UNIVERSITÀ DEGLI STUDI
DI MILANO – BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biotecnologie e Bioscienze

Dottorato di ricerca in Biotecnologie Industriali,
XXIV ciclo

Preparation and physicochemical characterization of glycoconjugate vaccines

Tutor:
Prof.ssa Silvia Maria Doglia
Tutor aziendale:
Dott. Francesco Berti
Coordinatore:
Prof. Marco Vanoni

Studente:
Dott. Gabriele Costantini

Anno Accademico 2011

Dottorato in Biotecnologie Industriali, XXIV ciclo

Dott. Gabriele Costantini
Matricola: 041908

Tutor: Prof.ssa Silvia Maria Doglia
Tutor aziendale: Dott. Francesco Berti

Il lavoro presentato in questa tesi è stato realizzato presso i laboratori del dipartimento Vaccine Chemistry della Novartis Vaccines and Diagnostics srl, sotto la supervisione del Dr. Francesco Berti.

Università degli Studi di Milano-Bicocca
Piazza dell'Ateneo Nuovo 1, 20126, Milano

Dipartimento di Biotecnologie e Bioscienze
Piazza della Scienza 2, 20126, Milano

Acknowledgements

I would like to express my sincere gratitude to Dr. Francesco Berti, Dr. Paolo Costantino and Prof. Silvia Maria Doglia, who drove my scientific education.

I would like to show my gratitude to all the members of the Vaccine Chemistry Research group (and previous members) from Novartis V&D in Siena - Vittoria Pinto, Barbara Brogioni, Stefano Crotti, Elisa Danieli, Roberto Adamo, Daniela Proietti, Anna Kabanova, Marta Tontini, Maria Rosaria Romano, Tommaso Niccolai, Giulia Brogioni, Emilia Cappelletti and Evita Balducci who helped with experiments and discussion of the present thesis.

I would you like to thank Prof. Mario Artur Monteiro at University of Guelph for having me in his lab for two months and for teaching me the fundamentals of bacterial polysaccharide characterization by GC-MS.

I was a recipient of a Novartis fellowship from the current PhD program of the University of Milano-Bibocca.

Riassunto

Il progetto del mio corso di dottorato ha riguardato principalmente la caratterizzazione di vaccini glicoconiugati. In questa categoria di vaccini, gli antigeni saccaridici sono legati covalentemente alla proteina carrier mediante l'utilizzo di gruppi funzionali presenti su entrambi, sia direttamente e sia attraverso l'impiego di linker. Una delle chimiche più comunemente usate coinvolge i gruppi amminici presenti sulla catena laterale delle lisine che risultano bene esposti. Questi possono reagire e legarsi al gruppo aldeidico presente sul terminale riducente del polisaccaride per amminazione riduttiva. È inoltre ampiamente diffuso anche l'utilizzo di appositi linker inseriti sul saccaride e in grado di reagire selettivamente con il gruppo amminico.

In particolare il mio dottorato si è focalizzato sullo sviluppo di una metodologia che permetta di identificare i siti di glicosilazione di diversi tipi coniugati, mediante l'uso della spettrometria di massa previa separazione in cromatografia liquida (LC-MS). Il punto di partenza per il mio progetto è stato un lavoro pubblicato in precedenza dal nostro gruppo, sulla caratterizzazione chimico-fisica di vaccini contro i serogruppi A, C, W135 e Y del batterio patogeno *Neisseiria meningitidis*. La parte di questa pubblicazione che descriveva un metodo per identificare le lisine glicosilate presenti in un glicoconiugato è risultata di particolare interesse per il mio progetto. Ogni coniugato, una volta ridotto e alchilato in modo da rompere e bloccare i ponti disolfuro delle cisteine, veniva digerito utilizzando l'enzima proteolitico tripsina, ottenendo una miscela di peptidi e glicopeptidi. Successivamente veniva eseguita una cromatografia di esclusione molecolare sul digerito triptico per arricchire la miscela in termini di glicopetidi, sfruttando la maggiore dimensione di questi ultimi rispetto ai peptidi. Questo passaggio, che comporta un arricchimento in termini di glicopeptidi era stato ritenuto necessario ai fini della migliore qualità dell'analisi di massa successiva. In un'analisi di una miscela di glicopeptidi e peptidi, allo spettrometro di massa, i glicopeptidi hanno infatti un'intensità relativa dei picchi molto più bassa, poiché possiedono una minor capacità di ionizzazione dovuta alla parte saccaridica. I glicopeptidi hanno quindi un segnale poco intenso rispetto ai peptidi e potrebbero essere confusi nel rumore di fondo dello spettro. L'altra problematica, riguardante l'analisi dei glicopeptidi derivati dalla

digestione del glicoconiugato, è la diversità nelle lunghezze delle catene saccaridiche, poiché questi polisaccaridi sono generalmente estratti dal patogeno e poi coniugati alla proteina carrier. Anche se questi antigeni, prima di essere coniugati, subiscono un processo di purificazione che ne diminuisce la lunghezza a un target medio specifico, non si otterrà mai una sola catena di lunghezza definita.

Nell'analisi di massa per distinguere un peptide glicosilato da uno non glicosilato, è necessario ricercare nello spettro un incremento di massa noto; per questo gli autori hanno effettuato sulla miscela un'idrolisi acida, capace di "tagliare" la catena polisaccaridica lasciando attaccata covalentemente al gruppo amminico della lisina solo il linker e la prima unità ripetente dell'antigene.

La metodologia è stata sviluppata basandosi sulle linee guida del articolo pubblicato e lavorando sulla caratterizzazione dei siti di glicosilazione di alcuni vaccini glicoconiugati contro *Candida albican*s, *Streptococcus* group A (GAS), *Streptococcus* group B (GBS) ed ancora *Neisseria meningitidis* gruppo A (MenA).

La nuova procedura è stata modificata e semplificata, permettendo di ridurre la quantità e i tempi di preparazione del campione. La prima differenza sta nell'utilizzo di un unico buffer per i processi di *unfolding*, di riduzione-alchilazione e di digestione triptica del materiale. Si ottiene questo risultato utilizzando un surfactante commerciale, il RapiGest SF, che risulta poi facilmente rimovibile con un trattamento acido blando prima della caratterizzazione finale. La seconda differenza risiede nell'eliminazione dello step di arricchimento dei glicopeptidi. Si è supposto, infatti, che la differenza della capacità di ionizzazione di un peptide glicosilato, privato della catena saccaridica nella sua quasi interezza (eccetto per linker e prima unità saccaridica), non sia poi così diversa da quella del medesimo peptide non glicosilato. La rimozione di questo step, che risultava il passaggio più oneroso in termini di tempo, ci ha permesso di ridurre enormemente il tempo di preparazione del campione. Per l'arricchimento era necessaria circa una giornata lavorativa per ogni campione analizzato. In aggiunta eliminando l'arricchimento, nelle cui varie corse cromatografiche si perdeva parte del campione, siamo stati in grado anche di diminuire la quantità di campione necessaria per l'analisi, passando da 500 a soli 300µg di coniugato. Per la fase di "taglio" del

polisaccaride, oltre all'idrolisi acida ripetuta nuovamente per l'analisi di MenA e introdotta per i coniugati di GBS, è stato sperimentato con successo, sui coniugati di *C. albicans,* un "taglio" enzimatico molto efficiente ottenuto con l'uso della *naringinasi*. Questo enzima è specifico per tagliare le strutture β(1,3)glucaniche di cui sono formati gli antigeni di questi coniugati.

Il lavoro sul coniugato di GAS ha invece evidenziato la criticità e l'importanza del processo di "taglio" del polisaccaride. Per questo coniugato, che possiede un antigene formato da un backbone di poli-ramnosio, non è stato possibile ottenere un risultato positivo di idrolisi, sia per via acida che per via enzimatica. Alla fine la caratterizzazione di questo coniugato è stata accantonata non potendo ottenere un'analisi che rivelasse un incremento di massa noto.

L'analisi di questi coniugati ha mostrato un'elevata similarità nella distribuzione dei siti di glicosilazione dei coniugati di *C. albicans* e MenA mentre i coniugati di GBS hanno evidenziato una più ristretta distribuzione dei residui glicosilati. Questi riscontri sono stati spiegati dalla similarità della chimica di coniugazione dei coniugati di *C. albicans* e MenA e dal maggiore peso molecolare dei polisaccaridi di GBS, quindi probabilmente dal maggiore ingombro sterico durante la coniugazione.

Nella ricerca di un vaccino contro *C. albicans*, durante il dottorato mi sono occupato anche della sintesi organica di due antigeni β(1,3)-glucanici che presentano un linker, un trisaccaride e un esasaccaride, e successivamente sono stati coniugati alla proteina CRM_{197}. Il linker è stato introdotto con una reazione radicalica, catalizzata da radiazione ultravioletta, tra un gruppo allilico presente sul carbonio anomerico del primo glucosio e la cisteamina. Inoltre l'antigene esasaccaridico è stato prima legato ad una struttura ramificata, il $PAMAM_4$, così da generare un antigene multivalente (un cluster) e poi coniugato al CRM_{197}. Per la coniugazione al CRM_{197} del trisaccaride, dell'esaccaride e del cluster esasaccaridico e per la funzionalizzazione del $PAMAM_4$ con l'esasaccaride, è stato utilizzato un linker simmetrico bifunzionale. Questo è derivato dall'acido adipico e possiede due esteri dell'*N*-idrossisuccinimide alle estremità. Uno dei due gruppi esterei reagisce con il gruppo amminico, precedentemente introdotto, degli antigeni: il materiale così funzionalizzato è stato infine coniugato alla proteina. Una strategia simile è stata utilizzata anche per la

preparazione dell'antigene multivalente e poi per la sua coniugazione. I quattro gruppi amminici liberi del $PAMAM_4$ sono stati attivati con il medesimo linker e fatti reagire con l'esasaccaride. Una volta preparato il cluster, la quinta funzionalità amminica del $PAMAM_{4,}$ che era protetta, è stata sbloccata e attivata nuovamente con il linker per la coniugazione al CRM_{197}.

Infine è stato coniugato al CRM_{197} un lipopolisaccaride (LPS) modificato di *Neisseria meningitidis* gruppo B (MenB), fornitoci dal laboratorio dell'Institute of Biological Science del National Research Council di Ottawa (Canada). Prima della coniugazione finale il lipopolisaccaride e la proteina sono stati attivati in parallelo con due linker differenti che hanno introdotto su l'uno e sull'altra un gruppo funzionale chimicamente complementare all'altro. Sull'LPS è stata introdotta una funzionalità maleimidica mentre sul CRM_{197} è stato inserito un gruppo tiolico. La preparazione di questo coniugato con l'LPS è stata ottenuta con successo ed è motivata dalla ricerca di un vaccino efficace contro il MenB.

In conclusione il lavoro effettuato durante il dottorato ha permesso di ottenere una valida metodologia per identificare i siti di glicosilazione di vari vaccini glicoconiugati. Tale metodologia è ad oggi molto utile per aumentare il livello di caratterizzazione dei prodotti in via di sviluppo; potrebbe però risultare ancora più utile come strumento per controllare coniugati appositamente disegnati mediante chimiche di coniugazione ancora più specifiche e tali da preservare il più possibile gli epitopi protettivi presenti sulla proteina carrier.

Abstract

The project of my PhD course has been focused on the characterization of glycoconjugate vaccines to develop a largely applicable methodology to identify their glycosylation sites.

The saccharide antigens are covalently attached to the carrier protein by a spacer or using specific functionalities available in both components. The general analytical strategy has involved a tryptic digestion and an enzymatic or acid hydrolysis of carbohydrate antigens in glycoconjugates. The resulting mixture of peptides and glycopeptides with well-defined mass increment has been analyzed by liquid chromatography interfaced with a mass spectrometer (LC-MS) technique to qualitatively understand which lysine residues have been involved in the glycosylation process. Several conjugate vaccines against *Candida albicans*, Group A *Streptococcus* (GAS), Group B *Streptococcus* (GBS) and *Neisseria meningitidis* group A (MenA) have been analyzed to estimate the distribution of glycosylated lysine residues. Among those, GBS resulted the less glycosylated in comparison to *Candida* and MenA ones. This aspect it is probably due to the different conjugation chemistries and GBS polysaccharide size. The second part of my PhD project has been the synthesis of two β(1,3)-glucans antigens, a trisaccharide and a hexasaccharide and their conjugation to CRM_{197}. The hexasaccharide has also been linked on a multimeric structure $PAMAM_4$ and then conjugated to CRM_{197}. The last part of this work has been the conjugation to CRM_{197} of a modified MenB LPS, provided from Institute of Biological Science (IBS) laboratory (National Research Council - Ottawa Canada).

The target sites of the carrier protein (CRM_{197}) and LPS have been activated by two different reactans, and the final glycoconjugate has been obtained by the reaction of these two.

The resulting conjugate has been characterized (saccharide and protein content, MALDI-TOF mass spectrometry and NMR) using an accurate analytical panel.

INDEX

ABBREVIATION

AB	ammonium bicarbonate
ADH	adipic acid dihydrazine
BCR	binding B cell receptors
Boc	tert-butyloxycarbonyl
Con A	concanavalin A
DMSO	dimethyl sulphosside
DTT	dithiothreitol
ESI	electron spray ionization
FF	flow filtration
GMBS	*N*-[γ-maleimidobutyryloxy]succinimide ester)
HPAEC	high performance anion exchange chromatography
Hib	*Haemophilus influenzae* type b
Ig	immunoglobulin
LO	late onset
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption/ionization
MALLS	multi-angle laser light scattering
Men	meningococcus
MHC	major histocompatibility complex
MS	mass spectrometry
MWCO	molecular weight cut off
NaP_i	sodium phosphate
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
EO	early onset
PBS	phosphate buffer saline
PEt	phosphoethanolamine
PS	polysaccharide
SATP	*N*-succinimidyl-*S*-acetylthiopropionate
SEC	size exclusion chromatography
TCR	T cell receptor
TD	T-cell dependent
TI	T-cell independent
TFA	trifluor acetic acid
TFF	tangential flow filtration

1. INTRODUCTION

Vaccinology has been and will be one of most important and promising field to improve human health quality. Vaccines have permitted to eradicate or block the spread of several dangerous diseases that affected and killed yearly millions of people such smallpox, tetanus, yellow fever, pertussis, *Haemophilus Influenzae* type b, rubella, polio, measles, mumps, chickenpox, andtyphoid.

The battlefield is wide and hard, actually vaccine research is working on many and different targets: from the best known anti-bacterial and antiviral vaccines to the antifungal and anticancer vaccines.

Vaccines confer protection by the stimulation of the immune system, inducing humoral and/or cellular response.

There are different categories of vaccines, the research group I worked is involved in the development and characterization of carbohydrate-protein conjugates vaccines. This vaccine category consists in a pathogen carbohydrate antigen covalently attached to a proper carrier protein, and that permits to obtain a strong and durable immune response with antibody against pathogen surface carbohydrates.

In fact pathogen, like bacteria, fungi, virus and parasites, are covered with unique and specific carbohydrate structures, which have different functions.

Pathogens use these saccharide structures as virulence factor to establish an infection and elude the host immune system response.
The polysaccharide capsules of bacteria prevent complement activation (Roitt 1997) and inhibit phagocytosis. In addition they form protective shields over conserved viral protein epitopes (Baghian et al. 2000; Scanlan et al. 2002; Sanders et al. 2002; Mori et al. 2003; Han et al. 2004; Barrientos, Gronenborn 2005; Goffard et al. 2005; Helle et al. 2006), and serve for interaction with host cell surface receptors leading to enhancement of viral spread (Pohlmann et al. 2001; Alvarez et al. 2002; Halary et al. 2002; Klimstra et al. 2003; Lozach et al. 2005; Davis et al. 2006) or induction of immunosuppressive response (Shan et al. 2007).
These antigen carbohydrate structures are very interesting and have long been used in the preparation of vaccines.

The first studies for vaccine development have been done on *Streptococcus pneumoniae* capsular polysaccharide. Immunogenic and protective

properties of pneumoccocal PS had been investigated starting from as early as 1920s. In 1980s two different formulations of anti-pneumococcal vaccine have been introduced containing capsular polysaccharide derived from 14 and 23 pneumonia serotypes. However, the vaccine was poorly immunogenic in infants under the ages of two, elderly and immunecompromised people. (Vliegenthart 2006). The same issues were demonstrated for meningococcal polysaccharide vaccines (Broker et al. 2009).

It is accepted that unconjugated polysaccharides are not the best category of vaccines, they cannot be presented in the context of MHC-antigen complex to the T-helper cells and thus are weak immunogens (Avci, Kasper 2009).

In 1931 O.T. Avery and W.F. Goebel prepared a conjugate of *S. pneumoniae* type 3 polysaccharide and horse serum globulin, which was able to induce specific antibodies in rabbits, unlike the pure polysaccharide (Avery and Goebel 1931). The two scientists discovered that linking a polysaccharide to an appropriate protein carrier enhances its immunogenicity.

Conjugate vaccines represent step further respect to polysaccharide vaccines, and overcome other limitations: poor immunogenicity and lack long-lasting protection.

The reason is that unconjugated polysaccharide antigen (Cobb *et al* 2005) generates a T-cell independent immune response, while glycoconjugate gives a T-cell dependent response due to the activation of the adaptive immune system.

The glycoconjugate enters in B cell by binding to the polysaccharide-specific B cell receptor, BCR, and is transported to the endosome. Inside the cell the proteic moieties are digested by proteases to release peptide epitopes that bind to MHC II by replacing the self-peptide. The peptide originated from carrier is presented to αβ T cell receptor (TCR) of $CD4^+$ T cells. The peptide/MHCII/T cell complex permits to T cell to release cytokines that stimulate B cell maturation and change the production of immunoglobulin from IgM to IgG (Fig.1) (Avgi et al 2010).

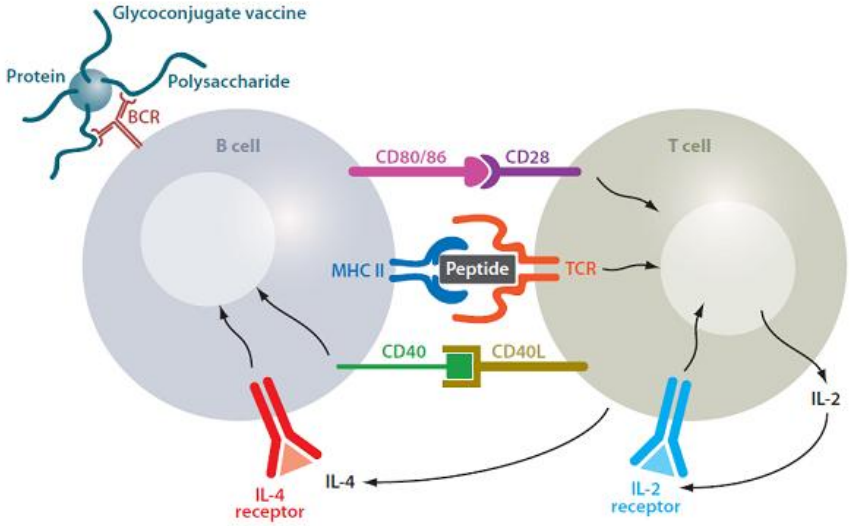


Fig 1. Schematic diagram of mechanism of action for conjugate vaccines. (Avci et al 2010)

In addition to the contact of the peptide/MCHII with TCR, other signals are necessary to elicit $CD4^+$ T cell help for the B cell. In particular repectors/ligands couples (e.g. CD80/86 and CD28, CD40 and CD40L) are fundamental for regulation, positive or negative.

In humans T-indipendent antigens, such as LPS and PS, gives an immune response 3-18 months after birth; commonly children under two years have a weak response and the majority of antibody is IgM type (>90%). Furthermore in these cases there are neither affinity maturation of antibody response, nor immunologic memory and nor enhancement effect of immune response by adjuvants. Unlike, for a T-dipendent antigen, there are either an immune response at (or shortly after) the birth and an affinity maturation; moreover adjuvants induce an enhanced response and the immunoglobulin production is heterogeneous.

The use of glycoconjugate vaccines in humans was introduced the late 80s The first goal was achieved on protection of infants and adults against *Haemophilus influenzae* type b (Hib). Hib was a leading cause of bacterial meningitis in USA, after market introduction of the glycoconjugate vaccine there was a reduction of 95% of meningitis cases due to this bacterium. This great result pushed research task to a quick development of glycoconjugate vaccines to prevent insidious and widespread pathogens such as *N. meningitidis*, *S.pneumoniae*, group b *Streptococcus* and *Salmonella typhi* Vi

(Jennings 1981, Robbins and Schneerson 1990). Generally common carrier proteins that have been utilized for conjugation are CRM_{197}, tetanus toxoid, diphtheria toxoid and outer membrane complexes from *Neisseria meningitidis* (Giannini et al. 1984; Verez-Bencomo et al. 2004, Jones 2005, Pace 2009).

Neisseria menengitidis , a commensal of human beings (5-10%), is one of the major cause of bacterial meningitis and sepsis both at endemic and epidemic level. When it becomes pathogenic the disease has a fast progression with mortality of 10-12%; moreover survivors often (20%) suffer of serious sequeale. Many successes were achieved in preparation and in efficacy of conjugated vaccines against four groups (A, C, W-135 and Y) of *N. meningitidis* (Giannini et al. 1984, Jones 2005, Pace et al. 2009), but not for group B to date, due to a poor immunogeniticy of its PS having similarity with sialic acids of self glycans present during normal development and in the central nervous system (Wyle 1972, Finne 1993).

Streptococcus pneumoniae is a primary cause of severe illness in young children, old adults and immunocompromised. This pathogen is the cause of both invasive diseases, especially affecting children under 2 years adults with more than 65 years (Anon 2007), correlated to a high morbidity and mortality and non-invasive diseases such as otitis media.

S. pneumoniae is also the greatest contributor to the increase in antibiotic resistance due to a high number of childhood hospital treatment of noninvasive mucosal infection incidence (Low 2005).

1.1 Preparation of glycoconjugates

Carbohydrate antigens are generally extracted from pathogens, purified and finally conjugates to the carrier. In addition to the natural source, synthetic oligosaccharide antigens with the same or similar structure of natives can be used. The amino group of lysine residues is one of the most used to functionality couple sugar and protein. Generally an excess of polysaccharide antigen is set to react with the carrier protein. The most common conjugation strategies are two: the is a direct reductive amination between lysine amino moiety and the aldehyde of sugar reducing end, or with an aldehyde group introduced by a partial oxidation of a hydroxyl group (Fig.2); second possibility is the use of bifunctional linkers (Fig.3).

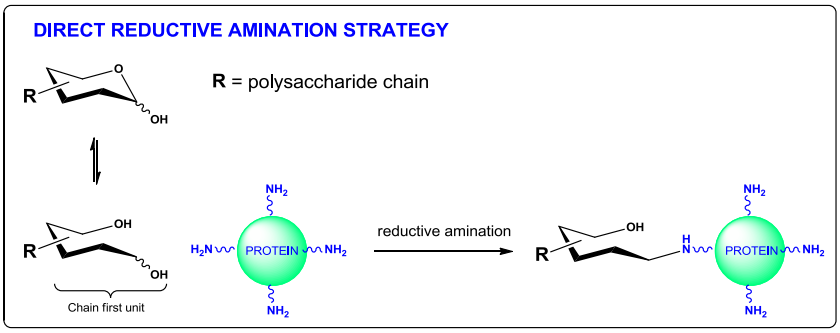


Fig.2 – Scheme of reductive amination for saccharide-protein coupling.

An almost infinite number of linkers exist and the activation of protein or carbohydrate or both cab be done before the conjugation, according to the antigenic structure and to desired conjugation strategy.

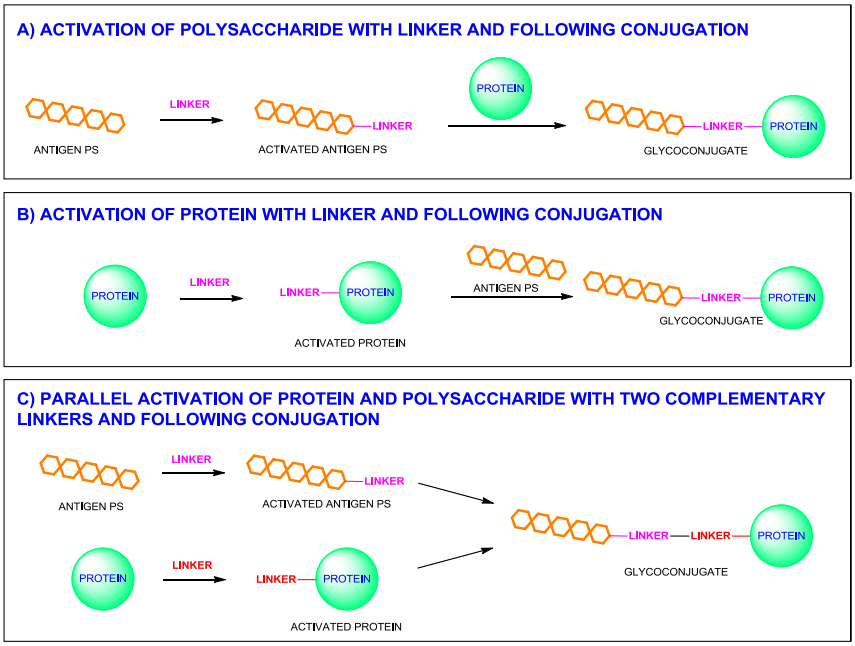


Fig.3 - Scheme of spacer-mediated coupling of saccharide-protein conjugates.

Mostly, linkers operates on the following groups:

→ **Primary amines**: This group is present at the N-terminus of each polypeptide chain and in the side chain of lysine residues (ε-amine). Primary amines are usually on the outer surface of proteins and

they are usually accessible for conjugation without denaturing protein. Amino groups can be insert by reductive amination on reducing end of PS.

→ **Carboxyl groups**: This group is present at the C-terminus of each polypeptide chain and in the side chains of aspartic acid and glutamic acid. Carboxyls are usually present also on the surface of protein structure. Moreover some sugars such as sialic acid have carboxyl moiety.

→ **Sulfhydryl groups**: This group is present in the side chain of cysteine. Normally cysteines are joined together between their side chains via disulfide bonds.When formed, disulfide bonds have to be reduced to sulfhydryls to make them available for coupling.

→ **Carbonyl groups**: Ketone or aldehyde groups are present at sugar reducing end or can be created by oxidizing the polysaccharide with sodium periodate.

Many different linkers with several functional moieties are commercially available; here I have reported a few examples of the most common chemistries that linkers can be employed (Costantino et al. 2011):

→ ***N*-hydroxysuccinimide esters** (NHS ester) are specific for primary amines. Reaction takes place at mild basic condition (7.2<pH<8.5) with the formation of an amide bond and releasing *N*-hydroxysuccinimide (Fig.4).

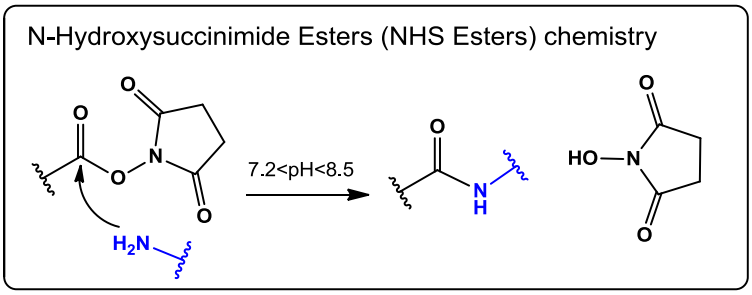


Fig.4 – N-hydroxysuccinimide chemistry.

→ **Maleimides** react specifically with sulfhydryl groups at pH between 6.5 and 7.5 forming a very stable thioether linkage (Fig.5).

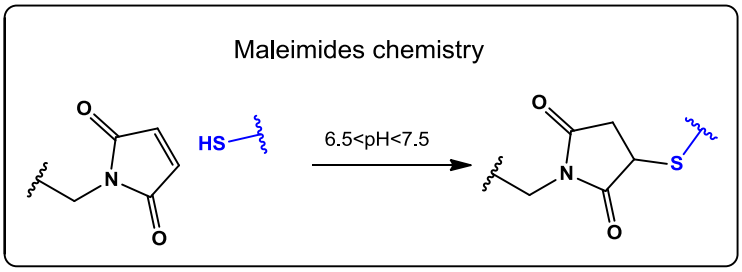


Fig.5 – Maleimide chemistry.

→ **Haloacetyls**, of Iodine or Bromine, react with sulfhydryl groups in mild basic condition obtaining a thioether bond (Fig.6).

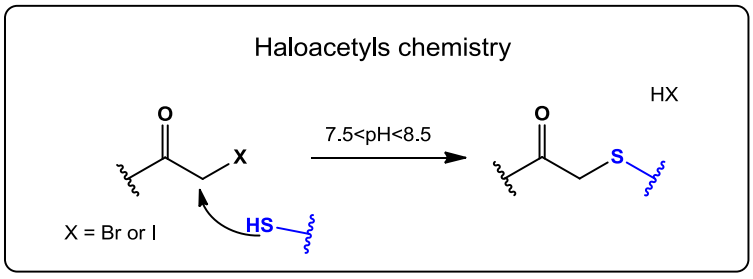


Fig.6 – Haloacetyls chemistry.

→ **Carbodiimides** are coupling agents that permit direct conjugation of carboxyls to primary amines. They activate carboxyl group *in situ* by the formation of *O*-acylisourea reactive intermediate. Primary amine can then easily react and form an amide bond, a urea side-product is released. Carbodiimides are also used to prepare NHS esters (Fig.7).

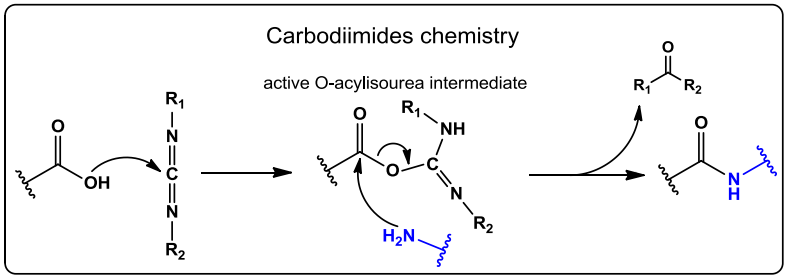


Fig. 7 – Carbodiimides chemistry.

Once conjugate is prepared there are several possibilities can be applied for its purification. One of the most common is the Size Exclusion Chromatography (SEC), this technique allows to elute the different molecule (glycoconjugate, unconjugated protein, salt, reactant) discriminating on base of size. Bigger structures elute earlier because can't penetrate in the solid phase matrix mesh, while smaller molecules are slowed down by mesh.

A second common methodology is Flow Filtration (FF), a membrane with a specific molecular weight cutoff (MWCO), allows passage only to the molecules smaller than MWCO. Since glycoconjugate is the largest structure of conjugation, by aimed membrane selection it is possible to eliminate all the other byproducts and salts. Byproducts and salts are eliminated by membrane selection according to their size.

1.2 Characterization

The characterization represents a key step in vaccine development.
Several physicochemical methods are currently applicable to obtain a fine characterization (Fig.8).
NMR spectroscopy is widely used for characterization of a polysaccharide, particularly to establish its identity, by assigning every single peak signal, and to assess its level of purity. Moreover, this technique can provide the identification of the PS average size, (polymerization degree of polysaccharide), and the percentage of its group activation (which are the hydroxyl groups activated in the repeating unit) (Jones 2005).
In addition, by size exclusion chromatography interfaced to a multi-angle laser light scattering (MALLS) detector, the PS molecular weight can be obtained. This detector works measuring the average intensity of light scattered by the sample at various angles from the beam, the result is a nearly instantaneous measurement of the weight-average molecular weight of the macromolecular species present in the sample.
Soft-ionization mass spectrometry techniques (MALDI, ESI) can be employed to estimate the mass of a glycoconjugate and its saccharide/protein average ratio, and it is also useful for carrier protein characterization (Monteiro 2009). Gas chromatography equipped with mass detector is very useful for the polysaccharide characterization (Fox et al. 1984, Ruiz-Matute et al. 2011).
Glycoconjugate vaccines need to be well characterized and controlled to consistent production and immunogenicity. Detailed characterization of conjugate vaccines is a long procedure that involves numerous tests on starting materials, purified polysaccharides and carrier proteins. For a correct characterization of glycoconjugate vaccines, several physicochemical methodologies including spectrophotometric methods, HPLC, mass spectrometry and NMR have been developed in order to achieve a strict control of the manufacturing process (Ravenscroft et al. 2000, Ravenscroft et al. 2010, Jones 2005b, Suker 2004).
An overview of the analytical tests applied to conjugate vaccines and related carrier proteins and polysaccharides is shown in figure 8.
The size of the saccharide chain, the saccharide/protein ratio, the size of conjugates and the level of free saccharide are fundamental aspects to guarantee manufacturing consistency and production batches with comparable immunogenicity to those used in clinical trials (Jones 2005). The level of free saccharide is the amount of saccharide that is not covalently linked to the protein carrier. Free saccharide can be present as process residual after the purification of the glycoconjugate, but can also be formed during vaccine storage by hydrolysis of the glycosidic linkages. Large value of free saccharide unfortunately results in the decrease of

glycoconjugate vaccines potency. Immunogenicity assays on glycoconjugates are performed in animal models to obtain preclinical data. Polysaccharides are not or poor immunogenic in mice and other animal models such as rabbit and guinea-pigs; differently after conjugation to protein carriers, polysaccharides become immunogenic and induce specific IgG anticarbohydrate antibodies.This change in immunological behavior reflects the acquisition of T-dipendent character. Immunogenicity potency estimated in animal model might not correlate with human efficacy/immunogenicity. In principle, in vivo potency tests could be used to assess manufacturing consistency, but physicochemical characterization methodologies are much more reliable due to their reproducibility, specificity and sensitivity. Glycoconjugate vaccines, especially those with a well-defined structure, can be appropriately characterized by physicochemical assays.

Process intermediates		Quality attributes	Typical tests
Polysaccharide	Structure	Identity Molecular size and polydispesity chemical composition *O* -acetyl	NMR, immunochemical methods SEC-UV/-RI/-MALLS HPAEC-PAD, colorimetric assay NMR, colorimetric assay
	Purity	Protein, nucleic acids Water and volatile substances Organic solvents resisuals Bioburden Endotoxins	colorimetric assay Thermogravimetry, Karl Fisher GC-Flame Ionization Detection Total viable aerobic count of microbial contamination LAL assay
Derivatizated poly-/oligosaccharide	Structure	Identity Molecular size and polydispesity Saccharide contents Degree of polymerization O-acetyl Derivatizing gropus content	NMR, immunochemical methods SEC-UV/-RI/-MALLS Colorimetric assey, HPAEC-PAD HPAEC-PAD, colorimetric assay, NMR NMR, colorimetric assay NMR, colorimetric assay
	Purity	Process-related residuals	GC, colorimetric assay
Carrier protein	Structure	Identity and molecular sized Protein sequence Protein folding Derivatizing groups content	SDS-Page, WB; SEC-UV/-RI/-MALLS, RPC MS CD, F MS, colorimetric
	Purity	Nucleic acids *E. coli* host cell proteins Other proteins as impurities Toxicity, endotoxins Bioburden	Colorimetric assay WB, ELISA SDS-Page, WB; SEC-UV/-F, RPC LAL assay Total viable aerobic count of microbial contamination
Bulk conjugate	Structure	Identity Conjugation proof Conjugated and unconjugated saccharide Conjugated and unconjugated protein *O* -Acetyl Molecular size	NMR, immunochemical methods SDS-PAGE, MS SPE or UF or HIC followed by HPAEC-PAD or colorimetrys analysis,CE SEC-UV/RI/-fluorescence, CE NMR SEC-UV/-RI/-F/-MALLS, MALDI-MS
	Purity	Process-related residuals Endotoxins Bioburden	Colorimetric assay,GC LAL assay Total viable aerobic count of microbial contamination
Formulated vaccine	Structure	Identity Conjugated and unconjugated saccharide Conjugated and unconjugated protein Adjuvan content Preservative content Residual moisture in lyphilized products	WB, immunochemical methods SPE or UF or HIC followed by HPAEC-PAD or colorimetrys analyses,CE, nephelometry SEC-UV/-IR/-F,CE Colorimetric,tritimetric, ICP-MS-AA Specif assay Specif assay
	Purity	Endotoxins Pyrogenicity Sterility	LAL assay Pyrogen test B&F

B&F: bacteriostasis and fungistasis test; CD: circular dichroism; CE: capillary electrophoresys; F:fluorescence; GC: gas chromatography; HPAEC-PAD: high performance anionic exchange cromatography-pulsed amperometric detection; ICP-MS-AA: inductively coupled plasma - mass spectrometry - atomic absorption; LAL: limulus amoebocyte lysate; MALDI: matrix-assisted laser desorpion/ionization; MALLS: multi angle light laser scattering; MS: mass spectrometry; RI refractive index; RPC: Reverse phase chromatography; SEC: size exclusion chromatrography; SPE: solid phase extraction; UV: ultra violet; WB: western blot

Fig.8 - Example of analytical tests for quality control of glycoconjugate vaccines (Costantino et al. 2011)

1.3 Conjugate antigen vaccines targets investigated

In this work, conjugate vaccines against Group A and Group B *Streptococcus*, Meningococcal serogroup A and B and *Candida albicans* infections have been investigated.

1.3.1 *Candida albicans*

Candida albicans is a commensal diploid fungus that can become an opportunistic pathogen agent of oral and genital infections in humans, and moreover it cans origin systemic fungal infections as important causes of morbidity and mortality in immunocompromised patients (HIV-positive, organ or bone marrow transplantation and cancer chemotherapy). The omnipresent presence of *Candida* in human flora and environment permits it to become invasive when the host immunity is compromised. *C. albicans* can easily form biofilms on the surface of implantable medical dentures and catheters and for this reason it is considered one of most important nosocomial disease.

The cell wall of *C. albicans* is composed primarily of the following polysaccharides, mannan, glucan, and chitin. Mannans represent about 23%, glucans 40-60%, proteins 6-25%, lipids 1-7%, and chitins 0.6-9% of the dry weight of the cell wall (Garzons et al. 1989). It is generally agreed that mannan is distributed throughout the cell wall, whereas the inner cell wall is composed mainly of chitin and glucan (Calderone et al. 1991). An interesting aspect is that fungi are characterized by a cell wall rich in carbohydrates in the form of polysaccharides or glycoproteins (Fig.9) (Bowman et al. 2006) and in particular both β-(1,3) and β-(1,6) glucans are highly conserved in the majority of pathogenic fungi for all their life cicle, and therefore it has been considered as an attractive target for vaccine development (Bromuro et al. 2002; Torosantucci et al. 2005).

Component	Structure	Abundance (% cell wall dry weight)	Feature
Glucan	β-(1,3)-D-glucan β-(1,6)-D-glucan	50-60	Conserved. Critical for fungal viability. Contains protective epitope
Glycoproteins	α-(1,2)–D-mannan α-(1,6)–D-mannan β-(1,2)–D-mannan	30-50 (> 50% is CHO)	Might induce blocking antibodies. Variable expression Protective epitope but only in Candida
Chitin	β-(1,4)-D-GluNAc	1-20	Conserved but less exposed

Fig.9 - Sugar composition of fungal cell walls.

Micotic phathongens, such as *C. albicans*, have often a relapsing nature, the development of prophylactic and/or therapeutic vaccines is considered as one of the most appropriate options to fight and prevent this kind of infections, given that antifungal drug resistance is an important problem in the nosocomial infections (Cutler et al. 2007). However, no such vaccine is currently available.

1.3.2 Group A *Streptococcus* (GAS)

Streptococcus pyogenes (group A *streptococcus*) is a species of gram-positive extracellular bacterial pathogens (Mitchell et al. 2003). Group A streptococci colonize the throat or skin and are responsible for a number of suppurative infections and non-suppurative sequelae. It is the most common cause of bacterial pharyngitis and is cause of scarlet fever and impetigo. Generally it is possible to distinguish between throat and skin strains, it became evident that there are serotypes of group A streptococci with a strong tendency to cause throat infection, and similarly, there are other serotypes often associated with impetigo (Carateptis et al. 2005). In the past, GAS was a common cause of puerperal sepsis or childbed fever. Today, the group A streptococcus is responsible for streptococcal toxic shock syndrome, and most recently it has gained notoriety as the "flesh-eating" bacterium which invades skin and soft tissues and in severe cases leaves infected tissues or limbs destroyed. The group A streptococcus has been investigated for its significant role in the development of post-streptococcal infection consequence, including acute rheumatic fever, reactive arthritis, and acute glomerulonephritis. Acute rheumatic fever and rheumatic heart disease are the most serious autoimmune sequelae of group A streptococcal infection and have afflicted children worldwide with severe disability and death (Cunningham et al. 2000, Cunningham et al. 2008). Group A streptococcal infections have recently been associated with Tourette's syndrome, tics, and movement and attention deficit disorders. The Lancefield classification scheme of serologic typing distinguished the beta-hemolytic streptococci based on their group A carbohydrate, composed of N-acetylglucosamine linked to a rhamnose polymer backbone, with alternated α(1,3)- and α(1,2)- bonds (Fig.10) (Pitner et al. 2000, Michon et al. 2005).

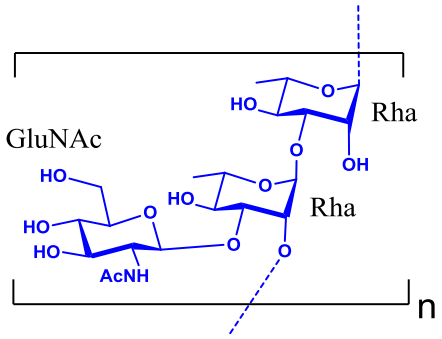


Fig.10 – Repeating unit of GAS cell wall polysaccharide.

Streptococci were also serologically separated into M protein serotypes based on a surface protein that could be extracted from the bacteria with boiling hydrochloric acid.

1.3.3 Group B *Streptococcus* (GBS)

Streptococcus agalactiae (group B streptococci (GBS)) is a significant cause of neonatal meningitis, septicemia, and pneumonia (Schrag et al. 2000), but GBS is not restricted to newborns, and it is increasingly recognized as an important cause of disease in adults, particularly those with underlying disease, and in the elderly (Skoff et al. 2009). The majority (60–70%) of GBS disease in the first three months of life actually occurs in the first six days of life (termed early onset (EO)). EO disease results from vertical transmission of GBS from mother to infant at or around the time of delivery. An estimated 20–30% of pregnant women are colonized with GBS in their Gastro Intestinal and/or genital tracts, approximately 50% of their infants become colonized and approximately 1% of these infants develop disease. Infection occurs rapidly, with clinical features of sepsis or pneumonia at birth or within 12–24 h in over 90% of cases (Heath et al. 2004, Heath et al. 2009). In contrast with EO disease, late onset (LO) disease (7–90 days old) is acquired nosocomially or from community sources and presents with meningitis in up to 50% of cases (Heath et al. 2004). GBS disease is associated with significant morbidity and mortality, GBS meningitis results in long-term neurodevelopmental handicap in 50% of those affected (Bedford et al. 2001). GBS is also a well-recognized cause of stillbirth and is strongly associated with prematurity, although these burdens are harder to

quantify (Schuchat et al. 1999). Out of the ten serotypes present in nature, serotypes Ia, Ib,, III and V (Fig.11a and 11b) account for 96% of neonatal cases in the USA and 88% of adult cases (Phares et al. 2008), with similar distributions seen in other western countries (Weisner et al. 2004).

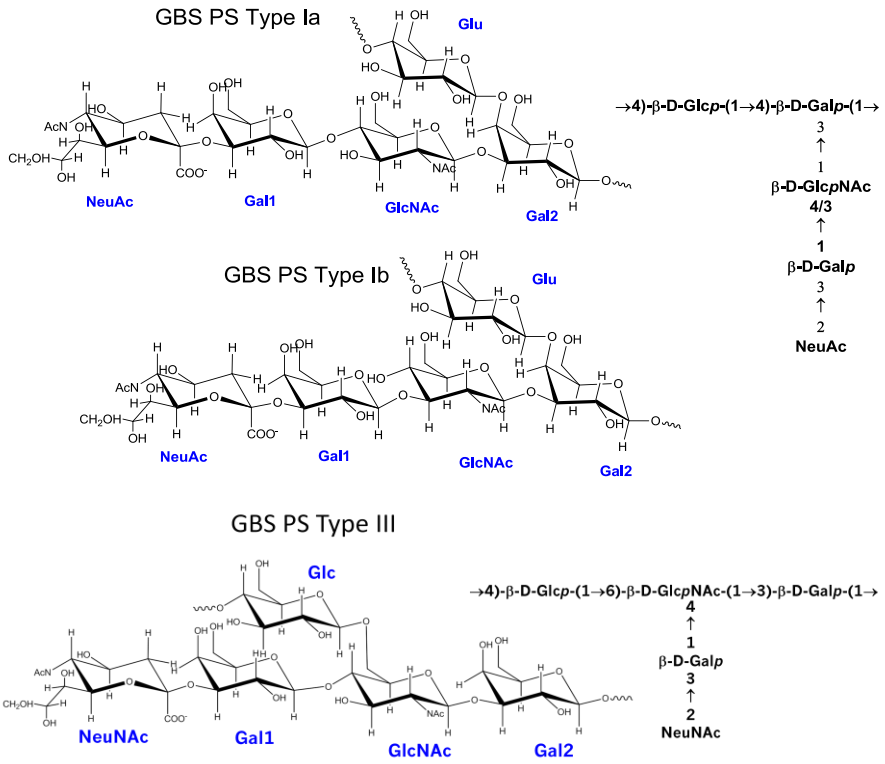


Fig.11a – GBS polysaccharides

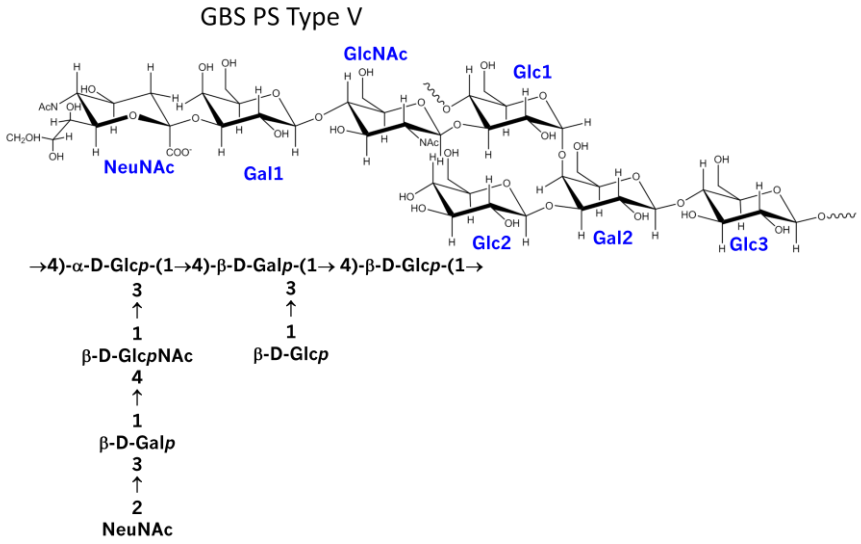


Fig.11b – GBS polysaccharide

1.3.4 *Neisseria meningitidis*

Neisseria meningitidis, the meningococcus, is a Gram-negative bacterium with a coccoid shape, and a pathogenic member of the *Neisseriae* family.

Meningococci are obligate commensals in man and colonize the nasopharyngeal mucosa without affecting the host, a phenomenon known as carriage. In nonepidemic settings, approximately 10% of healthy individuals at any time carry N. meningitidis in the upper airway (Cartwright et al. 1987; Stephens et al. 1999). Rates of transmission and carriage increase in closed and semi-closed populations, such as military recruits, university students and in the household contacts of a case of meningococcal disease (Olcén et al. 1981). The duration of the carrier state varies and it might be chronic, lasting for several months, intermittent or transient (Broome et al. 1986). Occasionally, shortly after the onset of colonization, N. meningitidis strains will penetrate the mucosal membrane and enter the bloodstream, then the bacterium may cause meningitis, severe sepsis with an often fatal outcome, and more rarely, other diseases such as septic arthritis, pneumonia, purulent pericarditis, conjunctivitis, otitis, sinusitis and urethritis (Tzeng et al. 2000).

Meningococcal diseases still now remain a major cause of meningitis and septicemia in infants, children and young adults. These invasive diseases

cause a relevant public health burden throughout the world, with about 500000 cases and more than 50000 deceases every year. Meningococcal strains can be divided into 13 serogroups: A, B, C, E-29, H, I, K, L, M, W-135, X, Y and Z. This subdivision is based on chemical and antigenic differences of polysaccharide capsule expressed by the bacteria. Six of these 13 serogroups, A, B, C, W135, Y and X constitute about all the cases of meningococcal disease. For four serogroups (A, C, W135, Y) (Fig.12) effective polysaccharide-based conjugate vaccines are available.

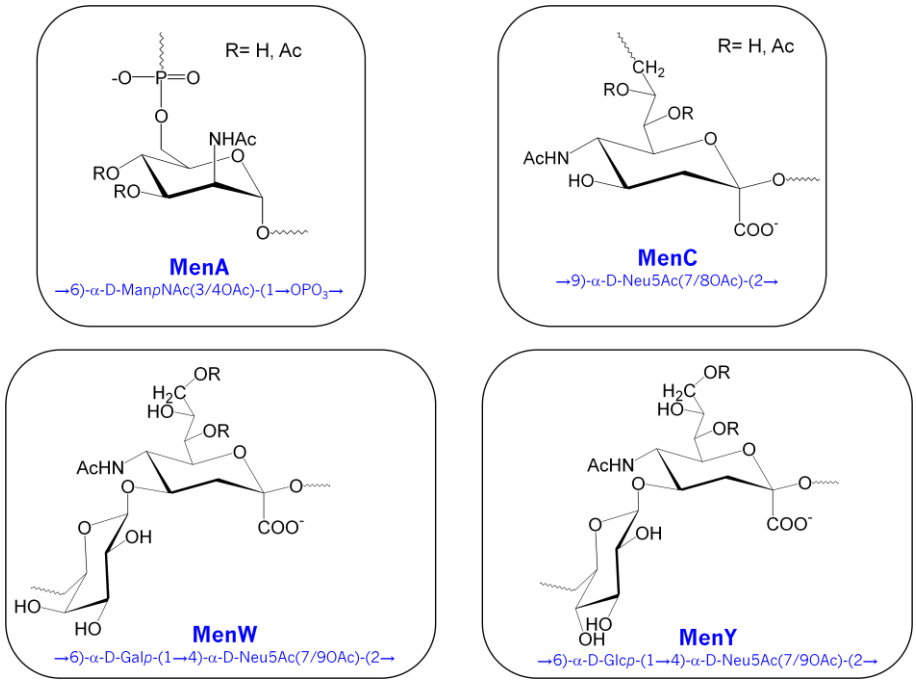


Fig.12 - Repeating units of meningococcal serogroup A, C, W135 and Y capsular polysaccharides.

Unfortunately this approach has not worked so far for the serogroup B which remains the most common cause of meningococcal meningitis in the developed world, so there is a huge requirement for a serogroup B vaccine. The meningococcal serogroup B polysaccharide capsule is composed of a homolinear polymer of α2-8 *N*-acetylneuraminic acid (sialic acid) (Fig13).

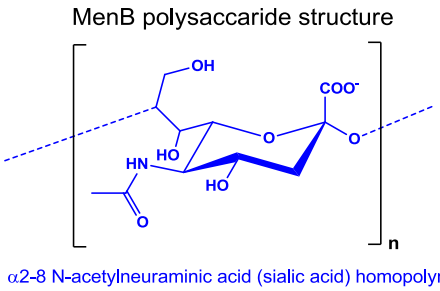


Fig.13 - Repeating unit of meningococcal serogroup B capsular polysaccharide.

The unique chemical structure and antigenic properties of the serogroup B capsule render it poorly immunogenic in humans. This has been attributed to the structural homology of the B polysaccharide to the polysialylated form of the neural cell adhesion molecule in the fetal brain and in small amounts in adult tissues.

2. GLYCOSYLATION SITES CHARACTERIZATION

Glycoconjugates vaccines consist in bacterial polysaccharide covalently attached to several amino acids of an appropriate carrier protein (Fig.14). The activated saccharide antigens are usually attached by a linker or by reductive amination on several carrier lysine residues or on other amino acids which can react with specific functionalities of the carbohydrates.

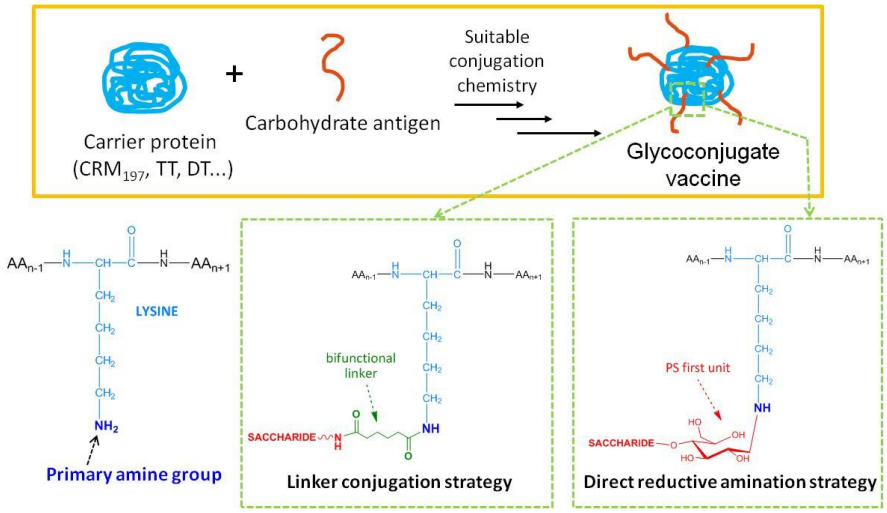


Fig.14 - Schematic representation of the carbohydrate-protein conjugation.

The mapping of glycosylation sites represents a fundamental key aspect to develop coupling procedures for preparation of well-defined conjugate vaccines and to understand the role of glycopeptides in the vaccine immunogenicity (Lai et al. 2009, Avci et al. 2010).

The analytical evaluation and structural characterization of these vaccines are highly dependent from the physicochemical methods (Jones 2005, Bardottti et al. 2008) and the mass spectroscopy undoubtedly represents a very powerful tool for this kind of characterization. A robust analytical procedure, that permits to find which amino acids are glycosylated, is consequently a key methodology.

It could be useful in pharmaceutical field to verify the comparability and reproducibility of different production batch.

The starting point of my PhD thesis was a work published in 2008 (Bardotti et al. 2008) on the characterization of A, C, W135 and Y polysaccharides

conjugated to CRM_{197} (amino acids sequence on Fig.15), wherein the glycosylation site identification was successfully applied.

GADDVVDSSK	SFVMENFSSY	HGTKPGYVDS	IQKGIQKPKS	GTQGNYDDDW	KEFYSTDNKY	60
DAAGYSVDNE	NPLSGKAGGV	VKVTYPGLTK	VLALKVDNAE	TIKKELGLSL	TEPLMEQVGT	120
EEFIKRFGDG	ASRVVLSLPF	AEGSSSVEYI	NNWEQAKALS	VELEINFETR	GKRGQDAMYE	180
YMAQACAGNR	VRRSVGSSLS	CINLDWDVIR	DKTKTKIESL	KEHGPIKNKM	SESPNKTVSE	240
EKAKQYLEEF	HQTALEHPEL	SELKTVTGTN	PVFAGANYAA	WAVNVAQVID	SETADNLEKT	300
TAALSILPGI	GSVMGIADGA	VHHNTEEIVA	QSIALSSLMV	AQAIPLVGEL	VDIGFAAYNF	360
VESIINLFQV	VHNSYNRPAY	SPGHKTQPFL	HDGYAVSWNT	VEDSIIRTGF	QGESGHDIKI	420
TAENTPLPIA	GVLLPTIPGK	LDVNKSKTHI	SVNGRKIRMR	CRAIDGDVTF	CRPKSPVYVG	480
NGVHANLHVA	FHRSSSEKIH	SNEISSDSIG	VLGYQKTVDH	TKVNSKLSLF	FEIKS	535

Fig.15 - CRM_{197} aminoacid sequence with lysine residues in red highlighted.

The analytical strategy which have been employed in published work consists in (Fig.16):

1) unfolding of conjugate in 6M urea, reduction with dithiothreitol (DTT) and alkylation with iodoacetic acid,

2) diafiltration to remove urea 6M and then trypsin digestion of conjugate,

3) the mixture of originated glycopeptides and peptides is enriched in glycopeptides part by a size exclusion chromatography,

4) the saccharide chains of glycopeptides have been cleaved by mild acid hydrolysis.

The final treated glycopeptide fraction was observed and analyzed by UPLC-MS.

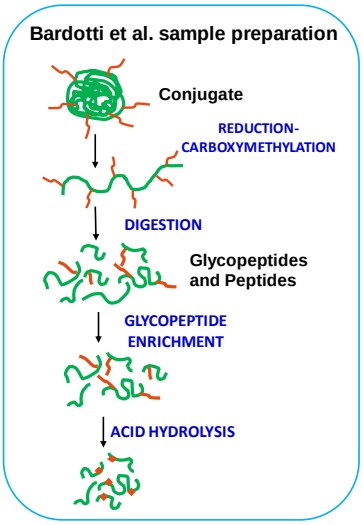


Fig.16 – Analytical scheme for glycosylation sites identification.

The enrichment of the glycopeptides in the pool would be helpful to improve the quality of mass spectrum signal in terms of sensitivity. If

analyzed in a mixture with peptides, the mass signals of glycopeptides are very weak. The issue is due to the worse ionization capacity of saccharide parts of glycopeptides; unlike the peptides signals are very strong and they sature the spectrum.

Furthermore the carbohydrate extracted from bacteria showed a chains with different length (wide polydispersion), to circumvent the issue of poly-dispersity of the coupled saccharide, an acid hydrolysis is performed to normalize the mass increment of the saccharide adduct, by cleaving the inter-residue glycosidic bonds. Only first units of each polysaccharide chain (plus linker if present) should remain bound to the lysine.

From the mass spectrum, the peaks of the modified peptides, with a mass increment, were identified and thus glycosylated lysine residues identified.

2.1 *Candida albicans* conjugates

Glycoconjugates against *Candida albicans* were investigated as first targets. It was considered three different β(1,3)-glucan antigens coupled to CRM_{197} by an adipic acid linker: laminarin, curdlan and a synthetic hexasaccharide (Fig. 17). The conjugations of these carbohydrate antigens are shown in chapter 3.1.

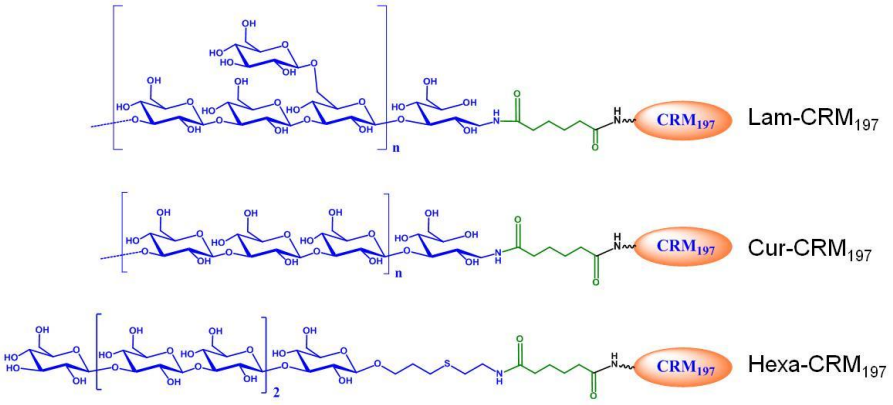


Fig.17 - Candidate vaccines against *Candida albicans.*

Two different approach for the cleaveage of the laminarin, as substrate have been used: 1) acid hydrolysis (HCl 0.1M, 4h, 60°C), 2) enzymatic process by the use of laminarinase. The second approach is very fast, almost instantaneous, while the first one was not enough hard to fully depolymerize.

The *Candida* conjugates have been digest by trypsin (37°C, 17h) in the same reduction-alkilation buffer, using a different reduction-alkylation procedure respect to the reference paper (Bardotti et al. 2008):

1) The protein unfolding has been performed by the use of a commercial surfactant, RapiGest SF (0.05-0.1% in 50mM of ammonium bicarbonate), instead of 6M urea buffer, while the reduction has been obtained with the same reactant, DTT.

2) The alkylation has been obtained by the adding the iodoacetamide (instead of iodoacetic acid).

The use of RapiGest SF have permitted to simplify the process, the same buffer solution (NH_3HCO_3 50mM + 0.05-0.1% RapiGest) has been used for reduction-alkilation and digestion phases, on the contrary in the previous published procedure (Bardotti et al. 2008) the reduction-alkylation was performed in urea 6M solution while trypsin digestion was in sodium phosphate buffer $(NaP)_i$ 10mM pH 7.2.

The buffer exchange step, carried by diafiltration, was one of critical point in which the conjugate could precipitate, losing partially or completely the sample material. The elimination of this step in our new procedure by the use of Rapigest, have enhanced the efficiency of the protocol, permitting the use of a lower amount of glycoconjugate as starting material (500µg to 300µg) reducing sample preparation time. After 17h trypsin not only has digested the glycoconjugate but it is inactivated by self digestion, thus it has been possible using directly another enzyme in the same reaction mixuture; in fact for the cleavage step of Candida conjugates an enzymatic digestion on glycopeptide/peptide mixture has been performed with the enzyme laminarinase (4h, 37°C).

Enzymatic and acidic treatments were previously tested to evaluate the cleavage step of laminarin and curdlan. Laminarinase is able to cut the (β1,3)-glucose-glucose bond, and the reaction on laminarin and curdlan, monitored by NMR, has been very efficient and rapid, almost instantaneous; otherwise the mild acidic treatment was slower. Accordingly with this result and the fact the acid hydrolysis can partially degrade the peptide moieties, the enzymatic cleavage solution by laminarinase has been preferred.

Last step before UPLC-MS analysis was RapiGest precipitation by very mild acid hydrolysis and then eliminated by centrifugation saving only supernatant.

The mixture was analyzed by UPLC-ESI-QTof instrument equipped with a BEH300 C18 100mm column. The acquired spectrum was finally processed by software *Biopharmalynx 1.2* (Waters) to find modified peptides that correspond to previous glycosylated peptides.

The main inputs to this software are the carrier protein sequence, the enzyme used for the digestion and the mass increments sought. In this case four masses have been set: the fully cleaved structure and the other three with some oligosaccharide residues. We tested four masses for hexasaccharide conjugate (Fig.18) and four masses for laminarin and curdlan ones (Fig.19).

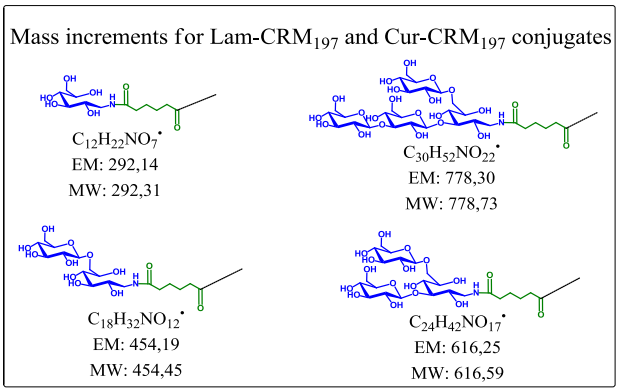


Fig. 18 - Mass increments for laminarin- and curdlan- conjugates.

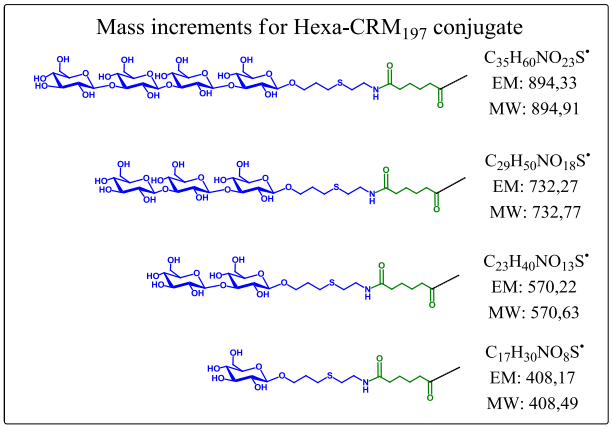


Fig. 19 - Mass increments for hexa-conjugate.

2.1.1. Materials and methods

Dithiothreitol (DTT), iodoacetamide, *laminarinase*, HCl, ammonium bicarbonate (AB), NH_3HCOOH, were purchased from Sigma-Aldrich, RapiGest SF from Waters, trypsin from Promega.

Analyzed samples have been Cur-CRM_{197} lot 8 and lot 9, Lam-CRM_{197} lot RS3002, Hexa-CRM_{197} lot 2.

Evaluation of efficiency of cleavage step with Laminarinase

^1H NMR spectra of two solutions of laminarin and curdlan both 1 mg/ml in AB 50mM deuterated were collected. Samples were treated with *laminarinase* (0.05mg in deuterated aqueous solution) and only in few minute, (about ten 10 minutes), the enzyme has cleaved almost all the β(1,3) bonds (Fig. 20) as shown below.

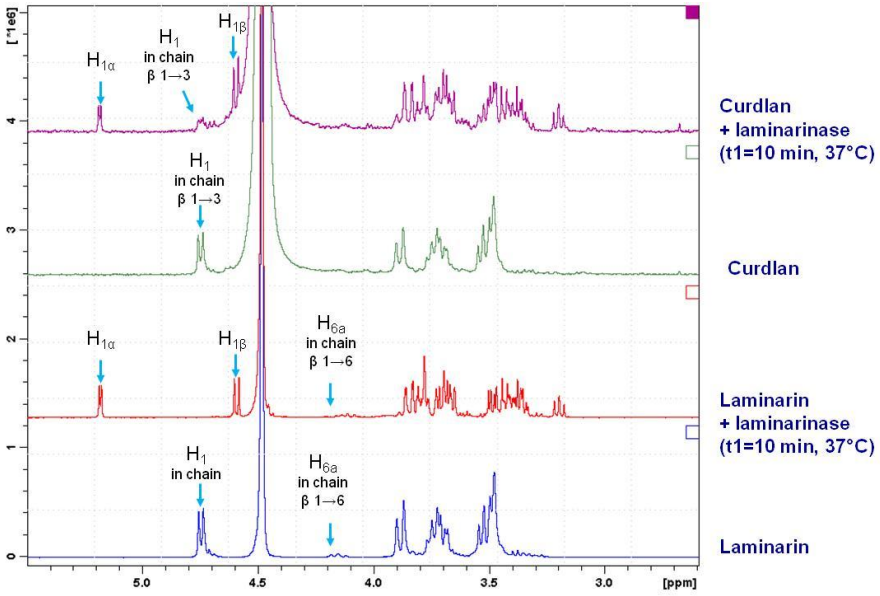


Fig. 20 – Proton NMR profiles collected on Laminarin and Curdlan treated with enzyme.

H_1 in chain disappear after the enzymatic treatment and H_1 (alfa and beta) appeared to show the cleavage. This enzyme has demonstrated an very high efficiency, an almost instantaneous action for cleavage of β(1,3) linkages. In any case the samples were monitored by NMR for many hours.

Reduction-alkylation and Trypsin digestion

300µg of conjugates have to be diafiltrated and concentrated with AB 50mM using a Amicon 30k MWCO filter and concentrated until to 125µl.

Sample has been transfer in a 1.5ml eppendorf. Rapigest has been resuspended (0.2%) in AB 50mM and 50µl of this concentrated Rapigest has been added to sample to obtain a final concentration of 0.06-0.07% of surfactant.

The sample was fluxed with N_2 (1 minute) and then 2µl of DTT 0.5M aqueous solution has been added. The reaction was left for 1 hour in a water bath at 60°C, and then the sample was left at RT temperature to cool down.

6µl of a aqueous solution of iodoacetamide 0.5 M has been added to the sample (30 minutes at RT in the dark).

Then a vial of trypsin (20µg) was resupended with 40µl of own acetic acid buffer and 4µg (8µl) were added to the sample and left for 17h at 37°C.

Saccharide cleavage for *Candida albicans* conjugates.

A solution of laminarinase was prepared with 50mM AB, about 0.1mg dissolved in 30µl of buffer. The solution was added to the mixture of peptides and glycopeptides and it has been carried out for 4 hours at 37°C. Sample has been dried.

Rapigest precipitation

Digested sample, has been dried by SpeedVac, resupended with 100µl TFA 0.5%, the reaction has been performed for 30 minutes at 37°C and then centrifugated for 1 minute at 12K g. Surnatant has been withdrawn and filtered with GHP Acrodisc filter 13mm 0.2µm; afterward it has been dried by speedvac (45°C), resuspended with 120µl of AB 50mM and frozen.

UPLC-MS analysis

The ultra-pressure liquid chromatographer was equipped with a BEH300 C18 100mm column.

The eluents were A=milliQ water plus 0.1% of formic acid, B= acetonitrile plus 0.1% of formic acid.

The LC run time was 80 minute and the gradient has been (Tab.1):

time [min]	A%	B%
0	98	2
5	50	50
60	50	50
65	2	98
72	2	98
77	98	2
80	98	2

Tab 1 - Eluent gradient used for the analysis

The elution of peptides, modified or not, has been shown between 0 and 60 mitutes; from 60 to 80 minutes column washing and rebalancing occur.
The mass equipment has calibrated with sodium cesium iodide (NaICs) calibrant ranging from 130 to 2500 dalton and each analysis was performed with lockmass with sodium acetate standard.
Lockmass is a technology of Waters that increases the quality of analysis in terms of analytical error.
The applied analytical mehod ESI-QTof used was composed of 2 functions:
First function ES+:
-acquisition time: 0 to 80 min
-mass range: 80-3000 dalton
-sample cone energy: 30V
-collision cell energy: 5V
Second function ES+:
-acquisition time: 0 to 80 min
-mass range: 80-3000 dalton
-sample cone energy: 200V
-collision cell energy: 5V
The first acquire a spectrum with a low collision energy, the second one acquires with high value that permits a fragmentation of peptides

Data Analysis
The data have been processed by software *Biopharmalynx 1.2* (Waters); this software is helpful in the search of modified peptides resulting from protein digestion. The request parameters used for this software are:

- amino acid protein sequence
- employed enzyme to perform *in silico* digestion
- number of missed cleavage
- data of modification(s) that have to be sought (mass value, amino acid site, frequency)
- accuracy threshold to set the error for the peak assignment (delta between theoretical and experimental mass peak)

All conjugates of *Candida albicans*, GBS and MenA analyzed were prepared with CRM_{197} carrier and employed enzyme for the digestion was always trypsin. CRM_{197} sequence, formed of 535 amino acids, was reported below:

GADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWKEFYSTDNKYDAAGYSVDNENPLSGK
AGGVVKVTYPGLTKVLALKVDNAETIKKELGLSLTEPLMEQVGTEEFIKRFGDGASRVVLSLPFAEGSSSVEYINNWEQA
KALSVELEINFETRGKRGQDAMYEYMAQACAGNRVRRSVGSSLSCINLDWDVIRDKTKTKIESLKEHGPIKNKMSESP
NKTVSEEKAKQYLEEFHQTALEHPELSELKTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSILPGIGSV
MGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDIGFAAYNFVESIINLFQVVHNSYNRPAYSPGHKTQPFLHD
GYAVSWNTVEDSIIRTGFQGESGHDIKITAENTPLPIAGVLLPTIPGKLDVNKSKTHISVNGRKIRMRCRAIDGDVTFCRP
KSPVYVGNGVHANLHVAFHRSSSEKIHSNEISSDSIGVLGYQKTVDHTKVNSKLSLFFEIKS

Fig. 21 - CRM_{197} aminoacid sequence.

The trypsin used for the analyses, cleaved after all not modified lysine residues. It's also necessary to set the number of missed cleavage sites that software considers to generate *in silico* peptides. The sought glycopeptide lysine residues are always modified and by the setting of 3 missed cleavages it could be possible to identify peptides having up to three internal modified lysine residues.

The structure and value of data modification were reported above; all the mass increment values, set in the software, have been reported subtracted of one proton, respect to the mass reported in the figures 18 and 19. It is due to the loss of lysine amino proton during the coupling reaction.

The accuracy threshold, expressed in ppm, represents the maximum mass difference error between theoretical mass and real mass spectrum value that software considers for peak assignation; considering various test analysis of trypsin digestion standard, a value of 60ppm was selected. The chromatograms of ULPC-MS analysis were processed and then results have been refined to eliminate false positives results. No terminal lysine can be glycosylated because trypsin can only cut after unmodified ones, all the peptides with terminal glycosylated lysine have to be discharged.

2.2.2 Results and discussion

protein coverage	78.2%		78.1%		80.3%		79.4%	
Lysine + N-t	Cur-CRM lot 8		Cur-CRM lot 9		Lam-CRM lot RS3002		Hexa-CRM lot2	
N-t	■	■	■	■	■		■	■
K10					■			
K24	■				■		■	■
K33								
K37	■	■	■	■	■		■	■
K39	■	■	■	■	■		■	■
K51	■	■	■	■	■		■	
K59	■	■	■	■	■		■	■
K76	■	■	■	■	■		■	■
K82	■	■	■	■	■			■
K90		■	■	■	■		■	■
K95	■	■	■	■	■		■	
K103	■	■	■	■	■		■	■
K104		■	■	■	■		■	■
K125	■	■	■	■	■			■
K157	■	■	■					
K172	■	■	■	■	■		■	■
K212	■	■	■	■	■		■	■
K214	■	■	■	■	■		■	■
K216	■	■	■	■	■		■	■
K221	■	■	■	■	■		■	■
K227	■	■	■	■	■		■	■
K229	■	■	■	■	■		■	■
K236	■	■	■	■	■		■	■
K242	■	■	■	■	■		■	■
K244	■	■	■	■	■		■	■
K264								
K299								
K385								
K419			■	■	■		■	
K440	■	■	■	■			■	■
K445	■	■	■	■	■			
K447	■	■	■	■	■		■	■
K456	■	■	■	■	■		■	■
K474								
K498	■	■	■	■			■	■
K516	■	■	■	■	■			
K522	■	■	■	■	■		■	■
K526	■	■	■	■	■		■	■
K534	■	■			■			

Tab 2 - Lysine glycosilation distribution of *C. albicans* conjugates

In table 2 is summarized the result for glycosilation sites characterization of *Candida albicans* conjugates. For all the sample seems that distribution has been similar. The mass spectra showed a very good quality, with high signal peaks and the peptides protein coverage was satisfactory.

2.2 GAS conjugate

To test the published procedure on a different conjugate but with the same carrier protein, the CRM_{197}, characterization of Group A *Streptococcus* (GAS) (Fig.22) has been another target.

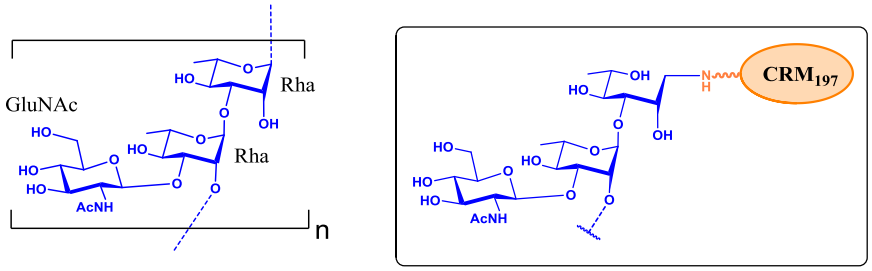


Fig. 22 - GAS PS repeating unit (on left) and GAS conjugate (on right).

This conjugate was previously prepared by direct reductive amination between the native polysaccharide and primary amino group of lysine residues.

The GAS polysaccharide consist in a linear polyrhamnose backbone with alternated α(1,3)- and α(1,2)- bonds, on which are present *N*-acetyl-glucosamine branches (Fig.22)

The conjugate has been reduced and carboxymethylated in denaturing condition (urea 6M) and then digested by trypsin. The enrichment was performed by a SEC column, Sephadex peptide PC 3.2/30; first run was performed to know the elution profile of glycopeptides, for the other runs the peak of glycopeptides was collected. A lectin enrichment by concanavalin A (Con A) resin kit was also tested but the result were not so good.

Following published procedure, this material was treated in mild acidic condition (0.1M HCl, 60°C, 4h) to cleave the GAS saccharide chains bound to glycopeptides; only the firs carbohydrate unit should remain attached to lysine residues (Fig.23).

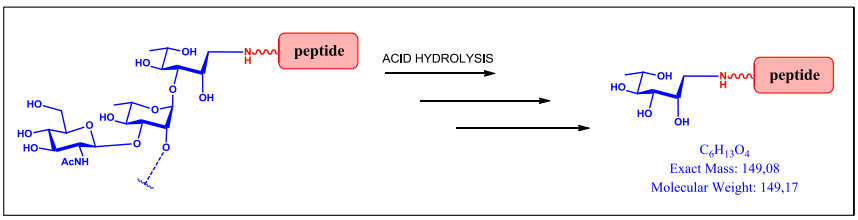


Fig.23 - Putative reaction for GAS saccharide cleavage.

The mixture was finally analyzed by UPLC-ESI-QTOF instrument; a table with mass incremented peptides (+148.08 Da) has been prepared from *in silico* trypsin digestion of CRM_{197} sequence, to identify the glycosylated lysine residues distribution.

From the first analytical session, performed without the use of data processing software Biopharmalynx, seven lysine of CRM_{197} (K39, K51, K59, K76, K90, K125 and K212) would have been identified as glycosylation sites.

The same analysis was repeated, except for the longer acid hydrolysis time (6h instead of 4h), and three glycosilated lysine were found, K39, K51 and K125.

The glycopeptides assigned have been also confirmed by using the software *Biopharmalynx 1.2* (Micromass - Waters. In addition the automated analysis carried out using this software detected many other peaks which could be associated to glycopeptides containing saccharide chains longer than one residue as supposed in the scheme of Fig.23.

At this point it has been necessary to verify the efficiency of hydrolysis condition on GAS oligosaccharide chains to properly understand if only monosaccharide units were formed. To investigate the hydrolysis step of GAS polysaccharide, a matrix set of 3x3 experiments has been planned: three different HCl concentrations (0.1 M, 0.2 M and 0.4 M) and three different reaction times (4 h, 8 h and 16 h). These nine analytical samples were analyzed by high performance anionic exchange chromatography interfaced with a pulsed amperometric detector (HPAEC-PAD) to measure the produced quantity of rhamnose and N-acetyl-glucosamine monomer. The results (Fig.22 and 23)confirmed that the hydrolysis condition used in the first experiments was not strong enough to fully depolymerized the saccharide chains leaving only one monosaccharide unit attached to the peptide moiety. Neither the strongest hydrolysis condition used (0.4 M HCl, 16 h) was not enough to get it. Only the GluNAc was partially cleaved and the percentage is proportional to reaction time and to acid concentration, instead the amount rhamnose was always under detection threshold in every reaction condition (Tab.3A and Tab.3B).

Rha HYDROLYZED

TIME	HCl CONCENTRATION 0,1M	0,2M	0,4M
4h	< 5 %	< 5 %	< 5 %
8h	< 5 %	< 5 %	< 5 %
16h	< 5 %	< 5 %	< 5 %

A

GluNAc HYDROLYZED

TIME	HCl CONCENTRATION 0,1M	0,2M	0,4M
4h	2,0%	6,8%	13,0%
8h	5,5%	10,7%	26,2%
16h	8,3%	12,0%	37,4%

B

Tab 3 – Percentage of GAS polysaccharide cleavage to monosaccharide units (A Rha; B GlcNAc) after acid hydrolysis.

Due to unsuccessfully efforts to get a complete hydrolysis, it has been decided to abandon the acid cleavage and to approach an enzymatic way.
First test was the use of naringinase, from *Penicillium decumbens*, on GAS polysaccharide. This enzyme has both a primary α-L-rhamnosidase (E.C. 3.2.1.40) and a secondary β-D-glucosidase (E.C. 3.2.1.21) activities, (Michon et al 1989). Unfortunately this tentative did not carry to good results and we can was suppose that GAS polysaccharide has a big structure whereas natural substrates are small molecules (e.g. nariginin), in fact the polysaccharide may not get in active site.

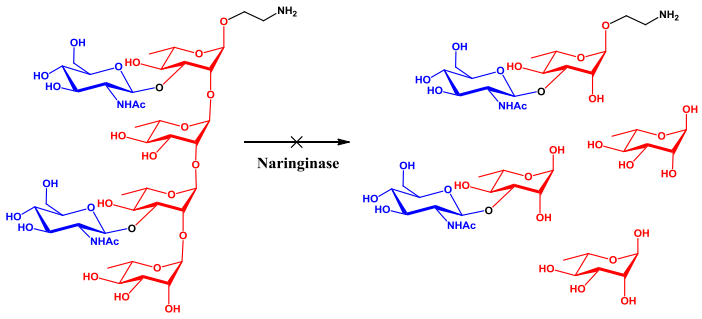


Fig.24 – Naringinase treatment on the molecule that mimics a GAS PS repeating unit.

A smaller synthetic molecule, purchased from Ancora Pharmaceutics, was used as model compound to test naringinase activity monitoring it by NMR. This molecule consists in two GAS PS repeating units equipped with a linker. We wanted to understand if naringinase could be able to cleave α(1,2)- and α(1,3)- rhamnose-rhamnose bonds, but also in this case the enzyme could not fully depolymerize the molecule (Fig.24). It was deduced that the enzyme could stop by the GluNAc branches present on the rhamnose backbone; thus a double digestion strategy was tried: first step was a reaction with β-N-Acetylglucosaminidase (from *Canavalia ensiformis*) to remove GluNAc residues and second step to depolymerize rhamnose scaffold by *naringinase* (Fig.25).

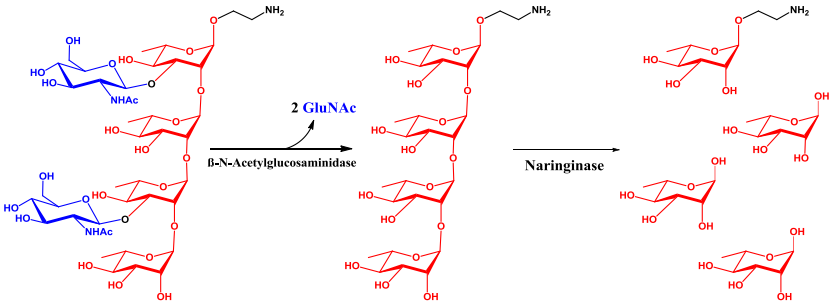


Fig. 25 – Double enzymatic strategy on the molecule that mimics a GAS PS repeating unit.

By this strategy it was possible to degrade the structure of the molecule. The double digestion was applied to GAS polysaccharide, cleavage of GlcNAc was achieved but with a longer reaction time, 3 day instead of few hours, but treating the de-GlcNAc-material with *naringinase*, the depolymerization was not obtained. Unfortunately it was decide to abandon the glycosylation sites characterization of GAS glycoconjugate, without an effective saccharide cleaving it is not possible to apply our analytical strategy.

2.2.1 Materials and methods

β-N-Acetylglucosaminidase and *naringinase* were purchased from Sigma-Aldrich, synthetic hexasaccharide was purchased by Ancora Pharmaceutical.

Determination of hydrolysis percentage of GAS polysaccharide by HCl

A stock solution of GAS polysaccharide (13µg/ml) was prepared; then to determinate the effect of HCl treatment on GAS polysaccharide a set of 9

experiments on GAS PS has been performed: three different HCl concentrations (0.1 M, 0.2 M and 0.4 M) combined with three different reaction times (4 h, 8 h and 16 h). An hydrolysis of GAS PS stock solution in TFA 4M at 100°C for 2h was made to obtain a reference control of full hydrolysis (100%) (obtaining only Rha and GlcNAc monomers). Sample after hydrolysis were dried by Speedvac (3h at 60°C, then ON at RT) and then resupended in milliQ water and filtered 0.45 µm for HPAEC analysis.

The effect of each hydrolysis has been detected by ionic chromatography using a CarboPac PA1 column (50mm×250mm) coupled to a CarboPac PA1 guard column and connected to a Dionex ICS3000 system.

The separation was performed with a flow rate of 1 mL/min using isocratic elution of 40 mM NaOH for 12 min, followed by a washing step with 500 mM NaOH for 5 min. The chromatography was monitored using the pulsed amperometric mode with a gold working electrode and an Ag/AgCl reference electrode, a quadruple-potential waveform for carbohydrates was applied. The chromatographic data were processed using Dionex ChromeleonTM software. Calibration curve, treated as samples, was built up with rhamnose and *N*-acetyl-glucosamine (Fluka) as standards in the range 0.5 - 10.0 µg/mL.

The following table reports the percentage of hydrolysis of each methods respectively for Rha and GlcNAc. Value of "<5%" for Rha indicates that its amount is under minor value of standard curve (Tab 1A).

2.2.2 Results and discussion

Due to the great stability, versus acid condition, of polyrhamnose backbone of GAS PS,it was not possible to obtain a valid procedure to characterize the glycosylation sites distribution. Neither a double enzymatic solution was useful. Another line of research to follow, to be sure of the cleavage efficacy, could be the use another rhamnosidases, instead of the *naringinase*.

2.3 GBS conjugates

The third approach was the glycosilation sites characterization of Group B Streptococcus (GBS) conjugates, in particular type Ia, Ib, and III (Fig.26).
The conjugation of the GBS polysaccharides is shown in chapter 3.3.

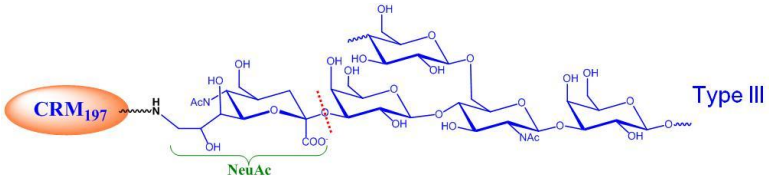


Fig. 26 - CRM_{197}-GBS Type III conjugate schematic structures as example.

The published analytical procedure, for GBS conjugates, was slightly modified to avoid gelification/precipitation problem. Applying the published procedure (Bardotti et al. 2008), during the sample concentration (diafiltration) the glycoconjugates were precipitated forming a transparent gel, probably due to the agglomeration of large GBS polysaccahrides (Ia 260, Ib 230, III 137, V 145 Kdalton). To avoid this issue our first step was the mild hydrolysis with acid condition (HCl 0.1M, 4h, 60°C) to cleave bond between sialic acid that is coupled to lysine residue and galactose. Then the sample was exchanged with ammonium bicarbonate buffer 50mM using a vivaspin500 10K MWCO to eliminate acid; finally the RapiGest surfactant was added.
Trypsin digestion was performed to obtain the mixture of peptides and modiefid peptides; the Rapigest was precipitated by soft acid condition (0.5% TFA, 37°C, 30 min) and wasted while surnatant, containing samples was maintained. A UPLC-MS was acquired with the same condition of *Candida albicans* and MenA conjugates. The mass increments for GBS sample were two (Fig.27):

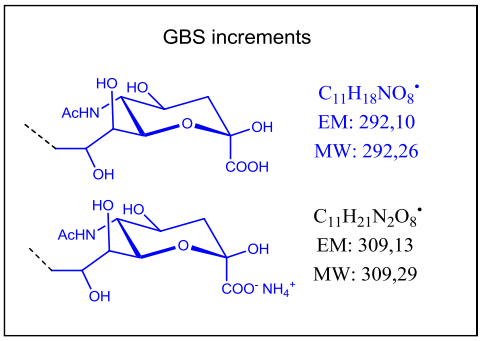


Fig.27 - GBS mass increments.

The first one corresponds to the cleaved sialic acid, the second ones is the same structure but with a ammonium bicarbonate counter ion. The table distribution of glycosylation sites for *Candida albicans*, MenA and GBS is reported in the results chapter.

2.3.1 Materials and methods

The sample analyzed were GBS Ia-CRM_{197} lot RS2502, GBS Ib-CRM_{197} lot RS2602, GBS III-CRM_{197} lot 2702.

Sample preparation was different respect Candida and MenA; the acid cleavage condition was the same as MenA conjugate.

The LC-MS analysis was the same of the other conjugates except for the mass increments (Fig.27).

Evalutation of HCl hydrolysis of GBS type III and V PS

Every GBS polysaccharide (Ia, Ib, II, III and V) is conjugated to carrier by a reductive amination between an oxidized sialic acid (NeuAc) and a lysine residue of CRM_{197} protein. The next residue, for each GBS type, after sialic acid is always a galactose, linked β(2,3).

Two mild acid (0.1M, 60°) hydrolysis have been performed on GBS PS type III and type V in deuterated solution [1mg/ml] and monitored by ^1H NMR. Sialic acid H_3 proton signals were employed to monitor the hydrolytic cleavage. In particular equatorial H_3 signals of bounded sialic acid were disappeared after 4h, while free sialic acid $H_{3\ eq}$ have been dectected (Fig.28). The β(1,3) linkage between the sialic acid and galactose has demonstrates to be enough labile in mild acid condition.

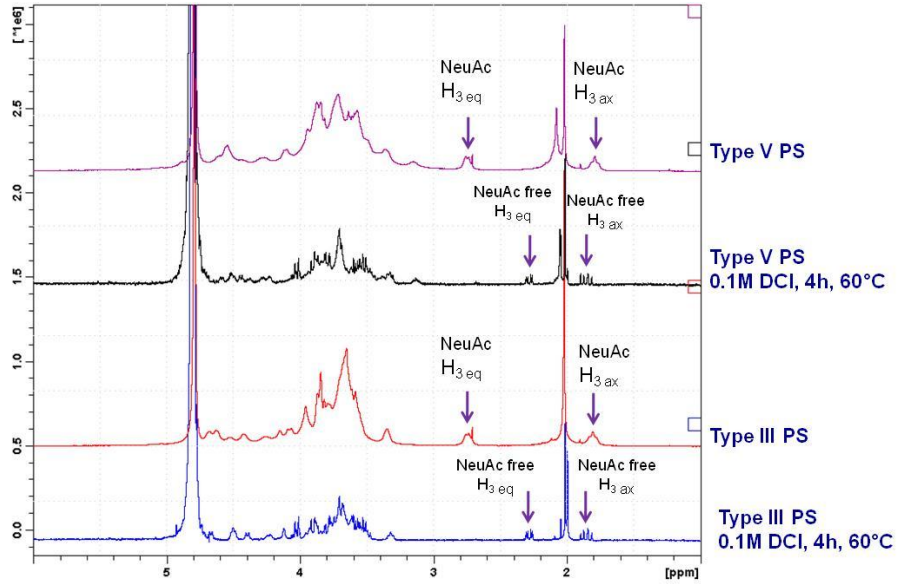


Fig.28 - Proton NMR profiles collected GBS polysaccharides after acid treatment.

Saccharide acid cleavage, reduction-alkylation and trypsin digestion for GBS conjugates

Cleavage: 300µg in protein of conjugate have been concentrated to a final volume of 380µl by Vivaspin500 30K MWCO 20µl of HCl 2M has been added and then gently stirred. Sample has been kept for 4 hours at 60°C and then cooled at room temperature.

1ml of AB 50mM was added and the sample was washed with AB 50mM pH 7 on a Vivaspin2 10K MWCO until the eluted buffer reached the same pH of the same of the washing solution); the final volume was about 200µl after washing.

Reduction-Alkylation: 62µl of RapiGest 0.2% (in AB 50mM) were added to cleaved GBS conjugate (200µl), to reach the final concentration of 0.065%.

The eppendorf with the sample was fluxed under N_2 for 1 minute and then 3µl of DTT 0.5M aqueous solution has been added. The mixture has been kept at 60° in a water bath for 1 hour. 9µl of a aqueous solution of iodoacetamide 0.5 M have been added to the sample, the mixture has been in the dark for 30 minutes at RT.

Digestion: 4µg of trypsin dissolved in 10µl of acetic acid buffer have been added to the sample (17 hours at 37°C). After reaction time Rapigest has been removed by described procedure in Candida section.

UPLC-MS analysis

The LC eluition protocol and mass analytical settings procedure were the same of *Candida albicans* conjugates.

Data Analysis

The data analysis, performed with *Biopharmalynx 1.2,* was the same of except that concerns mass increment values (Fig. 27).

2.3.2 Results and discussion

protein coverage	80.4%		77.2%		82.2%	
Lysine + N-t	GBS Ia-CRM RS2502		GBS Ib-CRM RS2602		GBS III-CRM RS2702	
N-t						
K10						
K24						
K33						
K37	■	■			■	■
K39	■	■			■	■
K51						
K59					■	
K76		■			■	■
K82		■			■	■
K90	■	■		■	■	■
K95	■	■		■	■	■
K103		■				
K104	■	■			■	
K125	■	■			■	■
K157						
K172	■	■		■	■	■
K212			■	■		
K214			■	■	■	■
K216					■	■
K221	■	■	■	■	■	■
K227	■	■	■	■	■	■
K229	■	■	■	■	■	■
K236	■	■	■	■	■	■
K242		■				
K244						
K264						
K299						
K385						
K419						
K440						
K445						
K447						
K456	■	■			■	■
K474						
K498			■	■	■	■
K516			■	■	■	■
K522	■	■	■	■	■	■
K526	■	■	■	■	■	■
K534	■	■	■	■	■	■

Tab.4 - Lysine residues glycosylation distribution of GBS conjugates.

In table 4 has been summarized the result for glycosylation sites characterization of GBS conjugates.

GBS type Ia and III conjugates have been analyzed and their distribution appeared similar.

The coverage obtained, reported in the Table 4, was good, but unfortunately the mass spectra quality was not excellent, probably due to the presence of the entire PS. There was not total depolymerization but only the cleavage of bond between sialic acid and next galactose residues.

2.4 MenA conjugates

A similar procedure was successfully applied for a meningococcal group A conjugate (Fig.29).

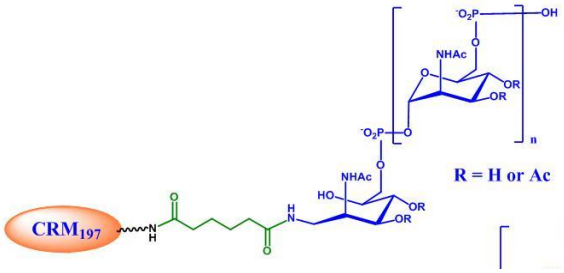


Fig.29 - CRM_{197}-MenA conjugate schematic structure.

The differences, in comparison to the Candida samples, were the acidic cleavage of saccharide chain (0,1M HCl, 4h, 37°C) and obviously the mass increments values that have been searched by software. Due to its own lability, a mild acid hydrolysis was sufficient to depolymerize MenA polysaccharide. In these analyses the precipitation of RapiGest surfactant occurred during the acid cleavage step. Considering the different possibilities of acetylation, the mass increments for MenA conjugate, utilized in *Biopharmalynx* , have been shown in figure30.

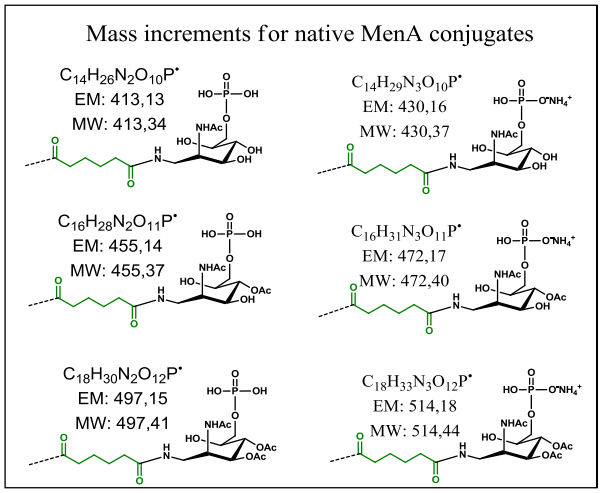


Fig. 30 - MenA mass increments.

2.4.1 Materials and methods

The reactants were the same of Candida section.

MenA-CRM_{197} lot GFB001A has been analysed.

Sample preparation and analysis have been same one reserved for Candida samples, except for saccharide cleavage and mass increments sought (Fig. 30).

Saccharide acid cleavage for MenA conjugates and Rapigest precipitation

Digested sample has been dried by SpeedVac and resupended with 380µl of milliQ waters and transferred to a 2ml glass vial. 20µl of HCl 2M have been added and the mixture has been stirred. Sample has been kept for 4 hours in an oven at 60°C and then cooled in refrigerator at 4°C.

During the acid step the RapiGest was precipitated; the material was centrifuged and the surnatant filtered with a GHP Acrodisc filter 13mm 0.2µm. At the end sample has been evaporated in a 1.5ml eppendorf by Speevac (45°C), resuspended in 120µl of AB 50mM and ready to be analyzed by UPLC-MS.

UPLC-MS analysis

The analytical methods for LC and MS have been the same employed for *Candida albicans* conjugates analysis.

Data Analysis

The parameter set in Biopharmalynx were the same used for *Candida albicans* analysis expect for mass increments (Fig.30).

2.4.2 Results and discussion

protein coverage	71.2%
Lysine + N-t	MenA-CRM lot GFB001A
N-t	
K10	
K24	
K33	
K37	
K39	
K51	
K59	
K76	
K82	
K90	
K95	
K103	
K104	
K125	
K157	
K172	
K212	
K214	
K216	
K221	
K227	
K229	
K236	
K242	
K244	
K264	
K299	
K385	
K419	
K440	
K445	
K447	
K456	
K474	
K498	
K516	
K522	
K526	
K534	

Tab 5 - Lysine residues glycosylation distribution of MenA conjugate.

In table 5 has been summarized the result for glycosylation sites characterization of MenA conjugate.

2.5 General discussion

A procedure to characterize the distribution of glycosylation sites has been developed and optimized following the published paper by Bardotti *et al* for the characterization on meningococcal conjugates.

A double scheme that summarizes and compares the preparation of two samples is reported in Fig.31.

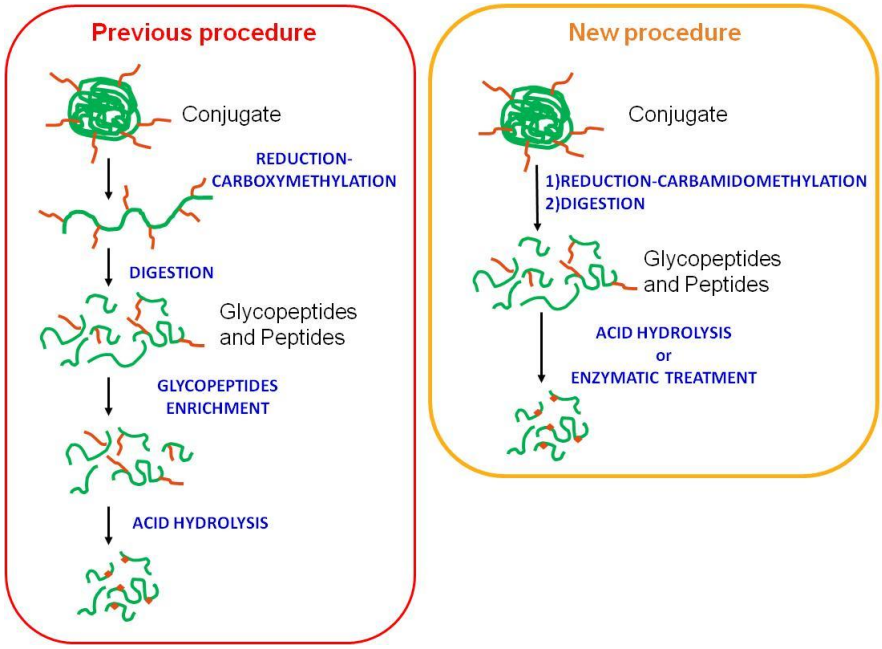


Fig.31 - Comparison of analytical sample preparations.

The old procedure needs of 500µg in protein of starting material consists in several steps: 1) a reduction-carboxymethylation with DTT, 2) iodoacetic acid in denaturing urea 6M buffer, 3) a digestion with trypsin in NaP_i 50mM pH 7.2 buffer, a size exclusion chromatography to improve in terms of glycopeptides enrichment, 5) an acid hydrolysis to cleave saccharide antigen chains.

The new procedure has been optimized as follow:

1) The reduction-alkylation and digestion have been performed in the same buffer (50mM AB) by the use of commercial RapiGest surfactant (0.05-0.1%), and with the presence of DTT and iodoacetamide *in situ.* The buffer exchange step from urea 6M to sodium phosphate buffer, necessary in the

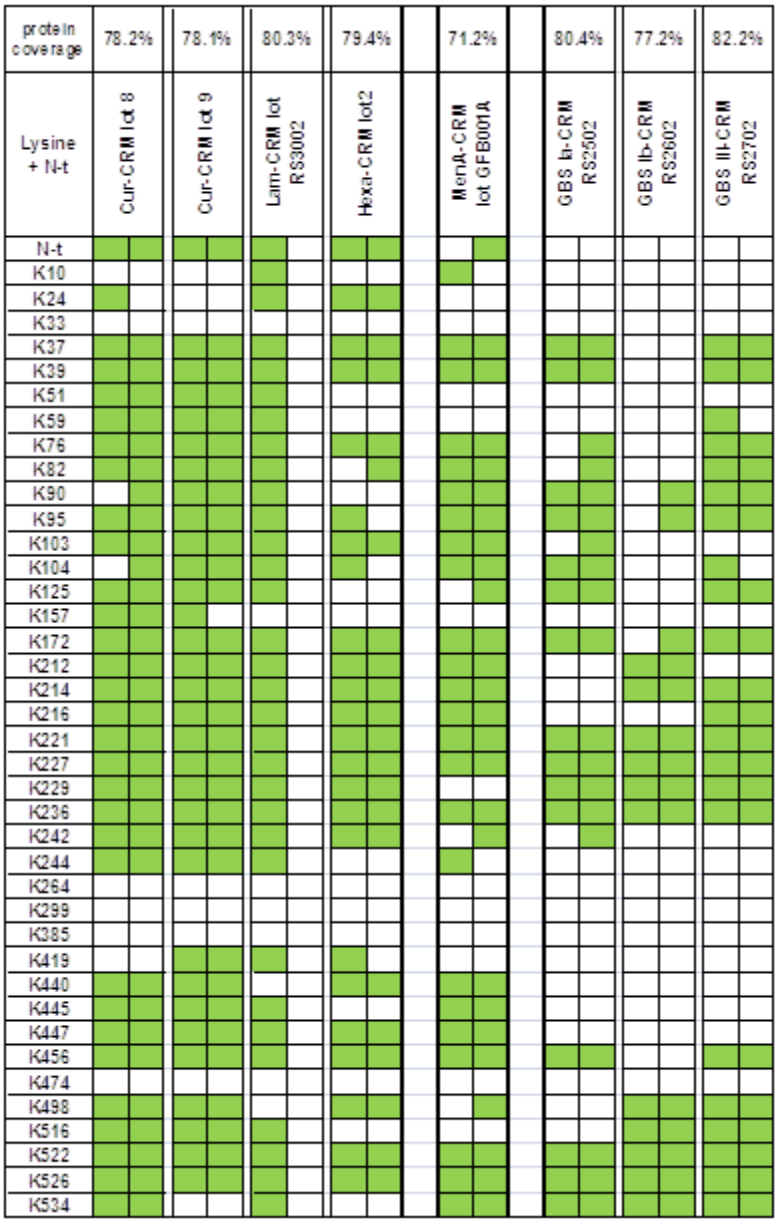
former procedure, has been eliminated, avoiding possible precipitation and loss of material.

2) The step of enrichment has been eliminated. It was not necessary because we thought the difference in the ionization capacity between a cleaved glycopeptide and a peptide was not relevant.

A gain in terms of time and material savings has been achieved: we could use only 300 μg of protein per sample instead of 500 μg and we could eliminate one day of enrichment per sample.

Another difference was the implementation of enzymatic saccharide cleavage for *Candida albicans* conjugates, and it has been immediately highlight for the higher quality of mass spectra in terms of signal intensity.

The results for *Candida albicans*, GBS and MenA have been reported in the next table. On the first row the percentage of peptide map coverage have been reported (Tab.6)



Tab 6 – Comparative summary of the analyzed conjugates.

The distributions of *Candida albicans* conjugates were very similar each other and the mass spectra obtained had a very nice intensity and high coverage, it was supposed that this aspect was due to enzymatic cleavage with *laminarinase* that not involved the amino acid part unlike acid treatment. The analyses of MenA and GBS conjugates have provided a

lower mass signal intensity and coverage but the behaviors were exhaustive for a good interpretation and for expected results.

During *Candida albicans* data analysis with Biopharmalynx, the peak intensity threshold was set to 8000 counts; that means every mass peak with intensity minor of 8000 counts was not considered. For GBS and MenA conjugates this value was lowered at 4000 counts, because the general intensity of spectra was worse and 8000 would be too high value.

The CRM_{197} sequence part that contain lysine (K) 264, 299, 385 was never covered by a trypsin digestion, for this reason it is impossible to know if those residues have been glycosylated or not.

In the future to circumvent this issue it could be possible to perform a double digestion strategy by the consecutive use of two different enzymes, e.g. first trypsin and then AspN. AspN is endoproteinase that hydrolyzes peptide bonds on the N-terminal side of aspartic and cysteic acid residues. This procedure could permit to generate different and smaller peptides and maybe cover a larger portion of protein sequence. However to easily process the mass spectrum of a double digestion we need a more updated software because ours can not effort this kind of elaboration. Other possibility for future works would be the application of a double complementary characterization of same sample first with trypsin and latter with different enzyme (e.g. AspN) that covers the missing sequence zone. Unfortunately in this case a double amount of sample would be necessary.

A smaller number of glycosylated lysine residues in GBS conjugate has been explained by bigger size of these polysaccharides and by the different conjugation chemistry that include an reductive amination. The large GBS polysaccharides have a greater issue of steric hindrance to reach the amino group of lysine respect smaller *Candida albicans* and MenA polysaccharide.

By the software PyMOL it was possible to build up the 3D structure of glycosylated lysine residues of analyzed conjugates on the CRM_{197} structure, thus we can have an idea of spatial and number distributions of glycosulations. The Fig.32 show a comparison of a Candida, a MenA and a GBS conjugates. It seems that Cur-CRM_{197} and MenA-CRM_{197} were very similar and with an higher amount of glycosylated residues respect to GBSIa-CRM_{197}.

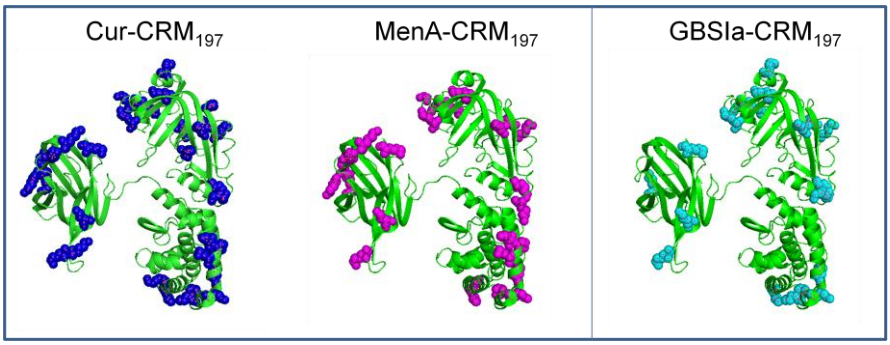


Fig. 32 - CRM_{197} protein structures with glycosylated lysine residues highlighted.

3. PREPARATION

3.1 *Candida albicans* conjugates

Our group has showed that when laminarin (Lam), a poorly immunogenic glucan extracted from the brown alga *Laminaria digitata*, was conjugated to CRM_{197} (Fig.33), the resulting Lam-CRM_{197} conjugate is both immunogenic and protective in a mouse challenge model against infections induced by *C. albicans* (Torosantucci et al. 2005). The conjugation strategy have consisted in: 1) a reductive amination of aldehyde of PS reductive terminus, 2) the vwith a bifunctional linker, 3) conjugation of activated PS to CRM_{197}.

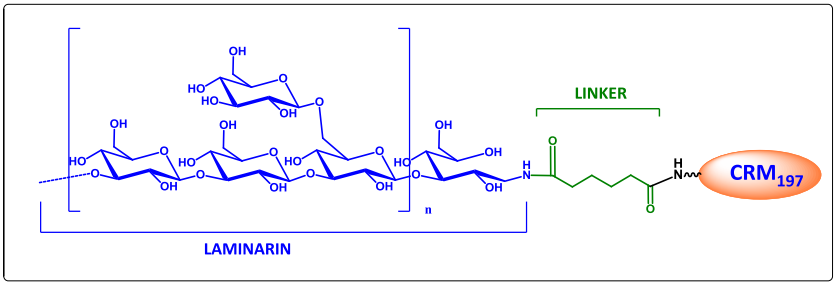


Fig.33 - Laminarin-CRM_{197} conjugate.

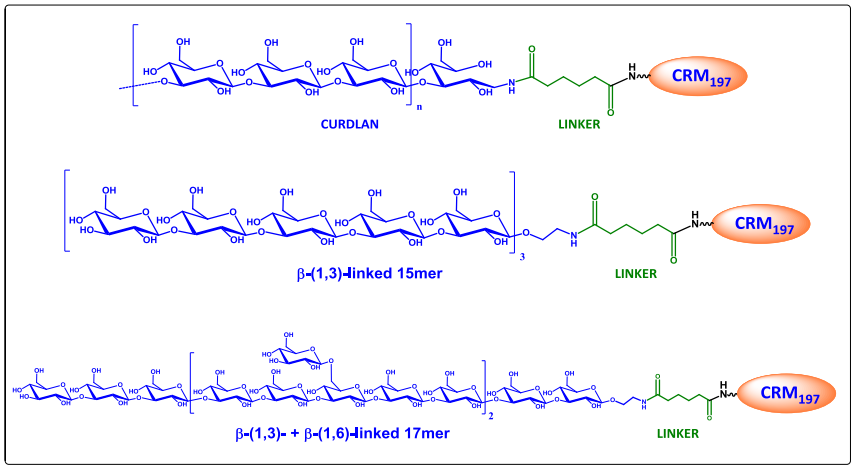


Fig.34 - Schematic structures of *Candida albicans* conjugates.

In a more recent study the conjugates of CRM_{197} two synthetic β-glucans have been compared to a linear glucan composed of 15 β-(1, 3) repeating units (15mer) and to an oligosaccharide with the same backbone but with two β-(1, 6) arms spaced by 5 repeats (17mer). Only the linear structure (Fig.34) has conferred protection against *C. albicans* (Bromuro et al. 2010).
In the same study it has observed also that conjugates of CRM_{197} with oligosaccharides derived from the linear β-(1,3) glucan curdlan

polysaccharide, were able to confer a significant level of protection against *C. albicans* in mice.
It has been further demonstrated by microarray analysis that the protective IgG2b mAb, named 2G8, specifically recognizes linear β-(1,3) glucan epitopes rather than β-(1,4) or β-(1, 6) (Torosantucci et al 2009).
This prompted us to assume that the protective epitope in *C. albicans* glucan cell wall is defined by a linear β-(1,3) glucan. Anyhow it is not still known which is the minimal epitope structure able to elicit protective antibodies against fungal cell wall.
With the aim to find a the minimal protective epitope for β(1,3), I have synthesized two linear β-(1,3) glucan structures a trisaccharide and a hexasaccharide, both equipped with a di-*N*-hydroxysuccinimidyl adipate linker. The two antigens have been conjugated to CRM_{197} (Fig.35), and tested in animal model.

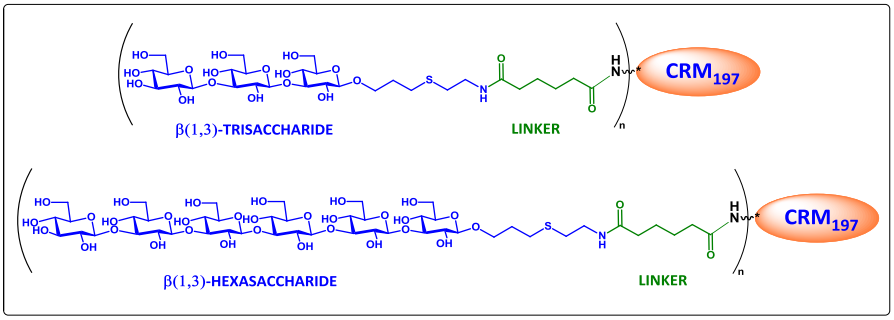


Fig. 35 - Structure of synthetic tri- and hexasaccharide *Candida albicans* conjugates.

Furthermore the hexasaccharide was mounted on a dendrimeric structure, a poly(amido amine) (PAMAM) molecule, $PAMAM_4$. This molecule has four amino functional groups which can bind up to four antigen molecules. In addition $PAMA_4$ shows a fifth protected amino group available for conjugation once deprotected. The conjugation of the hexasaccharide antigen to the cluster (Fig.36), and that of cluster to protein was obtained by the same chemistry as for trisaccharide and hexasaccharide. This conjugate was prepared to compare the effect of a clustered antigen to the monomeric hexasaccharide conjugate.

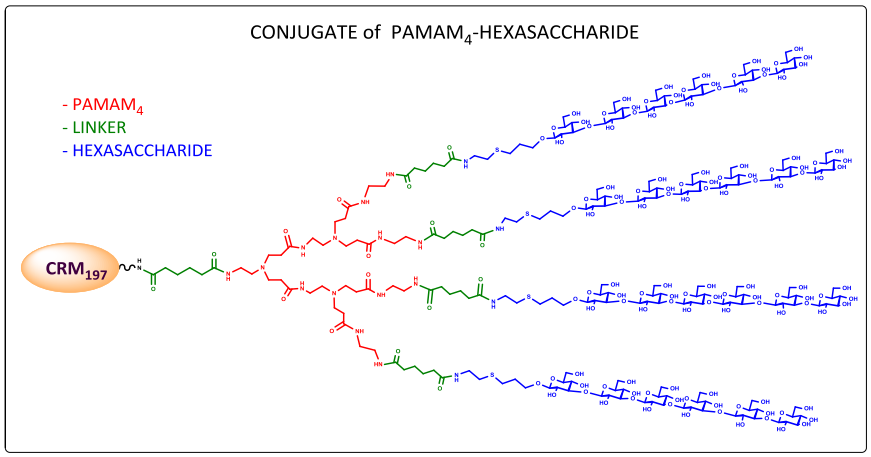


Fig. 36 - Structure of PAMAM$_4$-βGlucan conjugate

3.1.1 Synthesis of β(1, 3) glucan and conjugation

The glycosylation strategy was based on trichloroacedimidate donor compound to obtain the desired β-glucan bonds. Allyl-β-glucan antigens were elongated with cysteamine under UV radiation to insert an amino group suitable for conjugation (Fig.37).

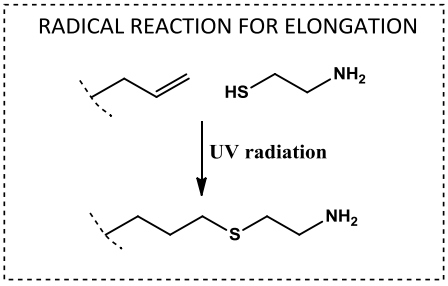


Fig. 37 - Radical reaction for elongation.

The linker employed for conjugation with carrier protein was an NHS ester of adipic acid (Fig.38).

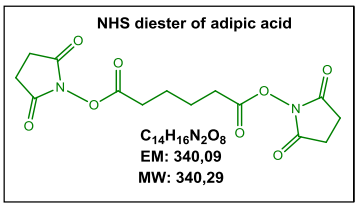


Fig.38 - NHS diester of adipic acid.

The complete synthetic pathways of trisaccharide and hexasaccharide synthesis have been reported in following schemes figure 39 and figure 40 respectively.

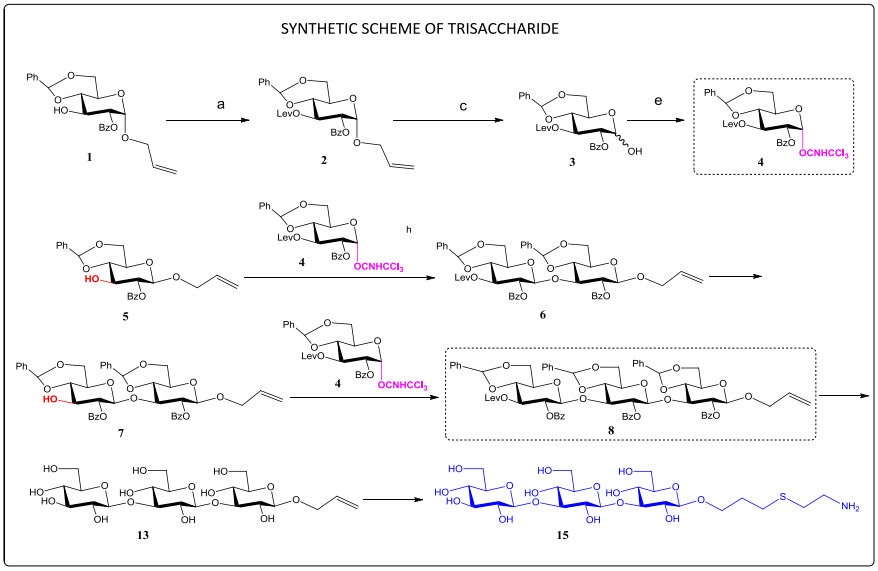


Fig.39 - Synthetic pathway of trisaccharide.

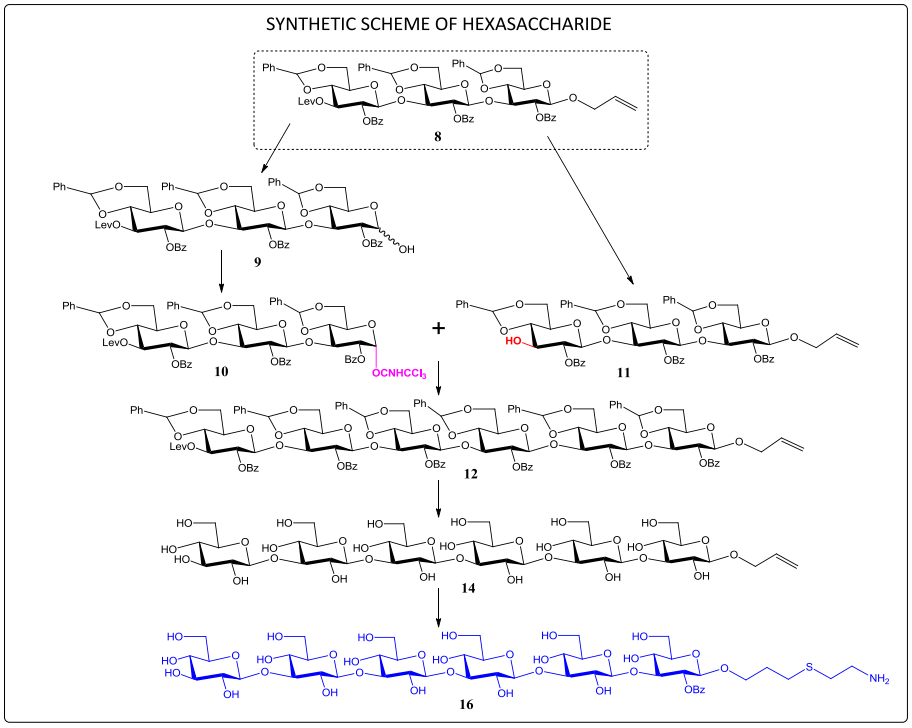


Fig.40 - Synthetic pathway for hexasaccharide.

Before the conjugations, antigens have been activated with a large excess of linker (10 equivalents) to avoid crosslinking side effect, that could due to its own symmetric functionalities (Fig.41).

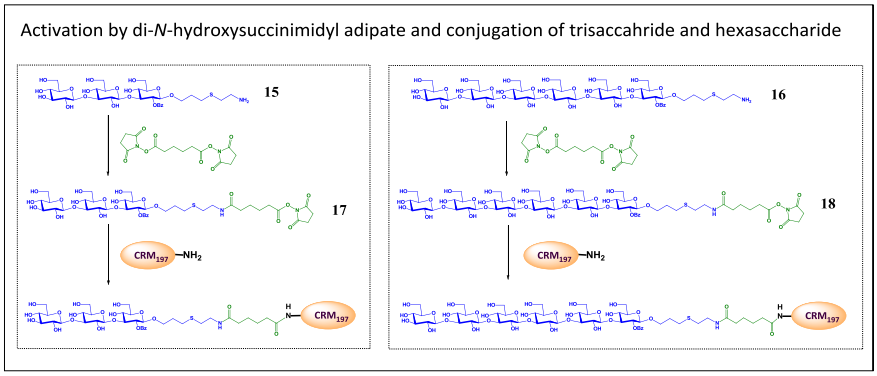


Fig.41 - Activation of tri- and hexa- saccharide with difunctional NHS ester linker of adipic acid.

The hexasaccharide antigen has been bound to a multimeric structures, $PAMAM_4$, and then conjugated to CRM_{197}, by the same conjugation chemistry: first the activation of antigen by the linker and the binding to cluster and then the final conjugation of purified activated antigen with lysine amino groups of protein (Fig.42).

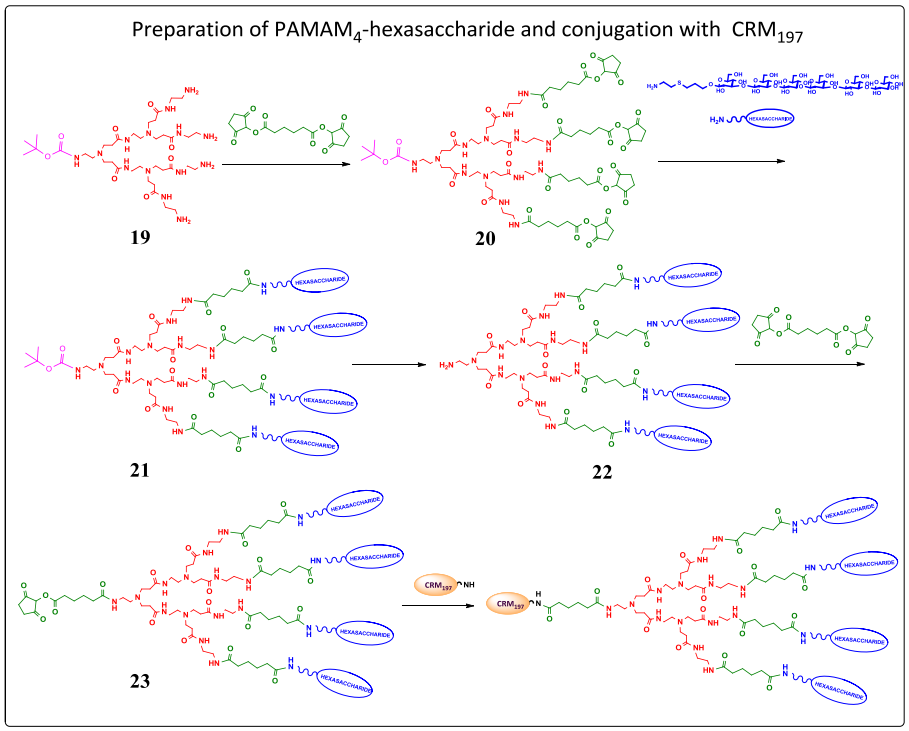


Fig.42 - Scheme of Hexasaccharide-$PAMAM_4$-CRM_{197}.

3.1.1.1 Materials and methods

All the chemicals were of reagent grade, and were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F254 (Merck); after exam under UV light, compounds were visualized by heating with 10% (v/v) ethanolic H_2SO_4. In the work up procedures, organic solutions were washed with the amounts of the indicated aqueous solutions, then dried with anhydrous Na_2SO_4, and concentrated under reduced pressure at 30–50°C on a water bath. Column chromatography was performed on Silica Gel 60 (Sigma Aldrich, 0.040–0.063 nm). Unless otherwise specified, a gradient 0 →100% hexane-EtOAc

was applied in a Combiflash R_f (Teledyne-Isco) or Flash Master Plus (Biotage) instrument. Solvent mixtures less polar than those used for TLC were used at the onset of separation. ^{1}H NMR spectra were measured at 300 MHz with a Varian Unity Inova 300 or 400 MHz with a Bruker Avance^{III} 400 spectrometer; δ_H values are reported in ppm, relative to internal Me_4Si (δ_H = 0.00, $CDCl_3$ and CD_3OD) or internal acetone (δ_H = 2.22, D_2O); when not suppressed solvent peak for D_2O was calibrated at 4.79 ppm. ^{13}C NMR spectra were measured at 75 MHz with a Varian Unity Inova 300 or 100 MHz with a Bruker Avance^{III} 400 spectrometer; δ_C values are reported in ppm relative to the signal of $CDCl_3$ (δ_C = 77.0, $CDCl_3$), CD_3OD (δ_C = 49.0, CD_3OD) or internal acetone (δ_C = 30.9, D_2O). Assignments of NMR signals were made by homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometers. Sugar moieties in the final compounds 117 and 118, and related building blocks are named using the letters A, B, C, D, E, F based on the hexasaccharide structure starting from the aglycon. Nuclei associated with the 3-(2- aminoethylthio)propyl linker are denoted with a prime. Exact masses were measured by electron spray ionization mass spectroscopy, using a Q-Tof micro Macromass (Waters) instrument. Palladium chloride and 1,5-Cyclooctadiene-bis(methyldiphenylphosphine)- Iridium-hexafluorophosphate catalyst was purchased from Sigma-Aldrich. Hydrogenation reactions were performed by flow chemistry on an H-Cube (Thalesnano) instrument.

General procedure for glycosylation with trichloroacedimidate donors

To a stirred solution of acceptor (1 mmol) and donor (1.2 mmol) in CH_2Cl_2 (15 ml) containing activated 4 Å MS (0.75 g), TMSOTf (0.1 mmol) was added at 0°C. The mixture was stirred for 30 min when TLC (2:1 hexane-EtOAc) showed the reaction was complete. Then the mixture was neutralized with triethylamine (0.1 μl), filtered through a celite pad, and the filtrate concentrated. Chromatography of the residue (hexane-EtOAc) gave the desired product.

General procedure for delevulinoylation

To a solution of the 3-*O*-Lev oligosaccharide (1 mmol) in CH_2Cl_2 (25 ml) ethylenediamine (0.26 ml, 4 mmol) and AcOH (0.29 ml, 5 mmol) were

added at 0°C. A white solid was formed and the suspension was stirred for 5-6 h at 50°C, when the deprotection was complete (TLC, hexane-EtOAc 2:1). The mixture was concentrated and chromatography of the residue (hexane-EtOAc) yielded the delevulinoylated product.

General procedure for deallylation

A mixture of the 1-*O*-allyl compound (1 mmol) and 1,5-Cyclooctadiene-bis(methyldiphenylphosphine)-Iridium-hexafluorophosphate catalyst (2 mg) in dry THF (7 mL) was carefully degassed.. The catalyst was activated under hydrogen atmosphere for 2 min, until the catalyst turned from red to pale yellow, and the reaction mixture was stirred at room temperature for 3 h. When NMR analysis of a small portion showed complete isomerization, water was added in order to get a 4:1 THF:H_2O mixture, followed by iodine (0.5 g, 2 mmol). After 15 min (TLC, 1:1 hexane-EtOAc) the solution was diluted with water and extracted with CH_2Cl_2 (3 $\times$ 10 mL). The organic layers were washed with a freshly prepared 5% solution of $Na_2S_2O_3$ until discoloration, dried (Na_2SO_4), filtered and concentrated. Chromatography of the residue (hexane-EtOAc) afforded the deallylated compound as a mixture of α/β anomers, which was used for the next step.

Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-α-D-glucopyranoside 2

To a solution of 3-OH sugar **1** (2.4 g, 5.8 mmol) in dichloromethane (100 ml), levulinic acid (810 mg, 7 mmol) was added followed by DMAP (850 mg, 7 mmol) and DCC (1.44 g, 7 mmol). The mixture was stirred for 3 h when TLC (2:1 hexane-EtOAc) showed the reaction was complete. The mixture was filtered, and the filtrate was partitioned with aq $NaHCO_3$. Combined organic layers were dried on $MgSO_4$ and concentrated. Chromatography of the residue with 5:1 hexane-EtOAc gave the 3-O-Lev product **2** (2.8 g, 94%). $[\alpha]_D$ = +77˚ (c = 0.6; $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.07–7.20 (m, 10 H, *Ph*CH, *Ph*CO), 5.88–5.75 (m, 2 H, CH_2CH=CH_2, incl. t, 5.84, *J* = 9.9 Hz, H-3), 5.56 (s, 1 H, PhC*H*), 5.31–5.09 (m, 4 H, H-2, CH_2CH=CH_2, incl. s, 5.28, $J_{1,2}$ = 3.9 Hz, H-1), 4.34 (dd, $J_{5,6a}$ = 5.1, $J_{6a,6b}$ = 10.1 Hz, H-6a), 4.25–3.97 (m, 2 H, CH_2CH=CH_2), 3.85–3.73 (m, 2 H, H-4,6b), 3.65–3.57 (m, 1 H, H-5), 2.65–2.50 (m, 4 H, CH_2CH_2), 2.00 (s, 3 H, CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 205.75 (CH_2*C*O), 171.77 (2 C, CH_2*C*OO, CH_3*C*O), 165.78 (Ph*C*O), 136.86, 134.40, 133.13, 132.53, 131.01, 129.86, 129.78, 129.50, 127.29, 125.24, 124.99

(PhCO, *Ph*CH, =*C*H), 117.65 (=*C*H_2), 101.44 (Ph*C*H), 95.77 (C-1), 79.08 (C-4), 72.17 (C-2), 69.07 (C-3), 68.71 (*C*H_2CH=CH_2), 68.65 (C-6), 62.56 (C-5), 37.87 (*C*H_2CO), 29.53 (CH_3), 27.3 (*C*H_2COO). ESI HR-MS ($C_{28}H_{30}O_9$): *m/z* = found ([*M*+H]$^+$ 511.1924; calc 511.1968).

Deallylation to obtain 3

The deprotection was obtained following the general deallylation procedure reported above.

Allyl group was removed from sugar **2** to give **3** (92%).

4,6-*O*-benzylidene-2-O-benzoyl-3-*O*-levulinoyl-α-D-glucopyranosyl trichloroacedimidate 4

The foregoing 1-OH sugar **3** (2.09 g, 4.46 mmol) was dissolved in CH_2Cl_2 (50 ml), to which trichloroacetonitrile (1.45 ml, 13.3 mmol) was added followed by DBU (74 μl, 0.89 mmol). The mixture was stirred for 2h when TLC (2:1 hexane-EtOAc) showed the reaction was finished. The mixture was concentrated and chromatographed (hexane-EtOAc) to afford 2.1 g of trichloroacedimidate **4** (84%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.59 (s, 1 H, NH), 8.05–7.26 (m, 5 H, *Ph*CO), 6.69 (d, $J_{1,2}$ = 3.9 Hz, H-1), 5.89 (t, 1 H, *J* = 9.6 Hz, H-3), 5.89 (s, 1 H, PhC*H*), 5.37 (dd, 1 H, $J_{2,3}$ =9.9 Hz, H-2), 4.40 (dd, 1 H, , $J_{5,6a}$ = 4.8, $J_{6a,6b}$ = 10.2 Hz, H-6a), 4.23–4.08 (m, 1 H, H-5), 3.88 (t, partially overlapped, 1 H, *J* = 9.9 Hz, H-4), 3.83 (t, partially overlapped, 1 H, *J* = 10.5 Hz, H-6b), 2.64–2.55 (m, 4 H, CH_2CH_2), 1.98 (s, 3 H, CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 205.64 (CH_2*C*O), 171.74 (2 C, CH_2*C*OO, CH_3*C*O), 165.46 (Ph*C*O), 160.70 (O*C*ONH), 136.56, 134.44, 132.49, 131.06, 129.93, 129.06, 128.86, 127.51, 127.32, 125.01 (PhCO, *Ph*CH), 101.51 (Ph*C*H), 100.94 (C-1), 93.34 (q, CCl_3), 78.40 (C-4), 70.93 (2 C, C-2,3), 68.81 (C-6), 65.17 (C-5), 37.85 (*C*H_2CO), 29.53 (CH_3), 27.86 (*C*H_2COO). ESI HR-MS ($C_{27}H_{27}Cl_3NO_9$): *m/z* = found ([*M*+Na]$^+$ 636.0529; calc 636.0571).

Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 6

The general procedure for glycosylation was applied to compounds **5** and **6**. Yield: 63 %. $[\alpha]_D$ = -6˚ (c = 0.1; $CHCl_3$).^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.76–7.25 (m, 20 H, 2 × *Ph*CO, 2 × *Ph*CH), 5.65–5.54 (m, 3 H, CH_2CH=CH_2, incl. s, 5.58, PhC*H*), 5.34, (s, 1 H, PhC*H*), 5.29 (t, partially overlapped, 1 H, *J* = 9.6

Hz, H-3^{B}), 5.27 (t, partially overlapped, 1 H, *J* = 8.2 Hz, H-2^{A}), 5.22 (t, partially overlapped, 1 H, *J* = 8.5 Hz, H-2^{B}), 5.13–5.00 (m, 2 H, $CH_2CH=CH_2$), 4.94 (d, 1 H, $J_{1,2}$ = 7.2 Hz, H-1^{B}), 4.61 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1^{A}), 4.37 (dd, 1 H, $J_{5,6a}$ = 4.9, $J_{6a,6b}$ = 10.5 Hz, H-6a^{B}), 4.24–4.15 (m, 3 H, H-6a^{A},3^{B},$CH_{2a}CH=CH_2$), 3.99–3.95 (m, 1 H, $CH_{2b}CH=CH_2$), 3.87–3.81 (m, 2 H, H-4^{B},6b^{B}), 3.77 (t, partially overlapped, 1 H, *J* = 9.6 Hz, H-4^{A}), 3.71 (t, partially overlapped, 1 H, *J* = 10.2 Hz, H-6b^{A}), 3.56–3.43 (m 2 H H-5^{A,B}), 2.49–2.33 (m, 4 H, CH_2CH_2), 1.94 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.69 (CH_2CO), 171.69 (2 C, CH_2COO, CH_3CO), 164.78, 164.47 (2 × Ph*C*O), 137.06–125.99 (2 × *Ph*CO, 2 × *Ph*CH, *C*H=), 117.54 (=CH_2), 101.34, 101.20 (2 × Ph*C*H), 101.34 (C-1^{B}), 100.05 (C-1^{A}), 79.19 (C-4^{B}), 78.45 (C-3^{A}), 77.91 (C-4^{A}), 73.21 (C-2^{A}), 72.73 (C-2^{B}), 71.95 (C-3^{B}), 69.80 ($CH_2CH=CH_2$), 68.68 (C-6^{B}), 68.52 (C-6^{A}), 66.35 66.35, (C-5^{B}), 66.13, (C-5^{A}), 37.80 (CH_2CO), 29.44 (CH_3), 27.90 (CH_2COO). ESI HR-MS ($C_{48}H_{48}O_{15}$): *m/z* = found ([*M*+Na]$^{+}$ 887.2902; calc 887.2891).

Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 7

Levulinic group was removed from **5** as described above. Yield: 85%. White crystals from EtOAc, m.p. 156–157°C. $[\alpha]_D$ = -15˚ (c = 1.0; $CHCl_3$).^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.83–7.26 (m, 20 H, 2 × *Ph*CO, 2 × *Ph*CH), 5.66–5.54 (m, 2 H, $CH_2CH=CH_2$, incl. s, 5.55, PhC*H*), 5.35, (s, 1 H, PhC*H*), 5.28 (t, 1 H, *J* = 7.5 Hz, H-2^{A}), 5.15–5.00 (m, 3 H, $CH_2CH=CH_2$, incl. t, 5.10, *J* = 7.5 Hz, H-2^{B}), 4.95 (d, 1 H, $J_{1,2}$ = 7.1 Hz, H-1^{B}), 4.63 (d, 1 H, $J_{1,2}$ = 7.5 Hz, H-1^{A}), 4.37 (dd, 1 H, $J_{5,6a}$ = 4.9, $J_{6a,6b}$ = 10.5 Hz, H-6a^{B}), 4.24–4.18 (m, 3 H, H-3^{A},6a^{B}, $CH_{2a}CH=CH_2$), 3.99–3.95 (m, 1 H, $CH_{2b}CH=CH_2$), 3.87–3.79 (m, 3 H, H-3^{B},4^{B},6b^{B}), 3.70 (t, partially overlapped, 1 H, *J* = 10.1 Hz, H-6b^{A}), 3.66 (t, partially overlapped, 1 H, *J* = 9.2 Hz, H-4^{A}), 3.58–3.52 (m 2 H H-5^{B}), 3.42–3.35 (m, 1 H, H-5^{A}), 2.35 (br. s, 1 H, OH-3^{B}). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 165.56, 164.59 (2 × Ph*C*O), 137.09–125.92 (2 × *Ph*CO, 2 × *Ph*CH, *C*H=), 117.57 (=CH_2), 101.63, 101.40 (2 × Ph*C*H), 100.23 (C-1^{B}), 99.95 (C-1^{A}), 80.46 (C-4^{A}), 79.23 (C-4^{B}), 79.91 (C-3^{A}), 75.22 (C-2^{A}), 73.45 (C-2^{B}), 72.57 (C-3^{B}), 69.77 ($CH_2CH=CH_2$), 68.73 (C-6^{B}), 68.58 (C-6^{A}), 66.32 66.35, (C-5^{B}), 66.00, (C-5^{A}). ESI HR-MS ($C_{43}H_{42}O_{13}$): *m/z* = found ([*M*+H]$^{+}$ 767.2756; calc 767.2702); found ([*M*+Na]$^{+}$ 789.2518; calc 789.2523); found ([*M*+K]$^{+}$ 805.2308; calc 805.2262).

Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene β-D-glucopyranoside 8

The reaction was carried out with **7** and **4** according to the general procedure for glycosylation. Yield: 53 %. $[\alpha]_D$ = -2˚ (c = 0.1; $CHCl_3$).^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.86–7.21 (m, 30 H, 3 × *Ph*CO, 3 × *Ph*CH), 5.66–5.53 (m, 1 H, CH_2CH=CH_2), 5.49 (s, 1 H, PhC*H*), 5.40–5.35 (m, 2 H, PhC*H*, H-3^C), 5.27 (t, 1 H, *J* = 8.6 Hz, H-2^C), 5.14–5.01 (m, 4 H, CH_2CH=CH_2, H-2^B, incl. d, 5.08, $J_{1,2}$ = 7.4 Hz, H-1^C), 4.92–4.88 (m, 2 H, H-2^A, H-1^B), 4.83 (s, 1 H, PhC*H*), 4.52 (d, 1 H, $J_{1,2}$ = 7.5 Hz, H-1^A), 4.32 (dd, 1 H, $J_{5,6a}$ = 4.7, $J_{6a,6b}$ = 10.4 Hz, H-6a^A), 4.24–3.99 (m, 6 H, H-6a^{B,C}, 6b^A,3^{A,B},CH_{2a}CH=CH_2), 3.96–3.91 (m, 1 H, CH_{2b}CH=CH_2), 3.77 (t, 1 H, *J* = 9.5 Hz, H-4^C), 3.73–3.51 (m, 5 H, H-4^B,5^{B,C},6b^{B,C}), 3.46–3.40 (m, 1 H H-5^A), 3.35 (t, 1 H, *J* = 9.2 Hz, H-4^A), 2.49–2.46 (m, 4 H, CH_2CH_2), 1.96 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.75 (CH_2CO), 171.63 (2 C, CH_2COO, CH_3CO), 164.97, 164.52, 164.31 (3 × Ph*C*O), 137.20–125.99 (3 × *Ph*CO, 3 × *Ph*CH, *C*H=), 117.44 (=CH_2), 101.66, 101.20, 100.59 (3 × Ph*C*H), 100.59 (C-1^A), 99.87 (C-1^C), 98.42 (C-1^B), 78.71 (C-4^A), 78.20 (C-4^B), 77.80 (C-4^C), 77.20 (C-3^B), 75.00 (C-3^A), 74.00 (C-2^A), 72.72 (C-2^B), 72.37 (C-2^C), 71.95 (C-3^C), 69.69, 68.63, 68.51 (4 C, CH_2CH=CH_2, C-6^{A,B,C}), 66.30 (C-5^A), 66.13, 65.48 (C-5^{B,C}), 37.86 (CH_2CO), 29.46 (CH_3), 27.93 (CH_2COO). ESI HR-MS ($C_{68}H_{66}O_{21}$): *m/z* = found ([*M*+Na]$^+$ 1218.4049; calc 1218.4097); found ([*M*+K]$^+$ 1257.3718; calc 1257.3734).

Deallylation to obtain 9

The removal of allyl group in trisaccharide **8** (1 g, 0.82 mmol) gave compound **9** (70%).

2-*O*-Benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl trichloroacedimidate 10

The 1-OH trisaccharide **9** (120 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (5 ml), to which trichloroacetonitrile (30 μl, 0.3 mmol) was added followed by DBU (3 μl, 0.02 mmol). The mixture was stirred for 1 h when TLC (2:1 hexane-EtOAc) showed the reaction was finished. The mixture was concentrated and chromatographed (hexane-EtOAc) to furnish 100 mg of trichloroacedimidate **10** (76%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.47 (s, 1 H, NH), 7.92–7.22 (m, 30 H, 3 × *Ph*CO, 3 × *Ph*CH), 6.43 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1^A), 5.57 (s, 1 H, PhC*H*), 5.46–5.41 (m, 2 H, PhC*H*, incl. t, 5.43, *J* = 9.1 Hz, H-3^C), 5.31 (t, 1 H, *J* = 8.7 Hz, H-2^C), 5.16–5.11 (m, 2 H, H-2^B, incl. d, 5.15, $J_{1,2}$ = 7.2 Hz, H-1^C), 5.07 (d, 1 H, $J_{1,2}$ = 4.4 Hz, H-1^B), 4.70 (s, 1 H, PhC*H*), 4.62 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2^A), 4.40 (t, 1 H, *J* = 9.7 Hz, H-3^A), 4.33–3.94 (m, 5 H, H-3^{A,B},6a^{A,B,C}), 4.08–3.88 (m, 2 H, H-5^{A,B}), 3.79 (t, 1 H, *J* = 9.6 Hz, H-4^C), 3.81–3.56 (m, 5 H, H-4^B,5^C,6b^{A,B,C}), 3.33 (t, 1 H, *J* = 9.3 Hz, H-4^A), 2.52–2.51 (m, 4 H, CH_2CH_2), 1.96 (s, 3 H, CH_3CO). ESI HR-MS ($C_{67}H_{62}Cl_3NO_{21}$): *m/z* = found ([*M*+Na]$^+$ 1344.2778; calc 1344.2778).

Allyl bis[2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)]-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 11

The general procedure for delevulinoylation was followed with **8**.

Yield: 82%.

$[\alpha]_D$ = +76° (c = 0.2; $CHCl_3$).^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.99–7.15 (m, 30 H, 3 × *Ph*CO, 3 × *Ph*CH), 5.67–5.57 (m, 1 H, CH_2CH=CH_2), 5.46 (s, 1 H, PhC*H*), 5.39 (s, 1 H, PhC*H*), 5.18 (t, 1 H, *J* = 7.9, H-3^C), 5.15–4.99 (m, 5 H, CH_2CH=CH_2, H-2^{B,C}, incl. d 5.07, $J_{1,2}$ = 7.4 Hz, H-1^C), 4.92 (d, 1 H, $J_{1,2}$ = 4.5 Hz, H-1^B), 4.89 (d, 1 H, *J* = 8.4, H-2^A), 4.64 (s, 1 H, PhC*H*), 4.50 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1^A), 4.28 (dd, 1 H, $J_{5,6a}$ = 4.7, $J_{6a,6b}$ = 10.4 Hz, H-6a^A), 4.22–3.99 (m, 5 H, H-6a^{B,C},3^{A,B},CH_{2a}CH=CH_2), 3.96–3.89 (m, 2 H, CH_{2b}CH=CH_2, incl. t, 3.91, *J* = 9.0 Hz, H-4^C), 3.69–3.42 (m, 6 H, H-4^B,5^{B,C},6b^{AB,C}), 3.41–3.36 (m, 1 H H-5^A), 3.21 (t, 1 H, *J* = 9.3 Hz, H-4^A). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 171.12 (CH_3*C*O), 165.68, 164.65, 164.42 (3 × Ph*C*O), 137.20–126.00 (3 × *Ph*CO, 3 × *Ph*CH, *C*H=), 117.38 (=*C*H$_2$), 101.79, 101.64, 100.44 (3 × Ph*C*H), 99.87 (C-1^A), 98.14 (C-1^C), 97.89 (C-1^B), 80.68 (C-4^B), 78.59 (C-4^A), 77.53 (C-4^C), 76.25 (C-3^B), 74.61 (C-3^C), 74.36 (C-3^A), 74.16 (C-2^A), 72.25, 72.32 (C-2^{B,C}), 69.68 (*C*H$_2$CH=CH$_2$), 68.64, 68.51 (3 C, , C-6^{A,B,C}), 66.29 (C-5^A), 65.96 (C-5^B). ESI HR-MS ($C_{63}H_{60}O_{19}$): *m/z* = found ([*M*+Na]$^+$ 1143.3581; calc 1143.3567).

Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-tetrakis[2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)]-2-*O*-benzoyl-4,6-*O*-benzylidene β-D-glucopyranoside 12

Glycosylation was performed according to the general procedure, using **10** and **11** (0.5 mmol of TMSOTf). Yield: 54%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.85–7.14 (m, 60 H, 6 × *Ph*CO, 6 × *Ph*CH), 5.66–5.57 (m, 1 H, CH_2CH=CH_2), 5.53 (s,

1 H, PhC*H*), 5.39 (t, 1 H, *J* = 9.0, H-3^{G}), 5.38 (s, 1 H, PhC*H*), 5.28 (dd, 1 H, $J_{1,2}$ = 8.3, $J_{1,2}$ = 9.3 Hz, H-2^{C}), 5.19 (t, *J* = 6.3 Hz, H-2^{B}), 5.15–5.02 (m, 3 H, CH_2CH=CH_2, incl. d 5.06, $J_{1,2}$ = 7.7 Hz, H-1^{G}), 4.99 (d, 1 H, $J_{1,2}$ = 6.0 Hz, H-1^{B}), 4.97–4.71 (m, 13 H, 4 × PhC*H*, H-1$^{C\text{-}F}$,2$^{A,C\text{-}F}$), 4.53 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1^{A}), 4.23 (dd, 1 H, $J_{5,6a}$ = 4.7, $J_{6a,6b}$ = 10.4 Hz, H-6a^{A}), 4.23–4.06 (m, 9 H, H-3^{A,B},6a$^{B\text{-}G}$, ,CH_{2a}CH=CH_2), 3.98–3.89 (m, 5 H, CH_{2b}CH=CH_2, H-3$_{C\text{-}F}$), 3.77 (t, 1 H, *J* = 9.3 Hz, H-4^{G}), 3.73–3.40 (m, 16 H, H-4$^{C\text{-}F}$,5$^{A\text{-}G}$,6b$^{A\text{-}G}$), 3.33 (t, 1 H, *J* = 8.5 Hz, H-4^{B}), 3.25 (t, 1 H, *J* = 9.0 Hz, H-4^{A}), 2.52–2.35 (m, 4 H, CH_2CH_2), 1.96 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.83 (CH_2CO), 171.69 (2 C, CH_2COO, CH_3CO), 165.06, 164.67, 164.55, 164.49 (6 × Ph*C*O), 137.22–126.06 (6 × *Ph*CO, 6 × *Ph*CH, *C*H=), 117.50 (=*C*H_2), 101.96, (Ph*C*H), 101.25 (2 , C-1), 101.13 (Ph*C*H), 100.96 (2 C, C-1), 100.77 (C-1), 99.94 (C-1^{A}), 99.16 (C-1^{G}), 98.26 (Ph*C*H), 97.28 (C-1^{B}), 96.94 (Ph*C*H), 96.79 (Ph*C*H), 78.79 (C-4^{A}), 78.36 (C-3), 78.27, 77.83, 77.40 (C-4), 74.94 (C-3^{A}), 74.57, 74.27,74.15, 74.00 (C-3), 73.01 (C-2$^{A,C\text{-}F}$), 72.48 (C-2^{B,G}), 72.02 (C-3^{G}), 69.79 (*C*H_2CH=CH_2), 68.67, 68.58 (6 C, C-6$^{A\text{-}G}$), 66.33, 66.13, 65.64, 65.47 (C-5$^{A\text{-}G}$), 37.92 (*C*H_2CO), 29.51 (CH_3), 27.98 (*C*H_2COO). ESI HR-MS ($C_{128}H_{120}NO_{39}$): *m/z* = found ([*M*+K]$^{+}$ 2319.6982; calc 2319.7044); found ([*M*+Na]$^{+}$ 2303.7092; calc 2303.7304).

General procedure for deprotection of glucans

The allyl oligosaccharide (**8** or **12**) (0.1 mmol) was dissolved in 80% AcOH (5 ml). After stirring for 6 h at 50° the solvent was evaporated and the material was dissolved in MeOH (5 ml) to which a solution of 1 M NaOMe was added until pH was strongly alkaline (TLC 9:1 CH_2Cl_2-MeOH). The mixture was stirred overnight, then concentrated. The residue was purified on a C18 Isolute SPE column with H_2O. Fractions containing the pure desired compound (NMR) were combined and freeze-dried.

Allyl bis[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside 13 from 8

Yield: 92%. ^{1}H NMR (400 MHz, D_2O) δ: 6.00–5.91 (m, 1 H, CH_2CH=CH_2), 5.39–5.26 (m, 2 H, CH_2CH=CH_2), 4.76 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^{C}), 4.73 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^{B}), 4.52 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^{A}), 4.39–4.35 (m, 1 H, H-1'a),), 4.23–4.18 (m, 1 H, H-1'b), 3.90 (d, 3 H, $J_{5,6}$ = 12.0 Hz, H-6a^{A,B,C}), 3.78–3.66 (m, 5 H, H-3^{A,C},6b^{A,B,C}), 3.53 (t, *J* = 9.3 Hz, H-2^{C}), 3.52–3.35 (m, 8 H, H-2^{A},3^{B},4^{A,B,C},5^{A,B,C}), 3.41–3.31 (m, 3 H, 2^{B},3^{A,C}). ^{13}C NMR (D_2O, 100 MHz) δ: 133.82 (*C*H=), 119.40 (=*C*H_2), 103.36 (C-1^{C}), 103.12 (C-1^{B}), 101.51 (C-1^{A}),

84.96, 84.77, 76.58, 76.20, 76.13, 74.03, 73.82, 73.47, 71.28 ($CH_2CH=CH_2$), 70.15, 68.75, 68.70, 61.28 (3 C, C-6^{A-C}). ESI HR-MS ($C_{21}H_{36}NO_{16}$): *m/z* = found ([*M*+Na]$^+$ 567.1930; calc 567.1901).

Allyl pentakis[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside 14 from 12

Yield: 93%. ^{1}H NMR (400 MHz, D_2O) δ: 6.00–5.91 (m, 1 H, $CH_2CH=CH_2$), 5.39–5.26 (m, 2 H, $CH_2CH=CH_2$), 4.76 (d, 4 H, $J_{1,2}$ = 8.0 Hz, H-1^{B-F}), 4.73 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1^G), 4.52 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^A), 4.40–4.39 (m, 1 H, H-1′a),), 4.24–4.19 (m, 1 H, H-1′b), 3.90 (d, 6 H, $J_{5,6}$ = 12.0 Hz, H-$6a^{A-G}$), 3.79–3.68 (m, 11 H), 3.57–3.30 (m, 19 H). ^{13}C NMR (D_2O, 100 MHz) δ: 133.82 (*C*H=), 119.40 (=*C*H_2), 103.40 (C-1^G), 103.12 (4 C, C-1^{B-F}), 101.51 (C-1^A), 84.94, 84.77, 76.59, 76.20, 76.13, 74.03, 73.89, 73.84, 73.48, 71.26 ($CH_2CH=CH_2$), 70.16, 68.75, 68.66, 61.25 (6 C, C-6^{A-G}). ESI HR-MS ($C_{39}H_{67}NO_{31}$): *m/z* = found ([*M*+H]$^+$ 1054.3602; calc 1054.3564).

General procedure for elongation with cysteamine

To a solution of allyl oligosaccharide **13** or **14** (0.1 mmol) in CH_2Cl_2 (0.2 ml), cysteamine (0.3 mmol) dissolved in MeOH (0.2 mL) was added, and the mixture was irradiated in a quartz vial under UV for 12 h. The residue was purified on a G10 Sephadex column run in an Akta GE Instrument, using water for elution. Fractions containing the pure desired compound (NMR) were combined and freeze-dried.

3-(2-aminoethylthio)propyl bis[β-D-glucopyranosyl-(1→3)]-β-D -glucopyranoside 15 from 13

Yield: 61%. ^{1}H NMR (400 MHz, D_2O) δ: 4.76 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^C), 4.74 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1^B), 4.49 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1^A), 4.04–3.99 (m, 1 H, H-1′a), 3.92 (d, 3 H, $J_{5,6}$ = 12.0 Hz, H-$6a^{A,B,C}$), 3.82–3.70 (m, 6 H, H-$3^{A,C}$,$6b^{A,B,C}$,1′b), 3.55 (t, *J* = 9.3 Hz, H-2^C), 3.54–3.40 (m, 8 H, H-2^A,3^B,$4^{A,B,C}$,$5^{A,B,C}$), 3.36 (t, 1 H, *J* = 9.1 Hz, H-2^B), 3.21 (t, 2 H, *J* = 6.8 Hz, H-5′), 2.86 (t, 2 H, *J* = 6.5 Hz, H-4′), 2.70 (t, 2 H, *J* = 7.1 Hz, H-3′), 1.96–1.90 (m, 2 H, H-2′). ESI HR-MS ($C_{23}H_{43}NO_{16}S$): *m/z* = found ([*M*+Na]$^+$ 622.2115; calc 622.2143).

3-(2-aminoethylthio)propyl pentakis[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside 16 from 14

Yield: 73%. ^{1}H NMR (400 MHz, D_2O) δ: 4.80–4.74 (m, 5 H, H-1^{B-F}), 4.48 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1^{A}), 4.02–3.96 (m, 1 H, H-1'a), 3.92 (d, 6 H, $J_{5,6}$ = 12.0 Hz, H-6a^{A-G}), 3.81–3.68 (m, 12 H, H-3$^{A,C-G}$,6b^{A-G},1'b), 3.56-3.38 (m, 18 H, H-2$^{A,C-G}$,3^{B},4^{A-G},5^{A-G}), 3.34 (t, 1 H, *J* = 8.8 Hz, H-2^{B}), 3.19 (t, 2 H, *J* = 7.1 Hz, H-5'), 2.84 (t, 2 H, *J* = 6.4 Hz, H-4'), 2.68 (t, 2 H, *J* = 7.1 Hz, H-3'), 1.95–1.88 (m, 2 H, H-2'). ^{1}H NMR (400 MHz, D_2O) at 50 °C showed the following anomeric signals, δ: 5.05 (d, 3 H, $J_{1,2}$ = 8.0 Hz, 3 × H-1), 5.04 (d, 1 H, $J_{1,2}$ = 5.3 Hz, H-1), 4.74 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1^{A}). ESI HR-MS ($C_{41}H_{73}NO_{31}S$): *m/z* = found ([*M*+H]$^{+}$ 1108.3982; calc 1108.3966).

Activation with linker of 15 and 16

The amino-oligosaccharide (**15** or **16**) was solubilized in water/DMSO (1:9, v/v) at a 4 mM amino groups concentration, then triethylamine (10 equiv) and adipic acid N-hydroxysuccinimido diester (10 equiv) were added. The reaction was kept under gentle stirring at room temperature for 2h. The activated oligosaccharides (**17** and **18**) were then separated from the reagents by precipitation with 9 volumes of EtOAc, followed by washing of the precipitate twice with EtOAc and drying under vacuum.

Compound 17: ESI HR-MS ($C_{33}H_{55}N_2O_{21}S$): *m/z* = found ([*M*+H]$^{+}$ 847.3033; calc 847.3018).

Compound 18: ESI HR-MS ($C_{51}H_{85}N_2O_{36}S$): *m/z* = found ([*M*+H]$^{+}$ 1333.4535; calc 1333.4603).

Conjugation of 17 and 18 to CRM_{197}

Conjugation was carried out by combining the activated oligosaccharides **17** or **18** with CRM_{197} at a molar ratio of 20:1 and 30:1 (moles of active ester per moles of protein)respectively in 10 mM phosphate buffer pH 7.0.

After reaction overnight at room temperature, the conjugate was purified by ammonium sulfate precipitation (500 mg/ml). Pellet was dissolved in 10 mM phosphate buffer pH 7.0, and again precipitated with ammonium sulfate (500 mg/ml). After repeating twice this operation, the solid was washed twice with a solution of ammonium sulfate (500 mg/ml) in 10

mM phosphate buffer pH 7.0, and finally reconstituted with 10 mM phosphate buffer pH 7.0.
SDS-PAGE shows the two conjugates (Fig.43):

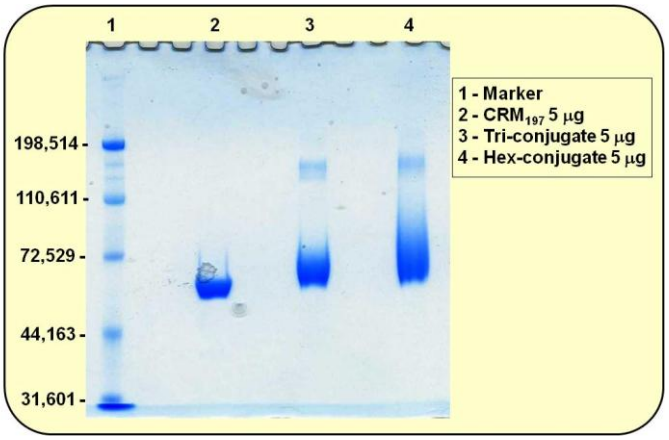


Fig.43 – SDS-Page of trisaccharide- and hexasaccharide *Candida albicans* conjugates.

MALDI-TOF mass spectra of CRM_{197} and final glycoconjugates were recorded by an UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics) in linear mode and with positive ion detection. The samples for analysis were prepared by mixing 2.5 µL of product and 2.5 µL of Super DHB matrix. 2.5 µL of each mixture were deposited on samples plate, dried at room temperature for 10 min and subjected to the spectrometer.

Activation of $PAMAM_4$ dendrimer by linker

The $PAMAM_4$ **19** (7 mg, 0.008mmol) 1eq was dissolved in 225µl DMSO dry, and then 18µl of triethylamine (16eq, 0.13mmol) were added, finally a solution of 70mg of adipic acid *N*-hydroxysuccinimido diester (25eq, 0.2mmol) in 225µl of DMSO dry was added dropwise to the reaction. The mixture was kept under gentle stirring at room temperature for 2h. Then the reaction raw was lyophilized, and the solid was extracted with 2ml of isopropanol, for 3 times; each washing was kept separated. The three fractions was analyzed by ESI-QTof, and the first one contained the activated $PAMAM_4$ **20** (9mg, 62%).

^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.44 (br. s, 8 H, CH_2^a), 3.15–3.01 (m, 12 H, $CH_2^{b,f}$), 2.99–2.92 (m, 2 H, CH_2^l), 2.79 (br. s, 16 H, CH_2^{NHS}), 2.71–2.55 (m, 16 H, $CH_2^{d,h}$, $CH_{2a}COO$), 2.46–2.37 (m, 6 H, $CH_2^{e,i}$), 2.24–1.99 (m, 24 H, $CH_2^{c,g}$, $CH_{2b}COO$, CH_2CONH), 1.64–1.40 (m, 16 H, CH_2CH_2), 1.34 (s, 9 H, tBoc). ESI

MS ($C_{77}H_{120}N_{18}O_{28}$): *m/z* = found ($[M+H]^{2+}$ 873.9; calc 873.4); found ($[M+H]^{3+}$ 583.0; calc 582.7)

Clusterization of hexasaccharide on activated $PAMAM_4$

Activated $PAMAM_4$ (1,3mg; 1eq) was dissolved in 100µl of DMSO dry, meanwhile hexasaccharide **16** (8.9mg; 10eq) was dissolved apart in 150 µl of DMSO dry. Activated $PAMAM_4$ was added to dissolved **16** by five additions of 20µl, stirring between each addition. The mixture was stirred for 2 h at room temperature, monitoring by LC-ESI-MS.

Then the material was purified by a G25 column eluting with water. Fractions contening the cluster, checked by ESI-MS, were concentrated on GeneVac to afford 1.8mg of compound **21** (42%).

^1H NMR analysis (D_2O, 600 MHz) using a Bruker instrument with cryoprobe at 298° K showed for peaks at 4.0 ppm corresponding to H-1a' and H-6a positions of the linker equipped hexasaccharide an integration for 25 H instead of the expected 28 H as compared to the CH_2CH_2 group in the adipate fragment of the cluster core, which integrated 16 H. An average loading of 3.5 carbohydrate chains was calculated.

ESI MS ($C_{225}H_{393}N_{18}O_{140}S_4$): *m/z* = found ($[M+H]^+$ 5715.4; calc 5715.3); = found ($[M+H_2O-C_{41}H_{73}O_{31}S)]^+$ 4626.0; calc 4625.9); found ($[M+2H_2O-2C_{41}H_{73}O_{31}S)]^+$ 3536.5; calc 3536.6).

***t*-Boc deprotection of $PAMAM_4$-hexasaccharide**

The material **21** was dissolved in 240µl of 20% TFA and stirred for 2h at room temperature.

ESI-QTof showed the Boc group has been removed. The sample **22** was freeze-dried (1.4mg, yeald 72%).

Activation of $PAMAM_4$-hexasaccharide by linker

The deprotected material **22**, 1.4mg, was dissolved in 200µl of DMSO dry, plus 2µl of triethylamine. N-hydroxysuccinimido adipic diester (2mg; 24eq) was dissolved to a concentration of 10mg/100µl in DMSO dry and added to the solution of cluster, keep the reaction for 2h at room temperature.

Then 1.8ml of EtOAc (9 volumes of it for 1 of DMSO), was added and a pellet of activated material was precipitated at 0°C. Surnatant was removed

and pellet was washed ten times with 0.5 ml of fresh EtOAc; it was obtained 0.7 mg of activated $PAMAM_4$-Hexasaccharide.
All EtOAc washings were combined and cooled at 0°C and other pellet was separated and washed again five times with volumes of 0.5ml of EtOAc, obtaining other 0.4mg of activated material **23**. Two precipitated materials were combined (1.1 mg, yield 85%)

Conjugation of activated $PAMAM_4$-hexasaccharide with CRM_{197}

Total material **23** (1.1mg, 0.189µmol, 1eq), was added of at a 8µl of NaP_i solution of 0.2mg (0.003µmol, 60eq) of CRM_{197}. The reaction was kept for 2h at RT.
SDS-PAGE shows the formation of a conjugate:

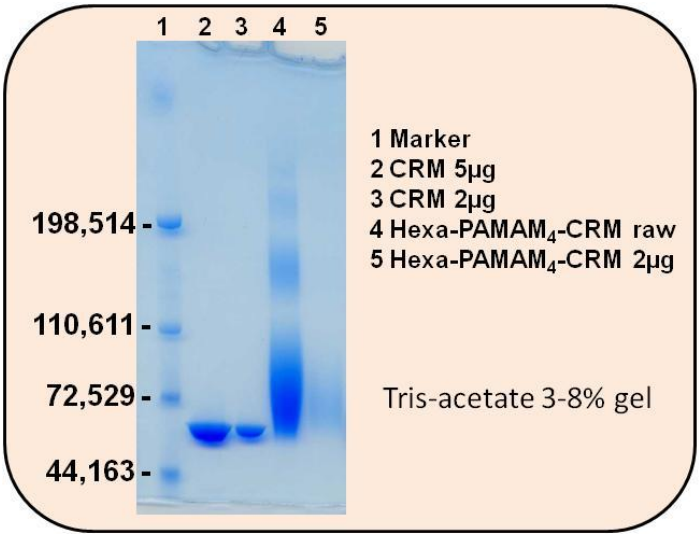


Fig. 44- SDS-Page of hexasaccharide-$PAMAM_4$ *Candida* conjugates.

For the purification (elimination of unconjugated free cluster), an ammonium sulfate precipitation was performed, it was repeated again after a solubilization in NaP_i 100mM pH 7.2; the precipitated material was washing with a ammonium sulfate 0.5M solution for two times.
The material was resuspended in NaP_i 100mM pH 7.2.

Determination of carbohydrate content in glycoconjugates

Total saccharide content in glycoconjugates was determined by ionic chromatography using a CarboPac PA1 column (50mm×250mm) coupled to a CarboPac PA1 guard column and connected to a Dionex ICS3000 system.

Dialyzed samples, where free oligosaccharide has been already removed, were diluted at 5 µg/mL of saccharide and treated with trifluoroacetic acid (TFA) at a final concentration of 2 M, heated at 100 ◦C for 2 h, dried, dissolved in 0.5 mL of distilled water and filtered 0.45 µm before the analysis.

The separation was performed with a flow rate of 1 mL/min using isocratic elution of 40 mM NaOH for 12 min, followed by a washing step with 500 mM NaOH for 5 min. The chromatography was monitored using the pulsed amperometric mode with a gold working electrode and an Ag/AgCl reference electrode, a quadruple-potential waveform for carbohydrates was applied. The chromatographic data were processed using Dionex ChromeleonTM software. Calibration curve, treated as samples, was set up with glucose (Fluka) in the range 0.5–10.0 µg/mL.

3.1.1.2 Results and discussion

A synthetic procedure to synthesize linear β(1,3) trisaccharide and hexasaccharide equipped with amino functionalized linker was optimized.

Moreover by this strategy could be possible obtain longer linear β(1,3) antigens to conjugated and tested. Then two antigens were conjugates to carrier protein CRM_{197} by a bifunctional NHS ester linker obtained from adipic acid and then purified.

The SDS-PAGEs of the two conjugation show smears, typical of conjugates, with higher mass respect to CRM_{197}:

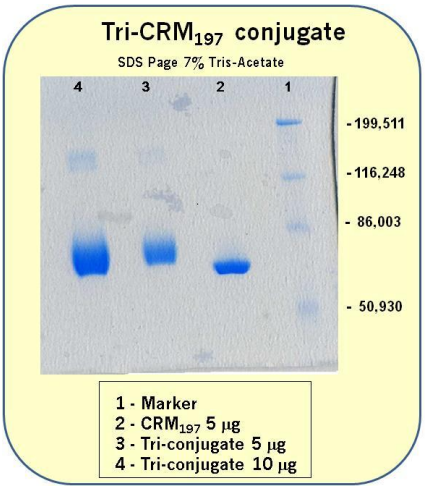


Fig.46 SDS-PAGE of trisaccharide conj..

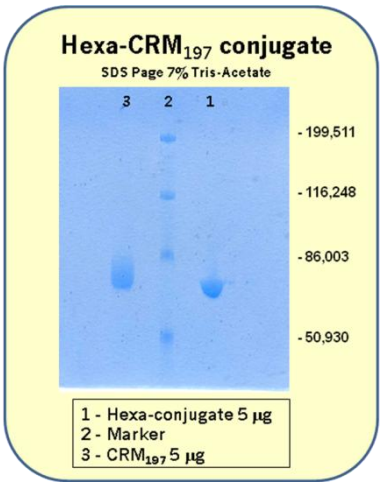


Fig.45 - SDS-PAGE of hexasaccharide conjugate.

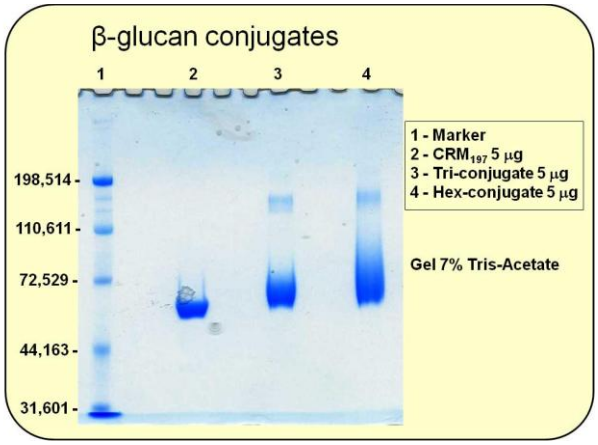


Fig.47 SDS-PAGE that compare hexasaccharide-CRM_{197} and trisaccharide-CRM_{197}.

The conjugates were characterized, for protein content by the colorimetric assay microBCA, for saccharide content by HPAEC-PAD.

The recovery in protein was almost complete for the trisaccharide conjugate, while for thehexasaccharide-conjugate has been 81%. The conjugates were also characterized by MALDI-Tof, the loadings estimated in this case were a bit lower in comparison to the results obtained by microBCA and HPAEC analyses. This evidence was explained by the presence of free oligosaccharide after conjugation, the NHS moiety of activated antigen have been hydrolyzed was not suitable for reaction anymore.

The loadings have been calculated from the three MALDI mass spectra (Fig. 48, 50 and 52), considering the mass of CRM_{197} (58388,756 estimated by MALDI-Tof) and the mass of conjugated antigens (Fig. 49, Fig. 51, Fig. 53)

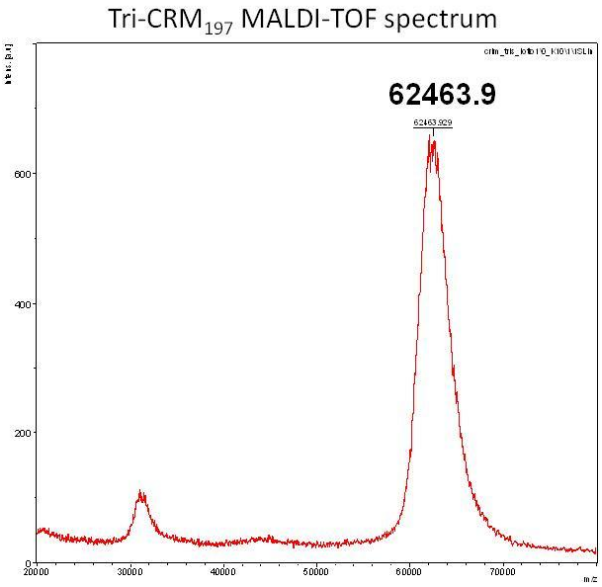


Fig. 48 - MALDI spectrum of Trisaccharide conjugate.

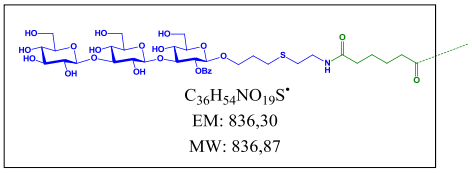


Fig. 49 – Tri-saccharide glucan structure.

For trisaccharide conjugate has been obtained an average loading of 5.5 chains of antigen per molecule of protein.

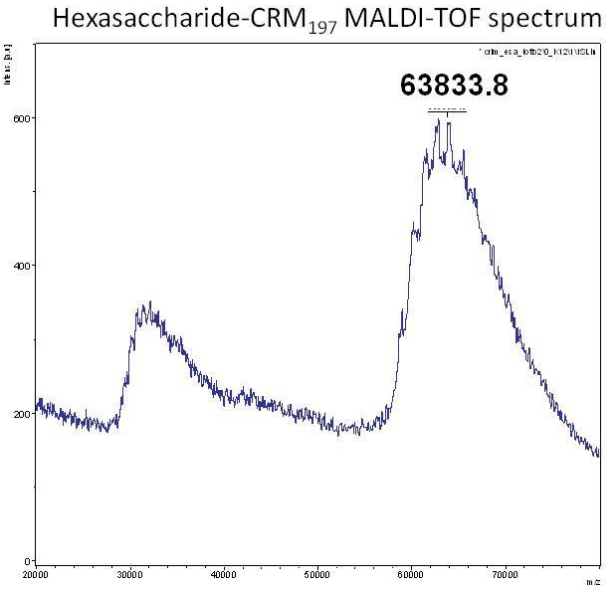


Fig. 50 MALDI spectrum of hexasaccharide conjugate.

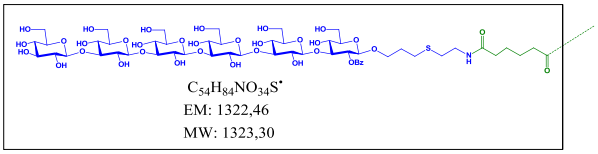


Fig. 51- Hexasaccharide glucan structure.

On the hand, for hexasaccharide conjugates was achieved an average loading of 3.6 chains per molecule of CRM_{197}.

The same approach was employed for the hexa-PAMAM$_4$-CRM$_{197}$ to calculated a loading, considering a fully derivatized PAMAM$_4$ (Fig.53) a loading was 1.7 conjugates cluster per molecule of protein. The cluster was not fully derivatized, only 3.5 chains of 4 branches.

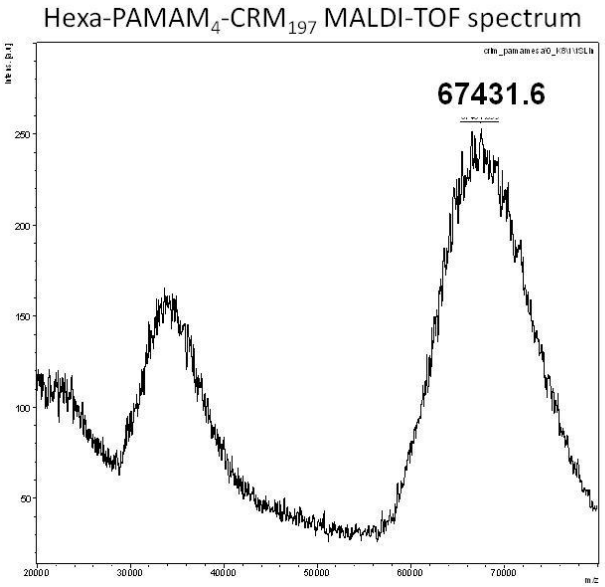


Fig. 52- MALDI spectrum of hexa-PAMAM$_4$ conjugate.

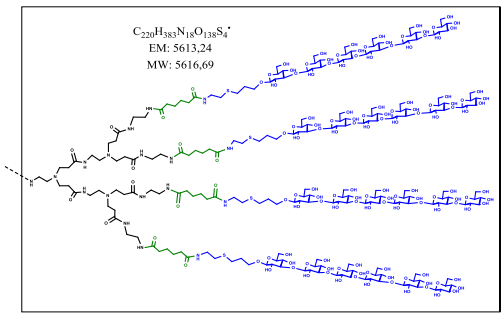


Fig. 53 – Hexasaccharide-PAMAM$_4$ structure.

The β-(1,3) glucan hexasaccharide-CRM_{197} conjugate was tested in Balb/c mice for its ability to induce anti-laminarin antibodies (Fig.54).

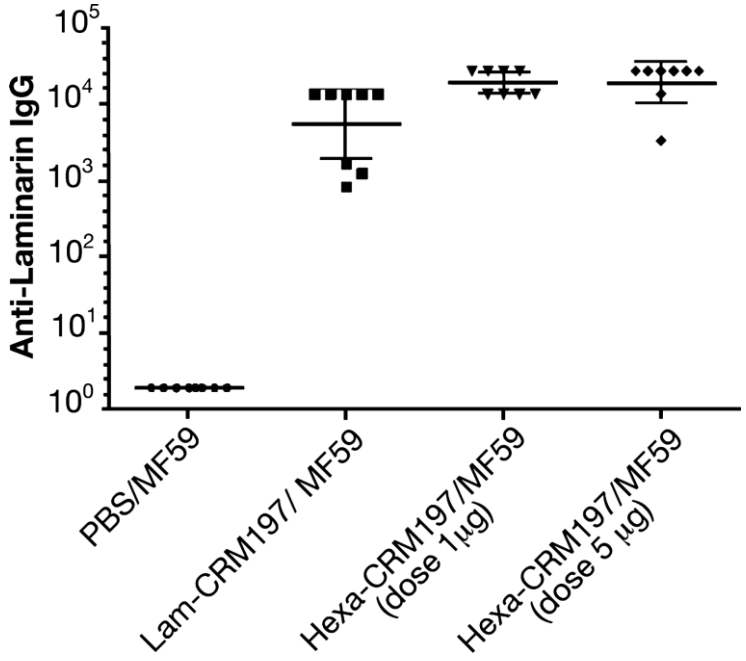


Fig. 54 - Antibody titers induced in mice by the synthetic β-(1,3) glucanhexasaccharide-CRM_{197} conjugate formulated with adjuvant MF59. Horizontal and verticalbars refer to the geometric means and 95% confidence interval, respectively (Adamo et al 2011)

3.2 GAS conjugates

The GAS-CRM_{197} conjugates were obtained from the Research group of Novartis Vaccines. This conjugate was prepared by a reductive amination of the aldehyde group at the reducing end terminus and the amino group of lysine residues of CRM_{197} (Fig.55).

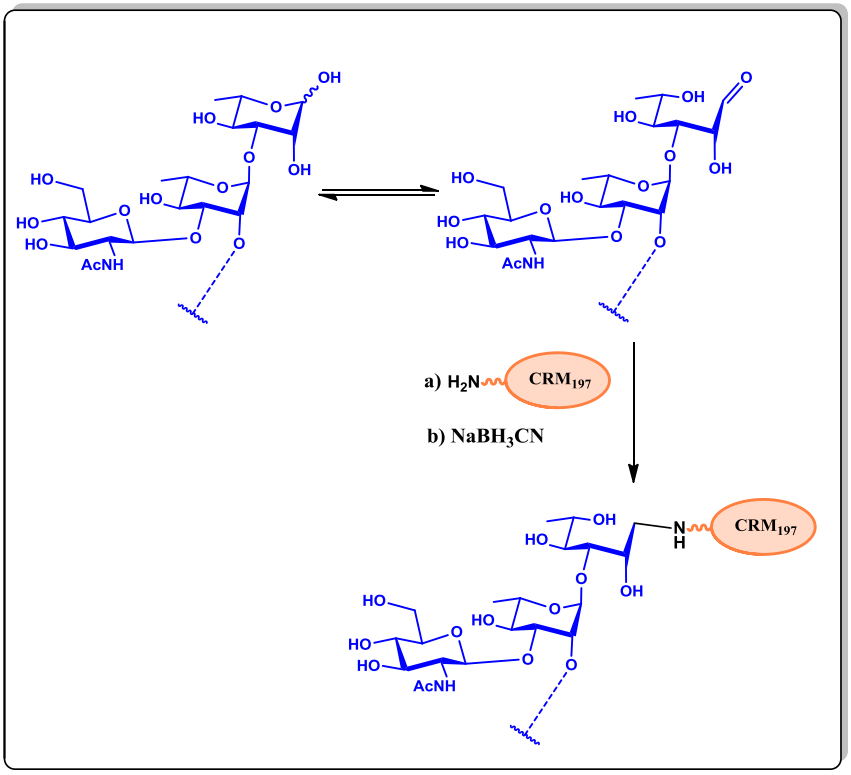


Fig.55 Conjugation of GAS PS to CRM_{197} by reductive amination.

3.3 GBS conjugates

The GBS conjugates were obtained from Technology Development Department of Novartis Vaccines.

The figures 11A and 11B report structures of type Ia, Ib, III of GBS polysaccharides.

The conjugation strategy consisted in a partially oxidation of PS sialic acid hydroxyl groups to obtain aldehyde moieties. These functionalities were used for reductive amination with carrier protein amino groups of lysine residues of CRM_{197} (Fig.56).

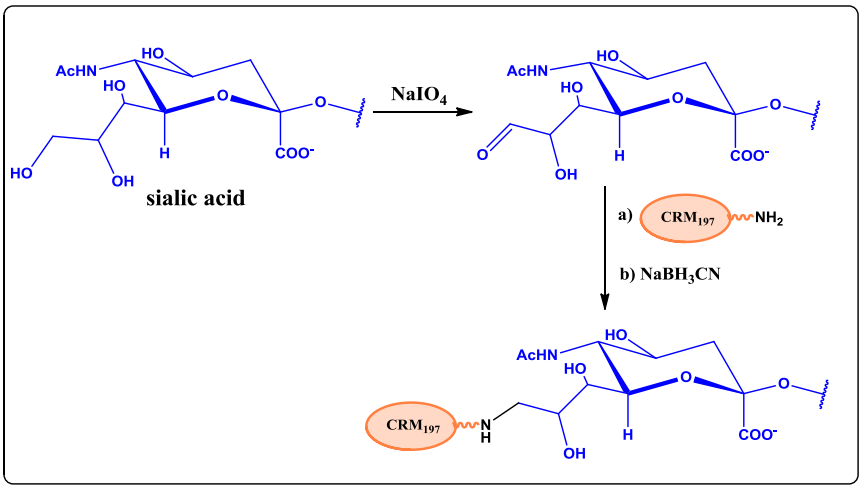


Fig.56 –GBS polysaccharide conjugation strategy.

3.4 MenA conjugate

The MenA conjugate was obtained from Manufacturing of Novartis Vaccines.

The MenA PS was aminated by ammonium acetate to introduce a amine on reducing end instead of an aldehyde. This aminated PS was activated with NHS ester bifunctional linker derived from adipic acid. Finally the activated PS was conjugates to CRM_{197} protein (Fig.57).

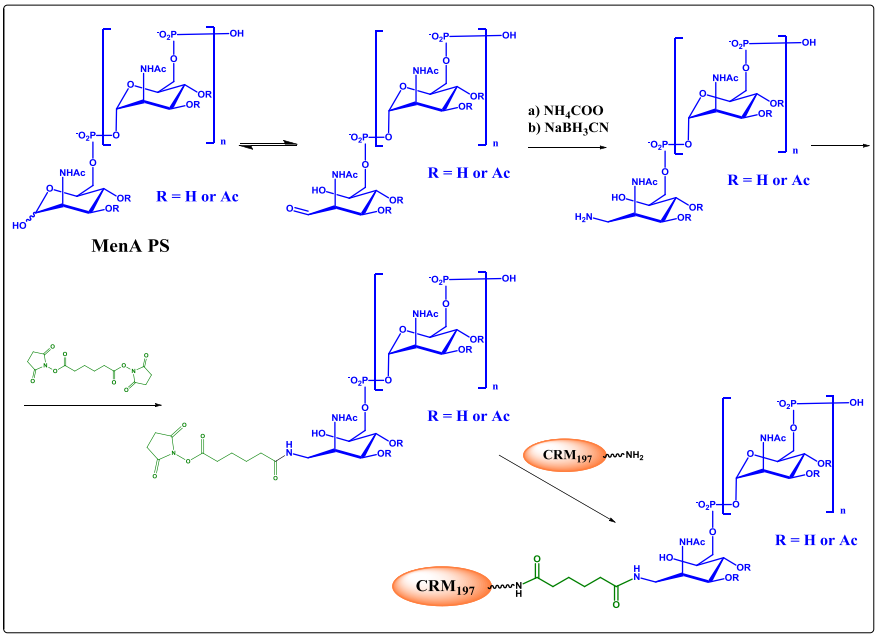


Fig.57 - Conjugation strategy to couple the MenA PS to CRM_{197}

3.5 MenB LPS conjugate conjugates

As described in the Introduction (chapter 1.3.4) the meningococcal serogroup B polysaccharide capsule is composed of a homolinear polymer of α2-8 N-acetylneuraminic acid (sialic acid), which is poorly immunogenic.

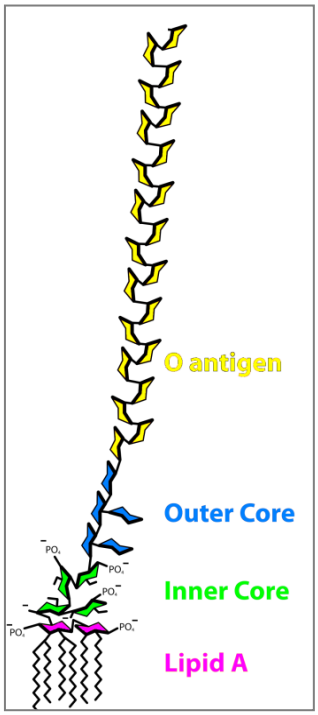


Fig.58 – Lipopolysaccharide (LPS) schematic structure.

To circumvent this limitation of this polysaccharide-conjugate, an approach that involves the use of inner core lipopolysaccharide (LPS) epitopes of MenB as vaccine candidate has been chosen.

LPS is one of the main components of Gram-negative bacterial outer membrane and it acts as a barrier, protecting the bacteria against adverse environmental factor. In addition LPS constitutes a major virulence factor and defends the cells from components of the immune system such as the complement system (Lerouge et al. 2002). The glycan structures on LPS mimic human epitopes resulting in modulation of immune responses (Bergman et al. 2004).

LPS is anchored to the membrane by the lipid A moiety widely known as endotoxin (Beutler et al. 2002; Heumann et al. 2002; Trent et al. 2006;

Freudenberg et al. 2008) and the structure continues with a core oligosaccharide, composed of inner-core and outer-core, and then with final O antigen (Fig.58).

Lipid A is a phosphorylated glucosamine disaccharide on which fatty acids chains are bind. These hydrophobic fatty acid chains anchor the LPS into the bacterial membrane, and the rest of the LPS is exposed on the cell surface. The lipid A moiety is responsible for the majority of toxicity of Gram-negative bacteria, in fact at the moment bacterial cells are lysed by the immune system, portions of membrane containing lipid A are released into the circulation, causing fever, diarrhea, and possible fatal septic shock. The inner-core always contains an oligosaccharide component which attaches directly to lipid A and commonly contains sugars such as heptose, mainly a L-glycero-D-mannoheptulose and 3-deoxy-D-mannooctulosonic acid (KDO or keto-deoxyoctulosonate).

The KDO residue is located at the reducing end of the oligosaccharide chain and is essential for its biological activity. These saccharide units are usually substituted by charged phosphate groups, resulting in an accumulation of charge in this inner region.

The last KDO is often modified with a phosphate or ethanolamine group. From the KDOs, there are attached two or three heptoses which are usually phosphorylated. The ketosidic bond between KDO and lipid A ($\alpha 2 \rightarrow 6$) is especially susceptible to acid cleavage. The outer core is made of hexose residues that are bound to the last heptose residue in the inner core. Hexoses generally found in the outer core are: D-glucose, D-mannose, D-galactose, etc.. There is usually at least three hexoses bound $\beta 1 \rightarrow 3$, with the O antigen being linked to the third hexose. Other hexoses are often found attached to the outer core, branching from the main oligomer. The LPS structure of Neisseria comprises a lipid A backbone attached to a core oligosaccharide unit with an inner core di-heptose-N-acetyl-glucosamine backbone, wherein the two heptose residues can provide a point of attachment for the outer core oligosaccharide residues.

At the moment, the meningococcal LPS has been classified into 12 immunotype (L1,…,L12) initially defined by mAb reactivities, and subsequently define by structural analyses. The structures of LPS from immunotypes L1/6, L2, L3 (Fig.59), L4/7, L5 and L9 are known.

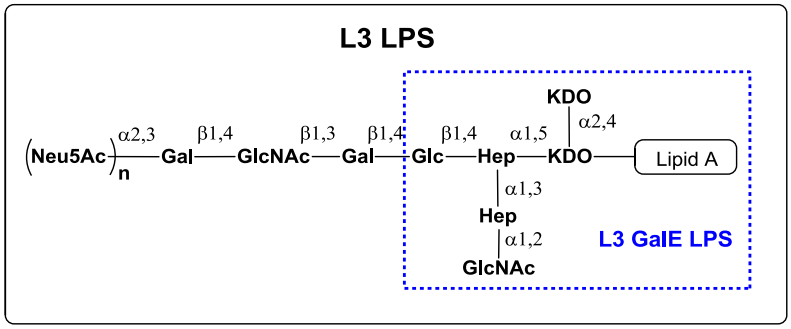


Fig.59 – LPS saccharide core structure.

This work has been performed in collaboration to the group of Andrew Cox and Jim Richards (Institute for Biological Sciences - IBS, National Research Council, Ottawa - Canada) and the group of Prof. Richard Moxon (University of Oxford, Oxford - UK).

A modified L3 LPS was provided by IBS laboratories and it was obtained from a mutant strain depleted of UDP glucose 4-epimerase. The enzyme UDP glucose 4-epimerase (GalE) is essential for *N. meningitidis* to synthesize UDP-Gal for incorporation of galactose into its LPS and is encoded by the gene *galE*. In the absence of this enzyme the structure is trimmed at galactose resulting in the loss the polysialylated mojety, which is similar to the neural cell adhesion molecule. This allows us to have an inner-core structure of meningococcal B LPS epitope (blue dashed box on Fig.59).

The L3 LPS extracted from bacterial growth was O-deacetylated with hydrazine and treated by enzyme from *Dictystelium discoides* to partially cleave the lipid chains: this process decreases the toxicity of LPS saccharide by partial elimination of the aliphatic chains of lipid A. In addition a free amine group is generated that will be used for conjugation to the carrier protein (Fig.60a).This intermediate has been supplied by IBS laboratory and it has represented my starting material for the preparation of the vaccine. Our synthetic strategy used the lipid A's free primary amine to bind the LPS to carrier protein. Basically the synthetic scheme leading to the conjugate consists of the activation of both LPS (Fig.60) and protein (Fig.62). The conjugation chemistry has involved two complementary linkers, one with thiol moiety, introducted on carrier protein, and the second with a maleimide group, placed on the LPS.

After the selective protection of the Phosphoethanolamine (PEt) amino groups with Boc (Fig.60b),the activation of saccharide has been obtained by reaction of GlcNAc lipid A amine groups with NHS ester group of GMBS (*N*-[γ-maleimidobutyryloxy] succinimide ester) linker (Fig.60c, Fig.61). Last step has been the final Boc deprotection (Fig.60d) of Phosphoethanolamine . The faster PEt amine reactivity has been exploited for the chemoselective protection with Boc, leaving the GlcNAc amine free. The activated lipooligosaccharide has maleimide group available to react with a thiol group inserted on activated carrier protein.

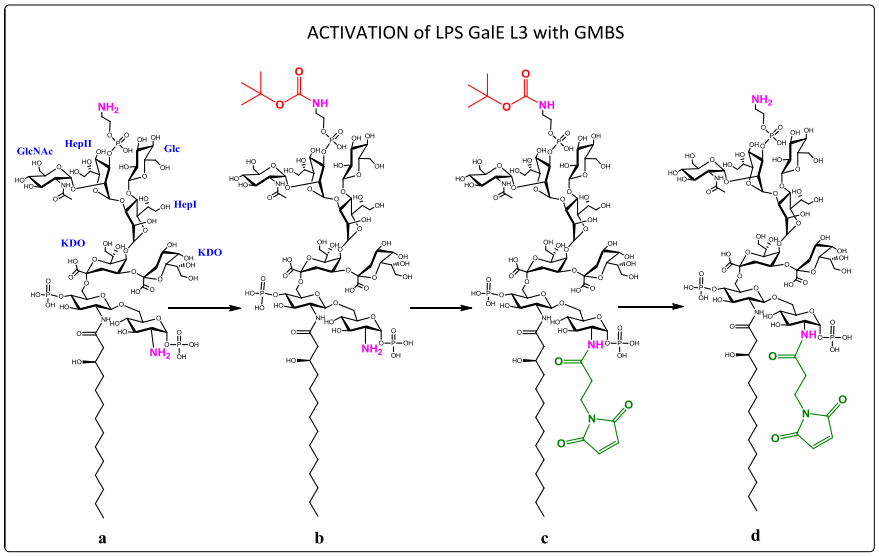


Fig.60 – Preparation scheme of LPS conjugate.

Consequently, the carrier protein CRM_{197} has been derivatized on acidic groups with adipic acid dihydrazide (ADH) to introduce highly nucleophilic amino groups, by EDC carbodiimide chemistry. The derivatized protein, CRM_{197}-ADH has been characterized by ESI-MS spectroscopy.

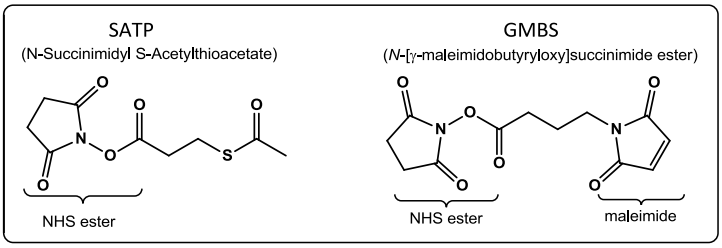


Fig.61– Linkers used for saccharide-protein coupling.

The CRM_{197}-ADH has been then functionalizated with SATP (*N*-succinimidyl *S*-acetylthiopropionate) (Fig.62) by the reaction of linker NHS ester group with ADH hydrazide moiety presents on the protein surface. The acyl protected thiol group of SATP linker was deprotected by hydroxylamine (Fig.62), and finally conjugated to maleimide (Fig.63) previously inserted on the LPS (Fig.63).

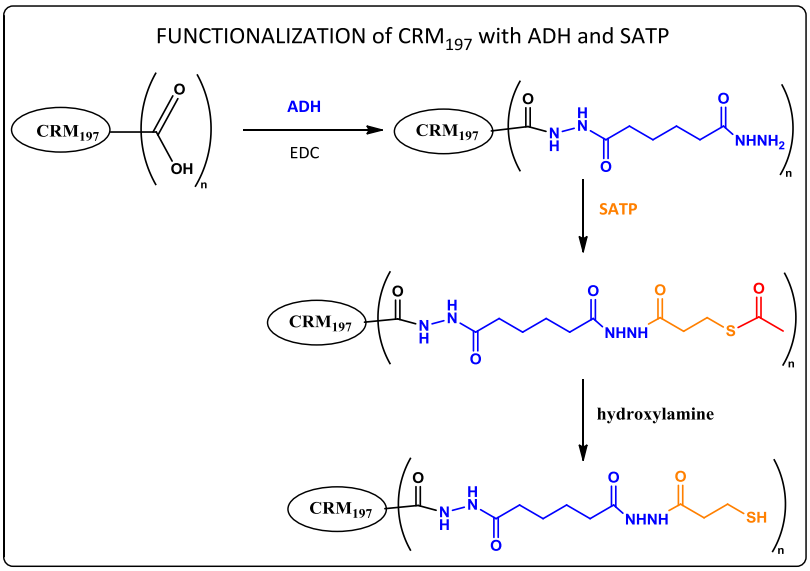


Fig.62 - Preparation scheme of LPS conjugate.

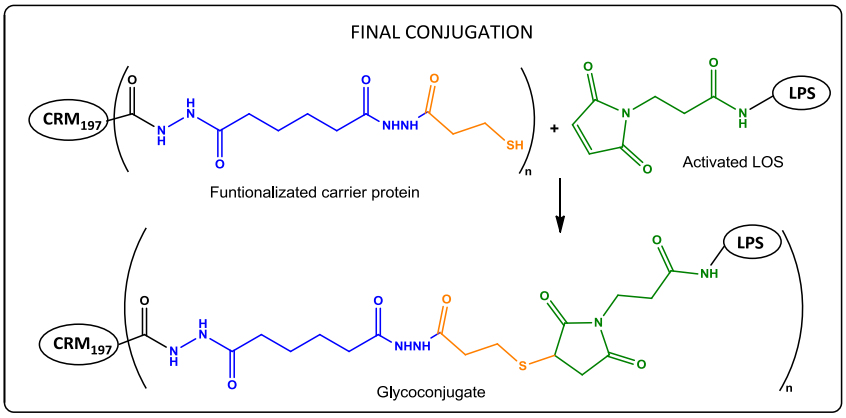


Fig.63 - Preparation scheme of LPS conjugate.

3.5.1 Materials and methods

L3 GalE lipopolysaccharide (Fig.65) was provided by Institute for Biological Sciences (IBS) of National Research Council (Ottawa - Canada). CRM_{197} was obtained from Manufacturing. Adipic acid dihydrazide (ADH), hydroxylamine hydrochlorid and di-*tert*-butyl dicarbonate (Boc anydryde), trifluoroacetic acid (TFA), 2-(*N*-morpholino) ethanesulfonic acid (MES) were purchased from Sigma Aldrich. The GMBS and SATP linkers were provided by Pierce-Thermo Scientific. Sephadex G-25 column resin was purchased from Amersham.

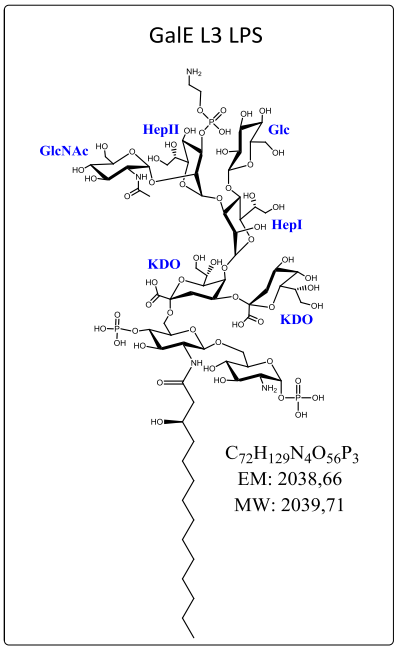


Fig.64 – GalE L3 LPS schematic structure.

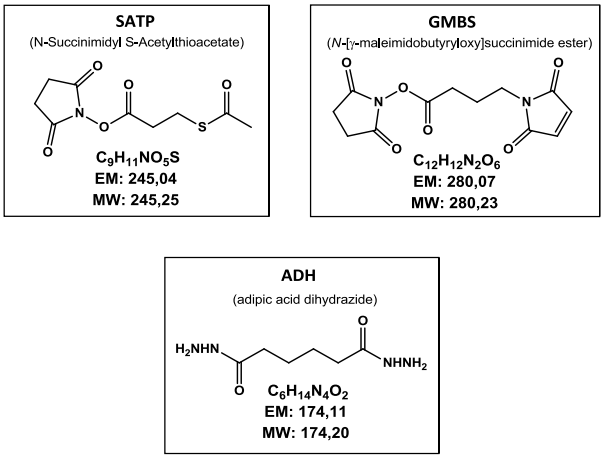


Fig.65 - Linkers used for coupling.

Boc protection

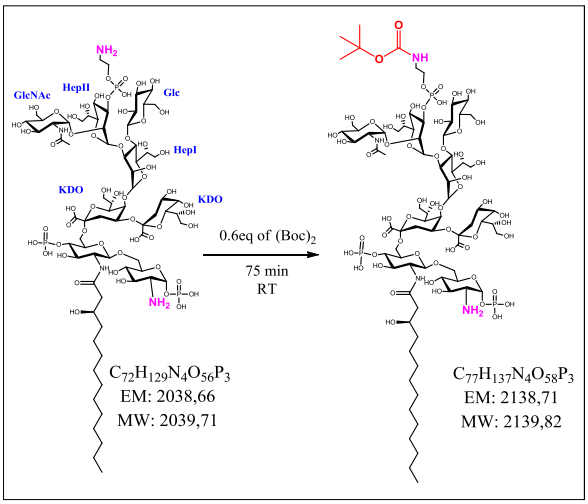


Fig.66 - Boc protection of LPS primary amine.

15mg of LPS (at 4mg/ml) were dissolved with 3 molar eq. of NaOH, diluting a 0.1M NaOH stock solution with an appropriate volume of water. (e.g. for a CHO with mol. wt of 2000 use 160µl of 0.1M NaOH and 0.84 ml of H_2O). Boc anhydride is solid at RT (mp=23°C) but becomes liquid with gently warming, making it easier to handle, d=0.95g/ml. It was added, to the LPS

solution, 0.6 molar eq. of Boc anhydride dissolved in the same volume of DMSO (i.e. if add 1ml Boc, dissolve in 1ml DMSO). The reaction was stirred for 75 minutes at RT. Then the aqueous mixture was washed three times, extracting with ethyl acetate (6ml/ 10 mg of LPS), spinning at 2k for 10min. The organic phase was discarded each time. The aqueous layer was lyophilized in a 100ml round bottom flask, after adding of 30ml of water.
The material (max 20mg per run) was purified using a 16mm x 60cm (V=120ml) Sephadex G-25 column (Amersham 17-0033-02, medium), eluting with water (flow 0.5min) and the product peak was collected and lyophilized. The recovery was approximately 100%.

Activation of Boc-LPS by GMBS

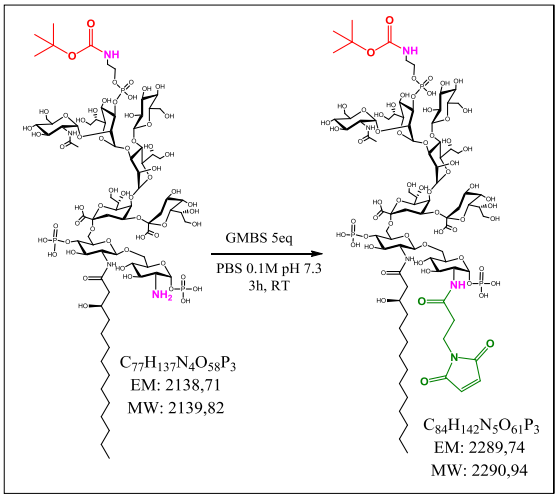


Fig.67 - GMBS linker coupling on the Boc-protected material.

Boc-protected LPS material was dissolved at 4mg/ml in PBS buffer 0.1M pH 7.3; meanwhile 5 equivalents of GMBS were dissolved apart in 3.75 ml of DMSO (2.8mg/ml) and then it were added, to the LPS, 1eq (750µl) of solubilized GMBS each time at t=0, 20, 40, 60, and 80min. The reaction was left at room temperature for 3 hours. The raw sample was purify (max 20mg per run) using a 16mm radius x 60cm (V=120ml) Sephadex G-25 column, eluding with water, flow 0.5ml/min. The product peak was pooled and lyophilized, and 12mg of product were obtained.

Boc deprotection of activated-LPS

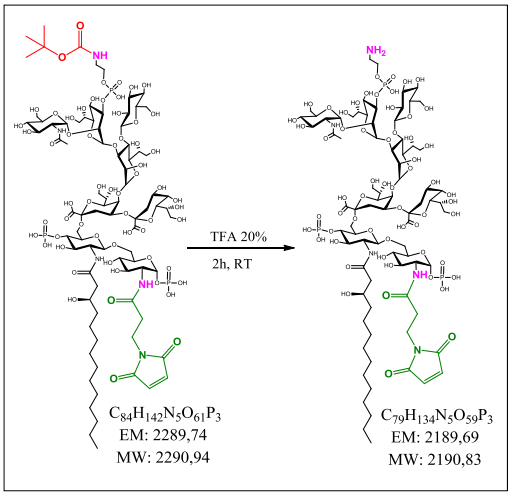


Fig.68 – Boc-Deprotection of PEt amino group.

Boc-protected and activated LPS (12mg, it was considered 80% yield of starting 15mg) were dissolve in 20% TFA (at 6mg/ml) and left at room temperature for 2hrs. The sample (max 20mg per run) was purified using a 16mm radius x 40cm Sephadex G-25 column, eluting with water (flow 0.5ml/min), the product pool was lyophilize, obtaing 9.6mg (80% of recovery). Check the fractions by NMR and mass spectrometry to confirm the Boc removal.

Functionalization of CRM_{197} with ADH

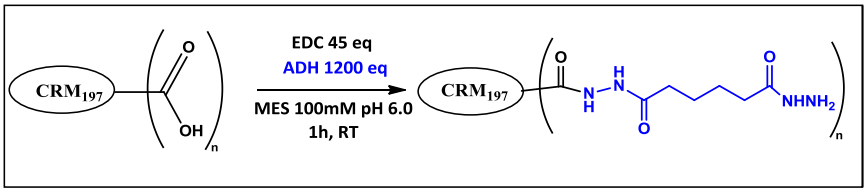


Fig.69– Reaction of acid side chain of CRM_{197} with adipic dihydrazide

CRM_{197} (10mg) was dissolved in 1ml of 100mM MES buffer (2-(*N*-morpholino) ethanesulfonic acid , Aldrich) pH 6.0, then 1200eq of adipic dihydrazide (ADH) was added under gently stirring until complete solubilization. It is important to be sure to check pH of this solution as pH tends to go below 5.0, if necessary to bring it back to 5.2 with small amount of 1M NaOH.

Then 45eq of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were added to CRM_{197}/ADH solution and placed at RT for 1h, and then the reaction was quenched adding 1ml of buffer NaPi 400mM pH 7.2.
The sample was purify using an Ultra-15 10kDa cut-off spin column (Millipore) into 100mM sodium phosphate pH 6.8 buffer (repeating dilute then spin four times, 4.9K 30min).
The resulting activated CRM protein was examined by MALDI-MS which revealed that 6-7 ADH molecules had been incorporated onto the CRM_{197} protein.

Functionalization of CRM_{197}-ADH with SATP linker

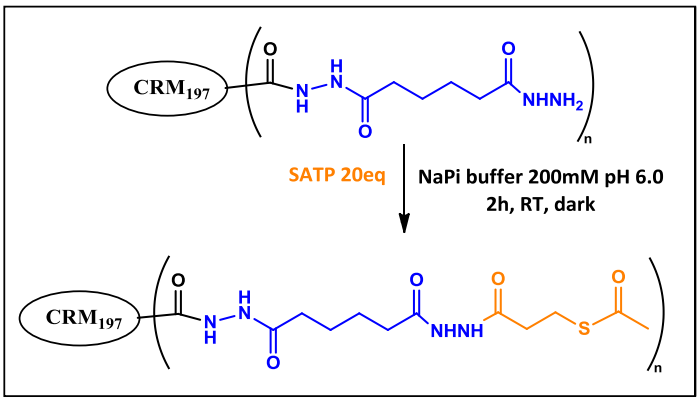


Fig.70 – Reaction between hydrazyde group and SATP linker.

The 10mg of concentrated CRM-ADH (7.3mg/ml, 1370µl) sample were diluted to 4ml with NaP_i buffer 200mM pH 6.0: the final concentration was 131.5 mM. Considering an average ADH loading of 6-7 chains each CRM_{197} molecule, 20eq *N*-Succinimidyl-*S*-acetylthiopropionate (SATP) previously dissolved in 0.1ml of DMSO were added under mild stirring. The reaction was incubated at room temperature for 45min in the dark with mild stirring. The sample was purified on Sephadex G-25) column (16mm X 60cm, V=120ml) and eluded with 100mM sodium phosphate pH 6.8 buffer.
The product peaks were pooled (18ml) and stored at 4°C.
The protein content estimated by microBCA was 470µg/ml.

Deprotection of thiol moiety of CRM-ADH-SATP

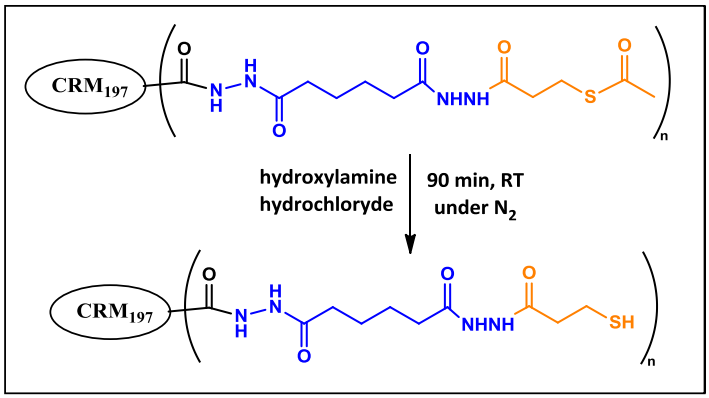


Fig.71 - Deprotection of thiol moiety by hydroxylamine hydrochloride

345µl of hydroxylamine hydrochloride 0.5M and 225µl, each 2mg of protein, were added to 3.07mg of CRM-ADH-SATP (470µg/ml, 6.54ml). The reaction was left under nitrogen flow for 90min at RT. The reaction was purified on on Sephadex G-25 column (16mm X 60cm, V=120ml) and eluded with sodium phosphate buffer 100mM pH 6.8. The product peaks were pooled (16ml) and stored at 4°C.

Conjugation of CRM-ADH-SATP with LPS-GMBS

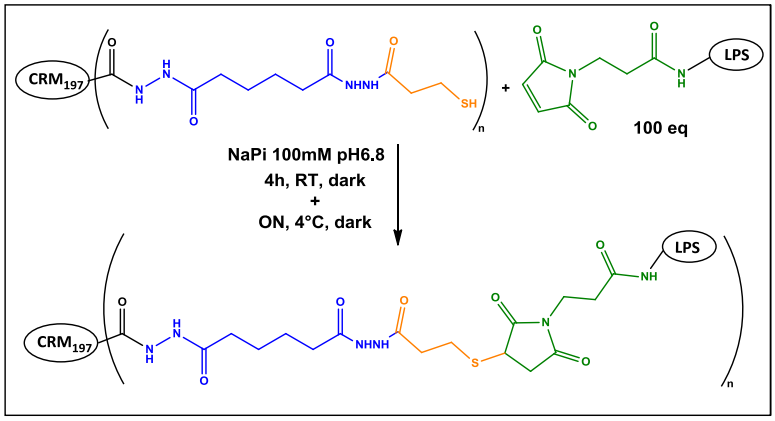


Fig.72– Final conjugation between functionalized LPS and carrier protein.

9.6mg of LPS-GMBS were dissolved in 1ml of NaP_i 100mM pH6.8, and then 500µl (50eq) was added to deprotected CRM-ADH-SATP all material (3mg, in 16ml of NaP_i 100mM pH6.8). The reaction was left in the dark at RT for

5h. Then the remained 500µl (50eq) of LPS-GMBS solution were added to react overnight at 4°C in the dark.
Final conjugate was concentrated to approximately 1ml on an Amicon ultra-15 10 MWCO centrifugal filter (Millipore UFC901096) and then on the same filter were performed three washing with sodium phosphate 100mM pH 7.2.

3.5.2 Results and discussion

The CRM_{197} has been functionalized with ADH to insert hydrazyde moieties that are more reactive of amine of lysine residues. The two complementary linker SATP and GBMS were choose because they are orthogonal respect other amino acid residues; other four thiol of cysteines of CRM_{197} are involved in disulfur bonds. Moreover the protected thiol moiety of SATP has permitted an easy purification, without risk of cross linking due to intermoleculear formations of disufur bonds.The conjugation of modified L3 GalE LPS was achieved as is shown on SDS-PAGE below, thus it has been demonstrated this coupling chemistry is a efficient way to bind a LPS structure to a carrier protein.

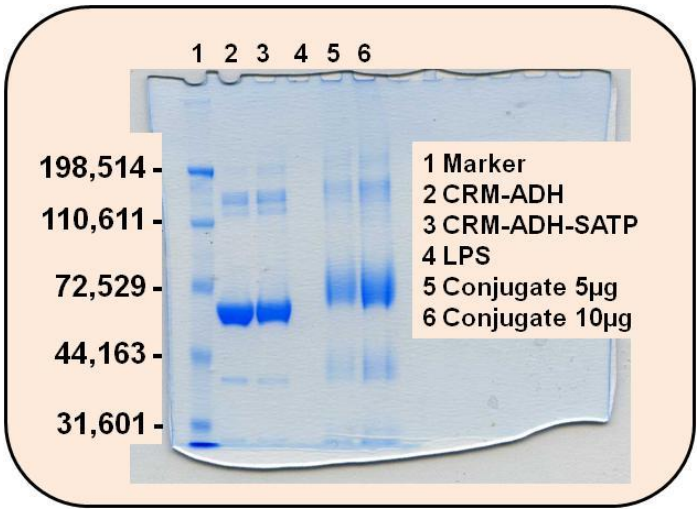


Fig.73– SDS-Page of LPS conjugate and activated protein.

In addition a MALDI-TOF spectrum (Fig.74) was collected to estimate the LPS loading on CRM_{197} considering a LPS mass increment 2189,64 Da.

The conjugate mass value has been subtracted of mass of CRM_{197} and then divided by LPS mass increment subtracted of one (2189,64 dalton), obtaining an average loading of 4 LPS molecules per CRM_{197} molecule.

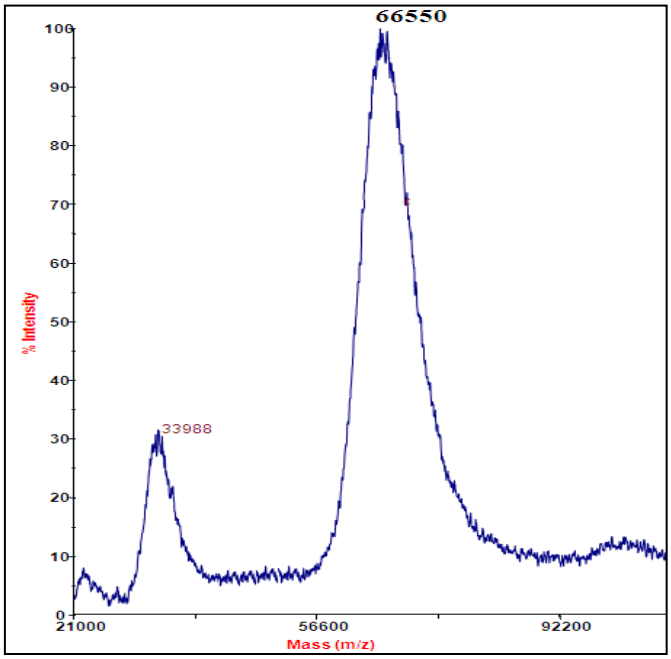


Fig.74 MALDI spectrum of LPS conjugate

4. REFERENCES

Ada G, Isaacs D. 2003. Carbohydrate-protein conjugate vaccines. Clin Microbiol Infect 9:79-85.

Adamo A, Tontini M, Brogioni G,Romano MR, Costantini G, Danieli E, Proietti P, Berti F, Costantino P. 2011. Synthesis of Laminarin fragments and evaluation of a β-(1,3) Glucan Hexasaccaride-CRM_{197} conjugate as vaccine candidate against *Candida albicans*; J. Carbohyd. Chem.; 30, 249-280.

Alvarez CP, Lasala F, Carrillo J, Muniz O, Corbi AL, Delgado R. 2002. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. J Virol 76:6841-6844.

Areschoug T, Carlsson F, Stalhammar-Carlemalm M, Lindahl G. 2004. Host-pathogen interactions in *Streptococcus pyogenes* infections, with special reference to puerperal fever and a comment on vaccine development. Vaccine 22 Suppl 1:S9-S14.

Avci F, Kasper D. 2010. How bacterial carbohydrates influence the adaptive immune system. Annu Rev Immunol 28:29-52.

Avery OT, Goebel WF. 1931. Chemo-Immunological Studies on Conjugated Carbohydrate-Proteins : V. the Immunological Specifity of an Antigen Prepared by Combining the Capsular Polysaccharide of Type III Pneumococcus with Foreign Protein. J Exp Med 54:437-447.

Baghian A, Luftig M, Black JB, Meng YX, Pau CP, Voss T, Pellett PE, Kousoulas KG. 2000. Glycoprotein B of human herpesvirus 8 is a component of the virion in a cleaved form composed of amino- and carboxyl-terminal fragments. Virology 269:18-25.

Bardotti A, Averani G, Berti F, Berti S, Carinci V, D'Ascenzi S, Fabbri B, Giannini S, Giannozzi A, Magagnoli C, Proietti D, Norelli F, Rappuoli R, Ricci S, Costantino P. 2008. Physicochemical characterization of glycoconjugate vaccines for prevention of meningococcal diseases. Vaccine 26:2284-2296.

Barrientos LG, Gronenborn AM. 2005. The highly specific carbohydrate-

binding protein cyanovirin-N: structure, anti-HIV/Ebola activity and possibilities for therapy. Mini Rev Med Chem 5:21-31.

Bedford H, de Louvois J, Halket S, Peckham C, Hurley R, Harvey D. 2001. Meningitis in infancy in England and Wales: follow up at age 5 years. BMJ; 323(7312):533-6.

Bergman MP, Engering A, Smits HH, van Vliet SJ, van Bodegraven AA, Wirth HP, Kapsenberg ML, Vandenbroucke-Grauls CM, van Kooyk Y, Appelmelk BJ. 2004. Helicobacter pylori modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. J Exp Med; 200(8):979-90.

Beutler B. 2002. LPS in microbial pathogenesis: promise and fulfilment. J Endotoxin Res; 8(5):329-35.

Bloom BR, Lambert PH. 2003. The vaccine book. Academic Press (Elsevier Science, London) p. 65.

Boas U, Christensen JB, Heegaard PMH. 2006. Dendrimers in medicine and biotechnology. In: New Molecular Tools. London (UK): Royal Society of Chemistry:56-61.

Bowman SM, Free SJ. 2006. The structure and synthesis of the fungal cell wall. Bioessays. 28(8):799-808.

Bloem A, Zenke G, Eichmann K, Emmrich F. 1988. Human immune response to group A streptococcal carbohydrate (A-CHO). II. Antigen-independent stimulation of IgM anti-A-CHO production in purified B cells by a monoclonal anti-idiotopic antibody. J Immunol 140:277-282.

Broker M, Dull PM, Rappuoli R, Costantino P. 2009. Chemistry of a new investigational quadrivalent meningococcal conjugate vaccine that is immunogenic at all ages. Vaccine 27:5574-5580.

Broome CV. 1986. Use of bacterial vaccines for prevention of pneumococcal and meningococcal disease in the day care setting. Rev Infect Dis; 8(4):584-8.

Bromuro C, Romano M, Chiani P, Berti F, Tontini M, Proietti D, Mori E, Torosantucci A, Costantino P, Rappuoli R, Cassone A. 2010. Beta-glucan-CRM_{197} conjugates as candidates antifungal vaccines. Vaccine; 28(14):2615-23.

Bromuro C, Torosantucci A, Chiani P, Conti S, Polonelli L, Cassone A. 2002. Interplay between protective and inhibitory antibodies dictates the outcome of experimentally disseminated Candidiasis in recipients of a Candida albicans vaccine. Infect Immun. 70(10):5462-70.

Calderone RA, Braun PC. 1991. Adherence and receptor relationships of Candida albicans. Microbiol Rev. 55(1):1-20.

Carapetis JR, Steer AC, Mulholland EK, Weber M. 2005. The global burden of group A streptococcal diseases. Lancet Infect Dis; 5(11):685-94.

Cartwright KA, Stuart JM, Jones DM, Noah ND. 1987. The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica. Epidemiol Infect.; 99(3):591-601.

Chabre YM, Roy R. 2008. Recent trends in glycodendrimer syntheses and applications. Curr Top Med Chem 8:1237-1285.

Chong P, Chan N, Kandil A, Tripet B, James O, Yang YP, Shi SP, Klein M. 1997. A strategy for rational design of fully synthetic glycopeptide conjugate vaccines. Infect Immun 65:4918-4925.

Cobb BA, Kasper DL. 2005. Zwitterionic capsular polysaccharides: the new MHCII-dependent antigens. Cell Microbiol.; 7(10):1398-403.

Costantino P, Rappuoli R, Berti F. 2011. The design of semi-synthetic and synthetic glycoconjugate vaccines. Expert Opinion on Drug Discovery; 6, 10: 1045-1066(22)

Cunningham MW. 2000. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13:470-511.

Cunningham MW. 2008. Pathogenesis of group A streptococcal infections and their sequelae. Adv Exp Med Biol; 609:29-42.

Cutler JE, Deepe GS Jr, Klein BS. 2006 Advances in combating fungal diseases: vaccines on the threshold. Nat Rev Microbiol.; 5(1):13-28.

Davis CW, Nguyen HY, Hanna SL, Sanchez MD, Doms RW, Pierson TC. 2006. West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. J Virol 80:1290-1301.

Fox A, Lau PY, Brown A, Morgan SL, Zhu ZT, Lema M, Walla MD. 1984. Capillary gas chromatographic analysis of carbohydrates of Legionella pneumophila and other members of the family Legionellaceae. J Clin Microbiol; 19(3):326-32.

Freudenberg MA, Tchaptchet S, Keck S, Fejer G, Huber M, Schütze N, Beutler B, Galanos C. 2008. Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. Immunobiology; 213(3-4):193-203.

Galonic DP, Gin DY. 2007. Chemical glycosylation in the synthesis of glycoconjugate antitumour vaccines. Nature 446:1000-1007.

Garzon S, Marquis G, Montplaisir S, Kurstak E, Benhamou N. 1989 Antigenic structure of Candida albicans. Electronmicroscopic localization of polysaccharide and immunodeterminants in the cell wall. Immunol Ser; 47:3-36.

Giannini G, Rappuoli R, Ratti G. 1984. The amino-acid sequence of two non-toxic mutants of diphtheria toxin: CRM_{45} and CRM_{197}. Nucleic Acids Res 12:4063-4069.

Giehm L, Christensen C, Boas U, Heegaard PM, Otzen DE. 2008. Dendrimers destabilize proteins in a generation-dependent manner involving electrostatic interactions. Biopolymers 89:522-529.

Goffard A, Callens N, Bartosch B, Wychowski C, Cosset FL, Montpellier C, Dubuisson J. 2005. Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. J Virol 79:8400-8409.

Goldblatt D. 1998. Recent developments in bacterial conjugate vaccines. J Med Microbiol 47:563-567.

Goridis C, Deagostini-Bazin H, Hirn M, Hirsch MR, Rougon G, Sadoul R, Langley OK, Gombos G, Finne J. Neural surface antigens during nervous system development Cold Spring Harb Symp Quant Biol. 1983;48 Pt 2:527-37

Guilherme L, Fae KC, Higa F, Chaves L, Oshiro SE, Freschi de Barros S, Puschel C, Juliano MA, Tanaka AC, Spina G, Kalil J. 2006. Towards a vaccine against rheumatic fever. Clin Dev Immunol 13:125-132.

Halary F, Amara A, Lortat-Jacob H, Messerle M, Delaunay T, Houles C, Fieschi F, Arenzana-Seisdedos F, Moreau JF, Dechanet-Merville J. 2002. Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. Immunity 17:653-664.

Han DP, Kim HG, Kim YB, Poon LL, Cho MW. 2004. Development of a safe neutralization assay for SARS-CoV and characterization of S-glycoprotein. Virology 326:140-149.

Haukness HA, Tanz RR, Thomson RB, Jr., Pierry DK, Kaplan EL, Beall B, Johnson D, Hoe NP, Musser JM, Shulman ST. 2002. The heterogeneity of endemic community pediatric group a streptococcal pharyngeal isolates and their relationship to invasive isolates. J Infect Dis 185:915-920.

Heath PT, Balfour G, Weisner AM, Efstratiou A, Lamagni TL, Tighe H, O'Connell LA, Cafferkey M, Verlander NQ, Nicoll A, McCartney AC; PHLS Group B *Streptococcus* Working Group. 2004. Group B streptococcal disease in UK and Irish infants younger than 90 days. Lancet; 363(9405):292-4.

Heath PT, Balfour GF, Tighe H, Verlander NQ, Lamagni TL, Efstratiou A. 2009. Group B streptococcal disease in infants: a case control study. Arch Dis Child; 94(9):674-80.

Helle F, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C, Dubuisson J. 2006. Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. J Biol Chem 281:25177-

Heumann D, Roger T. 2002. Initial responses to endotoxins and Gram-negative bacteria. Clin Chim Acta; 323(1-2):59-72.

Jackson LA, Jacobson RM, Reisinger KS, Anemona A, Danzig LE, Dull PM. 2009. A randomized trial to determine the tolerability and immunogenicity of a quadrivalent meningococcal glycoconjugate vaccine in healthy adolescents. Pediatr Infect Dis J 28:86-91.

Jennings HJ, Lugowski C. 1981. Immunochemistry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. J Immunol 127:1011-1018.

Jones C. 2005. Vaccines based on the cell surface carbohydrates of pathogenic bacteria. An Acad Bras Cienc 77:293-324.

Jones C. 2005 b. NMR assays for carbohydrate-based vaccines, J. of Pharmaceutical and Biomedical Analysis 38 (2005) 840–850.

Kalinski P, Moser M. 2005. Consensual immunity: success-driven development of T-helper-1 and T-helper-2 responses. Nat Rev Immunol 5:251-260.

Karakawa WW, Osterland CK, Krause R. 1965. Detection of Streptococcal Group-Specific Antibodies in Human Sera. J Exp Med 122:195-205.

Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD. 2003. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. J Virol 77:12022-12032.

Lai Z, Schreiber JR. 20009. Antigen processing of glycoconjugate vaccines; the polysaccharide portion of the pneumococcal CRM(197) conjugate vaccine co-localizes with MHC II on the antigen processing cell surface. Vaccine; 27(24):3137-44.

Lerouge I, Vanderleyden J. 2002. O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. FEMS Microbiol Rev; 26(1):17-47.

Lozach PY, Burleigh L, Staropoli I, Navarro-Sanchez E, Harriague J, Virelizier

JL, Rey FA, Despres P, Arenzana-Seisdedos F, Amara A. 2005. Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. J Biol Chem 280:23698-23708.

Lucas AH, Apicella MA, Taylor CE. 2005. Carbohydrate moieties as vaccine candidates. Clin Infect Dis 41:705-712.

Malkiel S, Liao L, Cunningham MW, Diamond B. 2000. T-Cell-dependent antibody response to the dominant epitope of streptococcal polysaccharide, N-acetyl-glucosamine, is cross-reactive with cardiac myosin. Infect Immun 68:5803-5808.

Mawas F, Peyre M, Beignon AS, Frost L, Del Giudice G, Rappuoli R, Muller S, Sesardic D, Partidos CD. 2004. Successful induction of protective antibody responses against Haemophilus influenzae type b and diphtheria after transcutaneous immunization with the glycoconjugate polyribosyl ribitol phosphate-cross-reacting material 197 vaccine. J Infect Dis 190:1177-1182.

Mc CM, Lancefield RC. 1955. Variation in the group-specific carbohydrate of group A streptococci. I. Immunochemical studies on the carbohydrates of variant strains. J Exp Med 102:11-28.

Michon F, Pozsgay V, Brisson JR, Jennings HJ. 1989. Substrate specificity of naringinase, an alpha-L-rhamnosidase from Penicillium decumbens. Carbohydr Res.; 194:321-4.

Michon F, Moore SL, Kim J, Blake MS, Auzanneau FI, Johnston BD, Johnson MA, Pinto BM. 2005. Doubly branched hexasaccharide epitope on the cell wall polysaccharide of group A streptococci recognized by human and rabbit antisera. Infect Immun; 73(10):6383-9.

Mitchell TJ. 2003. The pathogenesis of streptococcal infections: from tooth decay to meningitis. Nat Rev Microbiol; 1(3):219-30.

Mori Y, Akkapaiboon P, Yang X, Yamanishi K. 2003. The human herpesvirus 6 U100 gene product is the third component of the gH-gL

glycoprotein complex on the viral envelope. J Virol 77:2452-2458.

Monteiro MA, Baqar S, Hall ER, Chen YH, Porter CK, Bentzel DE, Applebee L, Guerry P. 2009. Capsule polysaccharide conjugate vaccine against diarrheal disease caused by *Campylobacter jejuni*. Infect Immun;77(3):1128-36.

Olcén P, Kjellander J, Danielsson D, Lindquist BL. 1981. Epidemiology of Neisseria meningitidis; prevalence and symptoms from the upper respiratory tract in family members to patients with meningococcal disease. Scand J Infect Dis. 13(2):105-9.

Pace D. 2009. Quadrivalent meningococcal ACYW-135 glycoconjugate vaccine for broader protection from infancy. Expert Rev Vaccines 8:529-542.

Paoletti LC, Madoff LC. 2002. Vaccines to prevent neonatal GBS infection. Semin Neonatol 7:315-323.

Paul JR. 1957. The epidemiology of rheumatic fever. American Heart Association (New York) 19-21.

Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ. 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA; 299(17):2056-65.

Pitner JB, Beyer WF, Venetta TM, Nycz C, Mitchell MJ, Harris SL, Mariño-Albernas JR, Auzanneau FI, Forooghian F, Pinto BM. 2000. Bivalency and epitope specificity of a high-affinity IgG3 monoclonal antibody to the Streptococcus group A carbohydrate antigen. Molecular modeling of a Fv fragment. Carbohydr Res; 324(1):17-29.

Pohlmann S, Baribaud F, Lee B, Leslie GJ, Sanchez MD, Hiebenthal-Millow K, Munch J, Kirchhoff F, Doms RW. 2001. DC-SIGN interactions with human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus. J Virol 75:4664-4672.

Pollard AJ, Perrett KP, Beverley PC. 2009. Maintaining protection against

invasive bacteria with protein-polysaccharide conjugate vaccines. Nat Rev Immunol 9:213-220.

Pozsgay V. 2000. Oligosaccharide-protein conjugates as vaccine candidates against bacteria. Adv Carbohydr Chem Biochem 56:153-199.

Pozsgay V, Chu C, Pannell L, Wolfe J, Robbins JB, Schneerson R. 1999. Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from Shigella dysenteriae type 1. Proc Natl Acad Sci U S A 96:5194-5197.

Pozsgay V, Kubler-Kielb J, Schneerson R, Robbins JB. 2007. Effect of the nonreducing end of Shigella dysenteriae type 1 O-specific oligosaccharides on their immunogenicity as conjugates in mice. Proc Natl Acad Sci USA 104:14478-14482.

Ravenscroft N, D'Ascenzi S, Proietti D, Norelli F Costantino P. 2000 Physicochemical characterisation of the oligosaccharide component of vaccines. Dev Biol (Basel); 103:35-47

Ravenscroft N, Wheeler JX, Jones C. 2010. Bioanalysis of meningococcal vaccines. Bioanalysis; 2(2):343-61

Reimer KB, Gidney MA, Bundle DR, Pinto BM. 1992. Immunochemical characterization of polyclonal and monoclonal Streptococcus group A antibodies by chemically defined glycoconjugates and synthetic oligosaccharides. Carbohydr Res 232:131-142.

Roitt I. 1997. Essential immunology, 9th edition. Blackwell Scientific (Oxford).

Robbins JB, Schneerson Polysaccharide-protein conjugates: a new generation of vaccines. 1990 J Infect Dis. **1990** May;161(5):821-32

Ruiz-Matute AI, Hernández-Hernández O, Rodríguez-Sánchez S, Sanz ML, Martínez-Castro I. 2011. Derivatization of carbohydrates for GC and GC-MS analyses. J Chromatogr B Analyt Technol Biomed Life Sci; 879(17-18):1226-40.

Saksena R, Adamo R, Kovac P. 2007. Immunogens related to the synthetic tetrasaccharide side chain of the Bacillus anthracis exosporium. Bioorg Med Chem 15:4283-4310.

Sanders RW, Venturi M, Schiffner L, Kalyanaraman R, Katinger H, Lloyd KO, Kwong PD, Moore JP. 2002. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J Virol 76:7293-7305.

Scanlan CN, Pantophlet R, Wormald MR, Ollmann Saphire E, Stanfield R, Wilson IA, Katinger H, Dwek RA, Rudd PM, Burton DR. 2002. The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120. J Virol 76:7306-7321.

Schrag SJ, Zywicki S, Farley MM, Reingold AL, Harrison LH, Lefkowitz LB, Hadler JL, Danila R, Cieslak PR, Schuchat A. 2000. Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. N Engl J Med; 342(1):15-20.

Schuchat A. 1999. Group B *Streptococcus*. Lancet; 353(9146):51-6.

Schwartz BL, Rockwood AL, Smith RD, Tomalia DA, Spindler R. 1995. Detection of high molecular weight starburst dendrimers by electrospay ionization mass spectrometry. Rapid communications in mass spectrometry 9:1552-1555.

Shan M, Klasse PJ, Banerjee K, Dey AK, Iyer SP, Dionisio R, Charles D, Campbell-Gardener L, Olson WC, Sanders RW, Moore JP. 2007. HIV-1 gp120 mannoses induce immunosuppressive responses from dendritic cells. PLoS Pathog 3:e169.

Skoff TH, Farley MM, Petit S, Craig AS, Schaffner W, Gershman K, Harrison LH, Lynfield R, Mohle-Boetani J, Zansky S, Albanese BA, Stefonek K, Zell ER, Jackson D, Thompson T, Schrag SJ. 2009. Increasing burden of invasive group B streptococcal disease in nonpregnant adults, 1990-2007. Clin Infect Dis; 49(1):85-92.

Stanley J, Linton D, Desai M, Efstratiou A, George R. 1995. Molecular

subtyping of prevalent M serotypes of Streptococcus pyogenes causing invasive disease. J Clin Microbiol 33:2850-2855.

Stephens DS. 1999 Uncloaking the meningococcus: dynamics of carriage and disease. Lancet.; 353(9157):941-2.

Suker J, Feavers IM, Corbel MJ, Jones C, Bolgiano B. Control and lot release of meningococcal group C conjugate vaccines. 2004. Expert Rev. Vaccines. Oct;3(5):533-40.

Tanford C. 1961. Physical Chemistry of macromolecules. Wiley (New York).

Torosantucci A, Bromuro C, Chiani P, De Bernardis F, Berti F, Galli C, Norelli F, Bellucci C, Polonelli L, Costantino P, Rappuoli R, Cassone A. 2005. A novel glyco-conjugate vaccine against fungal pathogens. J Exp Med 202:597-606.

Torosantucci A, Chiani P, Bromuro C, De Bernardis F, Palma AS, Liu Y, Mignogna G, Maras B, Colone M, Stringaro A, Zamboni S, Feizi T, Cassone A. 2009. Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence. PLoS One.; 4(4):e5392.

Trent MS, Stead CM, Tran AX, Hankins JV. 2006. Diversity of endotoxin and its impact on pathogenesis. J Endotoxin Res; 12(4):205-23.

Tzeng YL, Stephens DS. 2000. Epidemiology and pathogenesis of Neisseria meningitidis. Microbes Infect. 2(6):687-700.

Verez-Bencomo V, Fernandez-Santana V, Hardy E, Toledo ME, Rodriguez MC, Heynngnezz L, Rodriguez A, Baly A, Herrera L, Izquierdo M, Villar A, Valdes Y, Cosme K, Deler ML, Montane M, Garcia E, Ramos A, Aguilar A, Medina E, Torano G, Sosa I, Hernandez I, Martinez R, Muzachio A, Carmenates A, Costa L, Cardoso F, Campa C, Diaz M, Roy R. 2004. A synthetic conjugate polysaccharide vaccine against Haemophilus influenzae type b. Science 305:522-525.

Vliegenthart JF. 2006. Carbohydrate based vaccines. FEBS Lett 580:2945-2950.

Weisner AM, Johnson AP, Lamagni TL, Arnold E, Warner M, Heath PT, Efstratiou A. 2004. Characterization of group B streptococci recovered from infants with invasive disease in England and Wales. Clin Infect Dis; 38(9):1203-8.

Wyle FA, Artenstein MS, Brandt BL, Tramont EC, Kasper DL, Altieri PL, Berman SL, Lowenthal JP. J Infect Dis. 1972 Nov;126(5):514-21