trial, rabbits immunized with a disaccharide fragment of Type III *Pneumococcus* CPS covalently linked to a carrier protein were protected against challenge by the homologous organism [18]. Nevertheless, the first application of this concept to a vaccine for human use started in 1980 with the development of the first glycoconjugate vaccine against Hib [19]. Since then, glycoconjugates have been proven efficacious and cost effective to combat many life-threatening bacteria, such as *S. pneumoniae* (23 serotypes) [20], *N. meningitidis* (A, C, W and Y) [21] and *Salmonella* Typhi [22-24]. A summary list of all bacterial glycoconjugate vaccines licensed or in development has been recently reported (Table 1.1) [25].

Table 1.1 Glycoconjugate vaccines in the market or in development. *Adapted from [25].*

Type of glycan	Organism	Manufacturer (licensed:L) (clinical:C) (discovery:D)	Saccharide	Approach [a]	Carrier
Capsular poly-saccharide	*Haemophilus influenzae* serotype b	GSK (L)	PS	SS	TT
		Sanofi (L)	PS	SS	TT
		GSK (L)	Oligo	SS	CRM_{197}
		Merck (L)	Size reduced PS	SS	OMPC
		Pfizer (L)	Oligo	SS	CRM_{197}
		SIIL (L)	PS	SS	TT
		CIGB (L)	Oligo	ST	TT
		Hilleman Lab (D)	Size reduced PS	SS	TT
	Haemophilus influenzae serotype a	Bionet-Asia NRC Canada (D)	Size reduced PS	SS	CRM_{197} and Protein D
	Meningococcus	GSK (L)	Oligo MenC	SS	CRM_{197}
		Pfizer (Nuron) (L)	MenC size reduced PS	SS	CRM_{197}
		Baxter (L)	MenC PS De-OAc Size reduced	SS	TT
		Hilleman Lab (D)	MenX	ST	TT
		SIIL (L)	MenA Size reduced PS	SS	TT

Type of glycan	Organism	Manufacturer (licensed:L) (clinical:C) (discovery:D)	Saccharide	Approach [a]	Carrier
		GSK (D)	MenX Ps size reduced	SS	CRM_{197}
		GSK (L)	MenACWY Oligos	SS	CRM_{197}
		Pfizer (L) formerly GSK	MenACWY size reduced PS	SS	TT
		Sanofi (L)	MenACWY size reduced PS	SS	DT
		Sanofi (C)	MenACWY	SS	TT
		SIIL (C)	MenACWY X PS	SS	TT, CRM_{197}
	Pneumococcus	Pfizer (L)	4, 6B, 9V, 14, 18C, 19F, 23F, PS except 18C size reduced	SS	CRM_{197}
		Pfizer (L)	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F PS except 18C size reduced	SS	CRM_{197}
		GSK (L)	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F PS except 23F size reduced	SS	Protein D, TT(18C), DT(19F)

Type of glycan	Organism	Manufacturer (licensed:L) (clinical:C) (discovery:D)	Saccharide	Approach [a]	Carrier
		Limmatech Biologics (D)	Multivalent	B	rEPA
		Merck (C)	15 valent	SS	CRM_{197}
		CIGB (C)	1, 5, 6B, 14, 18C, 19F, 23F	NA	TT
	GBS	GSK (C)	Ia, Ib, III PS	SS	CRM_{197}
		GSK (D)	Ia, Ib, II, III, V PS	SS	CRM_{197}
		Various (D or C)	Ia, Ib, II, III, IV, V, VI, VII and VIII Ps	SS	TT and CRM_{197}
		Pfizer (C)	Multivalent	Platform developed for pneumo conjugates	CRM_{197}
	Staphylococcus aureus	GSK (C)	Type 5 and 8 PS	SS	TT
		Pfizer (C)	Type 5 and 8 PS	SS	CRM_{197}
		GlycoVaxyn (now Limmatech Biologics) (D)	Type 5 and 8 PS	B	rEPA
	Salmonella Typhi	NIH (C), GVGH/ Biological E (C), Biomed	Vi PS and Fragments	SS	CRM_{197}, TT, DT, rEPA

Type of glycan	Organism	Manufacturer (licensed:L) (clinical:C) (discovery:D)	Saccharide	Approach [a]	Carrier
		(L), Barath Biotech (L)			
	Burkholderia pseudomallei	DSTL (D)	Oligo	ST	TetHc
	Klebsiella pneumoniae	Max Plank Institute (D)	CPS repeating unit	ST	CRM_{197}
	Shigella	Limmatech Biologics (C)	*Sh. dysenteriae* type 1 PS	B	rEPA
		Limmatech Biologics (C)	*Sh. flexneri* 2a PS	B	rEPA
		NICHHD (C)	*S. sonnei* and *Sh. flexneri* 2a PS	SS	rEPA
		Institute Pasteur (C)	*Sh. flexneri* 2a oligo	ST	TT
O-Antigen	*Escherichia coli*	Limmatech Biologics/J&J (C)	O1, O2, O6, O25 Expec	B	rEPA
	Salmonella Paratyphi A and Non-typhoidal *Salmonella*	NVGH (D), NIH (C), IVI (D)	O2 *S. Paratyphi* A, O9 *S. Enteritidis*, O4, 5 *S.Typhimurium*	SS	TT, CRM_{197}, DT
	Pseudomonas aeruginosa	SSVI/WRAIR (C) program stopped	O1,2,3,4,5,6,11,12	SS	EPA

Type of glycan	Organism	Manufacturer (licensed:L) (clinical:C) (discovery:D)	Saccharide	Approach [a]	Carrier
	Klebsiella pneumoniae	University Maryland (D)	O1, O2a, O2a,c, O3, O4, O5, O7, O8, O12	SS	PA flagellin
	Vibrio cholerae	NIH, Institut Pasteur (D)	O1 (Inaba and Ogawa), O139	SS; ST	BSA, rEPA, TThc
	Francisella tularensis	CCRC-NRCC and DSTL (D)	O-Ag	ST; B	KLH; rEPA
	Burkholderia pseudomallei	Academic (D)	OPSII	B; ST	AcrA;
	Moraxella catarrhalis	NRC Canada (D)	Truncated LPS	SS	CRM_{197}
		NDCD/NIH (D)	Detox LPS serotype A, B and C	SS	TT, NTHi HMP, UspA, CD, CRM_{197}
Teichoic acids	*Enterococcus faecalis*	UML/Leiden University (D)	LTA	ST	BSA
PNAG	*Acinetobacter baumannii* and other pathogens	Harvard Medical School, Alopexx (D)	β-(1→6)-oligo glucosamine	ST	TT
ExoPS	*Pseudomonas aeruginosa*	Harvard Medical School (C and D)	Poly-mannuronic acid; alginate	ST	ExoA, Flagellin; TT, KLH, OMV, synthetic peptides
	Clostridium difficile	Guelph University, Max Planck Institute (D)	PS-I	ST	CRM_{197}

Type of glycan	Organism	Manufacturer (licensed:L) (clinical:C) (discovery:D)	Saccharide	Approach [a]	Carrier
		GSK, Guelph University, Max Planck Institute (D)	PS-II	ST; SS	CRM_{197}, *C.difficile* rToxins
		Max Planck Institute (D)	PS-III	ST	CRM_{197}
Cell Wall PS	Group A *Streptococcus* (GAS)	GSK (D)	GAC fragments	ST	CRM_{197}
		Rockefeller University (D)	PS	ST	TT
		Various Academic Institutions (D)	GlcNAc deficient PS	ST	Sp0435
	Aspergillus fumigatus	Zelinsky Inst. Org. Chem./Institute Pasteur (D)	α-(1→3)-glucans	ST	BSA
	Candida albicans	GSK, CCRC (D)	β-(1→3)/β-(1→6)-glucans	SS; ST	CRM_{197}
Fungal glycans		Alberta University/ Theracarb/ Novadigm (D)	β-(1→2)-mannotriose	ST	TT, *Candida* peptides
	Cryptococcus neoformans	Dublin University/J. Hopkins Bloomberg SPH (D)	GXM PS and oligosaccharides	SS; ST	HSA
Myco-bacterial glycans	*Mycobacterium tubercolosis*	Uppsala University/Eu	AM	SS	Ag85B, TT

Type of glycan	Organism	Manufacturer (licensed:L) (clinical:C) (discovery:D)	Saccharide	Approach [a]	Carrier
		rocine AB (D)			

Many studies have been performed to understand the mechanisms of internalization and processing of the glycoconjugate molecules by the immune system [26-28]. According to the classical mechanism proposed, the PS component of the vaccine binds to surface immunoglobulin of PS-specific B cells. Following internalization of the vaccine, the protein component is processed and the resulting peptide fragments are presented to the T-cell receptor of CD4+ peptide-specific T cells in the peptide-binding groove of MHCII (Figure 1.3). In addition to this cognate interaction, further signals are essential in eliciting CD4+ T-cell help for the B cell [29,30]. When B cells receive T-cell help, they proliferate and differentiate, with class switching from IgM to PS-specific IgG, into plasma cells, and memory B cells. These can rapidly proliferate and differentiate into plasma cells on subsequent encounter of the specific antigen producing high levels of antibodies [30-34]. Antibody avidity is increased through affinity maturation in germinal centers [35].

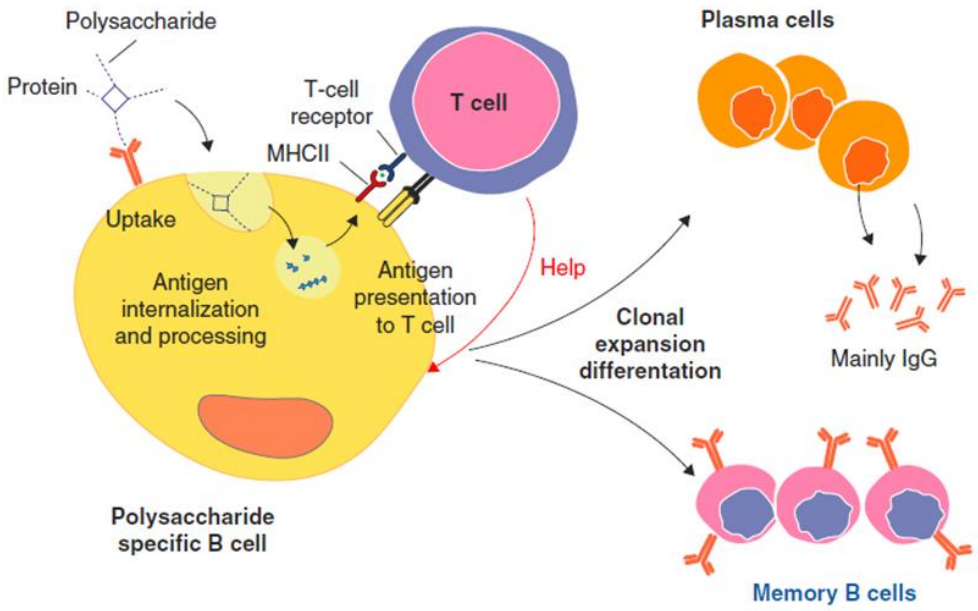


Figure 1.3. Mechanism of action traditionally proposed for glycoconjugate vaccines. *Reproduced from* [36].

Not all PSs are strictly T-independent-antigens and zwitterionic PSs, such as Polysaccharide A from *Bacteroides fragilis*, containing both positive and negative charges, are able to stimulate T cell help in the absence of a carrier protein [28,37-39]. Recently it has been proposed that the carbohydrate fragments of the glycopeptides, produced by glycoconjugate vaccine processing into B-cells, recognize CD4+ T cells with the peptide portions useful for binding to MHCII molecules [40,41]. This novel mechanism 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.

The process of developing a T cell mediated immunity by a glycoconjugate vaccine can be influenced by the formulation. Adjuvants can be used for various purposes: to increase the immunogenicity of the antigens, to reduce the amount of antigens or the number of immunizations needed for protective immunity, to improve the efficacy of vaccines in newborns, the elderly, or immunocompromised individuals and as delivery systems to facilitate the uptake of antigens by the mucosa [42].

1.4 Glycoconjugate vaccines preparation methods

The synthesis of a glycoconjugate traditionally requires a covalent linkage between the PS, extracted and purified directly from bacteria, and a purified carrier protein (Figure 1.4A). Two main approaches based on different conjugation chemistries have been used so far: one is based on the random chemical activation of the saccharide chain followed by covalent linkage to the carrier protein, resulting in high molecular weight (MW) cross-linked heterogenous structures; the second approach is based instead on the selective activation of the reducing end of the PS chain prior to conjugation to the protein, leading to a glycoconjugate with a radial structure [36,43-46]. In this second approach, the use of chemically or chemo-enzymatically synthesized carbohydrates, prepared starting from suitable monosaccharides, is increasingly attractive [47-50]. Synthetic OSs offer the advantage of being pure and well-defined molecules, typically bearing at the terminal residue a linker for coupling to the carrier protein. Starting from PSs purified from bacterial growth, the use of terminal selective chemistries usually requires fragmentation of the saccharide chain to produce shorter OSs. Different methods can be used at this scope [51]. Production of lower MW PS populations is typically achieved by chemical or mechanical PS fragmentation followed by size fractionation [52-54]. Depending on the PS structure, $NaIO_4$ oxidation results in simultaneous fragmentation and generation of terminal aldehyde groups available for conjugation (e.g., Hib CPS). Alternatively, the PSs can be fragmented by treatment with hydrogen peroxide [55] or via acid hydrolysis, and sized by chromatography or ultrafiltration techniques to isolate populations more defined in length for conjugation [54,56]. Individual bacterial strains may also express a wide range of saccharide sizes, as it is usually the case for O-PS, that, in some instances, may not include the desired target size for vaccine development. Also, in this case, isolation of the sugar with desired length is required, impacting on overall yield and complexity of the process.

Six proteins are currently used as carriers in licensed vaccines: Tetanus toxoid (TT), Diphtheria toxoid (DT), CRM_{197}, the Outer Membrane Protein Complex of Meningococcus B (OMPC), Protein D from *H. influenzae* and recombinant Exotoxin A of *P. aeruginosa* (rEPA) [36,57-60]. The choice of the chemical conjugation approach is often governed by structure, size, and composition of vaccine components [61]. For example, larger PSs are usually randomly activated, while smaller PSs are activated at the reducing end to preserve protective epitopes [62]. Also concerning the protein component, conjugation can be random (more often linking the PS to NH_2 or COOH groups of the protein) or selective (for example through introduction of non-natural aminoacids in specific points of the sequence) [63]. Selective approaches can be

important in case the protein is used with dual role of carrier and protein antigen. Depending on the conjugation chemistry employed, a chemical spacer can be used in order to facilitate the coupling of the protein to the saccharide antigen. Thus, glycan-protein conjugation can take place using the carbohydrate functional moieties (e.g., hydroxyl, carboxyl groups) or derivatizing the PS with linkers also to allow insertion of specific functionalities (e.g., thiols, bromide). In other cases the protein can be derivatized before conjugation. One of the most frequently used methods for conjugation entails the use of $NaIO_4$ oxidation which generates aldehydes from cis-diols. These chemical groups can be directly linked to the ε-amine of the protein lysine residues by reductive amination, or further derivatized before linkage to the protein [64-67]. The CDAP strategy (1-Cyano-4-dimethylaminopyridinium tetrafluoroborate) is also randomly applied to hydroxyl groups of PSs for subsequent condensation with the amines of the protein [68]. Moreover, OSs can be linked to the protein through the reducing end of the sugar directly, or via a spacer.

Although isolation of PSs from bacterial growth is the primary approach used for manufacturing licensed vaccines, other methods avoiding large scale pathogen fermentation have emerged over the last decades [69]. Organic synthesis has been shown to meet the demand of bacterial related OS, with the advantages of a more defined structure, ease of characterization, lack of bacterial contaminants, higher batch-to-batch reproducibility, and more robust correlation of the elicited immune response with the oligosaccharide chemical structure [70]. However, chemical synthesis of OSs remains challenging, expensive and time consuming, especially compared to the synthesis of other biopolymers such as peptides and oligonucleotides. A more recent approach, called bioconjugation, is also emerging (Figure 1.4B). The PS, synthesized by specific enzymes encoded by the inserted genes, is produced on a polyisoprenoid carrier and then transferred to an asparagine residue of the carrier protein containing at least one N-glycosylation site, either native or engineered, in *E. coli* periplasm by the oligosaccharyltransferase PglB [71,72].

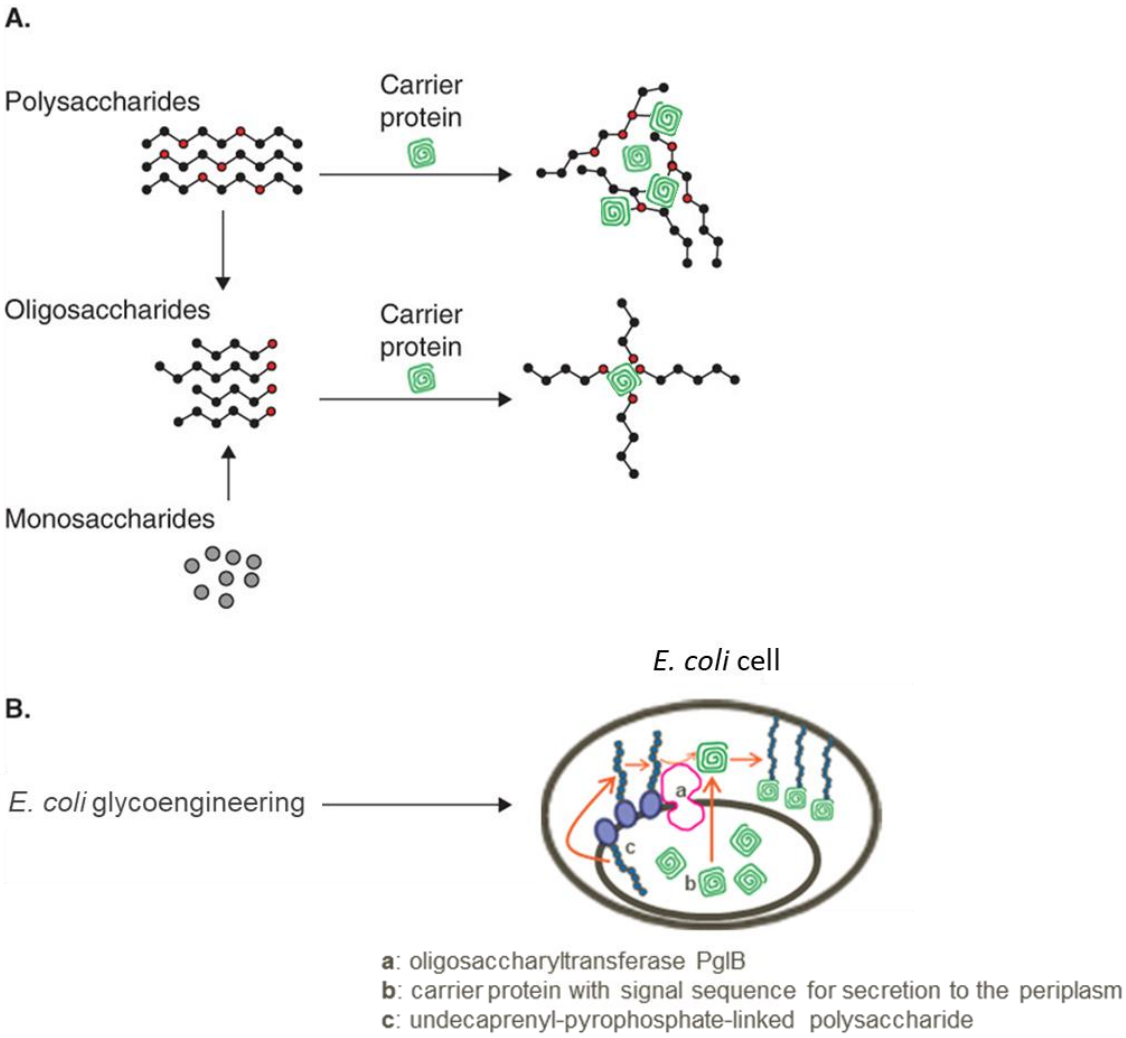


Figure 1.4. Schematic representation of **A**) poly- and oligosaccharide–protein chemical couplings: long PSs can be randomly attached to the protein or fragmented to generate shorter OSs and terminally linked to the protein. Short OSs can also be generated through chemical and/or enzymatic approaches from corresponding monosaccharides and **B**) novel technology to make glycoconjugate vaccines: the glycan encoding genes are transferred to *E. coli* (together with those for PglB and carrier protein expression) for the biosynthesis of the lipid-linked heterologous PS, which is coupled in the periplasm to the carrier protein containing the consensus acceptor sequon by PglB, forming the bioconjugate vaccine. *Adapted from* [36].

1.5 Variables influencing the immunogenicity of glycoconjugate vaccines

Different variables can influence the immunogenicity of conjugate vaccines: size of the saccharide chain, glycosylation degree (or saccharide to protein ratio), conjugation chemistry, the nature of the spacer between saccharide and protein and the carrier protein used [62].

Immunogenic epitopes involved in the interaction with specific antibodies usually comprise precise glycan structures, often no longer than six or eight sugar units (63 year-old paradigm established by Kabat) [69]. Several preclinical studies have investigated the impact of saccharide chain length and saccharide loading, highlighting difficulties in finding general rules, since results are often PS-specific, and interconnections between these two parameters. In fact, Pozsgay *et al*., studying the immunogenicity in mice of synthetic *Shigella dysenteriae* type 1 LPS OSs conjugated to human serum albumin (HSA), found that the optimal carbohydrate chain density differed with oligosaccharide length. The octa-, dodeca-, and hexadecasaccharide fragments induced high levels of LPS binding IgG antibodies in mice after three injections and were superior to a tetrasaccharide conjugate (11 moles of saccharide/HSA). Interestingly, the influence of the carbohydrate:protein ratio was different for the three conjugates according to the OS length: the octasaccharide-HSA conjugate with the highest density (20 moles of saccharide/HSA) evoked a good immune response, while in the case of dodeca- and hexadecasaccharide conjugates, the median density (9-10 saccharide chains) was optimal [70].

However, few studies have been performed in humans and there is no clarity of how preclinical data will translate in humans. Hib OS with an average of 8 or 20 RU conjugated to DT were equally immunogenic in rabbits and human adults, instead in human infants the 20-mer vaccine was significantly more immunogenic than the 8-mer vaccine [71]. A subsequent clinical study showed that glycoconjugates with Hib OS having an average of 7 or 20 RU were equally highly immunogenic in 1-year-old infants, but the 7-mer had a higher saccharide:protein ratio [72]. These studies suggest that OS chain length and number of saccharide chains loaded on the protein are interconnected antigen-specific parameters affecting the immunogenicity of glycoconjugate vaccines.

The spacer used for the conjugation could also have an impact on the immunogenicity. There are evidences in the literature that suggest that rigid, constrained spacers, like cyclohexyl maleimide, elicit a significant amount of undesirable antibodies, with the risk of driving the immune response away from the targeted epitope on the PS [73,74]. Similarly, the triazole

generated by click chemistry has been seen strongly immunogenic [75]. The use of a flexible alkyl type maleimide spacer has been reported as an alternative to overcome the previous observed immunogenicity of cyclic maleimide linkers [76].

A limited number of protein carriers has been used so far in preclinical and clinical evaluation of conjugate vaccines. Diphtheria and tetanus toxoids, which derive from the respective toxins after chemical detoxification with formaldehyde, were initially selected as carriers because of the safety track record accumulated with tetanus and diphtheria vaccination. CRM_{197}, a non-toxic mutant of diphtheria toxin [77], which instead does not need chemical detoxification, has been extensively used as carrier for licensed Hib, pneumococcal, meningococcal conjugate vaccines and for other vaccines being developed (Table 1.1). An outer membrane protein complex of serogroup B meningococcus has been used by Merck as carrier for their Hib conjugate vaccine [78]. GSK in their multivalent pneumococcal conjugate vaccine introduced the use of the Hib-related protein D as carrier for most of the PSs included into the vaccine [79,80]. The team of John Robbins made extensive use of the recombinant non-toxic form of *P. aeruginosa* exo-toxin as carrier for *Staphylococcus aureus* type 5 and 8 as well as for *S.* Typhi Vi conjugates.

A number of clinical trials have been conducted to compare the immunogenicity of different conjugate vaccines with different carrier proteins [81-85]. It is however very difficult to establish a direct comparison of the effect of the different protein carriers, due to the coexistence of other variables as conjugation chemistry, saccharide chain length, adjuvant, formulation technology, and previous or concomitant vaccination with other antigens.

In the last years many different studies highlighted the role of attachment site on the protein on immunogenicity [86]. The possibility to link the PS at specific sites on the carrier protein allows preservation of key protein epitopes, supporting the use of the protein with a dual role of carrier and antigen.

1.6 Genetic engineering strategies for site-selective protein modification

The repeated use of the same small set of carrier proteins could result in hyporesponsiveness toward new glycoantigens. Pre-existing immunity to a given carrier protein can blunt the immune response to a new carbohydrate conjugated to the same carrier. Such "carrier-induced epitopic suppression" was noticed when children who received Hib PS conjugated to diphtheria toxoid had superior immune responses to those who received Hib PS conjugated to tetanus toxoid when co-administered with a tetanus toxoid-conjugated pneumococcal vaccine [87]. An attractive alternative is to conjugate the bacterial PS antigen to a highly conserved and immunogenic protein antigen from the same bacterial species. With this "double-hit" approach, the glycoconjugate should ideally elicit functional antibodies targeting both the PS and the autologous carrier protein to protect against diseases. At this scope, selective conjugation approaches are likely to be necessary to preserve the antigenicity of the protein. Besides direct chemical modifications strategies [88-90], which still have some drawbacks related to selectivity/specificity, homogeneity and efficiency/stability of the reactions, genetic engineering has proved to be an indispensable methodology for the controlled introduction of a variety of abiotic and biotic chemical handles at designated sites for subsequent bioorthogonal reactions [91] and for the resulting production of homogeneous selective conjugates. Genetic engineering strategies used to achieve site-selective protein modification can be divided in three categories: insertion of canonical or non-canonical amino acids, insertion of motifs and enzymatic tags and fusion of proteins.

1.6.1 Insertion of canonical or non-canonical aminoacids (ncAAs)

Direct modification methods can be more successfully applied to genetically inserted aminoacid residue at specific positions in protein sequences. Out of all the canonical amino acids, cysteine (Cys) is the preferred residue for the installation of chemical handles in proteins, due to its low abundance (~1.9%), high nucleophilicity and ability to react in environments closer to neutral pH [88]. Commercial availability and ease of use and synthesis of maleimide derivatives [92], selectively reacting with Cys, have led to widespread use in conjugate synthesis [93]. Incorporation of non-canonical aminoacids (ncAAs) into proteins enables unique bioorthogonal chemistries which do not react with naturally occurring chemical functional groups. Schultz and colleagues spearheaded the movement towards the broadly applicable use of mutually orthogonal aminoacyl-tRNA synthetase–tRNA pairs for recognizing and inserting ncAAs at the site of the amber nonsense codon (UAG) in *E. coli* [94,95] (Figure 1.5). New methods for site-

specific ncAAs incorporation have been developed [96-98], but the amber suppression is still the most widely used. The copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC), resulting in the formation of a 1,2,3-triazole, is an example of a click reaction [99] that can be used to link an azido-functionalized ncAAs to an alkyne-functionalized polysaccharide, or vice versa. One disadvantage of CuAAC is that there is significant cytotoxicity with using copper as the catalyst, hampering utilization *in vivo* [100]. To overcome this limitation, Bertozzi and coworkers introduced a biocompatible catalyst-free [3 + 2] cycloaddition reaction between azides and cyclooctyne derivatives, known as strain promoted azide-alkyne cycloaddition (SPAAC) [100,101].

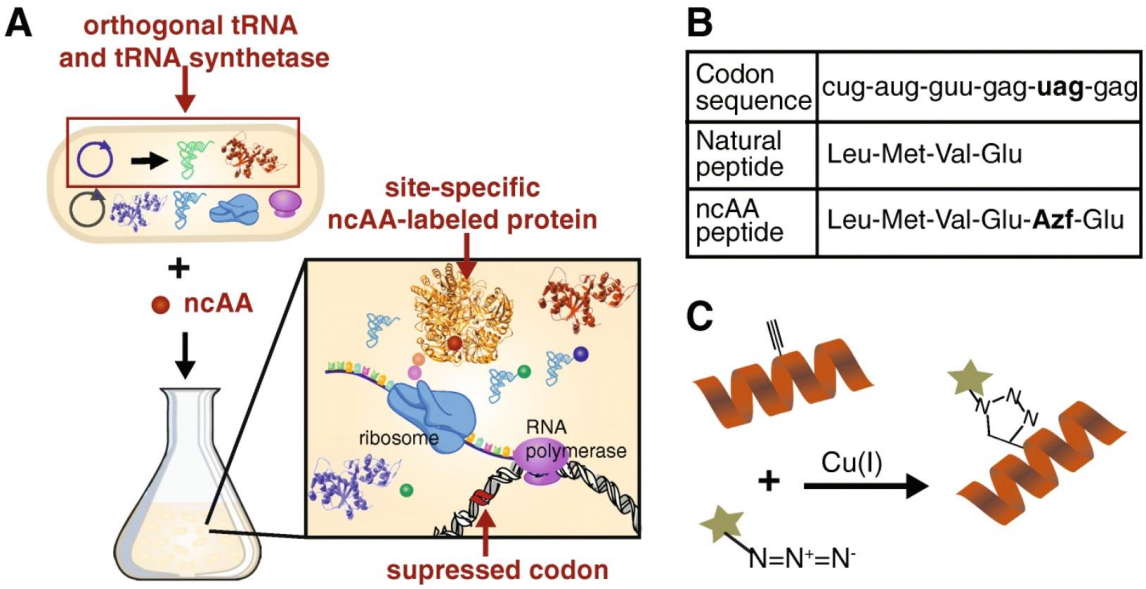


Figure 1.5. Overview of site-specific ncAA incorporation with amber suppression method. **A**) A plasmid that expresses the desired orthogonal tRNA and tRNA synthetase is transfected into cells along with the plasmid containing the protein of interest that has been engineered to carry the suppressed codon sequence at a specific site. ncAA is added to the system and the protein of interest is labeled site-specifically with the ncAA. **B**) An example of the codon sequence and corresponding peptides that result from either natural synthesis or synthesis in the presence of the orthogonal tRNA/tRNA synthetase and ncAA. **C**) A peptide labeled site-specifically with a ncAA carrying an alkyne functional group is conjugated to an azide-containing fluorophore via CuAAC. *Reproduced from* [102].

Recently, SPAAC has been used in a cell-free protein synthesis platform, for the precise conjugation of the dibenzocyclooctyne-derivatized form of Group A *Streptococcus* (GAS) carbohydrate at the level of incorporated p-azidomethyl phenylalanine residues into a C-terminally truncated Streptolysin O (SLO) toxoid. This selective double-hit vaccine generated functional antibodies against both conserved GAS virulence factors and provided protection

against systemic GAS challenges [103]. Another remarkably simple click reaction uses dialkyl squarate reagents to sequentially stitch two amino-functionalized biomolecules together [104]. As more vaccine candidates based on click reactions are beginning to emerge [105-107], further studies to elucidate the immunogenic potential of the aromatic rings formed following these reactions are required. Carrier-induced epitopic suppression resulting from immunodominant reactivity of aromatic linkers is in fact a known phenomenon [108,109].

1.6.2 Insertion of motifs and enzymatic tags

Modification of canonical amino acids can be also selectively achieved by enzymatic methods [110]. Enzymes can react with specific residues incorporated in a short amino acid tag which can be introduced either on the protein (Figure 1.6) or on the sugar to be conjugated. Among these enzymes, transglutaminases are a family of widely expressed enzymes that have been used to label lysines (Lys) [111] or glutamines (Gln) [112]. With regard to lysine modification, microbial transglutaminase enabled the selective acylation of only one CRM_{197} lysine at pH 8 after 18 h [113].

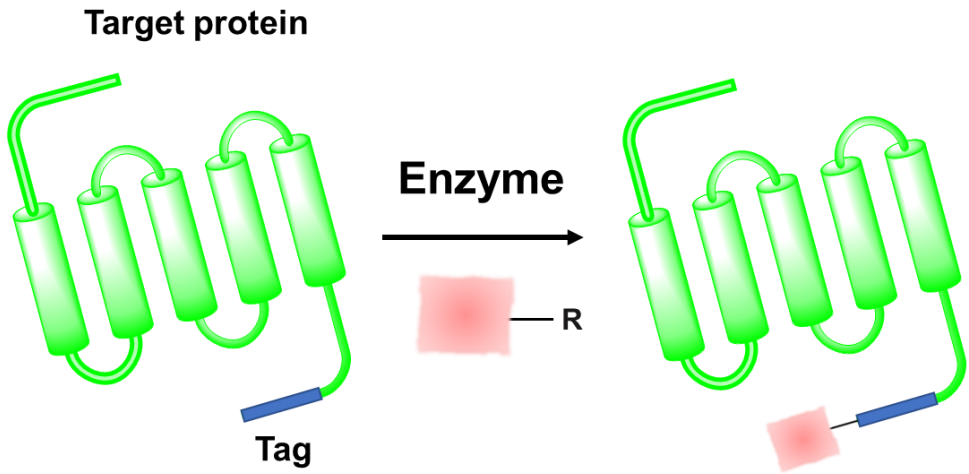


Figure 1.6. Chemoenzymatic reaction catalyzed by an appropriate enzyme tolerant of substrate analogs for the site-specific protein modification.

Sortases from *S. aureus* or *Streptococcus pyogenes* have been successfully applied for conjugation to an N-terminal oligoglycine or C-terminal LPXTG tag [114], which is the consensus sequence recognized by this transpeptidase. Ting an co-workers demonstrated that *E. coli* BirA, a biotin ligase recognizing an engineered 15-residue sequence, can be used as a ketone ligase, introducing a unique ketone group available for subsequent reactions [115].

However, the size and position of the inserted motifs could compromise protein activity. In some cases motifs can be added only at the extremities of proteins to be more accessible to the enzyme [116,117]. No less important, the enzyme must be easily obtained and achieve high conversions to be industrially useful [118].

1.6.3 Fusion of proteins

Based on the advantages of motif insertion, studies exploring novel fusion proteins have been reported recently. HaloTag and SNAP-tag, recognizing respectively functionalized chloroalkane and benzylguanine ligands, have proved valuable for fusion to termini of target proteins, but their use is currently limited to biochemical and bioimaging applications. Patterson and colleagues demonstrated that domain I of Human Serum Albumin (HSA) can be used as a fusion protein for the preparation of antibody conjugates [119], taking advantage of the preferential reactivity of a cyclohexene sulfonamide compound towards HSA Lys64 [120]. In Multiple Antigen-Presenting System (MAPS) technology, the protein of interest is genetically fused to a rhizavidin fragment for the subsequent generation of a macromolecular complex with a byotinilated PS, thanks to the high affinity interaction between rhizavidin and biotin [121] (Figure 1.7). This methodology was used for a pneumococcal vaccine prototype and has now been extended to *S.* Typhi [122], *S. aureus, K. pneumoniae* and *P. aeruginosa*.

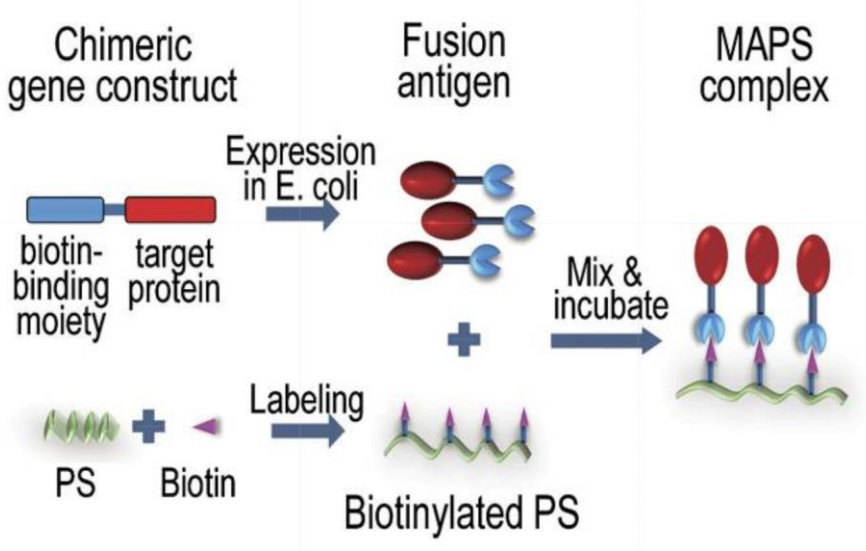


Figure 1.7. Schematic diagram of MAPS technology. *Adapted from* [121].

1.7 Bacterial glycoengineering as a new tool for site-selective protein glycosylation

Protein glycosylation is a complex enzymatic process that involves glycosyltransferases and activated substrates. Once thought to be exclusively present in eukaryotes, it is now evident, thanks to advances in analytical methods and genome sequencing, that protein glycosylation is a common feature in all three domains of life (Eukarya, Bacteria and Archaea) [123,124]**.** In 2002, the first *N*-linked protein glycosylation system was discovered in the human gastrointestinal pathogen *Campylobacter jejuni*, constituted by the *pgl* operon encoding enzymes that modify >80 proteins [125,126]. This pathway involves assembly of an OS precursor on a lipid carrier that is subsequently flipped across the inner membrane in the periplasm and the sugars are transferred *en bloc* by PglB to the asparagine residue of the D/E-X_1-N-X_2-S/T consensus sequon of the target protein, where X_1, X_2 cannot be prolines (Figure 1.8A) [127]. O-glycosylation mechanisms (involving serine, threonine and tyrosine residues) have been also described [127]. The functional transfer of N-glycosylation system in *E. coli*, together with the discovery of the relaxed substrate specificity of the oligosaccharyltransferase (OTase) PglB, opened the way to bacterial glycoengineering [128]. This led in fact to the development of Protein-Glycan Coupling Technology (PGCT) or bioconjugation, a novel technique for the production of glycoconjugate vaccines. Engineered *E. coli* strains, equipped with the machineries for the glycan synthesis and carrier protein and OTase expression, are

exploited for the *in vivo* production of the glycoconjugate. Several glycoconjugates have been produced with this new technology [129-135] and tested in clinical trials (Table 1.2). Cell-free variants of PGCT (decoupling the bioconjugation step from the glycan biosynthesis in *E. coli*) have been also developed [136,137].

Table 1.2 Glycoconjugate vaccine in development using bioconjugation technology. *Adapted from [138].*

Organism	**Glycan**	**Protein carrier**	**Status**	**Manufacturer**
Streptococcus pneumoniae	Capsule-multivalent	rEPA	Phase I clinical trials	Limmatech Biologics
Streptococcus pneumoniae	Capsule-serotype 4	piuA	Development	Academic-UCL/LSHTM UK
Staphylococcus aureus	Capsule-Type 5 and 8	rEPA	Development	GlycoVaxyn
Shigella dysenteriae	Capsule-Type 1	rEPA	Phase I clinical trials	Limmatech Biologics
Shigella flexneri	Capsule- 2a	rEPA	Phase IIb clinical trials	Limmatech Biologics
Escherichia coli	O-antigen-ExPEC	rEPA	Phase II clinical trials (4-valent); Phase III clinical trials (9-valent); Phase I/IIa clinical trials (10-valent);	Limmatech Biologics/ J&J
Francisella tularensis	O-antigen	rEPA	Development	Government/ Academic -DSTL/ LSHTM UK
Burkholderia pseudomallei	O-PSII	AcrA	Development	Government/ Academic- DRDC/ University of Alberta Canada

An alternative bacterial N-glycosylation pathway was more recently described in the Gram-negative γ-proteobacterium *Haemophilus influenzae* [139-141]. N-glycosyltransferases (NGTs) are soluble, cytoplasmic enzymes that catalyze asparagine glycosylation using nucleotide-activated monosaccharides as sugar donors (Figure 1.8B). The best characterized member of this protein family is the NGT of the porcine respiratory tract pathogen *Actinobacillus pleuropneumoniae* (ApNGT). It catalyzes the transfer of glucose or galactose from UDP to the side chain of an asparagine residue present in N-X-S/T sequon (X ≠ Pro) of proteins and peptides. Similar to the eukaryotic N-glycosylation system, the stereochemistry is inverted from an α-linkage in the nucleotide-activated sugar substrate to a β-linkage in the glycoprotein. In contrast to OSTs which contain a divalent cation in their active site, NGTs are metal-independent [142].

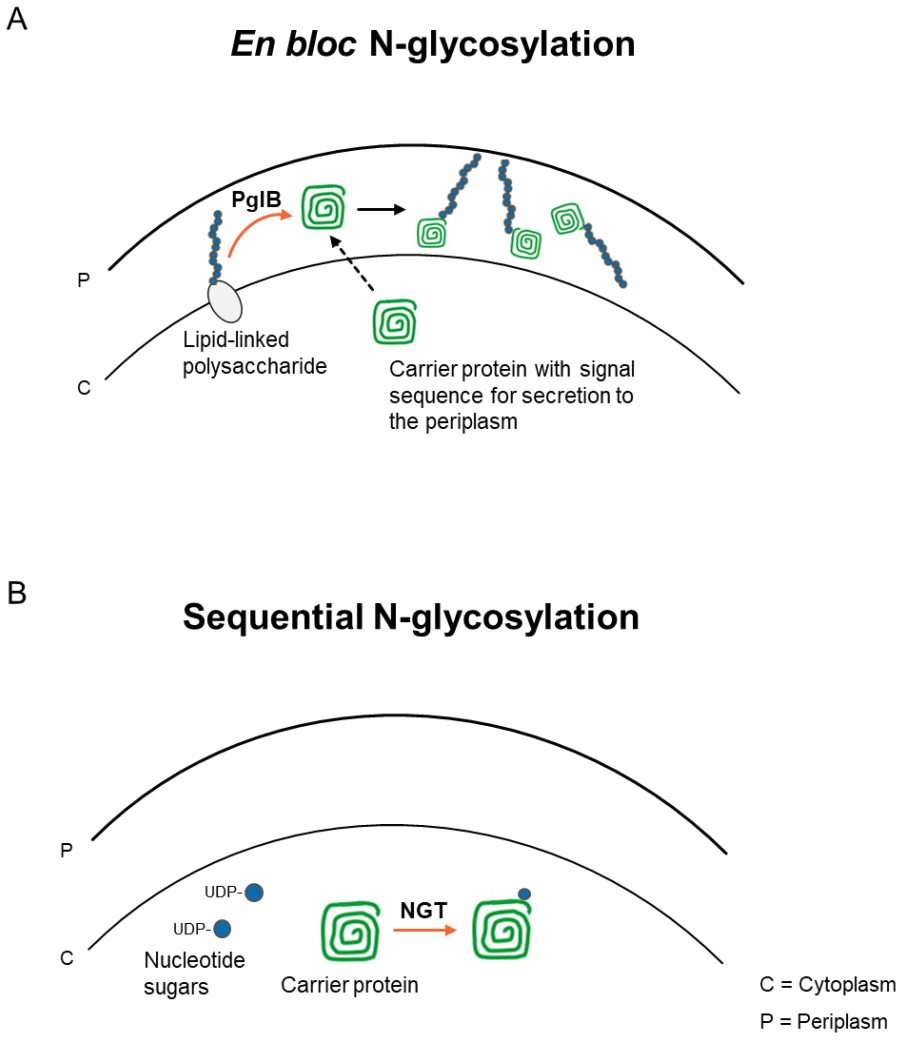


Figure 1.8. Schematic representations of periplasmic (A) and cytoplasmic (B) N-glycosylation systems occurring in bacteria.

Keys *et al.* developed a biosynthetic pathway for site-specific polysialylation of recombinant proteins in *E. coli* cytoplasm employing ApNGT for the installation of a priming β-glucose (Figure 1.9). By the addition of a series of appropriate enzymes (LgtB, SynB and a polysialyltransferase from *N. meningitidis* and CstII from *C. jejuni*), the authors were able to achieve not only polysialylation of a native substrate of ApNGT, but also of heterologous proteins like super folder Green Fluorescent Protein (sfGFP) or designed ankyrin repeat proteins (DARPin), a class of antibody mimetic with a number of emerging therapeutic application [143]. This technology platform has also been extended to self-assembling proteins for the production of glycosylated megadalton-scale nanoparticles in *E. coli* cytoplasm with diverse future biomedical applications [144]. The potential of NGT-catalyzed N-glycosylation as a tool for the site-specific modification of proteins with glucose sets in fact the basis for many interesting applications.

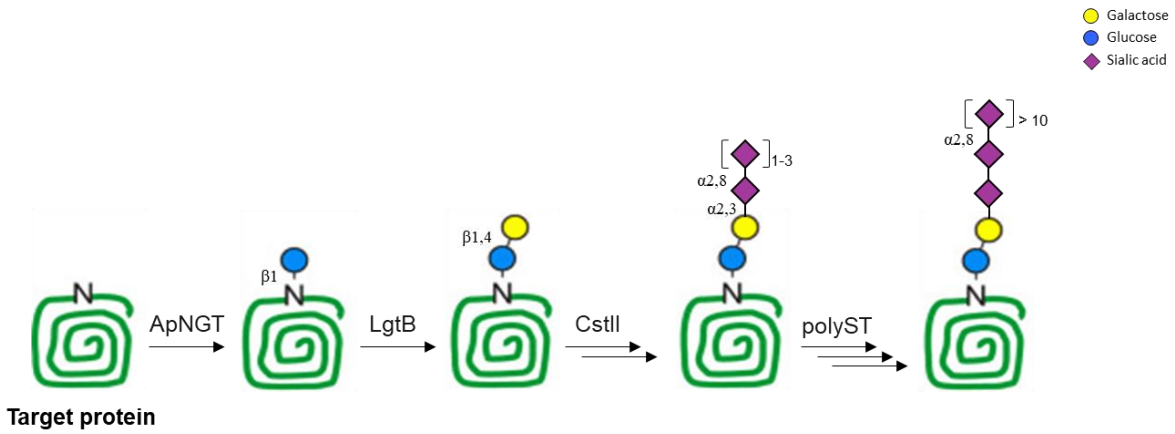


Figure 1.9. The biosynthetic pathway established by Tim Keys *et al.* for N-linked polysialylation of proteins in the bacterial cytoplasm [143]. Initial modification of the target protein is achieved by the ApNGT, sequential elongation of the glucose is operated by LgtB (β1,4-galactosyltransferase), CstII (α2,3/α2,8-oligo-sialyltransferase), and the polyST (α2,8-polysialyltransferase). SynB (CMP-sialic acid synthetase, not shown in the scheme) generates the activated nucleotide sugar, CMP-sialic acid, which is the donor substrate for sialyltransferases.

1.8 *Klebsiella pneumoniae*

Klebsiella pneumoniae (Kp) is a Gram-negative, encapsulated, nonmotile bacterium that resides in the environment, including soil and surface waters, and readily colonizes human mucosal surfaces and medical devices [145-147]. It belongs to the *Enterobacteriaceae* family and the genus *Klebsiella* derived its name from the German microbiologist Edwin Klebs, whose work on infectious diseases paved the way for modern bacteriology. In 1882, Kp was first identified as a causative agent of pneumonia, and during the 1960s and 1970s it became one of the most important causes of opportunistic healthcare-associated infections. In addition to pneumonia, Kp can also cause infections in the urinary and lower biliary tracts as well as bacteremia [148]. The most vulnerable patients are neonates and the elderly, especially those that are immunocompromised. Kp is naturally resistant to β-lactam antibiotics and its genome continued to acquire new resistances through horizontal gene transfer (HGT) [149], counting more than 400 antimicrobial resistance (AMR) genes so far [150]. In 2017, following the emergence of Kp strains resistant to last-line antibiotics such as carbapenems, WHO inserted Kp in the list of multidrug-resistant (MDR) pathogens for which the development of new interventions is a priority. Hypervirulent (hv) variants (Figure 1.10), displaying hypermucoviscosity and multiple siderophores as virulence factors, have also emerged. These hv-Kp strains are known to cause serious debilitating infections also in immunocompetent individuals [151,152]. The prevalence of AMR is lower in hv-Kp when compared to common Kp strains [153], but recent studies highlighted the increasing incidence of MDR hv-Kp with production of extended spectrum of beta-lactamases and carbapenemases, showing that classical Kp strains have acquired the mobilizable hv-Kp virulence plasmids via HGT and developed into MDR hv-Kp strains [154,155]. Epidemiologically, hv-Kp is more common in East and South-east Asia but is an emerging threat in Europe, particularly when associated with carbapenemase producing clones [155-157]. A systematic review and meta-analysis of available data from the African continent, with a focus on the past decade (2008–2018), identified *Klebsiella* spp (mostly Kp) as accountable for 21% of all reported cases of neonatal bacteremia or sepsis [158]. While more studies, including the Burden of Antibiotic Resistance in Neonates from Developing Societies study and the Global Neonatal Sepsis Observational Study, are currently collecting more field data, the available estimates clearly highlight the need to acknowledge Kp as one of the most important neonatal pathogens in low- and middle-income countries [159].

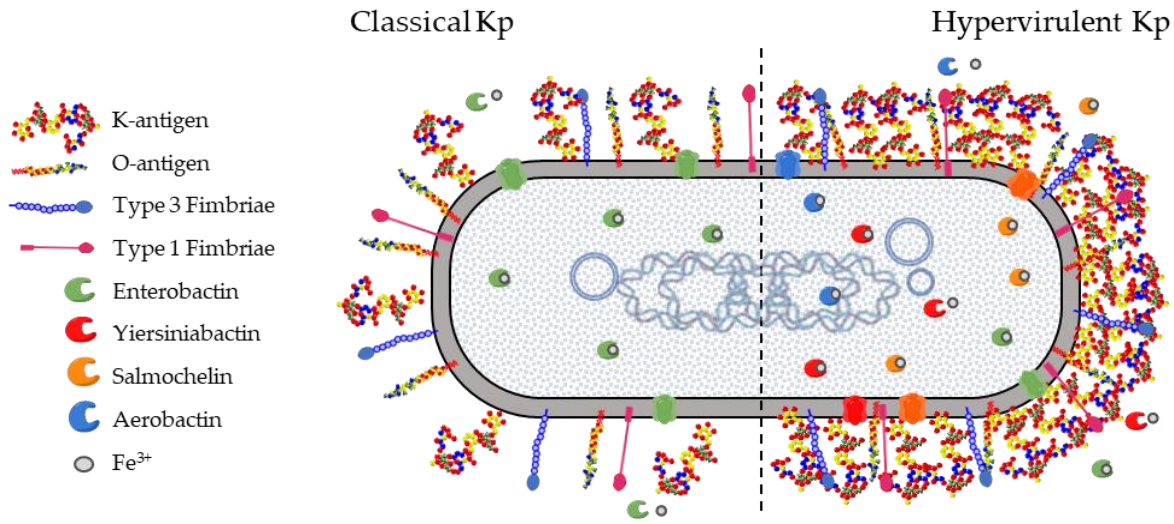


Figure 1.10. Virulence factors of classical and hypervirulent Kp strains. *Reproduced from* [159]. The capsule is an extracellular polysaccharide matrix that envelops the bacteria and is overproduced in hv-Kp strains. LPS is an integral part of the outer leaflet of the outer membrane and is produced by both classical and Hv-Kp strains. Type 1 and type 3 fimbriae are membrane-bound adhesive structures. Iron-scavenging siderophores are secreted small molecules recognized by specific membrane receptors mediating their uptake.

Besides siderophores, Kp possesses three other well characterized virulence factors: capsular polysaccharides (K-antigens), OAg portion of LPS and Type 1 and type 3 fimbriae. K-antigens (KAg) play a crucial role in protecting Kp from innate immune response mechanisms, evading complement deposition and opsonization, reducing recognition and adhesion to epithelial cells and phagocytes, and abrogating lysis by antimicrobial peptides and the complement cascade [160]. Traditionally, 77 KAg have been identified among *Klebsiella* spp. based on the diversity in their sugar composition, type of glycosidic linkages, and the nature of enantiomeric and epimeric forms [161,162] (Figure 1.11). Among the different KAg, K1, K2, K5, K16, K23, K27, K28, K54, K62 and K64 are some of the most commonly isolated serotypes globally [163], and approximately 70% of hv-Kp isolates are capsule types K1 or K2 [164]. Cryz *et al*. showed that anti-K1 IgG isolated from human volunteers are able to protect mice from Kp sepsis [165]. Additionally, a murine anti-K2 IgM monoclonal antibody was tested in rats against experimental Kp challenge and, even though the invasion was not prevented, mAb-treated animals showed a more rapid bacterial clearance resulting in an accelerated resolution of infection [166].

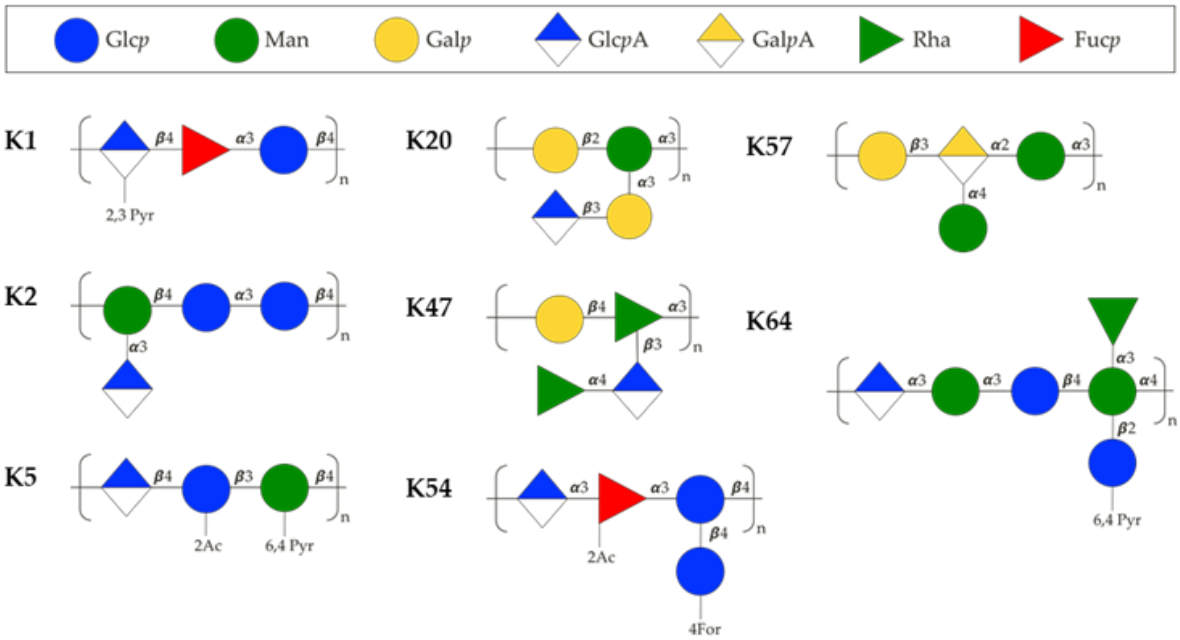


Figure 1.11. Structures of KAg most commonly found globally. *Adapted from* [159].

However, a following epidemiological study raised the awareness that a Kp vaccine able to cover around 70% of clinically relevant Kp strains should include at least 25 capsular PSs [167]. OAg are instead much lower in number compared to KAg: 11 serotypes have been described so far, and only four of them (O1, O2, O3 and O5) are predicted to cover over 80% of clinically relevant Kp strains [163,168-170] (Figure 1.12). For this reason, OAg could constitute valuable alternative targets for the development of a vaccine.

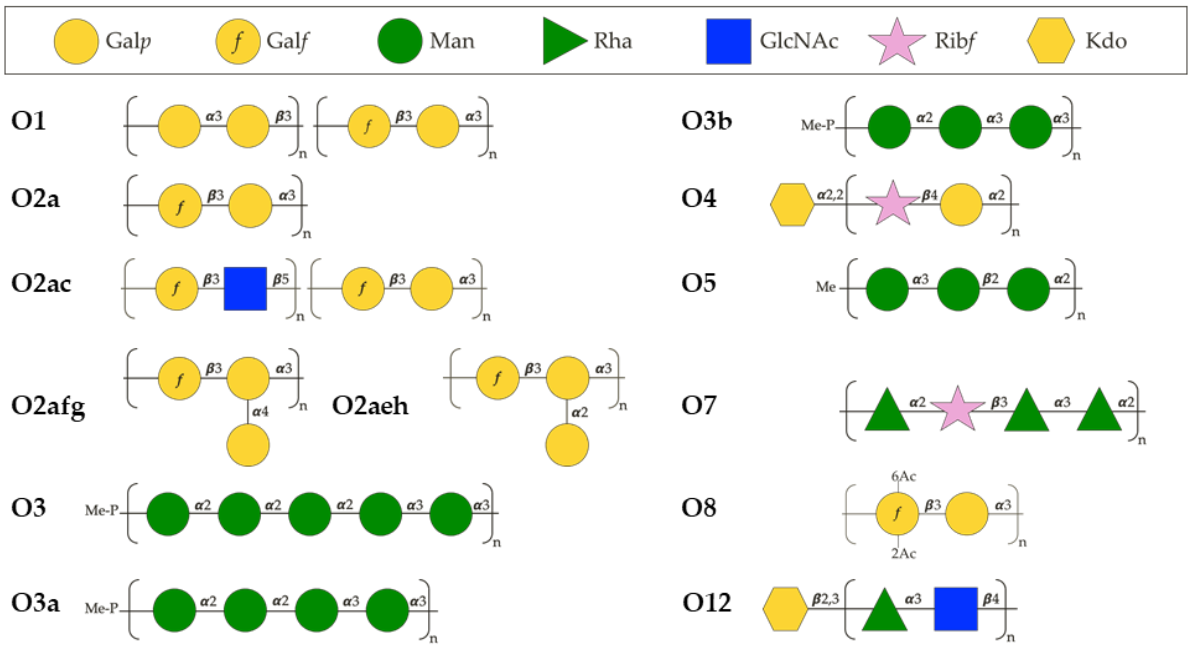


Figure 1.12. Structures of Kp OAg. *Adapted from* [159].

Preclinical studies have showed the efficacy of OAg conjugates in different animal models [171-173]. However, the accessibility of OAg to antibodies especially in hypercapsulated strains remains controversial. Interestingly, Cross and coauthors proposed the use of the four prevalent Kp OAg in MAPS complexes with the major structural component of Kp type 3 fimbriae, MrkA [174,175]. The type 3 fimbriae are the extracellular appendages most frequently expressed by strains of Kp [176,177]. These protein-based filaments are composed by MrkA monomers and intermittently minor subunits of MrkF, culminating with the tip adhesion protein MrkD. MrkA is a small (~ 20 kDa) hydrophobic protein, and this hydrophobicity may facilitate bacterial interactions leading to efficient growth as a biofilm [178]. MrkD instead does not appear to be necessary for rapid and efficient biofilm formation, although it is required for binding to extracellular matrix proteins [179]. MrkF is able to serve as an initiator for the growth of type 3 fimbriae [180]. It has been demonstrated that these fimbriae mediate adherence *in vitro* to basement membrane components and the submucosa of human lung tissue [181,182]. Following colonization, the bacteria can invade the systemic circulation and be disseminated from the respiratory tract. Consequently, humoral antibodies directed against type 3 fimbriae may play a role in preventing attachment within the submucosa of the respiratory tract and subsequent development of systemic infection. In the murine model of pneumonia virulent strains of Kp are rapidly disseminated from the site of inoculation leading to septicemia.

No vaccine against Kp has yet been licensed, but several vaccines, based on different approaches, are currently under development (Table 1.3) [183]. Given the complexity of Kp, multicomponent vaccine formulations could represent the most promising strategy to provide broad vaccine coverage. Currently, the only vaccine against Kp in clinical trials is a tetravalent candidate based on LPS OAg and adjuvanted with the squalene-based AS03, named Kleb4V, which entered Phase I/II in 2021 [184].

Table 1.3 Vaccines against Kp under devolopment. *Adapted from [185].*

Type of vaccine	**Antigen**	**Model**	**Measurement**	***In vivo* protection (Y/N)**
Whole cell vaccines	*K. pneumoniae tonB* deletion mutant	Mouse	Evaluated IgG level	Y
	Live attenuated *K. pneumoniae*	Mouse	Evaluated cytokines and IgA levels	ND
	Acetone-dried *K. pneumoniae*	Rabbit	Evaluated antibody level	ND
Subunit vaccines	*K. pneumoniae* cytotoxin	Mouse and Rabbit	Evaluated IgG level in mice and baby rabbit born from immunized mother	Y
	Purified type 3 fimbriae of *K. pneumoniae*	Mouse	Evaluated IgG level in mice	Y
	Recombinant AK36 protein comprised of antigens from OmpA and OmpK36	Mouse	Evaluated IgG, IgM, and IgA levels	Y
	Eight surface proteins Glycerophosphodiester phosphodiesterase (GlpQ), recombination regulator RecX, oxidoreductase (YhiN), nitrite reductase subunit (NirB), hypothetical protein (YfhM), DNA repair protein RecO (RecO), glutamine ABC transporter periplasmic protein (GlnH), and penicillin-binding protein 2 (MrdA)	Mouse	ND	Y
	Siderophore receptor protein	Cattle	Reduced KP mastitis and	ND

			increased milk production	
Capsular polysaccharide (CPS) vaccines	12 CPSs K1, K2, K3, K15, K20, K35, K36, K44, K50, K63, K70, and K74 antigens	Mouse	ND	Y
	Kl, K36, K44 and cross-reactive CPS	Mouse	ND	Y
	24 CPSs K2, K3, K5, K9, K10, K15, K16, K17,K18, K21, K22, K25, K28, K30, K35, K43, K52, K53, K55, K60, K61, K62,K63, and K64 antigens	Human	Evaluated IgG	ND
	K2, K3, K10, K21, K30, and K55	Human	Evaluated IgG level	Y
O polysaccharide (OPS) vaccines	*Klebsiella* O1 LPS into liposomes	Rat	ND	Y
	Klebsiella O1 LPS	Mouse	Evaluated IgG level	Y
Conjugate vaccines	Octasaccharide derived from CPS (K11) coupled to bovine serum albumin	Mouse	Evaluated IgG level	ND
	O1 OPS linked to tetanus toxoid	Rat	Alveolar macrophage activation	Y
	Hexasaccharide 1 coupled to diphtheria toxin mutant	Mouse and Rabbit	Evaluated IgG, and IgM levels	N
	K. pneumoniae OPS (O1,O2,O3,O5) linked to *P.*	Mouse	Evaluated IgG level	Y

	aeruginosa (PA) flagellin protein (FlaA, FlaB)			
MAPS vaccine	Four KP OPS (O1,O2,O3,O5) and MrkA	Mouse	Evaluated IgG level	Y
DNA vaccine	Vector pVAX1 expressing OmpA or OmpK36	Mouse	Evaluated cytokines and IgG levels	N
Vesicle vaccine	Extracellular vesicles	Mouse	Evaluated cytokines and IgG levels	Y

Progression in vaccine development may have been slowed down by the lack of fully standardized assays to measure immunity to Kp. Opsonophagocytosis and other mechanisms of antibody-induced killing have been shown to be important in protection against Kp. Both these complement-dependent assays are often used as correlates for protection to measure the functional capacities of vaccine-induced antibodies: the results of OPK are proven to correlate well with the functional efficacy of pneumococcal vaccines [186,187], and SBA is the gold standard for immunity against *N. meningitidis* [188,189]. Nevertheless, still little is known on correlates of protective immunity to Kp and work is ongoing to develop OPK and SBA assays against this pathogen and understanding role of anti-K- and anti-OAg antibodies in functionality. Since an important virulence trait of Kp is its ability to form biofilms, the inhibition of biofilm formation *via* antibody is another possible protective mechanism against Kp infections. Wang *et al.* showed that anti-MrkA antibodies induced a dose-dependent inhibition in biofilm formation *in vitro* and reduced bacterial attachment to human lung epithelial cells in culture, and found that these results correlated with the increased survival of mice after intranasal challenge and lower bacterial dissemination [190]. *In vivo* approaches are pivotal to develop new therapeutics to fight Kp infections. Kp disease manifestations in the respiratory and urinary tracts have been extensively modeled in animals [148]. In 1980, Fader *et al.* provided the first evidence of the attachment of Kp to the urinary epithelium using an *in vivo* model of infection of the rat bladder and showed the importance of fimbriae in adhesion to the urinary epithelium and in the severity of the urinary tract infection (UTI) [191]. Route and dose of infection are important elements of the infection/immunization outcomes, shifting the immune response to a protective or non-protective response. In fact, it is reported that the production of type 3 fimbria-specific antibodies in mice was associated with protection against

intranasal infection by low doses of bacteria, but not against challenges by large bacterial loads [192]. Since serious Kp pneumonias and UTIs may lead to bacteremia, which could eventually evolve to sepsis, the development and standardization of animal model of sepsis is also important. Differently from direct inoculation (e.g. via blood or peritoneal cavity), implantation of bacteria embedded in a fibrin clot allows a slower release of bacteria and a more sustained infection in preclinical models, better mimicking the human septic syndrome [193]. The successful establishment of sepsis by entrapping a dose of 150 colony forming units of Kp in a fibrin clot following implantation into the peritoneal cavity of mice is reported [194]. Inbred BALB/c mice are the most frequently used in challenge studies [195], even if they could not reflect the immune response variability of the human outbred population. Differences have been in fact highlighted in Kp pathogenicity between humans and animal models (e.g. the CR-Kp CG258 is responsible for serious infections in humans but is rapidly cleared in mice and rats [196]). The use of humanized mouse strains could help in reducing the gap between *in vivo* mouse models and clinical trials [197].

2 AIM OF THE PROJECT

The major aim of my PhD project was to develop alternative strategies for the generation of innovative selective glycoconjugate vaccines, combining protein glycoengineering to chemical conjugation. The major component of Kp type 3 fimbriae MrkA was chosen as model for its use as potential protective antigen and carrier for Kp capsular (KAg) and subcapsular (OAg) PSs.

The specific objectives of my work are the following:

1. Establishment of a pathway in *E. coli* for the cytoplasmic glycosylation of MrkA monomer, introducing a unique sugar handle onto the protein to be exploited for the selective conjugation of Kp K- and O-Ag.
2. Development of analytical methods suitable for glycoproteins characterization.
3. Immunogenicity studies in animal models to compare the immune response induced by these novel selective conjugates compared to more traditional random glycoconjugates of MrkA.

3 MATERIAL AND METHODS

3.1 Bacterial strains, mutants generation and growth condition

Kp strains NCTC11228 (K2:O1v1) and NCTC11679 (K61:O3) were obtained from Public Health England. NCTC11228 strain was mutated to delete the *tolR* gene for GMMA production. It was further mutated to obtain a GMMA-producer strain displaying O2a OAg, through deletion of the *wbbY* gene. *E. coli* K12 W3110 was acquired from ATCC (27325). *E. coli* K12 W3110 was selected for the generation of *lacZ* mutant, to be used as expression host for lactosylated proteins. To generate Kp and *E. coli* mutant strains, the kanamycin resistance gene *aph* was used to replace the target gene. The resistance cassette replacement constructs were amplified from the pKD4 vector using forward and reverse primers composed of 50 bp homologous to the flanking regions of the gene to be deleted and approximately 20 bp at the 3′ end matching the flanking region of the resistance gene. Primer sequences are listed in Table 3.1. PCR products were purified and were used to transform recombination-prone cells carrying pKD46 following methods described previously [198]. After gene deletion, the kanamycin resistance gene was removed through FLP-mediated recombination using the pCP20 plasmid to yield markerless mutant strains. Plasmids and protein coding sequences were ordered from GeneArt or Twist Biosciences and codons were optimized for protein expression in *E. coli*. Two expression system for cytoplasmic protein glycoengineering were adopted: one based on the use of two different expression vectors for the expression of carrier protein (pBAD myc/his B vector, Thermo Fisher Scientific) and enzymes (pACYCDuet vector, Novagen), as described previously [144], and a second one based on a single plasmid for the expression of both carrier protein and NGT (pET29b(+) vector, Thermo Fisher Scientific). All plasmids are listed in Table 3.2. All bacterial strains were grown at 30 °C in liquid Luria-Bertani (LB) medium in a rotary shaker for 16 hours. For GMMA production, overnight cultures were diluted in HTMC medium (15 g/L Glycerol, 30 g/L Yeast extract, 0.5 g/L $MgSO_4$, 5 g/L KH_2PO_4, 20 g/L K_2HPO_4) to an optical density at 600 nm (OD600) of 0.3 and grown at 30 °C in a rotary shaker for 8 hours using baffled flasks with a liquid to air volume ratio of 1:5. For recombinant protein production, overnight LB cultures were diluted in HTMC medium with 5 g/L glucose instead of 15 g/L glycerol, supplemented with appropriate antibiotics (kanamycin 50 μg/mL, chloramphenicol 20 μg/mL, ampicillin 100 μg/mL). Bacteria were grown for 7.5 at 30 °C and then protein

expression was induced with 0.4% Arabinose and/or with 1 mM IPTG, incubating cultures in baffled flasks (with a liquid to air volume ratio of 1:5) at 25 °C in a rotary shaker for 18 h.

Table 3.1. List of primers used in this study. Bold regions in the primer sequences correspond to the base pairs matching the flanking regions of the kanamycin resistance gene *aph* in the pKD4 vector.

Mutated gene	**Primers**	**Sequence (5' → 3')**	**Mutant strain generated**
tolR	Forward	ACAGGTTCAGCGCTTTAATAATTTCATCGTAA GGGACGTCTTTCGCACCG**GTGTAGGCTGGA GCTGCTTC**	Kp NCTC11228 Δ*tolR*
	Reverse	CATCGTTCCGCTGCTGGATGTCCTGCTGGTGC TGCTGCTGATCTTTATGG**CATATGAATATCC TCCTTAG**	
wbbY	Forward	CGGCGGTATTGGTACTGCTTTCACTGCCCTTG CCACTACTTTGGCAAAAA**GTGTAGGCTGGA GCTGCTTC**	Kp NCTC11228 Δ*tolR* Δ*wbbY*
	Reverse	ATAGAGGTTCCGGGACGATTTGCAATTTATA TCCCTGTAATGATATTTTG**CATATGAATATC CTCCTTAG**	
lacZ	Forward	TCGCCAGCTGGCGTAATAGCGAAGAGGCCCG CACCGATCGCCCTTCCCAA**GTGTAGGCTGG AGCTGCTTC**	*E. coli* K12 W3110 Δ*lacZ*
	Reverse	ATATGGAAACCGTCGATATTCAGCCATGTGC CTTCTTCCGCGTGCAGCAG**CATATGAATATC CTCCTTAG**	

Table 3.2. List of plasmids used in this study.

Plasmid	Resistance	Inducer
pBAD-sf*gfp*	Ampicillin	Arabinose
pBAD-*mrkA* version A	Ampicillin	Arabinose
pBAD-*mrkA* version B	Ampicillin	Arabinose
pACYCDuet-*ngt*	Chloramphenicol	IPTG
pACYCDuet-*ngt*+*lgtB*	Chloramphenicol	IPTG
pACYCDuet-ngt+*lgtB*+*lgtC*	Chloramphenicol	IPTG
pACYCDuet-*ngt*+*lgtB*+*wbbY*	Chloramphenicol	IPTG
pACYCDuet-*ngt*+*lgtB*+*lgtC*+*wbbY*	Chloramphenicol	IPTG
pACYCDuet-*ngt*+*lgtB*+*wbbY*+*wbbMNO*	Chloramphenicol	IPTG
pET29b(+)-*slo*	Kanamycin	IPTG
pET29b(+)-*slo*+*ngt*	Kanamycin	IPTG

3.2 Expression, purification and characterization of recombinant proteins in *E. coli*

Recombinant proteins were extracted from bacterial pellets stored at -20 °C. Cell lysis was performed either using CelLytic reagent (Sigma-Aldrich) or by sonication (10’ for g of biomass). The lysate was then the centrifuged to recover the soluble fraction (i.e., the supernatant). The supernatant was filtered and loaded onto HisTrap FF 1 mL or 5 mL (Cytiva) depending on the bacterial growth scale. After sample application, column was washed with 20 mM NaH_2PO_4, 500 mM NaCl and 20 mM imidazole pH 7.4, then the His-tagged protein was eluted with a linear gradient up to 500 mM imidazole. SDS-PAGE analysis was performed on Immobilized Metal Affinity Chromatography (IMAC) fractions to pool fractions containing the protein of interest. Imidazole was removed from the pooled fractions with Amicon ultrafiltration devices (cut-off 10 kDa for MrkA and sfGFP, 30 kDa for SLO), and proteins were exchanged in Phosphate-buffered saline (PBS) 1X. Sodium dodecyl sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE, 4-12% Bis-Tris gel run in MES buffer) and High-performance Liquid Chromatography–Size Exclusion Chromatography (HPLC–SEC) analyses were performed to check purity of the sample. Samples were analyzed with a TSK gel G3000 PWXL column (30 cm x 7.8 mm; particle size 7 µm; cod. 808021) with TSK gel PWXL guard column (4.0 cm x 6.0 mm; particle size 12 µm; cod.808033) (Tosoh Bioscience). Intact Mass Spectrometry (MS) analysis of proteins a 1 mM concentration was performed using direct infusion with QExactive HF-X.

3.3 Protein digestion and LC-MS/MS analysis for peptide mapping

Protein samples were reduced with dithiothreitol (DTT, Merck Life Science), alkylated with iodoacetamide, and digested using trypsin (Promega). The resulting peptides were then analyzed using an Ultimate 3000 RSLC nano coupled to a Q-Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an Easy Spray ESI ion source. Peptides were separated using gradient elution (phase A: 0.1% formic acid in water; phase B: 0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min and at 35 ° C on a PepMap TM RSLC C18 column, 75 µm × 150 mm, 2 µm, 100 Å (Thermo Fisher Scientific). Gradient starting condition was set to 5% phase B and it was increased up to 36% in one hour. The MS/MS spectra were obtained in "data-dependent scan" mode. Precursor ions were selected within an isolation window of 1.4 m/z. Fragment ion spectra were detected in the orbitrap at a resolving

power of 30 000. MS/MS was performed with a dynamic exclusion of 20 s and a total cycle time of 3 s. The raw LC-MS/MS data was analyzed using BioPharmaFinder software (version 2.0, Thermo Fisher Scientific) using as input he expected protein sequence. Only peptides identified with MS/MS confidence scores greater than 90% and mass tolerance within ±1.5 ppm were considered.

3.4 O-antigens purification and characterization

Briefly, GMMA were purified from the 0.22 μm filtered (Stericup filters, Millipore) supernant culture, after bacteria centrifugation at 5,000 x g for 45 minutes, through 2 rounds of ultracentrifugation (175,000 *x g* for 2 hours at 4° C) and finally resuspended in PBS. O1v1 and O2a OAg were extracted from GMMA as previously described [199,200]. Acetic acid hydrolysis is used to break the labile linkage between lipid A and KDO at the reducing end of the core region with release of OAg repeats attached to the core in the supernatant. Gel filtration chromatography was used to separate the OAg from core oligosaccharides with no OAg repeats and other impurities. Total extracted OAg was run on a HiPrep 16/60 Sephacryl S100 HR column (600 mm x 16 mm) (GE Healthcare). The mobile phase was PBS at flow rate of 0.5 mL/min. OAg was characterized by HPLC–SEC with differential refractive index (dRI) detection to estimate the molecular size distribution. OAg samples were run on a TSK gel G3000 PWXL column (30 cm x 7.8 mm; particle size 7 μm; cod. 808021) with TSK gel PWXL guard column (4.0 cm x 6.0 mm; particle size 12 μm; cod.808033) (Tosoh Bioscience). The mobile phase was 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% CH_3CN, pH 7.2 at the flow rate of 0.5 mL/min (isocratic method for 35 min). OAg peak molecular mass (MP) was calculated using dextrans as standards in the range 12 - 150 kDa. Sugar content was estimated by High-performance Anion-exchange Chromatography with Pulsed Amperometric Detection (HPAEC–PAD) analysis [201]. Nuclear Magnetic Resonance (NMR) spectroscopy was used to confirm PS identity and purity. All NMR experiments were performed with a Bruker Advance 800 MHz spectrometer equipped with a high-precision temperature controller using a 5 mm QCI CryoProbe. Spectra were weighted with 0.8 Hz line broadening and Fourier-transformed. NMR spectra were recorded at 50.0 ± 0.1 °C. The transmitter was set at the water frequency (4.70 ppm). Suppression of the water signal was achieved by excitation sculpting (2 ms selective square pulse). Proton spectra were acquired using a 90-degree pulse duration automatically calculated and collected with 32K data points over a 12 ppm spectral width,

accumulating 128 number of scans. Spectra were processed by applying an exponential function to the FID with a line broadening of 0.80 Hz to increase the signal-to-noise ratio and then Fourier transformed. Data acquisition and processing were performed with TopSpin 3.5 software package (Bruker BioSpin).

3.5 K-antigens purification and characterization

K2 was extracted directly from Kp strain NCTC11228 by acetic acid hydrolysis. It was then purified through precipitation with CTAB [202]. K2 was characterized by HPLC–SEC with dRI detection to estimate the molecular size distribution, using a TSK gel G3000 PWXL column (30 cm x 7.8 mm; particle size 7 µm; cod. 808021) with TSK gel PWXL guard column (4.0 cm x 6.0 mm; particle size 12 µm; cod.808033) (Tosoh Bioscience). K2 peak molecular mass (MP) was calculated using dextrans as standards in the range 50 - 410 kDa. DNA and protein impurities were determined measuring absorbance at 260 nm and through microBCA, respectively. Placing K2 in an ice bath, 30 cycles of sonication (VibraCell, Sonics and Materials Inc.) were performed to reduce its size (resulting PS is indicated as sK2). The sonication cycle consisted of 30'' of pulses followed by 30'' of rest. NMR spectroscopy was used to confirm PS identity and purity, as for OAg characterization.

3.6 Synthesis and purification of selective MrkA glycoconjugates

OAg and KAg were conjugated to Lac-MrkA using the following selective approach. PS were activated with CDAP and subsequently derivatized with adipic acid dihydrazide (ADH). Briefly, OAg (at 5 mg/mL in water) or K2 (2 mg/mL in water) was placed in an ice bath and the solution brought to pH 9. Reaction with CDAP (w/w ratio CDAP/PS of 0.2 for OAg and of 1 for K2) was performed at 0 °C for 15 min under stirring. After 15 min, 0.25 M ADH was added and reaction mixture was kept in agitation for 2 h at room temperature. Derivatized sugars were purified with PD10 Desalting column prepacked with Sephadex G-25 Superfine (GE Healthcare) to remove excess of free ADH. Conjugation reaction was performed via reductive amination between randomly ADH-derivatized Pss and oxidized lactose moiety on MrkA. After protein concentration (15-20 mg/mL) and buffer exchange in 100 mM Acetate pH 4.5, Lac-MrkA was oxidized with 5 mM sodium periodate ($NaIO_4$) for 30' at 25 °C. After that, reaction was quenched with 10 mM Na_2SO_3 for 15' at room temperature on a rotating wheel. Oxidized

protein was added to lyophilized PS-ADH (2:1 w/w protein to PS for K2, 1:1 w/w for OAg), $NaBH_3CN$ (5 molar excess respect to reactive aldehydes) was added for imine reduction and left ON at room temperature on a rotating wheel. Conjugation mixtures were purified through a first step of Size Exclusion Chromatography with HiPrep 16/60 Sephacryl S100 HR column (Cytiva), for OAg conjugates, or HiPrep 16/60 Sephacryl S300 HR column (Cytiva), for K2 conjugates, to remove unconjugated protein. Isocratic Elution at 0.5 mL/min in PBS 1x was used. Pooled SEC fractions containing the conjugate were then loaded onto a HisTrap Fast Flow 1 mL column (Cytiva) for the affinity purification of the conjugate taking advantage of the HisTag present on the protein, for the removal of the eventual free saccharide. The column was equilibrated and washed after sample application with 20 mM NaH_2PO_4, 500 mM NaCl pH 7.4, conjugate was then eluted with 500 mM of imidazole. Imidazole was removed using Amicon centrifugal filters (cut-off 30 kDa) and exchanged again in PBS 1X. Flow-through and elution pools were characterized by microBCA to verify presence of the conjugate in the eluates.

3.7 Synthesis and purification of traditional random glycoconjugates

For random chemistry, sugar chains were randomly activated with CDAP at pH 9, as previously described, and directly conjugated to amino groups of Lys residues of proteins, MrkA or CRM_{197}, the latter kindly provided by GSK. Proteins were exchanged in saline and concentrated to 20-25 mg/mL prior conjugation reaction with CDAP-activated PS. Conjugation was conducted at room temperature for 2 h under stirring, with PS:protein w/w ratio of 1:1. Reaction was then quenched with 0.5 M glycine pH 7.2 and kept in agitation ON at 4 °C. Conjugate purification was performed with Sephacryl HR 16/60 S100 column (Cytiva) for O1v1-MrkA conjugate, while sK2-MrkA conjugate was purified following the same strategy used for the selective one. sK2-CRM_{197} was instead purified with Sephacryl HR 16/60 S1000 column. Isocratic Elution at 0.5 mL/min in PBS 1x was used.

3.8 Glycoconjugates characterization

Intermediates of conjugation were characterized as previously described [203]. Purified conjugates were characterized by HPAEC–PAD analysis for sugar quantification and by micro BCA for protein quantification, using BSA as a reference following the manufacturer's instructions (Thermo Fisher Scientific); the ratio of saccharide to protein was then calculated. HPLC–SEC was used to verify conjugates formation and detect the presence of unreacted

protein [203]. OAg glycoconjugates were run on a TSK gel G3000 PW XL column (Tosoh Bioscience), while KAg glycoconjugates on TSKgel G6000PW XL and G5000PW XL columns (Tosoh Bioscience) connected in series. SDS-PAGE/western blot analyses were performed on SEC fractions and on final purified OAg conjugates. In brief, samples were first mixed with LDS Sample Buffer supplemented with reducing reagent (Thermo Fisher Scientific) and heated at 100 °C for 3 min. Heated samples were then resolved by a 4%–12% Bis-Tris SDS-PAGE gel (Thermo Fisher Scientific). For SDS-PAGE gel staining, the gel was stained with ProBlue Safe Stain (Giotto Biotech) for 2 h at room temperature with agitation, followed by three washes with deionized water to remove excess dye. The stained gel was then imaged by a CCD camera under white light (Chemidoc Imaging System, Bio-Rad). For Western blot, the resolved proteins were transferred from the SDS-PAGE gel onto a PVDF membrane using the iBlot (Thermo Fisher Scientific) according to the manufacturer's instructions. The membrane was then blocked in 10% non-fat milk (diluted in 1X PBS 0.05% TWEEN 20) for 1 h at room temperature and sequentially incubated with primary antibodies and HRP-conjugated secondary antibodies, both for 1 h at room temperature. Following extensive washes with 1X PBS 0.05% TWEEN 20, the membrane was incubated with luminol-based chemiluminescent substrate (Thermo Fisher Scientific) and the immuno-bands were captured by a CCD camera (Chemidoc Imaging System, Bio-Rad). The following primary antibodies were used: mouse anti-His (1:2000, Qiagen), mouse anti-O1v1 mAb (KPE33, 1:2000), mouse anti-O2a mAb (KPN42, 1:2000) and mouse anti-MrkA mAb (KP3, 1:2000). As secondary antibody, rabbit anti-mouse IgG-HRP was used (1:5000). All antibodies were diluted in 3% non-fat milk 1X PBS 0.05% TWEEN 20.

3.9 Immunogenicity studies in mice

Animal studies were performed at GSK Animal Resources Centre in Siena under the animal project 479/2017-PR 09/06/2017 approved by the Italian Ministry of Health. All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK policy on the Care, Welfare and Treatment of Animals. In the first study with the two alternative MrkA constructs, 10 female CD1 mice (4-6 weeks old) were immunized subcutaneously (SC) with 10 μg of protein formulated in AS03 at day 0, 21 and 35. Sera were collected at days -1, 27, and 42. In the study with MrkA conjugates, 10 female CD1 mice (4-6 weeks old) were SC immunized at day 0 and 28 at 5 μg MrkA dose formulated in Alhydrogel

(Aluminium hydroxide at 0.7 mg/mL Al^{3+}). Sera were collected at days 27 and 42. Individual mouse sera were tested for anti-MrkA, anti-O1v1 and anti-K2 total IgG by enzyme-linked immunosorbent assay (ELISA) as previously described [204]. MrkA at a concentration of 15 μg/mL, O1v1 at 0.6 μg/mL and K2 at 15 μg/mL in PBS pH 7.4 were used as coating antigens. Results are reported as anti-antigen specific IgG EU/mL: one ELISA unit is defined as the reciprocal of the standard serum dilution that gives an absorbance value equal to 1. Bars in the graph represent geometric mean titers and each dot results from each single animal.

3.10 Flow cytometry

Kp strain NCTC11679 (K61:O3) was grown ON in LB medium at 37 °C and 5% CO_2. Bacteria were pelleted at 4000 × *g* for 5 min and washed with PBS. Bacteria were then blocked with PBS + 3% (w/V) BSA for 20 min and incubated with pooled mice or rabbit sera diluted 1:10,000 in PBS + 1% (w/V) BSA for one hour. After washes, samples were incubated with FITC anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA) diluted in PBS + 0.1% (w/V) BSA for 45 min at 4°C. Finally, bacteria were fixed with 4% (w/V) formaldehyde for 20 min. Flow cytometry analyses were performed on a FACS Canto II flow cytometer (BD Biosciences). Results are reported as overlaid histograms with the relative fluorescence intensity on the X axis and the percentage of the maximum number of events on the Y axis.

3.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7. The non-parametric Mann-Whitney two-tailed test and Kruskal-Wallis analysis with post-hoc Dunn's test were used respectively to compare two or multiple groups. Wilcoxon matched-pairs signed rank two-tailed test was used to compare results from the same group at different time points.

4 RESULTS

4.1 Design and production of a stabilized MrkA monomer

Intramolecular stabilization strategy was applied to stabilize MrkA in a monomeric form. Indeed, neighbouring subunits in the fimbriae interact via donor strand complementation (Figure 4.1). Based on this information, two alternative constructs of stabilized MrkA monomer were designed, namely MrkA version A (vA) and MrkA version B (vB). A 6xHistidine tag was added at the N-term to make easy protein purification through IMAC.

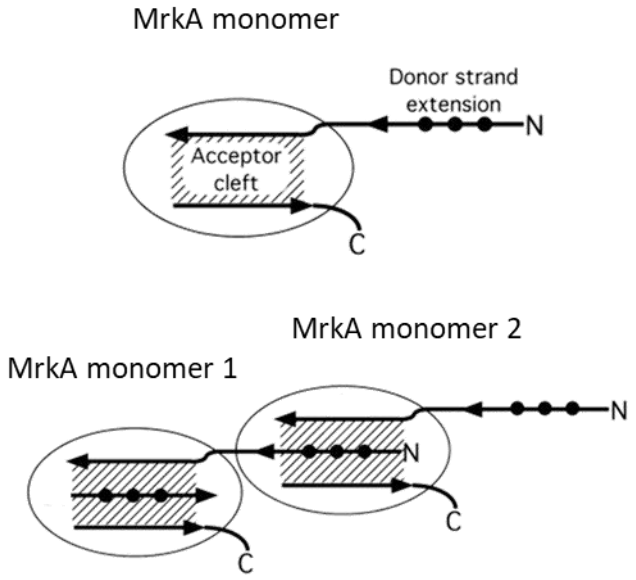
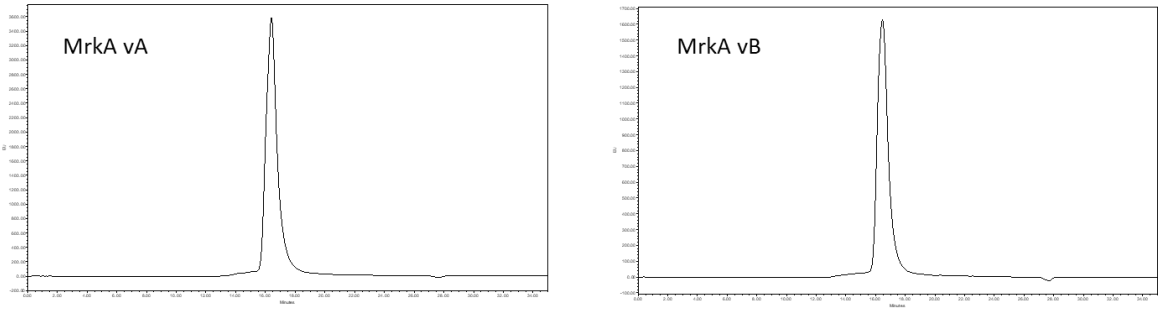


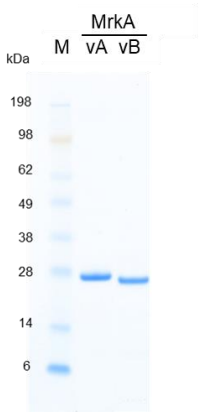
Figure 4.1. Schematic representation of fimbrial filament assembly. Type 3 fimbriae are assembled through the donor strand complementation mechanism, meaning that N-term donor strand extension of one MrkA subunit interacts with the acceptor cleft of a second MrkA subunit.

MrkA vA and vB were recombinantly expressed in *E. coli*, growing bacteria in yeast extract medium (HTMC) and inducing protein expression over night at 25 °C. Both proteins were obtained with a good yield (~ 60 mg/L) and purity, as shown by HPLC-SEC (Figure 4.2A) and SDS-PAGE (Figure 4.2B) analyses. Proteins thermal stability was relatively good (Tm close to 90 °C). Also, both constructs resulted to be stable after 5 cycles of freeze-thaw from -20 to RT, as no variation in Tm occurred (Figure 4.2C).

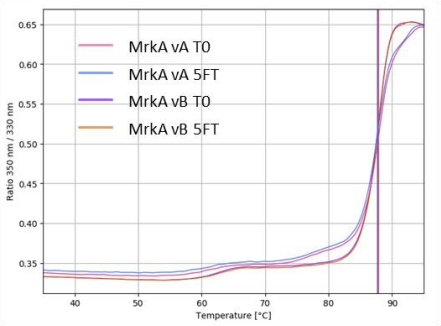
A



B



C



Sample	Tm (°C)	
	T0	Post 5 FT
MrkA vA	87.9	87.6
MrkA vB	87.7	87.7

Figure 4.2. Characterization of purified MrkA version A and version B. **A**) HPLC-SEC fluorescence emission profiles. **B**) SDS-PAGE showing proteins bands at the expected MW (~ 23 kDa for MrkA vA and ~ 21 kDa for MrkA vB). **C**) Thermal unfolding curves at T0 and after 5 cycles of FT by nano-Differential Scanning Fluorimetry (nano-DSF).

4.2 Testing the two different MrkA monomers in mice

MrkA vA and MrkA vB were tested in a first study in mice to investigate MrkA monomer antigenicity and also to understand if the two constructs could induce a different immune response. Mice were immunized subcutaneously three times (at day 0, 21, 35) with 10 µg of MrkA vA or vB in presence of AS03, an Adjuvant System (AS) containing α-tocopherol and squalene in an oil-in-water emulsion. MrkA vA induced a better immune response compared to MrkA vB, eliciting significantly higher anti-MrkA IgG titers with respect to MrkA vB already after 2 injections (Figure 4.3). For this reason, MrkA vA was chosen as carrier for the generation of selective glycoconjugates exploiting protein cytoplasmic glycosylation.

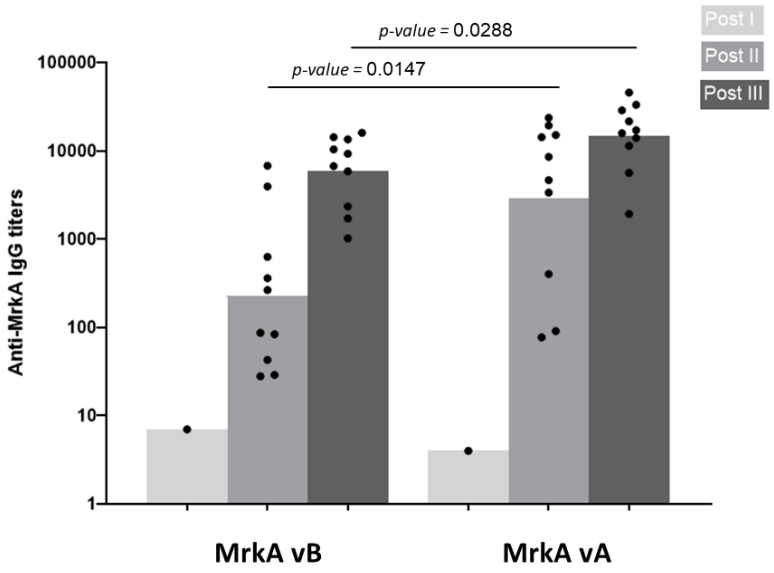


Figure 4.3. Immunogenicity in mice of MrkA vA and MrkA vB. Ten mice per group were subcutaneously immunized at days 0, 21 and 35 with 10 µg of protein adjuvanted with AS03. Bleeds at day 15, 36 and 51. Summary graphs of anti-MrkA specific IgG reporting individual antibody levels (dots) and geometric mean with 95% confidence interval (bars) for post II and post III sera. Sera post I were analyzed in pool. ELISA has been performed using MrkA vB as coating antigen.

4.3 Generation of molecular tools for protein glycoengineering

The biosynthetic pathway for the lactosylation of a model protein, sfGFP, in *E. coli* cytoplasm was first established. This pathway consisted of a first enzyme, ApNGT from *Actinobacillus pleuropneumoniae*, required for protein modification with single β-linked glucose at target asparagine (Asn, N) residue in the N-A-T sequon recognized by this glycosyltransferase. For generating the N-linked lactose, the β1,4-galactosyltransferase LgtB from *N. meningitidis* [205], having a demonstrated substrate promiscuity and active expression in *E. coli*, was used to transfer galactose from the corresponding nucleotide-activates sugar to Glcβ1-Asn handle. These two enzymes were cloned in a pACYCDuet plasmid, each one under the IPTG-inducible control of LacUV5 promoter, a mutated variant of lac promoter (Figure 4.4B). sfGFP expression was instead under the control of the arabinose-inducible promoter in a compatible vector plasmid to allow protein and enzymes co-expression (Figure 4.4A). This dual plasmid expression system should ensure a good balance between substrate protein and glycosyltransferases synthesis.

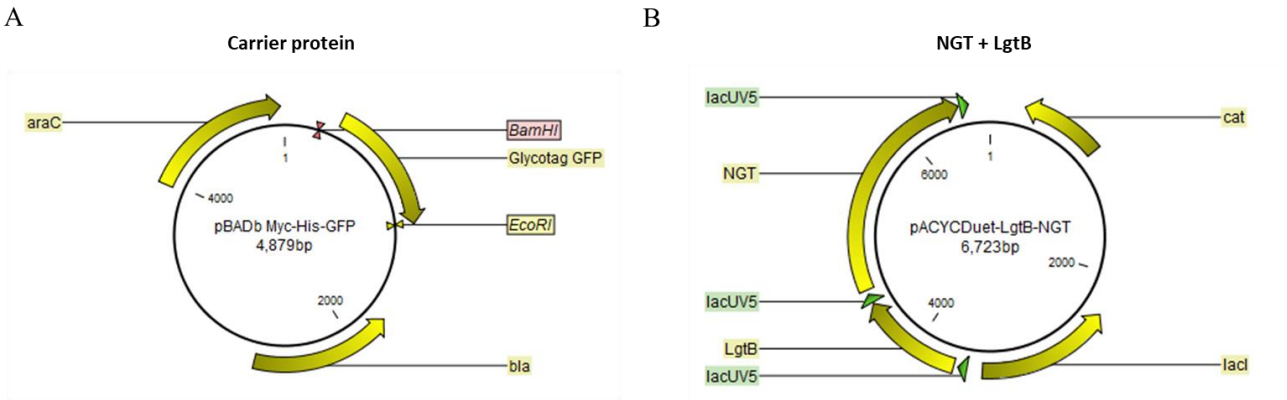


Figure 4.4. Plasmid maps of vectors used for expression of sfGFP (or carrier protein) (**A**) and glycosyltransferases (**B**).

As expression host, an *E. coli* strain widely used for recombinant protein production, that has been also employed by Tytgat and colleagues in their work on cytoplasmic protein glycoengineering [206] was chosen. The *lacZ* gene was deleted from *E. coli* K-12 derivative W3110 genome, in order to prevent lactose catabolism from the encoded β-galactosidase. In figure 4.5, the agarose gel confirming the deletion of the gene is reported. The *E. coli* mutant

strain was transformed with the plasmid encoding the substrate protein sfGFP (with an engineered N-A-T at the C-term) and the one carrying the two glycosyltransferases. Protein and enzymes expression was induced in the presence of Arabinose and IPTG, respectively, and verified by SDS-PAGE analysis on whole cell lysates, as shown in Figure 4.6A. GFP expression was also confirmed by FACS analysis, taking advantage of its intrinsic fluorescence (Figure 4.6B).

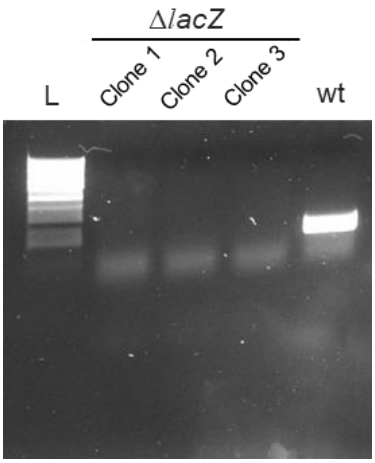


Figure 4.5. Agarose gel of PCR amplicons showing the absence of *lacZ* gene in the three clones of the mutated *E. coli* K12 W3110 strain and its presence instead in the wild-type positive control strain.

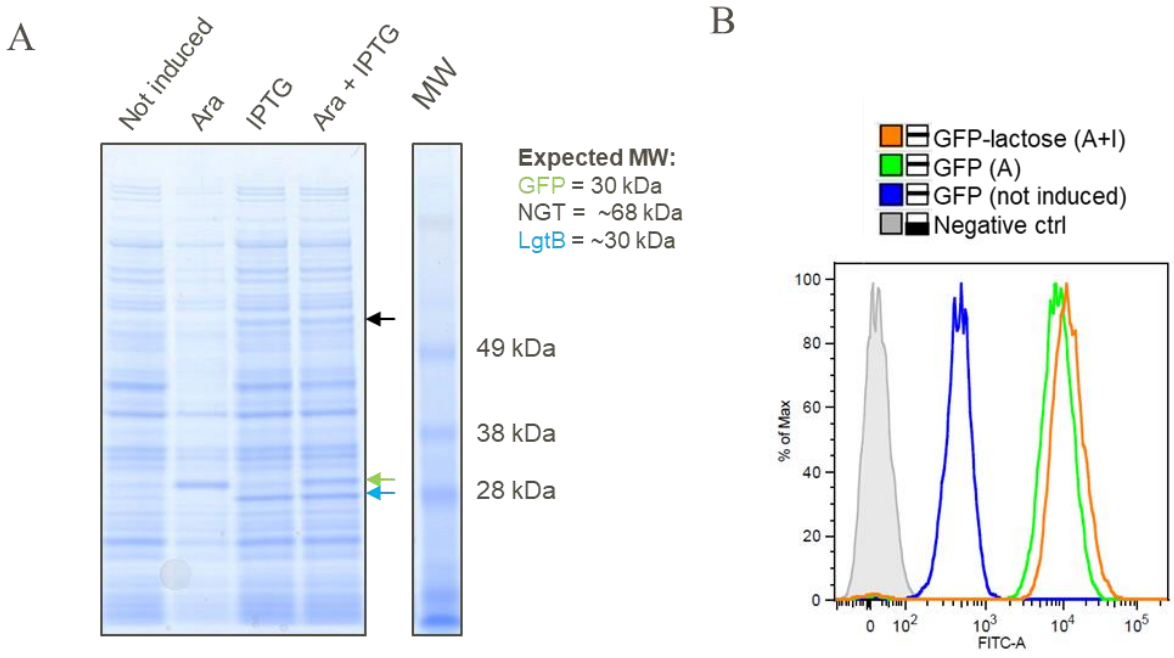


Figure 4.6. sfGFP and sfGFP-lactose expression after overnight induction (ON) with 0.4% arabinose (to induce sfGFP expression) and 1 mM IPTG (to induce lactosylation enzymes): SDS-PAGE analyses on whole cell lysates

(**A**) and FACS on bacteria (**B**). In the SDS-PAGE, each arrow indicates the gel band corresponding to one of three different expressed proteins: green arrow for sfGFP (lane Ara and Ara + IPTG), and black and blue ones for NGT and LgtB, respectively (lane IPTG and Ara + IPTG). Minimal degree of sfGFP expression in absence of the inducer (Arabinose, A) is shown by FACS analysis (the slightly positive signal in blue). Higher expression levels were confirmed after induction (Ara and Ara + IPTG).

After purification by IMAC, intact mass spectrometry confirmed the successful introduction of N-linked lactose onto sfGFP in *E. coli* (Figure 4.7). This same biosynthetic pathway was thus used for MrkA glycoengineering.

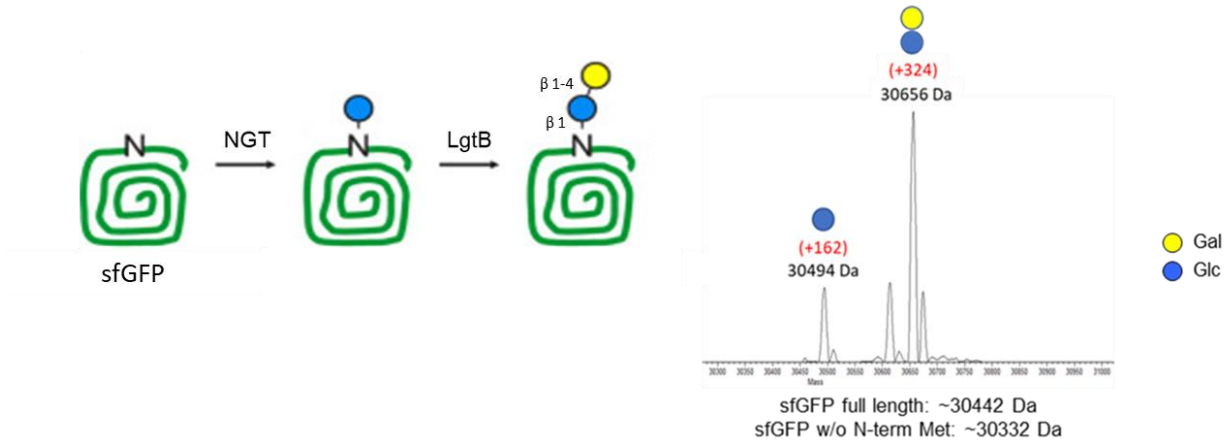


Figure 4.7. MS spectrum of Lac-sfGFP. Two main species are present: the expected one with a 324 Da increase corresponding to the added lactose, and a second one (Δ MW = + 162) related to glucose modification only.

4.4 Expression and characterization of Glyco-MrkA

MrkA vA, with N-A-T sequon inserted at the N-term, was cloned in the designed plasmid for carrier protein expression. The recombinant protein was then co-expressed in the *E. coli* K-12 derivative W3110 Δ*lacZ* strain with ApNGT and LgtB enzymes or with ApNGT only. In fact, a truncated pathway construct was produced in order to follow the sequential MrkA modification and to evaluate which type of sugar handle could better work for the selective conjugation strategy. The resulting His-tagged proteins were purified through affinity chromatography after cell lysis and their concentration determined by microBCA. As for the unmodified MrkA, Glyco-MrkA recombinant proteins were obtained with a good yield (~70 mg/L) and purity as shown by SDS-PAGE and HPLC-SEC analyses (Figure 4.8A and 4.8B). In the protein gel a slight shift of Lac-MrkA band to higher MW can be observed, most likely suggesting modification occurrence. Successful modification with glucose and lactose was indeed confirmed by intact mass analysis, and through HPAEC-PAD analysis it was possible to quantitatively determine the level of each modification introduced *in vivo* (Figure 4.8C).

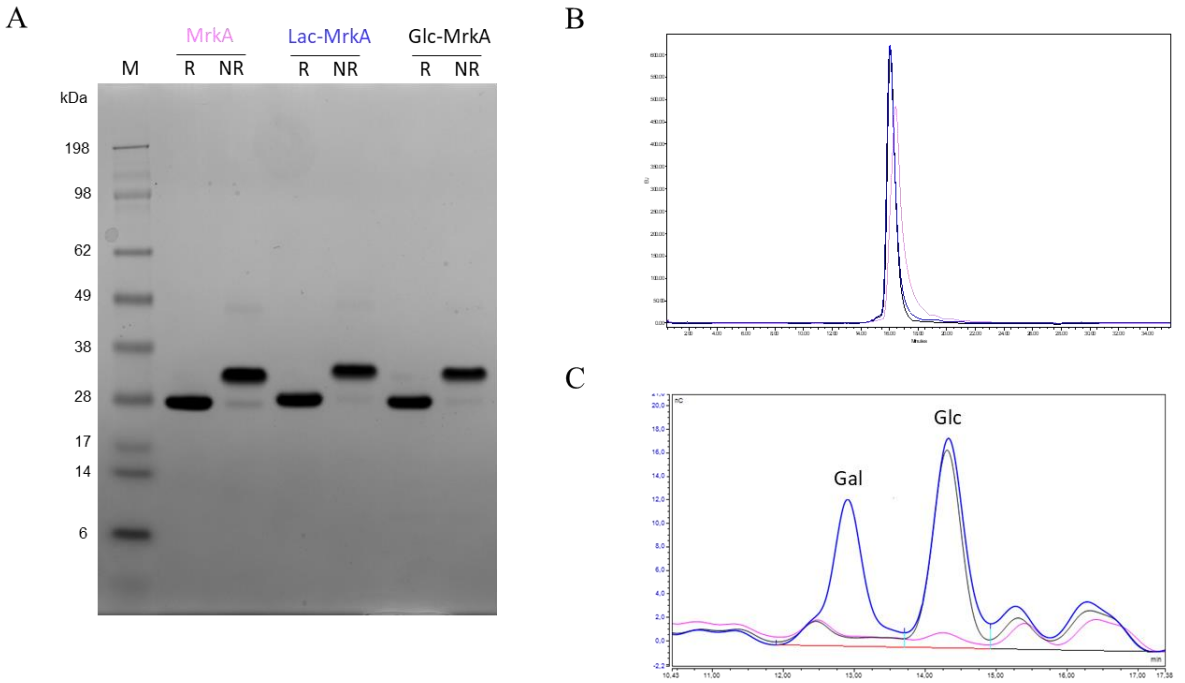


Figure 4.8. Characterization of purified Glyco-MrkA recombinant proteins. **A**) SDS-PAGE analysis of MrkA co-expressed with NGT+LgtB (Lac-MrkA) or with NGT only (Glc-MrkA), compared to the unmodified protein. Due to the presence of a disulfide bond in MrkA, non reduced (NR) samples bands appear shifted toward higher MW respect to the corresponding ones treated with DTT (R). Lac-MrkA band is also slightly higher respect to that of

MrkA, supporting protein modification evidence. **B**) HPLC-SEC overlaid fluorescence emission profiles of MrkA (pink line), Lac-MrkA (blue line) and Glc-MrkA (black line). **C**) HPAEC-PAD chromatograms showing Galactose (Gal) and Glucose (Glc) content in the three proteins (same color code). Quantification of glucose in Lac- and Glc-MrkA and galactose in Lac-MrkA was performed in relation to a calibration curve containing a mixture of these neutral sugars, after performing the acid hydrolysis of MrkA samples for the release of the above-mentioned monosaccharides.

Lactose occupancy was improved by modifying HTMC medium for the expression of Lac-MrkA recombinant protein. More specifically, 15 g/L of glycerol were substituted with 5 g/L of dextrose as a carbon source. This allowed to improve efficiency of modification and reduce batch-to-batch variability, as shown in Table 4.1. Lactose to protein molar ratio was in fact increased up to a 100%, maintaining 100% of Glc modification in Glc-MrkA.

Table 4.1. Molar percentage of lactose or glucose in MrkA recombinant protein co-expressed with NGT and LgtB in *E. coli* W3110 Δ*lacZ* grown in HTMC or modified HTMC

Batch/Growth medium	***Molar ratio Gal=Lac/protein (%)***	***Molar ratio Glc/protein (%)***
Batch 1/HTMC	***6***	***96***
Batch 2/HTMC	***21***	***100***
Batch 1/Modified HTMC	***70***	***100***
Batch 2/Modified HTMC	***100***	***100***
Batch 3/Modified HTMC	***82***	***98***

However, from peptide mapping analysis it was found that glucose and lactose modification did not occur at the level of the engineered sequon at protein N-terminus (underlined in Figure 4.9), but a sequon naturally present in MrkA primary structure was instead recognized by NGT (Figure 4.9).

MrkA vA AA sequence

MGSHHHHHHHHHHGSSNATGSADTTVGGGQVNFFGKVTDVSCTVSVNGQGSDANVYLSPVTLTE
VKAAAADTYLKPKSFTIDVSNCQAADGTKQDDVSKLGVNWTGGNLLAGATSKQQGYLANTEAS
GAQNIQLVLSTDNATALTNKIIPGDSTQPKAKGDASAVADGARFTYYVGYATSAPTTVTTGVVNSY
ATYEITYQGGGGGGADTTVGGGQVNFFGKVTDVS

Glyco-MrkA	Peptide Sequence	Modification	Site	Delta (ppm)	Confidence Score	RT	Mono Mass Exp.	Mono Mass Theo.
Glc-MrkA	QQGYLANTEASGAQNIQLVLSTDNATALTNKIIPGDSTQPK	N-glucose	N140	-0.91	100	53.8	4432.2207	4432.2247
	QQGYLANTEASGAQNIQLVLSTDNATALTNK	N-glucose	N140	-1.14	100	51.19	3395.6655	3395.6694
	LANTEASGAQNIQLVLSTDNATALTNK	N-glucose	N140	-0.65	99.1	49.63	2941.4475	2941.4494
Lac-MrkA	QQGYLANTEASGAQNIQLVLSTDNATALTNK	N-lactose	N140	0.07	100	47.49	3557.7227	3557.7224
	QDDVSKLGVNWTGGNLLAGATSKQQGYLANTEAS GAQNIQLVLSTDNATALTNKIIPGDSTQPK	N-lactose	N140	0.04	90.9	51.48	6906.4541	6906.4538
	QDDVSKLGVNWTGGNLLAGATSKQQGYLANTEAS GAQNIQLVLSTDNATALTNKIIPGDSTQPK	N-lactose, Na+	N140	-3.2	84.3	51.49	6928.4136	6928.4358

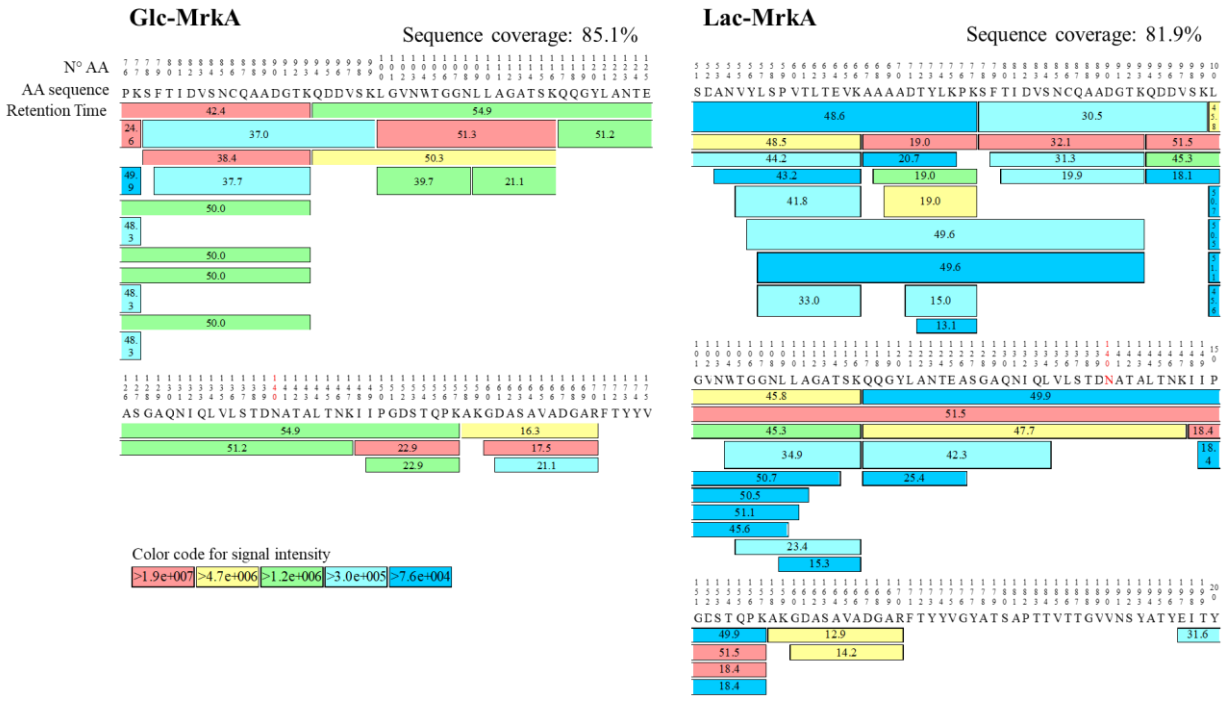


Figure 4.9. LC-MS/MS analysis of Glc-MrkA and Lac-MrkA tryptic digests revealing the actual N residue modified by NGT: Asn140, highlighted in red in MrkA vA AA sequence.

4.5 Preliminary tests for MrkA glycoforms oxidation

Preliminary tests were performed in order to select the best condition for glucose or lactose oxidation, as this step is required for the formation of adelhyde groups involved in the subsequent conjugation reaction *via* reductive amination. Glc-MrkA and Lac-MrkA were oxidized at two different $NaIO_4$ concentration: 2 mM and 5 mM. Prior oxidation, proteins were exchanged in 100 mM acetate buffer pH 4.5 and concentrated to approximately 12 mg/mL; after 30 minutes at 25 °C, the reaction was quenched with Na_2SO_3. Oxidized glyco-proteins were then analyzed through anion exchange chromatography in order to determine sugars oxidation level. At higher $NaIO_4$ concentration, the oxidation level increased (Table 4.2). Interestingly, galactose seemed to be more susceptible to oxidation with respect to glucose, probably being more solvent-exposed as part of a disaccharide unit. Thus, the lactose glycoform was chosen for the development of the selective conjugation chemistry, since with approximately 50% of sugar oxidation in Glc-MrkA, only half of MrkA molecules wouldbe actually available for the conjugation.

Table 4.2. Oxidation percentages of glucose (in Glc- and Lac-MrkA) and galactose (in Lac-MrkA) from oxidation tests with $NaIO_4$

Glyco-protein	**2 mM $NaIO_4$**		**5 mM $NaIO_4$**	
	Glc ox (%)	**Gal ox (%)**	**Glc ox (%)**	**Gal ox (%)**
Glc-MrkA	*41*	-	*58*	-
Lac-MrkA	*0*	*83*	*8*	*96*

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4.6 Characterization of purified Kp O- and K-antigens

O1v1 and O2a polygalactans were selected as model subcapsular PSs to test the selective conjugation strategy on OAg of differente size. The two OAg share the same [→3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→] disaccharide repeat structure (Galactan I), with O1v1 presenting an additional capping [→3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→] repeat unit (Galactan II) [207]. Together with O3 and O5 polymannans, O1 and O2 are responsible for approximately 80% of all Kp infections [208].

After O1v1 and O2a extraction by acetic acid hydrolysis [199], which cleaves the linkage between the terminal KDO of the core region and the lipid A, thus causing the release of the OAg chains (attached to the core region), SEC was performed in order to separate the OAg from core oligosaccharides with no OAg repeats and other impurities (Figure 4.10). Fractions containing OAg were pooled and further characterized. By HPLC-SEC analysis, using a dextrans calibration curve, a MM of approximately 20 kDa for O1v1 and 10 kDa for O2a was determined (Figure 4.11A and 4.11C). O1v1 and O2a OAg structures were confirmed by ^{1}H NMR spectroscopy (Figure 4.11B). By ^{1}H-NMR spectrum an equal number of cap and backbone repeating units in O1v1 OAg was estimated. The average number of repeating units was calculated by HPAEC-PAD analysis, through the quantification of Glc present in the core structure (Figure 4.11C). Protein impurities, quantified through microBCA, were < 1% and < 3% in O1v1 and O2a, respectively. The characterization summary of the two purified OAg, available in good amounts (~ 40 mg of O1v1 and ~ 30 mg of O2av1 obtained from 700-mL bacteria growths), is reported in Figure 4.11.

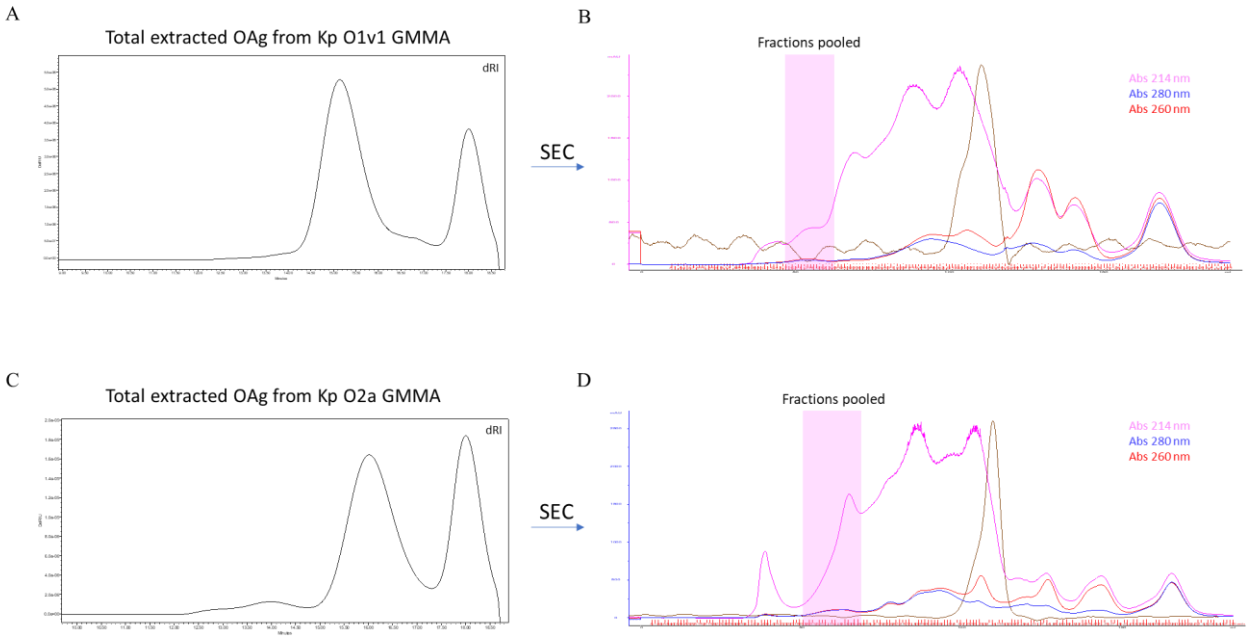
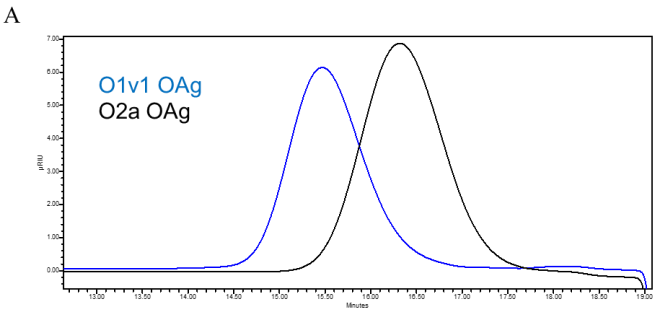
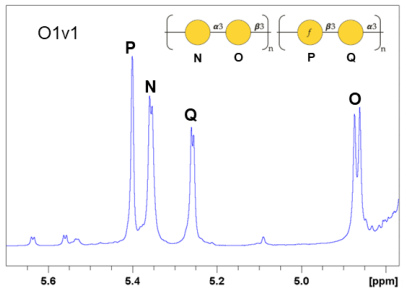
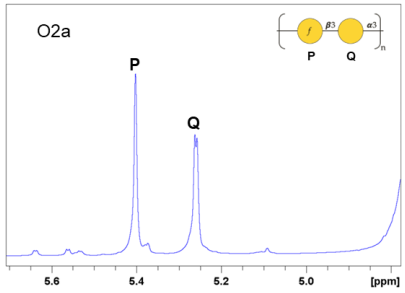


Figure 4.10. Purification of O1v1 and O2a OAg extracted from GMMA. **A** and **C**) HPLC-SEC dRI profile of total extracted OAg. **C** and **D**) S100 purification profile of extracted OAg. Pooled fractions containing the purified OAg are highlighted in pink.







C

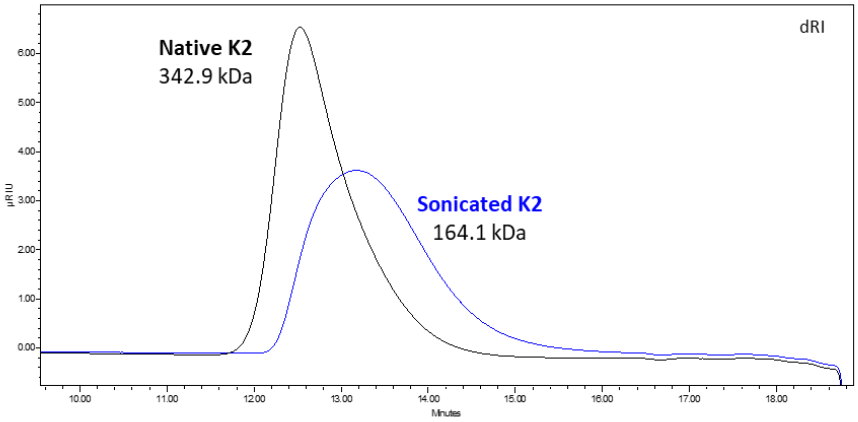
Sample	OAg conc. (µg/mL Gal)	% soluble proteins (on Gal content)	Molecular Mass (kDa)	Average number RU
O1v1	3016.1	0.91	22.8	45
O2a	1651.4	2.8	10.7	26

Figure 4.11. Characterization of purified MMM O1v1 and O2a OAg. **A**) HPLC-SEC dRI profiles. **B**) ^{1}H NMR spectra of OAg anomeric region. **C**) Summary table reporting OAg (Gal) concentration, average number of repeating units per OAg chain, size (MM, kDa) and % of residual soluble proteins.

As model capsular polysaccharide K2 KAg was selected, being often found in hv-Kp strains and known to be among the most prevalent serotypes responsible for neonatal sepsis in LMICs [117,164].

K2 was extracted through acid hydrolysis directly from bacterial culture and isolated through precipitation with cetyltrimethylammonium bromide (CTAB) [202]. Purified K2 was characterized by < 1% proteins and DNA impurities. From HPLC-SEC analysis, K2 resulted to have a MM of ~ 340 kDa. As for the OAg, in order to test the selective conjugation approach on capsular polysaccharides of different size, native K2 (nK2) was slightly fragmented to obtain a batch of shorter KAg. Through ultrasonication its MM was reduced to ~ 160 kDa, as shown by HPLC-SEC dRI profiles in Figure 4.12A. Diffusion-Ordered NMR Spectroscopy (DOSY) of sonicated K2 (sK2) confirmed (Figure 4.12B) the expected polysaccharide structure as reported in literature [209]. The ^{1}H-NMR spectrum also showed the presence of an O-acetyl substituent in the K2 repeating unit (signal at 2.15 ppm, Figure 4.12B). This substitution was estimated to be 95%.

A



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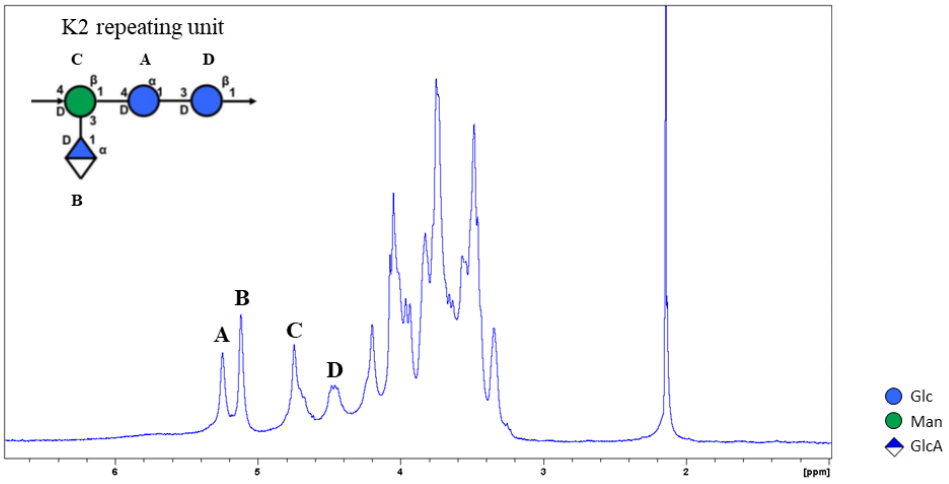


Figure 4.12. Characterization of purified K2 polysaccharide. **A**) HPLC-SEC profiles of native and sonicated K2 KAg detected by refractive index. **B**) ^{1}H monodimensional DOSY NMR spectrum (400 MHz Bruker) of sonicated K2 with the assigned anomeric signals and the O-acetyl group at 2.15 ppm.

4.7 Exploiting Lac-MrkA for the generation of site-selective glycoconjugates

Lac-MrkA was used to develop a selective conjugation approach aiming at preserving MrkA antigenicity. The unique sugar anchor present on the recombinant MrkA monomer has been indeed exploited as specific target for the site-selective conjugation of Kp PSs, both OAg and KAg. Purified PSs were randomly activated with a cyanilating agent (CDAP), derivatized with the aldehydes-reactive homobifunctional linker ADH and then conjugated via reductive amination to the oxidized lactose on MrkA monomer, as schematized in Figure 4.13. Similar activation percentages per repeating unit were obtained for O1v1 and O2a OAg, and for native and sonicated K2 Kag (Figure 4.14B). For the OAg, a w/w protein to PS ratio of 1:1 was used, while for KAg of 2:1. These conditions were selected after preliminary tests to reduce the percentage of unconjugated protein, as determined by HPLC-SEC analysis of the reaction mixtures.

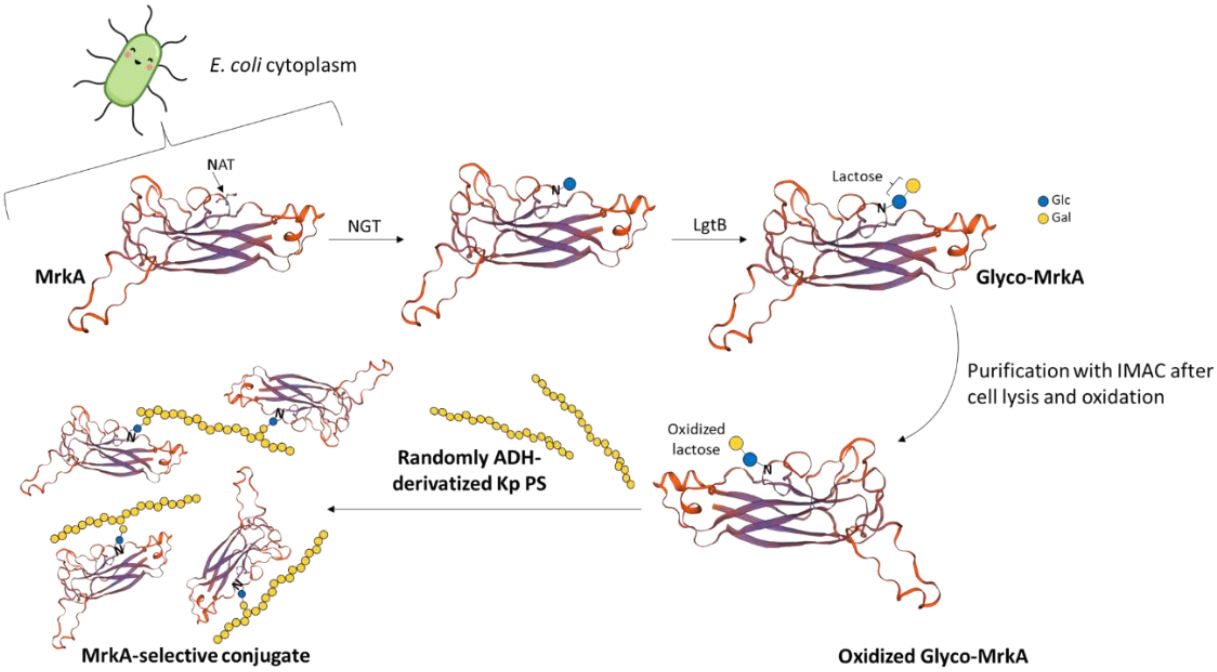
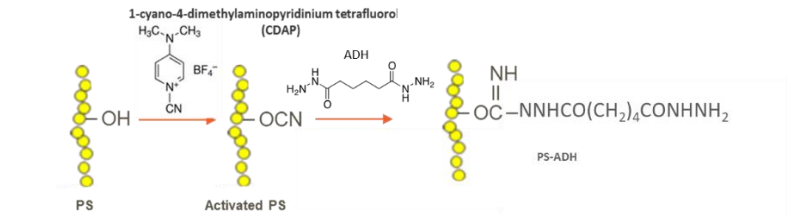


Figure 4.13. Engineered glycosylation pathway of MrkA in the *E. coli* cytoplasm and selective conjugation of Kp PS on oxidized Glyco-MrkA via reductive amination chemistry.

A



B

PS	Activation % per RU
O1v1	9.4
O2av1	11.1
Native K2	37
Sonicated K2	34

Figure 4.14. Polysaccharides derivatization reaction. **A**) Polysaccharide hydroxyls groups are activated with CDAP leading to the formation of a cyanoester intermediate for the subsequent reaction with ADH. **B**) % activation per repeating unit of O- and K-Ag.

4.7.1 O-antigens conjugates

The resulting OAg conjugates were purified through size exclusion chromatography (Figure 4.15A and 4.15B), to remove unconjugated protein, followed by a second purification step with IMAC, to isolate conjugate population from free saccharide. Western Blot analysis of SEC fractions showed the formation of conjugates with MM distribution reflecting the number of MrkA monomers covalently attached along the OAg chain (Figure 4.15C and 4.15D).

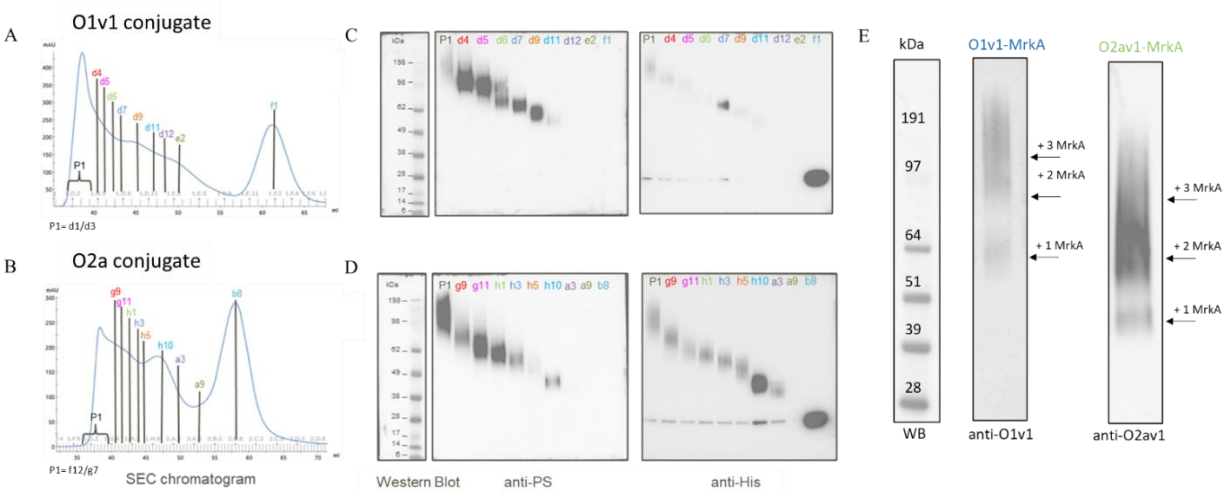


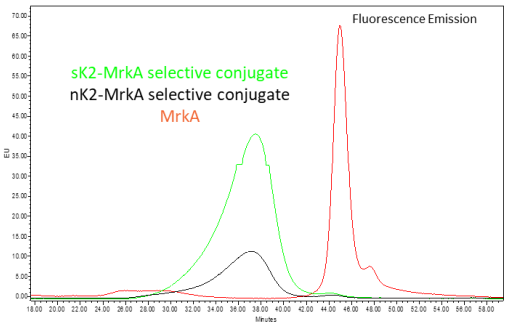
Figure 4.15. Purification and characterization of O1v1- and O2a-MrkA selective conjugates. **A** and **B**) S100 purification profiles. **C** and **D**) WB analyses anti-PS and anti-His of S100 fractions. **E**) WB anti-PS of final purified conjugates.

Conjugates were entirely recovered in the elution pools of the second purification step with HisTrap column. With HPAEC-PAD, combined with a protein quantification method (microBCA), the molar ratio between MrkA and OAg was determined. An average of 3 MrkA molecules were linked to O1v1 OAg, as highlighted also by WB analysis of final purified conjugate, while for O2a-MrkA an average close to 1 was calculated, even if the conjugate smear appeared more intense at the MW corresponding to 2 linked MrkA molecules in the WB (Figure 4.15E).

4.7.2 K-antigens conjugates

K2 selective conjugates were purified following the same strategy used for OAg conjugates, thus performing first a size exclusion chromatography with S300, more suitable for separation of higher MM compounds compared to S100 used for OAg conjugates, and then a HisTrap purification. K2 conjugates recovery in the elution pool were lower compared to that of OAg conjugates (70% for sK2-MrkA and 50% for nK2-MrkA), probably due to the reduced accessibility of the His-tag to Ni^{2+} ions in a conjugate with a long PS like K2. Characterization of purified K2-MrkA selective conjugates is reported Figure 4.16. In this case, an average number of almost 40 MrkA molecules was estimated to be linked to native K2, while approximately 20 to sonicated KAg.

A



B

Selective conjugates	K2/protein w/w ratio	Protein/PS molar ratio
sK2-MrkA	0.33	21.8
nK2-MrkA	0.41	36.6

Figure 4.16. Characterization of purified K2-MrkA selective conjugates. **A**) HPLC-SEC fluorescence emission profiles of native and sonicated K2-MrkA conjugates compared to unconjugated MrkA. **B**) Summary table reporting the PS to protein w/w ratio and the number of MrkA molecules linked to K2 polysaccharide for each conjugate.

4.8 Conjugation reaction is selective at the level of Lactose handle

To verify if the PS was selectively conjugated to the sugar moiety of the protein, a blank reaction between O1v1-ADH and the unmodified MrkA was performed. MrkA was oxidized in the exact same condition of Lac-MrkA and then mixed with the randomly derivatized PS for the reaction to occur (ON at room temperature). Both HPLC-SEC and WB analyses confirmed the selectivity of the reaction as no conjugate formation was observed with unmodified MrkA (Figure 4.17).

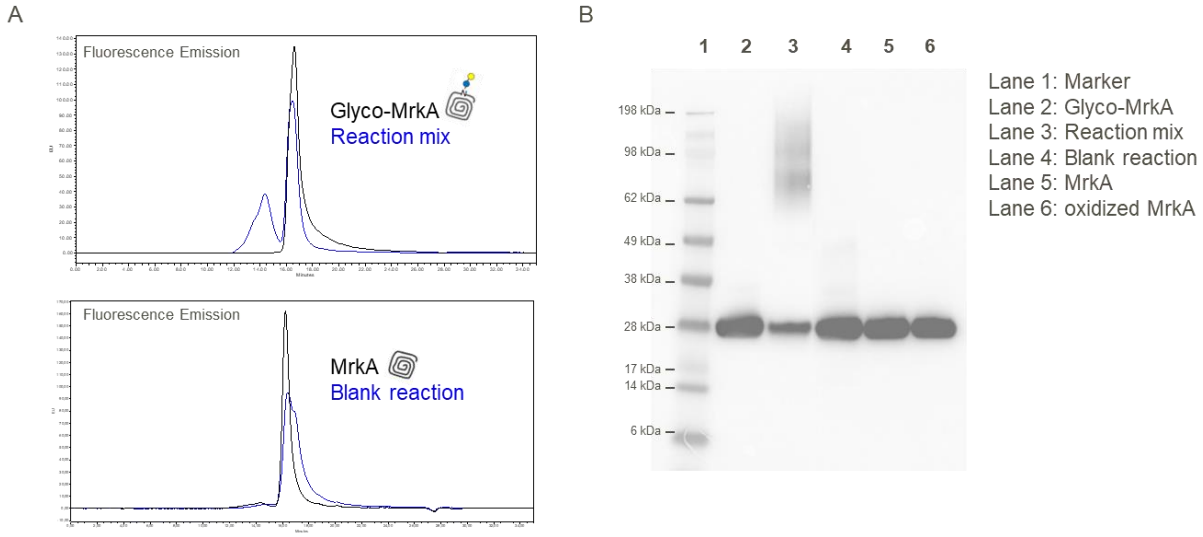


Figure 4.17. HPLC-SEC and SDS-PAGE/WB analyses confirming the selectivity of the reaction: (**A**) absence of a peak at conjugate retention time (RT) in the Blank reaction chromatogram (second plot) and (**B**) no smear visible at higher MW in the anti-MrkA WB (lane 4 vs. lane 3).

4.9 Synthesis of MrkA random conjugates

In order to compare the immunogenicity of these selective glycoconjugates to that of more traditional ones, a random conjugation approach was also used with MrkA. Polysaccharides were randomly activated with CDAP and directly conjugated to primary amino groups on the carrier protein. For the animal study we decided to test the selective MrkA conjugate with O1v1, for the bigger size of the OAg compared to O2a, and the one with the shorter K2, for its better yields post purification and sterile filtration with respect to nK2-MrkA. For these reasons, MrkA random conjugates with O1v1 OAg and sK2 K-antigen were produced. HPLC-SEC analysis showed similar retention times for sK2-MrkA conjugates, regardless of the chemistry (Figure 4.18A). O1-MrkA random conjugate had instead a slightly bigger size with respect to the selective one, as suggested by the lower RT in the HPLC-SEC profile (Figure 4.18B). Higher PS to protein ratios were calculated for the random conjugates: 0.76 for O1v1-MrkA (vs 0.32 for the corresponding selective conjugate) and 0.55 for sK2-MrkA (vs 0.33 for the corresponding selective conjugate). Characterization of all conjugates is reported in table 4.3.

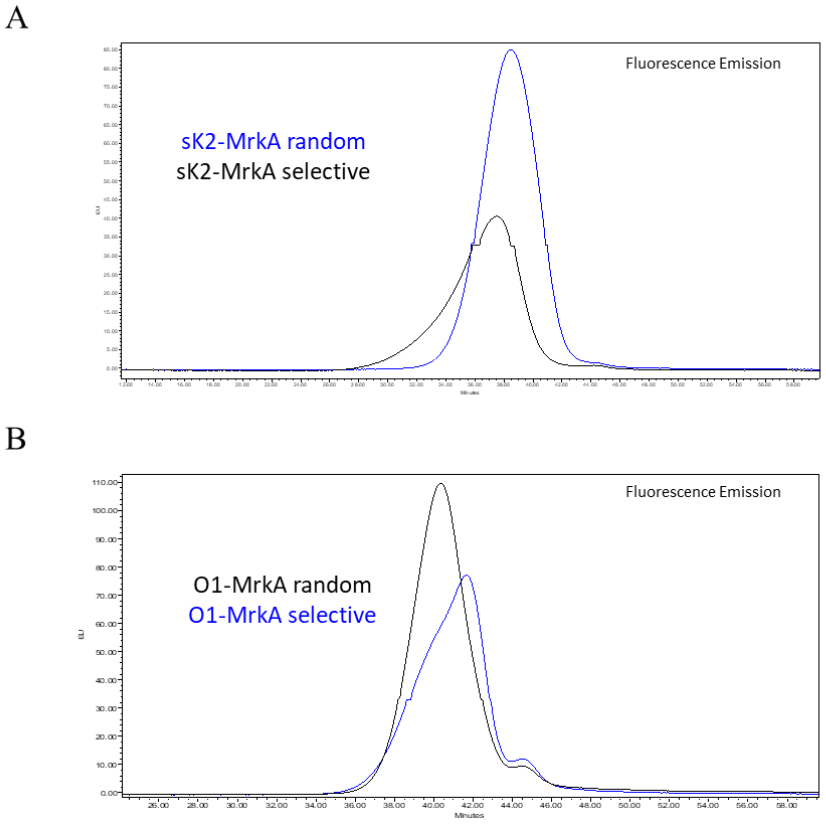


Figure 4.18. HPLC-SEC fluorescence emission profiles of random and selective MrkA conjugates. **A**) Overlaid chromatograms of sK2 conjugates. **B**) Overlaid chromatograms of O1v1 conjugates.

Table 4.3. Polysaccharide to protein w/w ratio of random and selective conjugates prepared for the animal study

Conjugate	PS/protein w/w ratio	
	Random	**Selective**
sK2-MrkA	0.55	0.33
O1-MrkA	0.76	0.32

4.10 Immunogenicity study in mice to evaluate MrkA as carrier for Kp polysaccharide antigens

O1v1 and sK2 selective and random conjugates were then tested in mice to investigate the ability of MrkA to work as antigen and carrier for Kp PSs. The the impact of conjugation chemistry (random vs selective) not only on anti-PS as well as anti-MrkA immune responses induced by the different glycoconjugates. Mice were immunized subcutaneously twice (at day 0 and 28) at the same MrkA dose of 5 µg in presence of Alhydrogel, normalizing also the PS dose of random conjugates to that of the selective ones for subsequent statistical comparison. Alhydrogel is commonly used as adjuvant for glycoconjugate vaccines. To verify if the oxidation step required in the selective conjugation reaction could affect MrkA antigenicity, Lac-MrkA was oxidized and also tested in animals. A CRM_{197} conjugate with sK2 was synthesized to be tested in the study as positive control. As negative control, a physical mixture of sK2 with MrkA was also included in the immunization scheme. All the antigens tested in the study are reported in table 4.4.

Table 4.4. Polysaccharide to protein w/w ratio of random and selective conjugates

Antigen	Protein dose (µg)	PS dose (µg)
MrkA	5	-
Oxidized Lac-MrkA	5	-
O1-MrkA random	5	3.8
O1-MrkA selective	5	1.6
O1-MrkA random	2.1	1.6
sK2-MrkA random	5	2.8
sK2-MrkA selective	5	1.7
sK2-MrkA random	3	1.7
sK2-CRM_{197}	2.4	1.7
MrkA + sK2	5	2.8

Sonicated K2 randomly conjugated to MrkA induced significantly lower anti-K2 IgG response than sK2-CRM_{197} conjugate, but significantly higher compared to the physical mixture of sK2 and MrkA (Figure 4.19A and 4.19B).

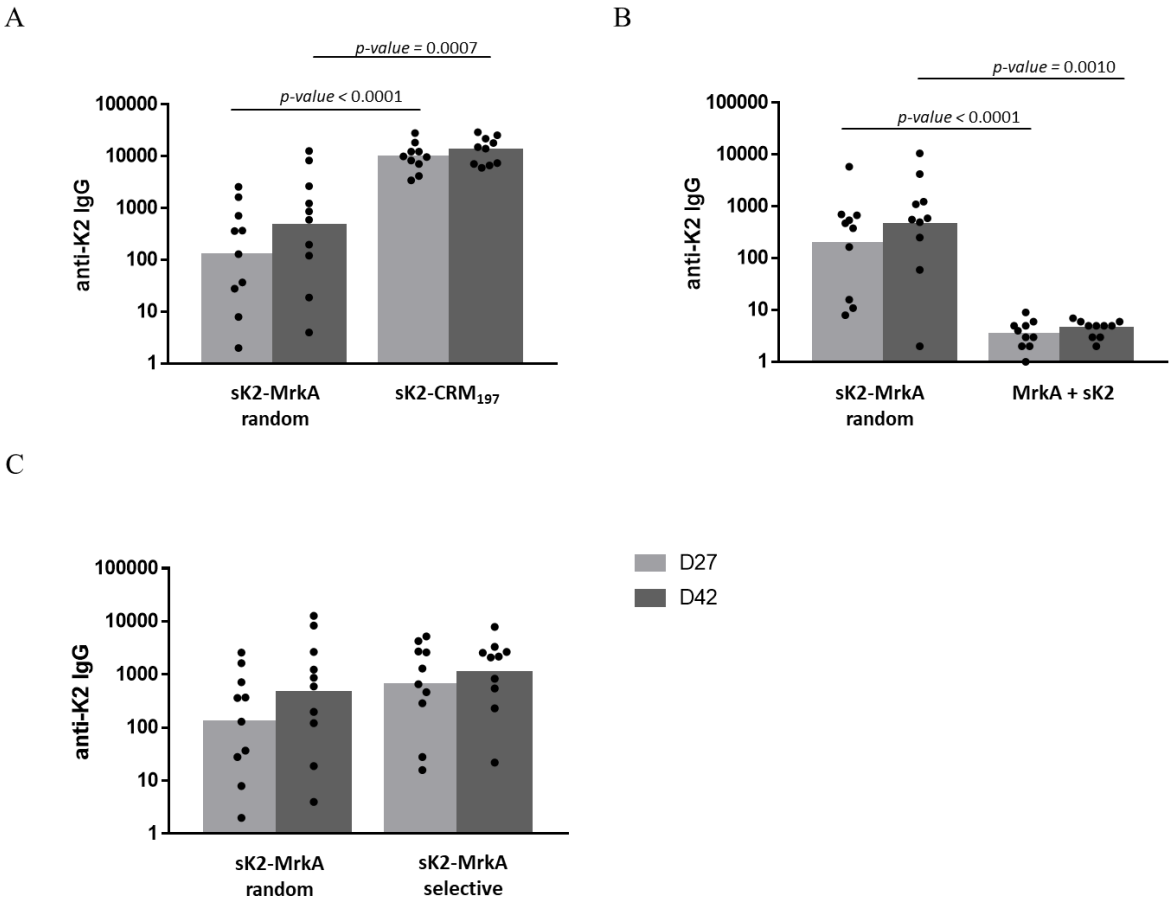


Figure 4.19. Immunogenicity of random K2 conjugates. Ten CD1 mice per group were subcutaneously immunized at days 0 and 28 with 2.8 or 1.7 µg PS dose adjuvanted with Alhydrogel 0.7 mg/mL Al^{3+}. Summary graphs of anti-K2 specific IgG reporting individual antibody levels (dots) and geometric mean with 95% confidence interval (bars) induced by sK2-MrkA conjugate compared to: sK2-CRM197 (**A**), sK2 physically mixed with MrkA (**B**) and to sK2-MrkA selective conjugate (**C**).

Looking at the impact of conjugation chemistry on anti-K2 immune response, no statistically significant differences were observed between the random and the selective sK2-MrkA conjugate (Figure 4.20C). For what concerns instead the OAg conjugates, the O1v1-MrkA random induced significantly higher anti-OAg IgG titers with respect to O1v1-MrkA selective (Figure 4.20).

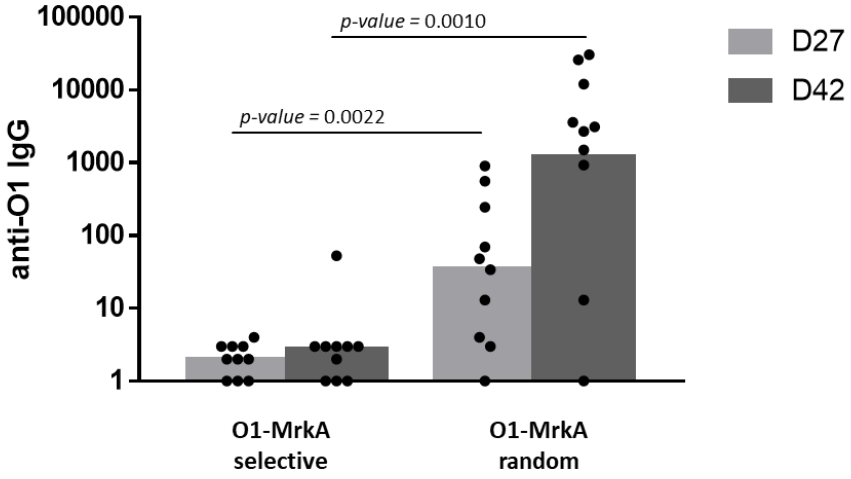


Figure 4.20. Anti-O1v1 immune response induced by random and selective MrkA conjugates. Ten CD1 mice per group were subcutaneously immunized at days 0 and 28 at the same PS dose adjuvanted with Alhydrogel 0.7 mg/mL Al^{3}+. Summary graphs of anti-O1v1 specific IgG reporting individual antibody levels (dots) and geometric mean with 95% confidence interval (bars).

Before proceeding with the analysis of the anti-MrkA IgG response induced by the different MrkA conjugates, it was verified if oxidation of Lac-MrkA had any impact on the anti-protein immune response: no difference was observed by comparing MrkA and oxidized Lac-MrkA at both day 27 and 42 (Figure 4.21). To be noted that many mice were no responders in both groups.

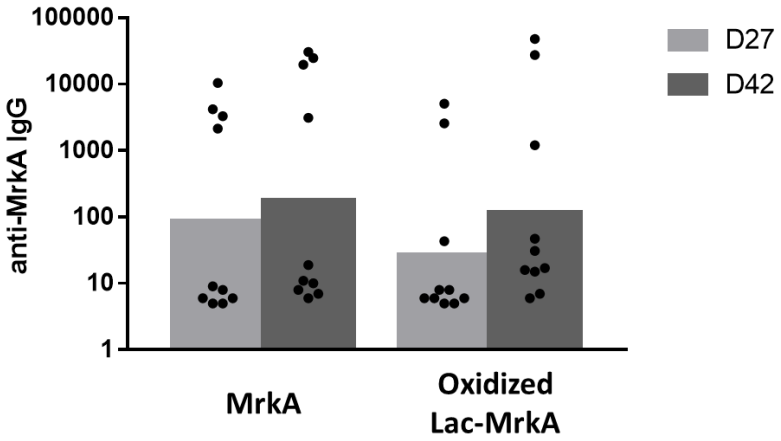


Figure 4.21. Immunogenicity of Lac-MrkA after $NaIO_4$ oxidation compared to recombinant MrkA. Ten CD1 mice per group were subcutaneously immunized at days 0 and 28 with 5 µg of protein adjuvanted with Alhydrogel 0.7 mg/mL Al^{3+}. Summary graph of anti-MrkA specific IgG reporting individual antibody levels (dots) and geometric mean with 95% confidence interval (bars).

Comparing the anti-MrkA IgG response induced by the OAg conjugates (random and selective) and MrkA, no statistically significant differences were observed (Figure 4.22A). However, a higher number of non-responders was found in the selective conjugate group (6) and MrkA alone (5), compared to the random conjugate (1). At day 42, the random conjugate elicited anti-MrkA IgG GeoMean 17 fold higher than MrkA alone, while the GeoMean ratio was of 0.5 for the selective conjugate. With K2, on the contrary, anti-MrkA IgG response increased when the polysaccharide was selectively linked to MrkA molecules, instead of randomly conjugated (Figure 4.22B). At day 27, anti-MrkA IgG response elicited by the selective conjugate was significantly higher than that induced by the random conjugate ($p = 0.0242$). At day 42, the GeoMean of the selective conjugate group was 14-fold higher than MrkA group, while only 1.5-fold higher for the random conjugate. Furthermore, number of non-responders was 5 in the MrkA group, 4 in the random conjugate group and only 1 for the selective conjugate.

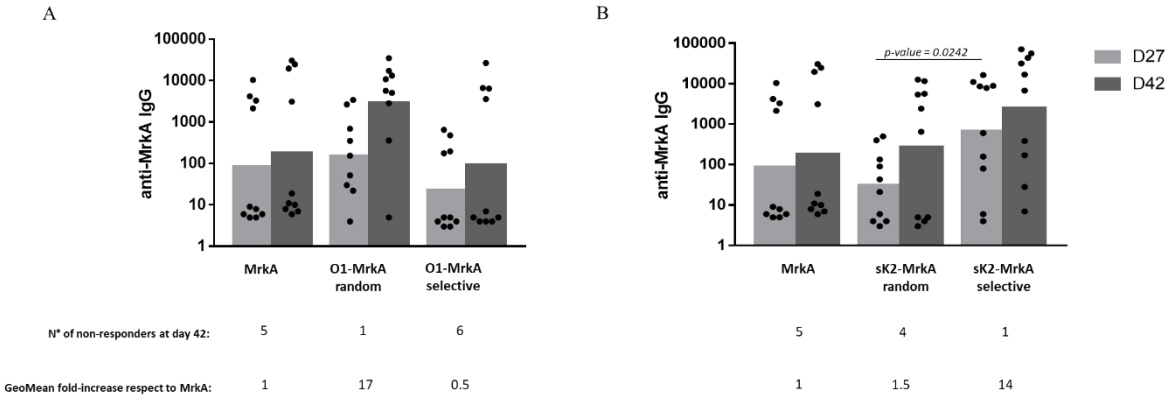


Figure 4.22. Anti-MrkA immune response induced by random and selective MrkA conjugates. Ten CD1 mice per group were subcutaneously immunized at days 0 and 28 at the same protein dose adjuvanted with Alhydrogel 0.7 mg/mL Al^{3+}. Summary graphs of anti-MrkA specific IgG reporting individual antibody levels (dots) and geometric mean with 95% confidence interval (bars) for O1 (**A**) and sK2 (**B**) conjugates. For each group, numbers of non-responders and GeoMean fold-increase respect to MrkA alone at day 42 are also reported.

Anti-MrkA sera were also tested by FACS for their ability to bind MrkA expressed as fimbriae (and not recombinant monomer) on a Kp strain, expressing K61 K-antigen and O3 long OAg. In agreement with anti-MrkA ELISA results, sera from the selective conjugate group showed a higher binding capacity compared to those of random conjugate and protein alone (Figure 4.23).

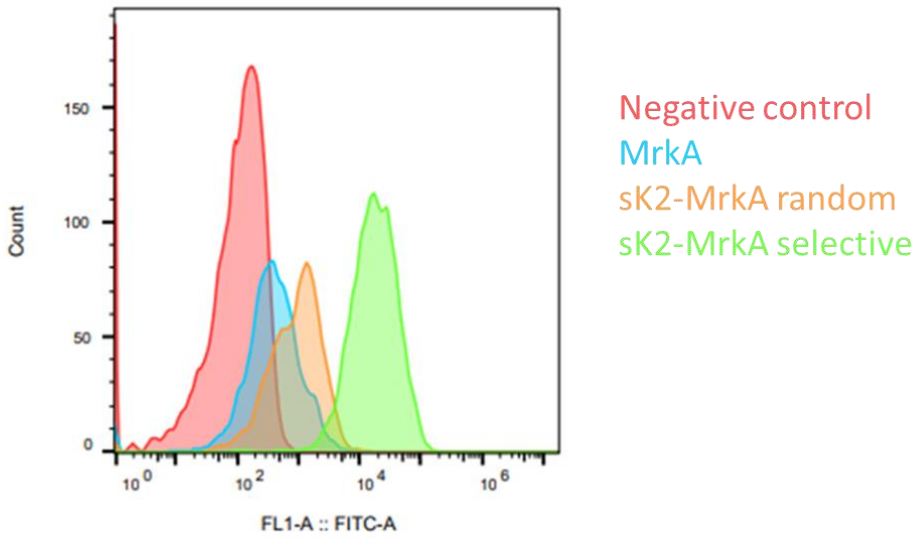
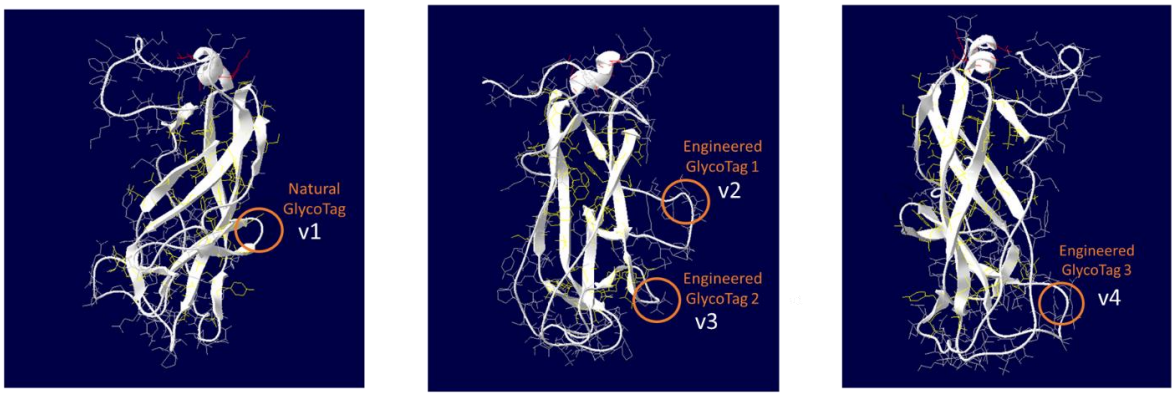


Figure 4.23. FACS histograms of Day 42 pooled sera (1:500 dilution) of MrkA alone, sK2-MrkA random and selective conjugates groups with negative control represented by PBS 1x.

4.11 Design of alternative mono- and multi-sequons MrkA versions

Having discovered through LC-MS/MS that Lac modification occurred at the level of a natural sequon in MrkA and not on the added one at the N-term of the protein sequence, different MrkA versions were designed to better characterize the activity of the glycosyltransferases, also with the future aim to verify if conjugation site can have an impact on conjugate immunogenicity and on the antigenicity of the protein. To reduce the complexity of the system, only NGT activity was studied, knowing that glucose modification was complete and consistent (Table 4.1). Four versions were produced: v1 is the native version and v2-4 carry each one an engineered sequon in a different position and are deprived of the natural one (Figure 4.24 A and 4.24B). HPAEC-PAD analysis confirmed 100% of Glc modification in all the four constructs (Figure 4.24C). By LC-MS/MS, it was verified that modification occurred at the level of the expected Asn residue in each construct. A fifth construct (MrkA v5) was also designed carrying all these four sequons: by HPAEC-PAD analysis a molar ratio of Glc to protein of 4 was verified, meaning that all sequons were 100% glucosylated (black line in Figure 4.24C).

A



B

MrkA version	AA sequence
1 (natural sequon)	MGSHHHHHHHHHHGSSCTVSVNGQGSDANVYLSPVTLTEVKAAAADTYLKPKSFTIDVSNCQAADGTKQDDVS KLGVNWTGGNLLAGATSKQQGYLANTEASGAQNIQLVLSTD**N**ATALTNKIIPGDSTQPKAKGDASAVADGARFTYY VGYATSAPTTVTTGVVNSYATYEITYQGGGGGGADTTVGGGQVNFFGKVTDVS
2 (engineered GlycoTag 1)	MGSHHHHHHHHHHGSSCTVSVNGQGSDANVYLSPVTLTEVKAAAADTYLKPKSFTIDVSNCQAADGTKQDDVS KLGVNWTGGNLLAGATSKQQGYLANTEASGAQNIQLVLSTDGATALTNKIIPG**N**ATQPKAKGDASAVADGARFTYY VGYATSAPTTVTTGVVNSYATYEITYQGGGGGGADTTVGGGQVNFFGKVTDVS
3 (engineered GlycoTag 2)	MGSHHHHHHHHHHGSSCTVSVNGQGSDANVYLSPVTLTEVKAAAADTYLKPKSFTIDVSNCQAADGTKQDDVS KLGVNWTGGNLLAGATSKQQGYLA**N**ATASGAQNIQLVLSTDGATALTNKIIPGDSTQPKAKGDASAVADGARFTYY VGYATSAPTTVTTGVVNSYATYEITYQGGGGGGADTTVGGGQVNFFGKVTDVS
4 (engineered GlycoTag 3)	MGSHHHHHHHHHHGSSCTVSVNGQGSDANVYLSPVTLTEVKAAAADTYLKPKSFTIDVSNCQAADGTKQDDVS KLGVNWTGGNLLA**N**ATSKQQGYLANTEASGAQNIQLVLSTDGATALTNKIIPGDSTQPKAKGDASAVADGARFTYY VGYATSAPTTVTTGVVNSYATYEITYQGGGGGGADTTVGGGQVNFFGKVTDVS

C

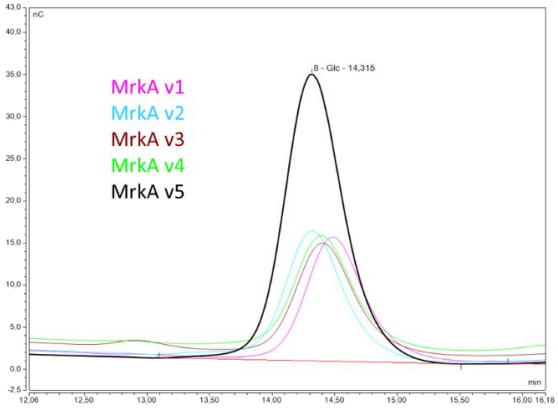


Figure 4.24. Characterization of new MrkA versions. **A**) Phyre2 models showing the position of each sequon, natural or engineered, in protein predicted loops. **B**) Table reporting the four mono-sequon (underlined) sequences. Mutated AA for the generation of v2-v4 sequons are highlighted in grey in v1 sequence. The natural sequon was removed in the other mono-sequon versions (v2-v4), substituting Asn with Gly (highlighted in green). **C**) HPAEC-PAD chromatograms showing the amount of glucose in the different MrkA versions.

4.12 Application of cytoplasmic glycoengineering to other target pathogens

To verify if this strategy is easily applicable to other proteins from different pathogens, cytoplasmic glycoengineering was extended to Streptolysin O (SLO), a highly conserved virulence factor from Group A *Streptococcus* (GAS). This hemolytic exotoxin has been tested as carrier for Group A carbohydrate (GAC) verifying that it worked well as carrier, but its antigenicity was negatively impacted by random conjugation [210]. SLO naturally presents multiple glycotags (Figure 4.25) in sites of the protein more or less accessible to the solvent. To start investigating this protein for cytoplasmic glycoengineering, all the 8 sequons were maintained and assessed for their tendency to be modified. Only a 6xHistidine tag was added at the N-term of SLO sequence for purification purposes. A single expression vector, pET29b(+), was used for the co-expression of SLO and NGT, both under the control of the strong T7 promoter in *E. coli* BL21(DE3) cells. In this case, the expression of T7 RNA polymerase, required for gene transcription, was induced with IPTG.

SLO AA sequence

MGSHHHHHHASESNKQNTASTETTTTNEQPKPESSELTTEKAGQKTDDMLNSNDMIKLAPKEMPLESAEKEEKKSEDKKKSEEDHTEEIN
DKIYSLNYNELEVLAKNGETIENFVPKEGVKKADKFIVIERKKKNINTTPVDISIIDSVTDRTYPAALQLANKGFTENKPDAVVTKRNPQKIHID
LPGMGDKATVEVNDPTYANVSTAIDNLVNQWHDNYSGGNTLPARTQYTESMVYSKSQIEAALNVNSKILDGTLGIDFKSISKGEKKVMIA
AYKQIFYTVSANLPNNPADVFDKSVTFKELQRKGVSNEAPPLFVSNVAYGRTVFVKLETSSKSNDVEAAFSAALKGTDVKTNGKYSDILENSS
FTAVVLGGDAAEHNKVVTKDFDVIRNVIKDNATFSRKNLAYPISYTSVFLKNNKIAGVNNRTEYVETTSTEYTSGKINLSHQGAYVAQYEILW
DEINYDDKGKEVITKRRWDNNWYSKTSPFSTVIPLGANSRNIRIMARECTGLAFEWWRKVIDERDVKLSKEINVNISGSTLSPYGSITYK

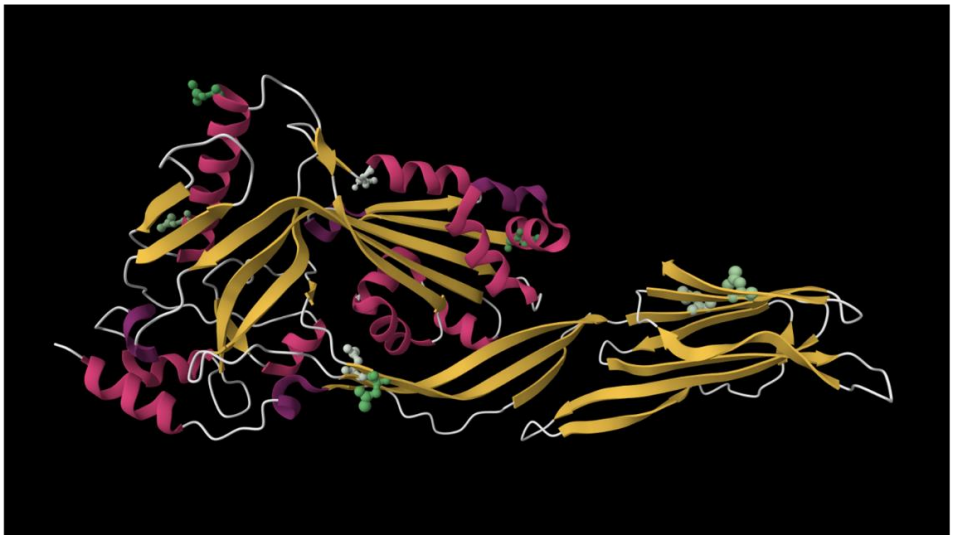


Figure 4.25. Crystal structure of SLO (PDB ID 4HSC). Ribbon diagram with the N residues of the 8 natural sequons (underlined in the AA sequence) highlighted in green. Color intensity indicates accessible surface area ($Å^2$).

SLO was expressed with or without NGT, growing bacteria in the same conditions used for MrkA, and purified through affinity chromatotography. Proteins were obtained with a good purity, as shown by SDS-PAGE analysis (Figure 4.26A), and yield (~ 50 mg/L for SLO and ~ 80 for SLO + NGT). By HPAEC-PAD analysis the successful modification SLO was confirmed (Figure 4.26B): a molar ratio of Glc to protein of 2 was calculated. Mass analysis is ongoing to verify position of modifications introduced on the protein.

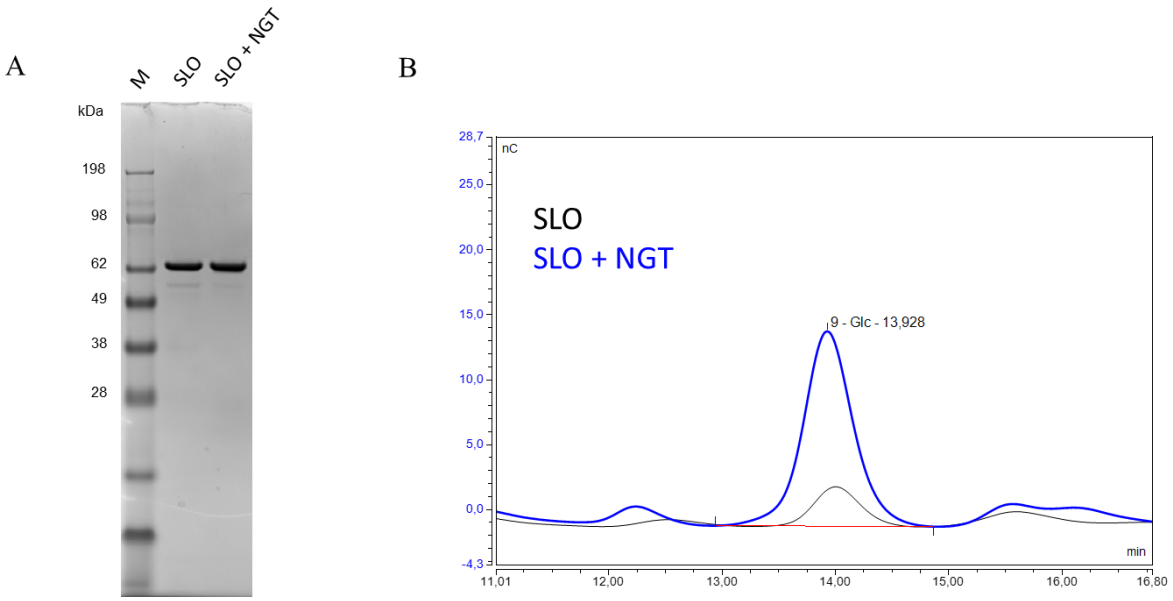


Figure 4.26. Characterization of purified SLO expressed with or without NGT. **A**) SDS-PAGE analysis showing proteins bands at the expected MW of ~ 63 kDa. **B**) HPAEC-PAD analysis showing the Glc peak in the chromatogram of SLO + NGT.

4.13 Attempts for lactose elongation in *E. coli*

To fully exploit the potential of cytoplasmic glycoengineering, possible strategies for the stepwise biosynthesis of O1v1 and O2a polygalactoses, using lactose on MrkA as primer, were designed (Figure 4.27). In the literature it is reported that WbbY, possessing two glycosyltransferase catalytic sites, is responsible for O1v1 polymerization [211], while WbbO, WbbM and WbbN seem to be involved in O2a biosynthesis [212].

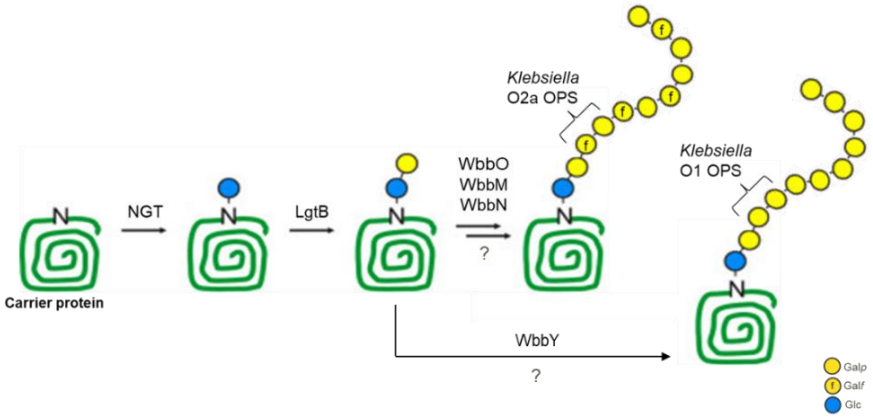


Figure 4.27. Proposed artificial pathway for O1v1 and O2a biosynthesis. After the addition of the first priming glucose by NGT, LgtB β-1,4-galactosyltransferase adds a galactose residue, that can be used as an anchor for the subsequent addition of the desired sugar monomers. WbbO, WbbM and WbbN are enzymes involved in the biosynthetic pathway of O2a, instead WbbY alone is retained to be responsible for O1v1 biosynthesis.

Selected genes were cloned in the additional multicloning site of pACYCDuet plasmid carrying already NGT and LgtB ezymes (Figure 4.28).

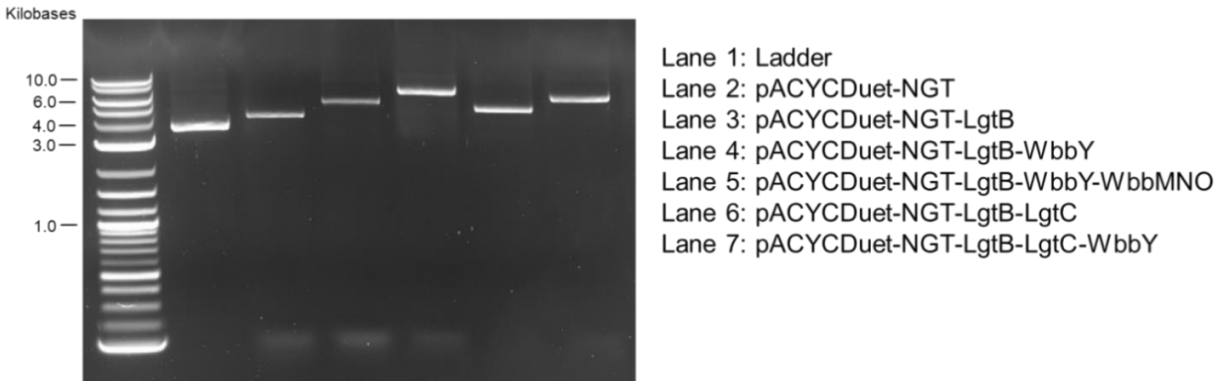


Figure 4.28. Agarose gel showing the new plasmids produced (lane 4-7).

The route for the generation of O1v1-MrkA bioconjugate was investigated for starting, as only one enzyme (WbbY) is sufficient for O1v1 polimerization [211]; in O2a biosynthesis more galactosyltransferases are involved and an undecaprenyl-diphospho-*N*-acetylglucosamine acceptor is required for its initiation [213]. *E. coli* K12 W3310 Δ*lacZ* strain expressing MrkA was transformed with the new plasmids carrying the information for the expression of LgtC only or LgtC and WbbY, in addition to NGT and LgtB. LgtC is α-1,4-galactosyltransferase from *N. meningitidis* catalyzing the transfer of an α-galactose from UDP-Gal to a terminal lactose [214]. The presence of an additional galactose could favour the activity of WbbY, considering that it uses α-Gal*p*-(1→3)-β-Gal*f* as an acceptor for *in vitro* polymerization of O1 [211]. WB analysis on whole cell lysates after induction with Arabinose or Arabinose+IPTG was performed (Figure 4.29). When NGT and LgtB were co-expressed, a shift towards higher MW was observed, corresponding to the linkage of lactose (lane 5). Adding LgtC, a slight shift in MW from that of Lac-MrkA was noticeable (lane 7). Analysis to confirm the presence of two galactose residues is ongoing. Unfortunately, with the addition of WbbY, the desired galactose polymerization was not obtained (lane 9). The α-Gal*p*-(1→4)-β-Gal*p* disaccharide could not work as acceptor substrate for the WbbY enzyme. However, additional experiments are needed to understand also if WbbY enzyme is correctly expressed and soluble, since the SDS-PAGE on soluble and insoluble fractions after cell lysis of WbbY-expressing *E. coli* strain was not clear.

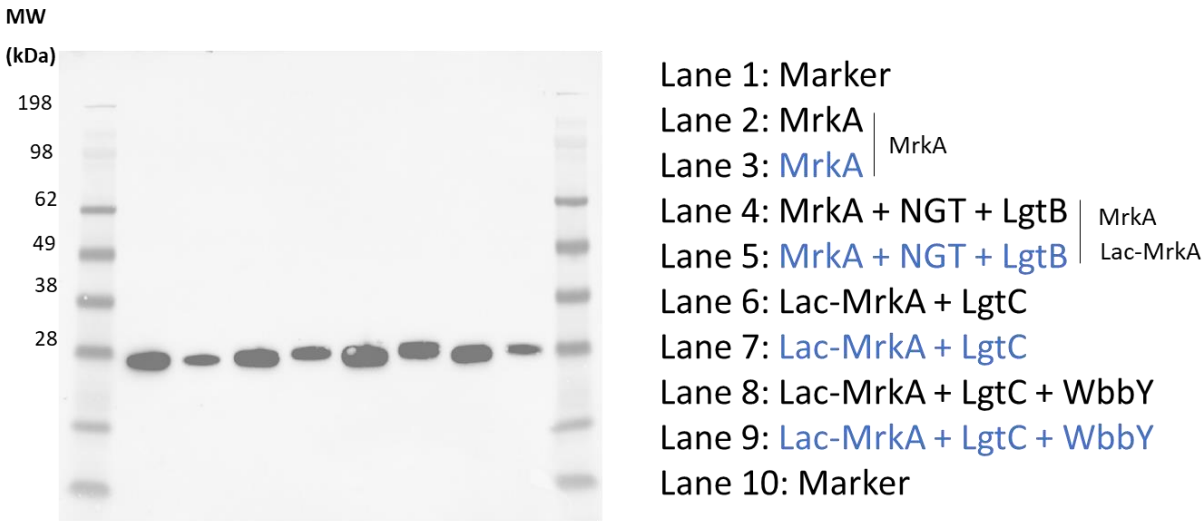


Figure 4.29. WB analysis on whole cell lysates of *E. coli* co-expressing MrkA (Ara) and GTs (IPTG) for O1v1 artificial biosynthesis. In blue, Ara+IPTG induced samples, in black Ara only.

5 DISCUSSION

Glycoconjugates are a well-established category of bacterial vaccines that revolutionized vaccinology history since their development started nearly 40 years ago. Conjugation of PSs to appropriate carrier proteins allowed in fact to improve the immunogenicity of these T-cell independent antigens naturally containing only B-cell epitopes, enhancing memory response, class-switching and antibody production in infants [215]. Currently, only six carrier proteins are used in licensed glycoconjugate vaccines [60], and there are increasing concerns about the pre- or co-exposure to the same carrier that could lead to the reduction of the anti-carbohydrate immune response [216]. The risk of this so-called carrier-induced epitope suppression phenomenon highlighted the need for new carrier proteins [60,217]. The research of new carriers is also driven by the interest in exploring the ability of proteins to function simultaneously as carrier and antigen against a specific pathogen. In this perspective, selective conjugation chemistries might be necessary to preserve the antigenicity of the protein. Site-selective chemical and enzymatic approaches or bioconjugation have been investigated in order to have a limited impact on protein structures and conformation. In bioconjugation, the OTase PglB, possessing a relaxed substrate specificity, catalyzes the *en bloc* transfer of a diverse range of glycans from preassembled lipid-bound precursors to the asparagine residue of the N-glycosylation site of the engineered carrier protein in the periplasm of the *E. coli* cells [218-220]. Thus, to obtain a bioconjugate, three heterologously expressed components are needed: the coupling enzyme (PglB), the glycan encoding genes and the acceptor protein, which needs to be in the periplasm in order to be conjugated by the OTase. This puts a limitation on the choice of the carrier protein, and the periplasmic compartment might be characterized by lower protein expression yields compared to the cytoplasm, given that signal peptides, required for protein secretion to the periplasm, have unpredictable effects on protein synthesis and secretion [221]. Peptide library screening for the selection of the optimal signal peptide, which can vary from a protein to another protein, is also costly and time-consuming.

Recently, an alternative bacterial N-glycosylation mechanism, whose central enzyme is the cytoplasmic N-glycosyltransferase NGT, has been exploited to obtain glycosylated proteins in *E. coli* cytoplasm [222]. This system involves the sequential transfer of monosaccharides from nucleotide activated donors to asparagine side chains of the N-X-S/T sequon, rather than block

transfer of preassembled OS. The possibility to obtain in *E. coli* site-specifically functionalized proteins can be exploited for the generation of innovative glycoconjugates.

Indeed, in my PhD project, I have developed a novel selective conjugation strategy based on cytoplasmic protein glycoengineering, testing Kp type 3 fimbriae MrkA as protein carrier and antigen. A double-hit approach, meaning that both PS and carrier protein belong to the same pathogen, may be very useful for a bacterial species with large glycan variability like Kp. For this reason, double-hit vaccines were generated by selectively conjugating Kp model capsular and subcapsular PSs at the level of the unique sugar handle introduced onto MrkA. MrkA has gained strong interest as a potential vaccine target, being expressed by most of Kp strains, including hv-Kp. It is in fact present with a sequence homology of above 85% in approximately 95% of Kp isolates from the Institute Pasteur database (~ 40,000 genomes). Moreover, there are already preclinical data showing that immunization with purified fimbriae was able to protect mice against a lethal challenge in a model of acute pneumonia [223], and that mice vaccinated subcutaneously with both monomeric and oligomeric MrkA showed a reduction in bacterial burden after intranasal challenge with Kp [190].

Our preliminary study in mice with the two alternative designs of stabilized MrkA monomer highlighted the poor immunogenicity of this small recombinant protein. However, MrkA vA induced a significantly higher level of anti-MrkA IgG antibodies already after two injections compared to MrkA vB. Based on these results, MrkA vA was selected to develop the selective conjugation strategy.

Cytoplasmic glycoengineering was successfully applied for the modification of MrkA vA. In fact, a biosynthetic pathway for MrkA glucosylation and lactosylation in *E. coli* was successfully established. These modifications resulted to be consistent, as verified by the quantification of Glc and Gal through anion exchange chromatography. Moreover, it seems that glycosylation conferred higher solubility and stability to the protein, having obtained better yields for Glyco-MrkA in every single batch produced compared to the unmodified one. Glycosylation has been shown to increase the solubility of many proteins and also prevents aggregates formation [224]. Peptide mapping analysis revealed that MrkA was modified at the level of a natural sequon, located in a solvent-exposed loop as revealed by the homology-based structural model, so no intervention on its sequence was needed. Moreover, possibly for a matter of solvent exposure, the lactose glycoform was better oxidized with respect to Glc-MrkA, so oxidized Lac-MrkA was used for the reductive amination reaction with the randomly ADH-derivatized PSs. Among the prevalent O- and K- serotypes, O1v1 and O2a OAg and K2

KAg were selected as model subcapsular and capsular PSs, respectively [163,164]. This selective conjugation reaction led to the formation of glycoconjugates characterized by several protein molecules linked through their single attachment point to the PS chains, differently from bioconjugates where one protein carries few glycan chains. In our immunogenicity study in mice comparing these novel selective conjugates to more traditional random glycoconjugates, we decided to test the one with the longer OAg (O1v1), having also a higher average number of linked MrkA molecules compared to O2a-MrkA, and the one with the sonicated K2, considering the advantages related to the conjugation of a PS with a lower viscosity. The ability of MrkA to work as carrier for PSs was proportional to the molecular mass of the final glycoconjugate. In fact, both random conjugates, as well as the K2-MrkA selective conjugate, were able to induce a good level of anti-PS IgG antibodies, while the selective OAg conjugate did not elicit any response. This could be due to the small dimension of this conjugate, composed by an average of 3 MrkA molecules selectively linked to O1v1, that may not contain an adequate density of accessible epitope [225]. Concerning the anti-MrkA IgG response, MrkA recombinant protein was scarcely immunogenic, however conjugation of PSs resulting in an increase of the conjugate size improved the immune response against MrkA as well. This was not true in fact for the selective O1v1-MrkA conjugate, suggesting that with the OAg a cross-linked structure might be needed for optimal B-cell receptor recognition. Interestingly, this study highlighted the ability of a long polysaccharide like K2 to work as "carrier" for MrkA increasing the immunogenicity, poor *per se*, of the protein selectively linked along its chain. In fact, looking at the GeoMean fold increase and at the number of non-responders, it seems that selective conjugation of MrkA to a long PS like K2 can be more effective at enhancing anti-MrkA immune response. Random conjugation of long CPS instead could mask or alter immunologically relevant protein epitopes. In fact, in the case of a carrier with a concomitant role of protective antigen, the protein provides not only T-cell helper epitopes but also protective B-cell epitopes, so the extent and location of the saccharide chains on the protein carrier might be relevant in terms of preservation of its key B-cell epitopes [226]. Opposed to T cell epitopes that are linear sequences of a minimum of 8-12 aminoacids that bind to MHC class II and interact with T cell receptors on the surface of CD4+ T cells [227], B-cell epitopes are often of a conformational nature, so a selective approach becomes crucial to maintain the antigenicity of the protein antigen. Knowing that Kp type 3 fimbriae play an important role in the ability of the bacteria to form biofilms, a key method to evaluate the functionality of anti-MrkA antibodies, would be the biofilm formation inhibition assay. Interestingly, literature data report that the anti-MrkA mAb KP3 was able to determine OPK activity against different Kp

serotypes and to display protective activity in various *in vivo* models, showing that OPK may be a reliable assay to measure *in vitro* antibodies ability to mediate *in vivo* protection [190]. Since the development of these functional assays is still on going, the binding capacity of anti-MrkA antibodies from the mice study to a wild type Kp strain expressing high levels of MrkA was verified via flow cytometry. In agreement with anti-MrkA ELISA results, sera from sK2 selective conjugate immunized mice showed a higher binding capacity, compared to sera from random conjugate and protein alone groups. This result is important as antibodies generated were not only able to recognize the recombinant protein monomer in ELISA, but also the natural MrkA polymer displayed on bacteria surface.

Another limitation of this work is that MrkA functional epitopes are still unknown, therefore we can not rule out that MrkA lactosylation in its natural sequon is impacting its overall antigenicity. However, sequons can be easily engineered in different sites of the protein supporting additional studies to understand if the glycosylation position can affect the immunogenicity of resulting selective conjugates. At this scope, alternative mono-sequon MrkA versions with engineered glycotags in predicted exposed loops were designed and co-expressed with NGT. The new three engineered sequons of MrkA v2, v3 and v4 were fully glycosylated, and modification site was confirmed by LC-MS/MS analysis. Binding studies via Surface Plasmon Resonance (SPR) can be useful to understand the impact of glycosylation site on the recognition by anti-MrkA mAbs, also to guide the design of the glycoconjugate vaccine in a more rational way. An additional version, carrying the natural and the three engineered sequons, was also produced, and as expected, modification of all glycotags occurred at 100%. Given the results from the mice study with the selective O1v1-MrkA conjugate, more than one sequon, once the proper sites not affecting MrkA antigenicity are identified, could be necessary in order to induce good anti-PS as well as anti-protein immune responses. In fact, in the literature is reported that a high glycan loading could be needed for conjugation of short OSs to result in an optimal immune response [62].

The new selective conjugation approach developed in this work could be also extended to other pathogens, combining PS and protein antigens in novel effective glycoconjugate vaccines. Here cytoplasmic glycoengineering was applied to SLO, a conserved virulence factor from the Gram-positive bacteria GAS, against which no vaccine is yet available. SLO was identified as a promising vaccine candidate through a reverse vaccinology approach [228], together with two highly conserved GAS proteins, SpyAD and SpyCEP. These three protein antigens are prevalent in clinical collections and, together with GAC, could virtually cover all GAS clinical

isolates [229]. Di Benedetto and colleagues tested the ability of these proteins to work as protective antigens and carrier for GAC, aiming at reducing the complexity of the final vaccine formulation containing a GAC-CRM_{197} conjugate and the three recombinant proteins, but the random approach used for GAC conjugation impacted anti-protein antibodies functionality [210]. SLO presents already several sequons in its sequence, so no glycotags were engineered, in order to evaluate its natural predisposition as NGT substrate. As occurred with Glyco-MrkA, a higher protein yield was obtained when SLO was co-expressed with NGT. From HPAEC-PAD analysis, a molar ratio of Glc to protein of 2 was found. LC-MS/MS analysis will identify which are the glycosylated sequons. SLO glycoengineering would be also facilitated by the fact that its structure has been solved with X-ray crystallography [230]. Not less important, the availability of already developed functional assay (e.g. hemolysis inhibition assay) would be an advantage in deciphering the immunogenicity of SLO glycoconjugates. Interestingly, a study in literature reporting a double-hit approach used for a GAC glycoconjugate demonstrated that conjugation of modified GAC to GAS protein ADI (arginine deiminase protein) gave a robust immune response to both components, without a loss of immunogenicity to the protein component compared with a protein alone response, despite the use of a random CDAP chemistry [231]. In this case it is possible that random conjugation did not impair ADI B-cell epitopes, but it also true that GAC is a rather small PS with an average MM of 7 kDa [232]. In principle, selective conjugation is likely to be the most effective method for double-hit glycoconjugates, as demonstrated also by the selective SpyAD-GAC conjugate synthesized through click-chemistry coupled to ncAAs: IgG titers against SpyAD were high for both conjugate and protein alone, and improved shifts in antibody binding by flow cytometry for SpyAD-GAC compared with SpyAD alone was obtained [233], as observed with the selective MrkA conjugates.

Moreover, it has been shown that the GAC rhamnose backbone can be built successfully in *E. coli* with the correct chemical structure, setting the basis for the development of bioconjugation approaches [234]. GAC has a N-acetylglucosamine (GlcNAc) at the reducing end, suggesting it would be suitably recognized by OTase; however, the following sugar in the chain, rhamnose, is attached to the GlcNAc by a β-1,4 linkage whichmay not be recognized or transferred by the OTase efficiently [235,236]. Therefore, alternative strategies may be required for the generation of a GAC bioconjugate, like cytoplasmic glycoengineering.

To further advance the NGT technology, we tried to extend the lactose handle on MrkA with enzymes for the biosynthesis of the immunodominant domain of O1v1, D-galactan II. O1

antigen is extended from the non reducing terminus of O2a antigen by the enzyme WbbY, which possesses two distinct catalytic domains (C-term adding a β-1,3–linked D-Galp, N-term adding α-1,3–linked D-Galp) and uses an acceptor composed of at least one O2a repeat unit [212]. LgtC, a galactosyltransferase from *N. meningitidis* [215], was added to the biosynthetic pathway in the attempt to build an acceptor for WbbY more similar to the natural one, adding an α-1,4–linked D-Galp to the terminal lactose. This galactosyltransferase, as LgtB, is involved in the biosynthesis of *N. meningitidis* LOS outer core [237]. Unfortunately, with the addition of LgtC and WbbY to the pathway for MrkA lactosylation, Gal polymerization was not observed. Characterization of the purified product is ongoing to verify the presence at least of two Gal residues. However, this first experiment highlighted the scarce promiscuity of WbbY, compared to the OTases used in bioconjugation. The transition to total bioconjugation using cytoplasmic glycoengineering, compared to the PGCT, appear more challenging, as for each different polysaccharide a new strategy has to be put in place for the identification of the proper enzymes. The advantages in terms of production process and costs of a bioconjugate would be enormous, but on the other hand immunogenicity might not be ideal with a low saccharide density [36]. However, the insertion of multiple sequons in the protein could allow to overcome this potential issue.

In conclusion, I exploited the bacterial cytoplasmic protein glycosylation system for the selective modification of MrkA in *E. coli*, aiming at testing its ability to work as carrier for Kp polysaccharides while preserving its antigenicity through a selective conjugation strategy. The selective conjugation of a long PS like K2 to Lac-MrkA resulted successful, not only to maintain, but to improve the immune response of a poorly immunogenic protein like MrkA and to make MrkA working as carrier for the PS. Size and macromolecular/multimolecular structure of the conjugate play indeed a significant role in the production of a good anti-polysaccharide IgG response. A study in a different animal model (i.e., rabbits) will help to consolidate the results obtained in mice and the development of functional assays (i.e. biofilm inhibition assay, OPK, SBA) will allow to assess sera functionality. The selective conjugation approach developed here can be extended to other pathogens for the synthesis of innovative glycoconjugates selectively linking long polysaccharides to proteins with the double role of carrier and antigen. The application of cytoplasmic glycoengineering for the generation of novel bioconjugates will have numerous advantages over chemically synthesized glycoconjugates, but it remains rather challenging.

6 REFERENCES

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