
Major Advances in the Development of Synthetic Oligosaccharide-Based Vaccines

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Abstract

Because of their involvement in a variety of different biological processes and their occurrence onto pathogens and malignant cell surface, carbohydrates have been identified as ideal candidates for vaccine formulation. However, as free oligosaccharides are poorly immunogenic and do not induce immunological memory in the most at risk population (infants and young children, elderly and immunocompromised patients), glycoconjugate vaccines containing the same carbohydrate antigen covalently linked to an immunogenic carrier protein have gained a prominent role. Accordingly, a number of glycoconjugate vaccines mostly directed against infections caused by bacterial pathogens have been licensed and are currently available on the market. However, also glycoconjugate vaccines suffer from significant drawbacks. The challenging procedures required for the isolation and purification of the carbohydrate antigen from its natural source often lead to poor homogeneity and presence of biological contaminants, resulting in batch-to-batch variability. Moreover, in some cases, the overwhelming immunogenicity of the carrier protein may induce the

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carbohydrate epitope suppression, causing hyporesponsiveness. The development of synthetic oligosaccharide-based vaccine candidates, characterized by the presence of pure and well-defined synthetic oligosaccharide structures, is expected to meet the requirement of homogeneous and highly reproducible preparations.

In the present chapter, we report on the major advances in the development of synthetic carbohydrate-based vaccines. First of all, we describe different strategies developed during the last years to circumvent the inherent difficulties of classical oligosaccharide synthesis, such as the one-pot glycosylation and the solid-phase synthesis, and their application to the preparation of carbohydrate antigens apt to conjugation with protein carriers. Next, we discuss the most representative methodologies employed for the chemical ligation of oligosaccharide structures to proteins. Finally, in the last section, we report significant examples of fully synthetic vaccines exploiting the multivalency effect. These constructs are based on the concept that the conjugation of multiple copies of synthetic oligosaccharide antigens to multivalent scaffolds, such as dendrimers, (cyclo)peptides, gold nanoparticles, and calixarenes, raises cooperative interactions between carbohydrates and immune receptors, leading to strong enhancement of the saccharide antigen immunogenicity.

Keywords

Vaccines • Immunology • Oligosaccharide • One-pot oligosaccharide synthesis • Solid-phase oligosaccharide synthesis • Glycoconjugates • Protein conjugation • Multivalency

Abbreviations

Ac ₂ O	Acetic anhydride
AcOH	Acetic acid
CDI	Carbonyldiimidazole
CPS	Capsular polysaccharide
CRM ₁₉₇	Nontoxic cross-reactive material of diphtheria toxoid
CuAAC	Cu(I)-catalyzed azide-alkyne cycloaddition
DMAP	<i>N,N</i> -dimethylaminopyridine
DMTST	Dimethyl(thiomethyl)sulfonium trifluoromethanesulfonate
DT	Diphtheria toxoid
ECA	<i>Erythrina cristagalli</i> agglutinin
EDC or EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Fmoc	Fluorenylmethyloxycarbonyl
GNP	Gold nanoparticle
GPI	Glycosylphosphatidylinositol
GSL	Glycosphingolipid
HbPG	Hyperbranched polyglycerol

HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
KLH	Keyhole limpet hemocyanin
LAM	Lipoarabinomannan
Lev	Levulinoyl
LM	Lipomannan
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization with time of flight detector
mRNA	Messenger ribonucleic Acid
NCL	Native chemical ligation
NIS	<i>N</i> -iodosuccinimide
NMR	Nuclear magnetic resonance
OMP	Outer membrane protein from <i>N. meningitidis</i> serogroup B
OST	Oligosaccharyltransferase
OVA	Ovalbumin
PADRE	Pan HL ADR binding epitope
PAMP	Pathogen-associated molecular pattern
PEG	Polyethylene glycol
PGCT	Protein glycan coupling technology
PV	Polio virus
RAFT	Regioselective Addressable Functionalized Template or Reversible Addition Fragmentation chain Transfer polymerization Technology
rEPA	Recombinant exoprotein from <i>Pseudomonas aeruginosa</i>
RRV	Relative reactivity value
SAM	Self-assembled monolayer
SAR	Structure-activity relationship
SPOS	Solid-phase oligosaccharide synthesis
SPPS	Solid-phase peptide synthesis
STol	ρ -methylphenylthio-
TACA	Tumor-associated carbohydrate antigen
TEC	Thiol-ene coupling
TfOH	Trifluoromethanesulfonic acid
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
tRNA	Transfer ribonucleic acid
TT	Tetanus toxoid
TYC	Thiol-yne coupling
UAAs	Nonnatural Amino Acids
VNTR	Variable number of tandem repeats
WHO	World Health Organization
ZPS	Zwitterionic polysaccharide

Glossary

- Adaptive immune response** The (antigen-specific) **immune response** is the response of antigen-specific lymphocytes to antigen, including the development of the immunological memory. The adaptive response is initiated and promoted by the innate immune system cells. The activated APCs travel into the draining lymph nodes where they present the peptide antigen to recirculating naïve T cells (immunological synapse), eliciting a T cell-dependent immune response. Depending on the antigen exposed on APC surface, the immunological synapse raises a complex cascade of events culminating in the activation and differentiation of T cells into **cytotoxic T lymphocytes** (CTL or CD8+ cells) and/or **T helper cells** (T_H or CD4+ cells). CTL are effector T cells responsible for the **cellular immunity** that destroy target cells infected by intracellular viruses and bacteria. T_H cells initiate the maturation process of resting B cells driving their differentiation into **plasma cells** (antibody-forming cells) and **memory B cells (humoral immunity)**. While plasma cells secrete low-affinity IgM-type antibodies, memory B cells survive for a long time in the body and respond rapidly to subsequent exposures of antigen by eliciting high-affinity IgG antibodies, which represents the overall objective of the vaccination practice.
- Adjuvant** **Adjuvant** is any substance that enhances the immune response to an antigen. Adjuvants can be categorized in delivery systems and immune potentiators. The delivery systems play the main function to localize vaccine components and to target them to APCs, whereas immune potentiators directly activate APCs acting as agonists of PRRs (mostly TLRs) facilitating the antigen uptake.
- Antibody** An **antibody** is a protein that binds specifically to a particular substance, called **antigen**. While each antibody has a unique structure that enables it to bind specifically to its corresponding antigen, all antibodies have the same overall structure and are known collectively as **immunoglobulins** (Ig).

Antigenic determinant	An antigenic determinant , or epitope , is the portion of an antigenic molecule that is bound by a given antibody.
Antigen-presenting cells	Antigen-presenting cells (APCs): highly specialized cells that can process antigens and display their peptide on the cell surface. Antigen-presenting cells include macrophages and dendritic cells (DC), but also B cells can act as APCs.
B cell epitope	B cell epitope is the minimal portion of an antigenic molecule needed to induce the production of specific antibodies in the humoral immune response.
Hapten	Hapten is any small molecule usually unable to activate an immune response unless conjugated to an immunogenic carrier.
Innate immunity	The innate immunity is based on a variety of innate resistance mechanisms that recognize and respond to the presence of a pathogen. The innate response is mediated by APCs and it acts during the early stages of the infection. APCs detect and respond to pathogen-associated molecular patterns (PAMPs), structurally and chemically diverse compounds that are unique to foreign microorganisms, via a multiple set of pathogen recognition receptors (PRRs), including peptidoglycan recognition proteins, scavenger receptors, C-type lectin receptors typically including a carbohydr-binding domain, and Toll-like receptors (TLRs) . Binding to PRRs induces the pathogen engulfment by APC and its intracellular processing, i.e., enzymatic degradation into short peptides that eventually bind the major histocompatibility complex (MHC) molecule and are exposed on cell surface to be presented to T cells.
T and B cells	T and B cells are the two subsets of lymphocytes defined by their development in the thymus and bone marrow, respectively.
T cell epitope	T cell epitope is the minimal portion of an antigenic molecule (typically a peptide) needed to elicit a T cell-dependent immune response.

1 Introduction

Together with proteins and nucleic acids, carbohydrates are a major class of biopolymers that mediate a wide range of biological processes in living organisms. It is now well established that, for example, carbohydrates play crucial roles in viral and bacterial invasion, angiogenesis and tumor cells metastasis, embryogenesis, inflammatory reactions, and, more generally speaking, in a large number of fundamental molecular recognition phenomena (Varki et al. 2009; Dwek 1996; Varki 1993). In particular, there is a growing interest towards what is known as glycoimmunology: this term derives from the merger of glycobiology with immunology, and it deals with the peculiar interactions of carbohydrates with the immune system, emphasizing the importance of using carbohydrates to induce protective immunity against microbial infections but also cancer metastasis. This relatively new field is based on the concept that diverse pathogens and malignant cells expose on their surface a dense array of often unique glycan structures that exert a protective function against the host's immune defense and are essential for their pathogenicity. Typical examples are lipopolysaccharides (LPS) of Gram-negative bacteria, the polysaccharide coat (capsular polysaccharides, CPS) of encapsulated bacteria, and diverse glycoproteins/glycolipids specifically expressed on viral and cancer cell surface. On the other hand, all these glycoforms are capable of interacting with the immune system, acting as cell antigenic determinants, and raising carbohydrate-specific antibodies production, and therefore they represent attractive targets for vaccine development.

Vaccination has certainly been a key breakthrough in the history of medicine and produced a tremendous impact on health-care systems. It is therefore not surprising that, despite the advent of more and more sophisticated antibiotics, vaccination is still considered by the World Health Organization as the most cost-effective approach for preventing infectious diseases and protecting public health, especially in developing countries. There is however a general concern about carbohydrate-based vaccines. Vaccines based on free and purified polysaccharides are poorly immunogenic in infants, in young children (under 2 years of age), in the elderly, and in immunocompromised patients, since they induce only short-lasting antibody responses in adults and fail to generate conventional B cell-mediated immunological memory (Gonzalez-Fernandez et al. 2008; Segal and Pollard 2004). Polysaccharide-based vaccines thereby possess limited clinical efficacy that has been largely ascribed to the T cell-independent immune response, hallmarked by the exclusive production of low-affinity IgM antibodies and no class switch to high-affinity protective IgG antibodies. This kind of immune response is typically triggered by antigens with polymeric structure composed of multiple repeating units, such as polysaccharides (Mond et al. 1995). The discovery that the polysaccharide immunogenicity can be strongly enhanced by their conjugation to an immunogenic carrier protein led to the development of glycoconjugate vaccines (Lesinski and Westerink 2001; Weintraub 2003). This strategy opened a new era in the field of vaccinology. Glycoconjugate antigens are able to induce T cell recruitment and immune memory B cell proliferation, with the production of long-lasting IgG antibodies specifically directed towards the carbohydrate strictly associated to

the pathogen (Ada and Isaacs 2003). The isolation and purification of carbohydrate antigens from their natural sources, and their subsequent manipulation for protein conjugation, are however a challenging task. In addition, the material available is often heterogeneous and not sufficient for the inclusion in a vaccine, and these represent major limitations to further expansion in this field. Hence, the development of cost-effective, fully synthetic carbohydrate vaccines would be of great importance, as witnessed by the spectacular success of the Cuban vaccine against *Haemophilus influenzae* type b (Verez-Bencomo et al. 2004). Synthetic carbohydrate antigens have, indeed, defined composition, and they can be produced as homogeneous compounds in a controlled manner with little or no batch-to-batch variability, affording highly reproducible biological properties. In addition, some microorganisms express carbohydrate structures that have close similarity to mammalian tissue-specific structures, and this molecular mimicry may induce tolerance by the host's immune system. One approach to evade this immune tolerance is to use a chemically modified version of the carbohydrate, an option available only with the recourse to the chemical synthesis. The unnatural structure will be perceived as a foreign antigen by the host, but at the same time, it should elicit antibodies able to cross-react with the natural glycan expressed on the pathogen cell surface.

On the basis of these considerations, the present chapter deals with the major advances in the formulation of synthetic carbohydrate-based vaccines, with a particular focus on the strategies and relevant synthetic methodologies that emerged during the last years.

First and foremost, for the sake of clarity and with the purpose to facilitate the comprehension of the contents by nonspecialized readers, we report a glossary defining the most significant terms referred to the complex machinery of the immune response and used throughout this chapter.

The following section highlights the most significant synthetic methodologies applied to the preparation of carbohydrate-based vaccine candidates. Finally, the new strategies (or new applications of the existing ones) carried out for the design of new, more efficient, and safer vaccines based on the conjugation of synthetic oligosaccharides with protein carriers and polyfunctional scaffolds (in order to exploit the multivalency effect) are described and critically discussed. It should be mentioned that a comprehensive review focused on the preparation of vaccines or vaccine candidates based on fully synthetic carbohydrate antigens but classified according to the target disease (infections from bacteria, viruses, parasites, fungi, and cancer) has been published by our group in 2011 (Morelli et al. 2011).

2 New Methodologies for the Synthesis of Oligosaccharides as Vaccine Candidates

Due to the enormous importance of carbohydrates in different biological processes (Varki et al. 2009; Dwek 1996; Varki 1993), the study of their functions and structure-activity relationships (SARs) needs homogeneous and well-defined

oligosaccharides. As stated above, the achievement of carbohydrate structures in high purity and quantity from natural sources is a tough and complex process. To overcome these problems, significant efforts have been carried out for the development of chemical and enzymatic synthesis of well-defined oligosaccharides and conjugates (Zhu and Schmidt 2009; Boltje et al. 2009; Fraser-Reid et al. 2008; Kamerling 2007; Wong 2003; Ernst et al. 2000; Nicolaou and Mitchell 2001; Koeller and Wong 2001; Seeberger and Werz 2007; Bertozzi and Kiessling 2001).

The synthesis of carbohydrates is more difficult than the synthesis of the other two major classes of biopolymers (peptides/proteins and nucleotides/DNA and RNA). In the construction of oligosaccharides, two major challenges have to be taken into consideration: the regioselective protection/deprotection of polyhydroxy groups and the stereoselective formation of glycosidic linkages.

The chemical synthesis of oligosaccharides requires monosaccharide building blocks with appropriate protecting groups and anomeric leaving group. The mammalian glycome includes a limited number of monosaccharides and linkages, so using only a limited number of building blocks, a large variety of oligosaccharides could be prepared by a proven synthetic strategy (Werz et al. 2007a). This will also be true for viruses, which use the host glycan machinery for the synthesis of their oligosaccharides. On the contrary, the bacteria glycome comprises much more complex structures (Adibekian et al. 2011), attainable only in assembling a large number of building blocks.

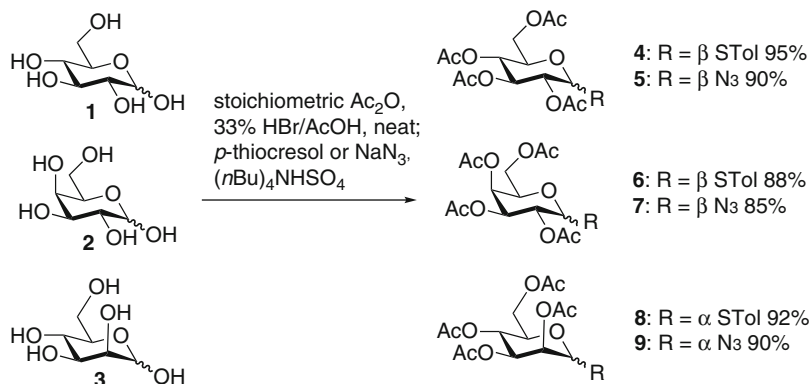
The chemical synthesis of oligosaccharides, as well as the development of new methodologies for the orthogonal protection of building blocks used in saccharide chain assembly, made enormous progress during the last two decades. Some significant examples of these techniques and their application to the preparation of carbohydrate antigen are described in the following section.

2.1 Regioselective Protection of Monosaccharides

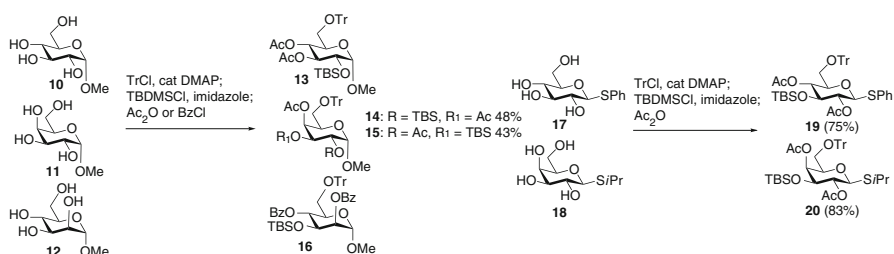
The correct choice of the protecting groups is the basis to design an ideal synthesis of complex oligosaccharides. One of the most common problems is the regioselective installation of orthogonal protecting groups. The protection of the different hydroxy groups of a monosaccharide influences the reactivity of a glycosyl donor/acceptor, the stereoselectivity of the newly formed glycosidic bond, and the ease of the final deprotection.

Besides the classical stepwise protection strategy, during the last years, different methodologies for the one-pot protection of monosaccharides have been developed.

An example of one-pot protection is the *per-O*-acetylation of sugars: with this reaction it is possible to obtain important precursors of monosaccharide synthons. In 2003 Hung and coworkers developed a procedure to obtain fully acetylated hexoses: the reaction proceeds under solvent-free conditions using a stoichiometric amount of acetic anhydride and a catalytic amount of $\text{Cu}(\text{OTf})_2$ (Tai et al. 2003). Subsequently, another one-pot procedure to prepare thioglycosides and glycosyl azides from unprotected sugars was developed (Kumar et al. 2006). The reaction



Scheme 1 One-pot full acetylation and subsequent synthesis of thioglycosides or glycosyl azides



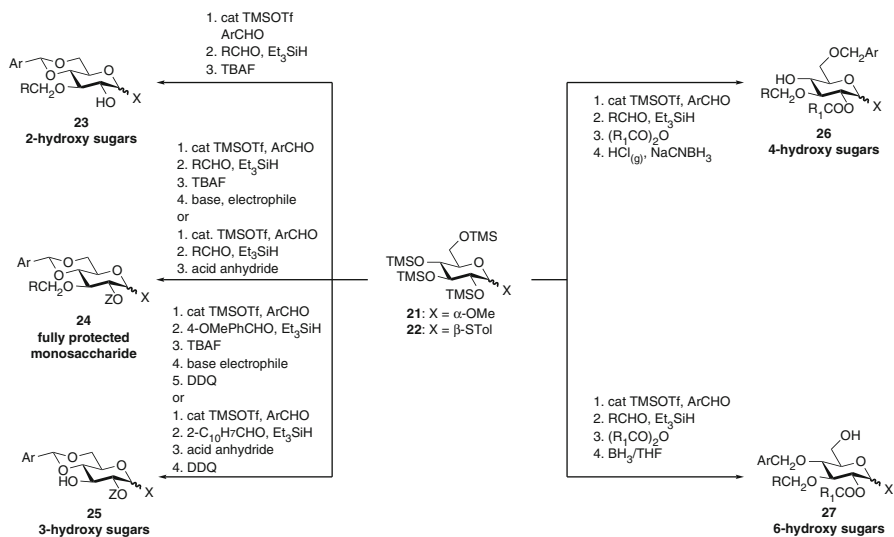
Scheme 2 One-pot tritylation/silylation/acetylation

proceeds under phase-transfer conditions using a stoichiometric amount of Ac_2O and 33 % HBr in AcOH: thioglycosides **4**, **6**, and **8** were obtained by further addition of *p*-thiocresol and $(n\text{Bu})_4\text{NHSO}_4$, while glycosyl azides **5**, **7**, and **9** were prepared by adding NaN_3 instead of *p*-thiocresol (Scheme 1).

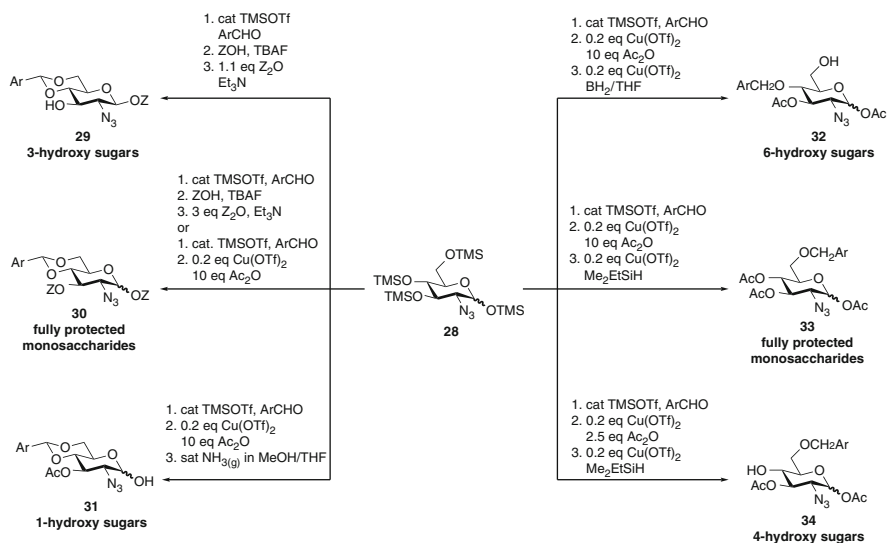
In order to prepare monosaccharides containing different and orthogonal protecting group, a one-pot tritylation/silylation/acetylation sequence was employed (Scheme 2) by treatment with a catalytic amount of DMAP. Different results were obtained using methyl glycosides (**10**, **11**, **12**) or thioglycosides (**17**, **18**), but in all cases the fully protected monosaccharide was obtained in good yield (71–86 %) (Du et al. 2000).

Another possibility to obtain partially or fully protected glycosides is the combinatorial and regioselective one-pot protection. In this case the reaction is performed using 2,3,4,6-tetra-*O*-trimethylsilylated glucosides (α -Me-glucopyranoside **21** and β -*p*-tolylthioglucoopyranoside **22**) (Wang et al. 2007, 2008). Starting from the fully silylated sugar, it is possible to follow five different routes to obtain 2-hydroxy sugars **23**, fully protected monosaccharides **24**, 3-hydroxy sugars **25**, 4-hydroxy sugars **26**, and 6-hydroxy sugars **27** (Scheme 3).

The same procedure can be also applied to the synthesis of fully protected monosaccharides (**30** and **33**) and 1-, 3-, 4-, 6-OH sugars (**31**, **29**, **34**,



Scheme 3 Synthesis of different orthogonally protected monosaccharide building blocks using combinatorial and regioselective one-pot protection



Scheme 4 Synthesis of different orthogonally protected glycosyl azide building blocks using combinatorial and regioselective one-pot protection

32, respectively) starting from 2-azido glucopyranoside **28** (Scheme 4) (Chang et al. 2010). The authors selected the azido group at C-2 because it can be converted into an *N*-acetyl or free amino group (found in different biologically important compounds) and it can control the stereoselective formation of the glycosidic bond.

2.2 Programmable One-Pot Oligosaccharide Synthesis

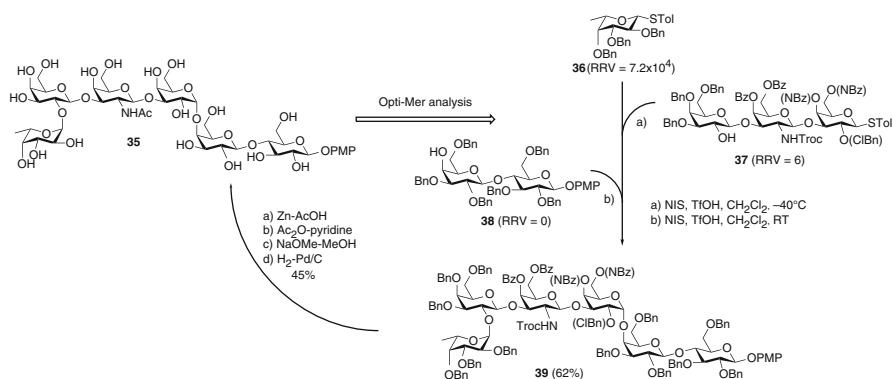
The one-pot glycosylation strategies enable the preparation of oligosaccharides without the isolation and purification of reaction intermediates that are time-consuming procedures typically affecting classical synthesis. For this purpose three different one-pot methodologies have been developed:

1. The chemoselective strategy: the less reactive donor is glycosylated with the more reactive activated donor providing a new glycoside that can act as a new donor itself (Raghavan and Kahne 1993; Ley and Priepeke 1994; Yamada et al. 1994a).
2. The preactivation strategy: a donor is activated alone; then it is reacted with a second donor/acceptor with the same aglycon at the reducing end (Crich and Sun 1998; Codee et al. 2003; Huang et al. 2004).
3. The orthogonal strategy: a donor is selectively activated over another donor with different leaving group (Kanie et al. 1994; Yamada et al. 1994b).

Here we focus our attention on the first methodology, which in principle can become an automatic process.

The reactivity of the glycosyl donor and acceptor can be tuned by the manipulation of the protecting groups. The basic concept of the chemoselective glycosylation is that the product obtained using this strategy can be directly used in a subsequent glycosylation without modification of the aglycon at the reducing end. In 1990 the Fraser-Reid group developed the concept of “armed/disarmed” donor. Monosaccharides containing 2-*O*-ether protecting groups (armed) react preferentially than those with 2-*O*-ester group (disarmed) (Fraserreid et al. 1990). In 1998 the Ley research group developed a new concept, the “semi-disarmed” sugar. In this case the reactivity of the thioglycosyl donor is tuned by using a diacetal protecting group (Douglas et al. 1998).

The one-pot glycosylation proceeds starting from the nonreducing end to the reducing one. The first donor used is the most reactive one, while the last one is the less reactive. To design an ideal one-pot glycosylation, it is important to know the “relative reactivity” of a variety of glycosides. In 1999 Wong and coworkers performed a competitive HPLC experiment in order to obtain informations on the reactivity of different glycosyl donors (Zhang et al. 1999). The study was carried out using STol glycosides (easy to prepare) for their intrinsic characteristics, such as stability during protecting groups manipulations and the possibility to study the reactivity via HPLC due to the presence of an aromatic ring. The relative reactivity values (RRVs) for each thioglycosyl donor were obtained by comparison of peracetylated tolylthiomannoside (RRV = 1.0). In particular, high values of RRV represent donors with high reactivity. The authors revealed interesting trends in the reactivity of thioglycoside donors, in particular: (1) pyranosides show reactivities that differ as a function of the sugar (fucose > galactose > mannose > glucose > sialic acid), (2) the reactivity of amino sugars can be tuned by the *N*-protecting groups (NHCBz > NHTroc > NHPhth > N₃ > NHAc), (3) the protecting groups



Scheme 5 Programmable one-pot synthesis of Globo H antigen via [1 + 3 + 2] protocol using the OptiMer analysis

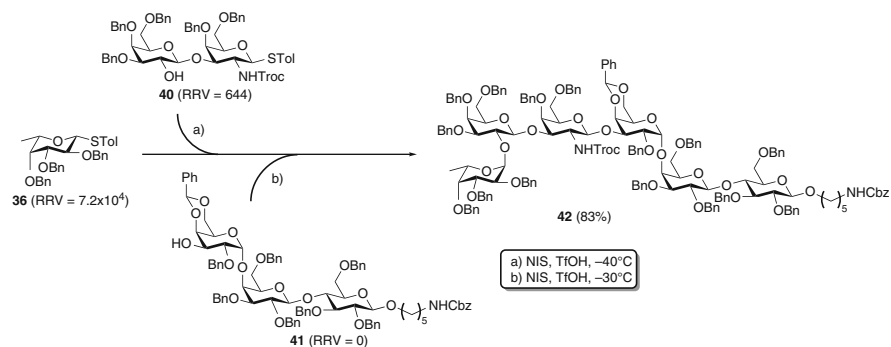
greatly influence the reactivity properties (in particular the substitution at C-2 plays a significant role), (4) the position that mostly affects pyranosides reactivity is not always the same for all sugars, (5) the magnitude of any protecting group reactivity effect is influenced by its position on the pyranoside, and (6) influence of leaving group like steric effects (the reactivity of the glycosyl donor can be tuned by changing the size of its leaving group) and electronic effects. In addition, it was found that, for each sugar, conformational effects (torsional effects) and solvent effects may be crucial to determine the RRVs.

The results of this study led to a computer database (OptiMer) in which RRVs of many donors and donors/acceptors are reported. Starting from the structure of the target oligosaccharide, the computer generates the best combination of building blocks for its preparation. Using this program/database, it is possible to prepare oligosaccharides containing three to six monosaccharides in minutes or hours, without isolation and purification of the intermediates.

An example is the synthesis of the Globo H hexasaccharide **35**, a carbohydrate antigen occurring in many epithelial tumors. The analysis of the oligosaccharide by the OptiMer program showed that the synthesis can be performed using three building blocks (**36**, **37**, **38**), tuning the different reactivity by electron-donating and electron-withdrawing groups (Scheme 5) (Burkhart et al. 2001).

The trisaccharide donor/acceptor **37** can be prepared by one-pot glycosylation. The glycosylation between the fucosyl donor **36** and the trisaccharide **37** and the subsequent glycosylation with the disaccharide acceptor **38** promoted by NIS/TfOH system provided the fully protected hexasaccharide **39** in 62 % yield that after deprotection led to the desired Globo H antigen **35**. The same authors developed another approach for the one-pot synthesis of Globo H hexasaccharide (Huang et al. 2006). In this case the synthetic strategy is based on [1 + 2 + 3] one-pot synthesis (Scheme 6). The challenging Gal α (1 \rightarrow 4)Gal bond was formed in a previous preparation.

Using this approach the fully protected hexasaccharide **42**, precursor of Globo H antigen, was obtained in 82 % yield. The trisaccharide building block **41**, bearing a



Scheme 6 Programmable one-pot synthesis of Globo H hexasaccharide via [1 + 2 + 3] protocol. Conditions: (a) NIS, TfOH, -40°C and (b) NIS, TfOH, -30°C

proper linker, is a valuable building block for the synthesis of a set of truncated Globo H sequences, used for the fluorescent-based binding analysis of the two monoclonal antibodies VK9 and Mbr1 and the identification of the optimal structure for the development of vaccines.

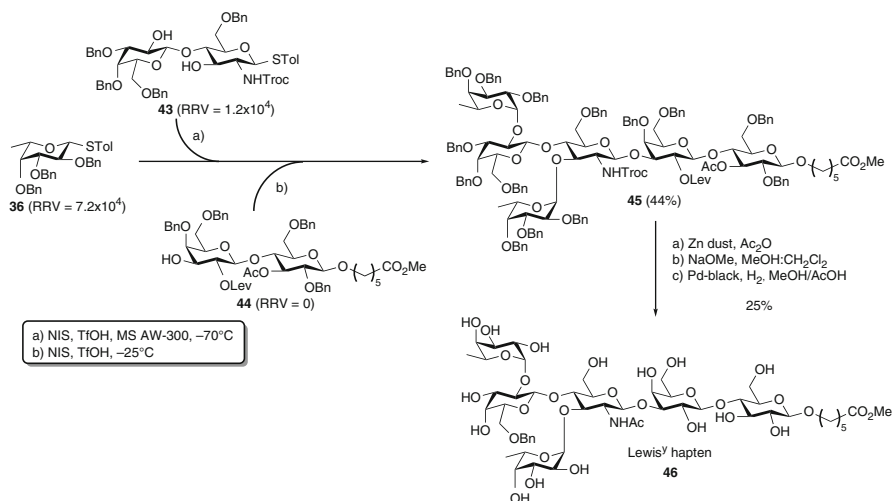
Based on the high reactivity of the fucosyl donor **36** used in the synthesis of Globo H fragment, Wong and coworkers developed another one-pot synthesis of fucose-containing oligosaccharides. In particular they synthesized the Lewis^y hapten **46**, a carbohydrate antigen expressed on the surface of many carcinoma cells. With the OptiMer analysis, three building blocks have been identified for the hexasaccharide synthesis (Scheme 7) (Mong and Wong 2002).

Also in this case the one-pot synthesis was performed using the NIS/TfOH system as a promoter. The first glycosylation was carried out at -70°C in order to obtain the α glycosidic bond, while the second reaction was performed at -25°C to facilitate the coupling between the two large and less reactive sugars. The hexasaccharide **45** was obtained in 44 % yield (81 % per glycosylation).

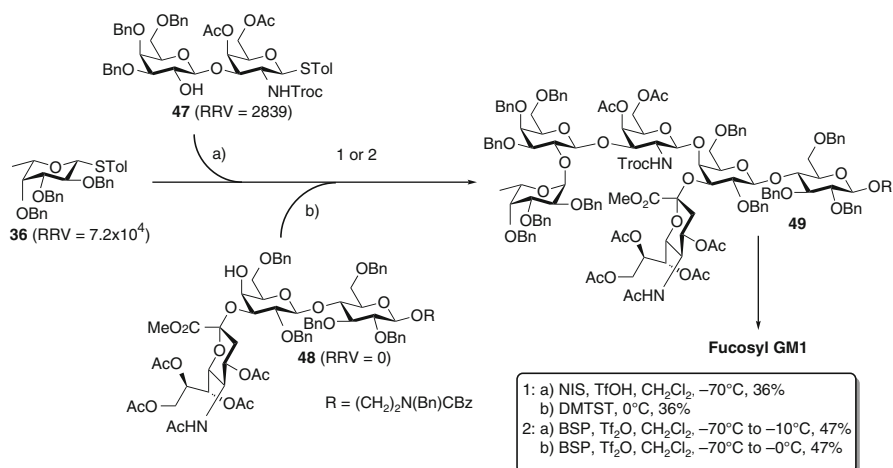
Although the NIS/TfOH system is used to promote the programmable one-pot glycosylation of thioglycosyl donors, in some cases, the succinimide generated by a stoichiometric amount of NIS can compete in the subsequent glycosylation. To overcome this problem, Crich and Smith used the reagent system 1-(benzylsulfonyl) piperidine/trifluoromethanesulfonic anhydride (BSP/Tf₂O). The applicability of this reagent mixture was confirmed in the one-pot synthesis of fucosyl-GM1 **49** (Scheme 8), a tumor marker for small-cell lung cancer (Mong et al. 2003; Lee et al. 2006a).

Using the BSP/Tf₂O system, the overall yield increased from 13 % to 22 % and the reaction time decreased from 1 day to 5 h. This promoter system was also applied to the one-pot synthesis of the tumor-associated antigen N3 octasaccharide **52** (Scheme 9) (Lee et al. 2006b).

The one-pot oligosaccharide synthesis was then used for the preparation of oligomannoses, precursors of antigens recognized by 2G12, a human monoclonal antibody (mAb) specific for the oligomannose residues present on gp120



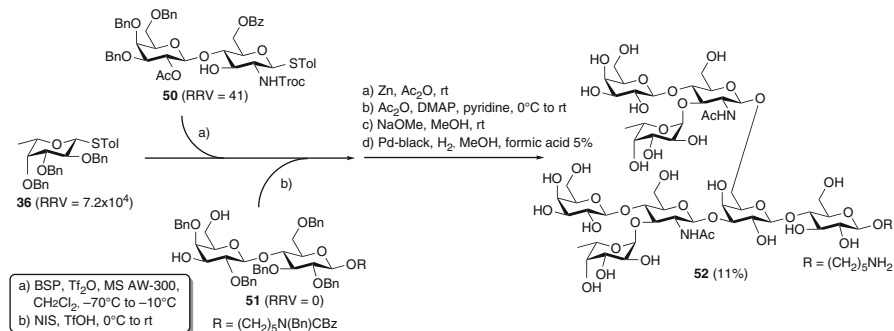
Scheme 7 Programmable one-pot synthesis of Lewis^y hapten



Scheme 8 Programmable one-pot synthesis of fucosyl-GM1 and comparison of NIS/TfOH and DMTST route and BSP/Tf₂O strategy

(the surface glycoprotein of HIV-1 virus) (Lee et al. 2004). On the basis of binding study between 2G12 and gp120, Wong and coworkers synthesized five different oligomannose structures **53–57** (Fig. 1) using the one-pot synthesis and a minimal number of building blocks.

The dimannose **59** and trimannose **60** structures, precursors of compounds **53–57**, were synthesized using a novel one-pot methodology based on the self-condensation of a mannosyl donor/acceptor (Scheme 10). The authors obtained



Scheme 9 Programmable one-pot synthesis of N3 antigen

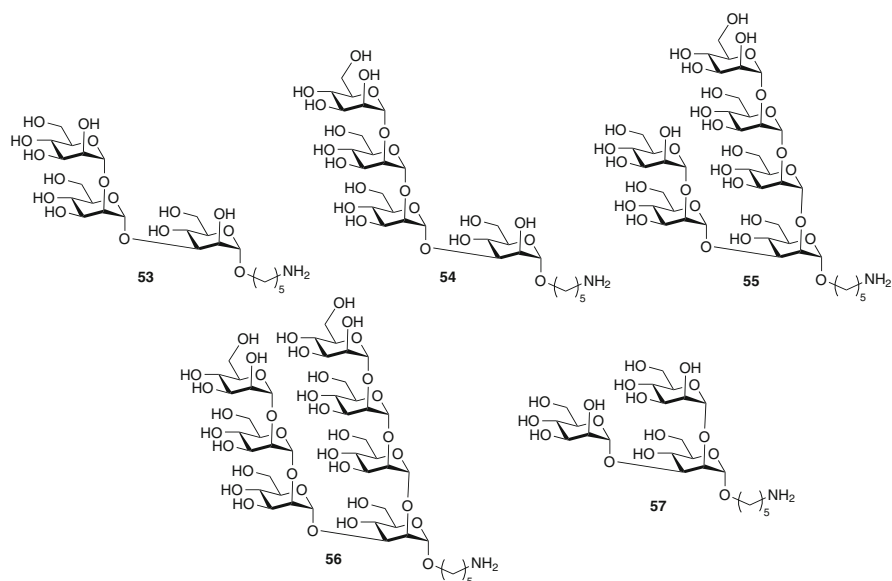
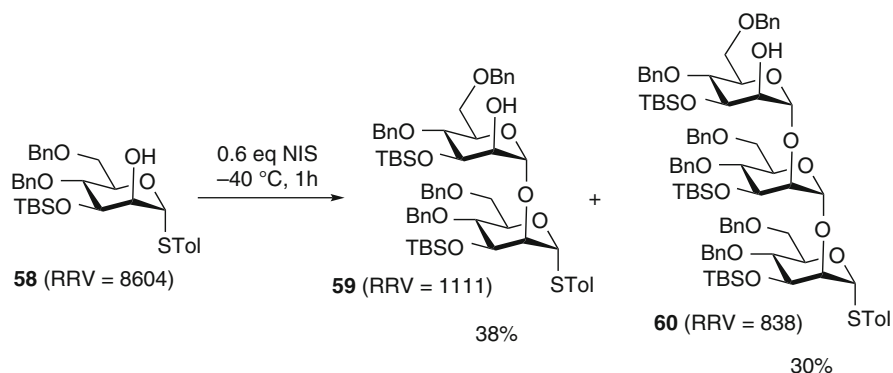


Fig. 1 Structures of oligomannose targets A–E

disaccharide **59** in 38 % yield and trisaccharide **60** in 30 % yield. As expected by RRV values, the most reactive monosaccharide provided the less reactive disaccharide by self-condensation, which could react with another monosaccharide providing the trimer. With the three donors in hand (mono-, di-, and trisaccharide), compounds **53–57** were prepared using a mannosyl acceptor bearing a suitable linker. Next the inhibition in the interaction between 2G12 and gp120 was studied using **53–57**. Compounds **53** and **57** were the less active, while **54** and **55** showed the highest inhibition values (79 % inhibition at 2.0 nM). Surprisingly, the heptamannose **56** showed a low value of inhibition, probably due to a nonoptimal conformation for 2G12 recognition.

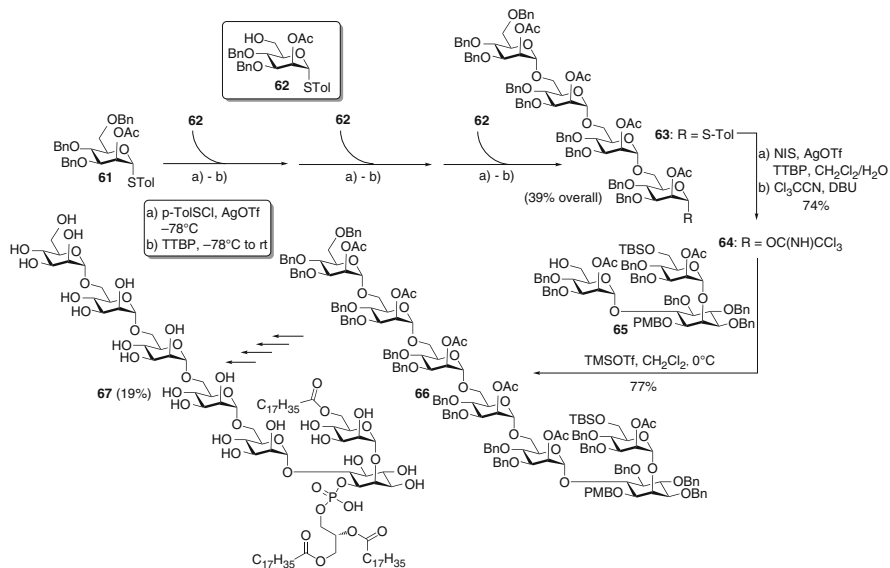


Scheme 10 One-pot self-condensation of mannosyl donor/acceptor

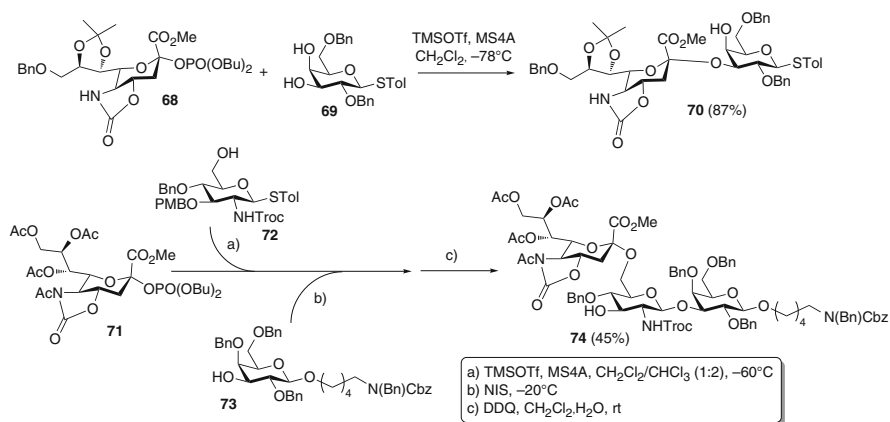
More recently, the synthesis of a lipomannan (LM), suitable for vaccine formulation, was developed using a convergent and efficient methodology (Gao and Guo 2013). Lipomannans (LMs), together with lipoarabinomannans (LAMs), are the major virulent factors of *Mycobacterium tuberculosis* (*Mtb*), the pathogen causing tuberculosis (TB), one of the most life-threatening diseases worldwide (more than two million deaths per year). The LM synthesized in this work is composed by a tetramannose residue and a trisaccharide containing a *myo*inositol derivative **67** (Scheme 11). The one-pot synthesis of the tetramannose building block **63** was carried out using thioglycoside monosaccharides. Each donor was preactivated with *p*-TolSOTf (generated from *p*-TolSCl and AgOTf) at $-78\text{ }^{\circ}\text{C}$; then the glycosylation was performed by addition of 2,4,6-tri-*tert*-butylpyridine (TTBP) as a scavenger for TMSOTf generated during the reaction. Tetramannose **63** was obtained in 39 % overall yield (73 % for each glycosylation step) in a total time of 6 h. Due to the low reactivity of the tetramannose thioglycoside **63**, this compound was first converted into trichloroacetimidate **64** and then coupled with the trisaccharide acceptor **65** providing the fully protected LM precursor **66** in 77 % yield. The desired LM compound **67** was obtained after deprotection and derivatization, and its structure was confirmed by NMR and MALDI-TOF analysis. Starting from the fully protected intermediate **66**, it is possible to regioselectively introduce a proper linker in order to prepare LM-based vaccine by conjugation with carrier protein or other scaffolds.

In 2013 Wong and coworkers reported an elegant synthesis of the RM2 antigen, a glycosphingolipid (GSL) occurring in renal cell carcinoma (cell line TOS-1), and prostate cancer cell lines LNCap and PC-3 (Chuang et al. 2013). The authors developed a [1 + 2 + 3] one-pot synthesis of the hexasaccharide precursor. Initially, the disaccharide donor/acceptor **70** was synthesized using a regioselective glycosylation and the trisaccharide acceptor **74** was prepared itself by a one-pot strategy (Scheme 12).

Next an orthogonal [1 + 2 + 3] glycosylation allowed to obtain the desired hexasaccharide **76** in 32 % overall yield in a total time of 23 h (Scheme 13).

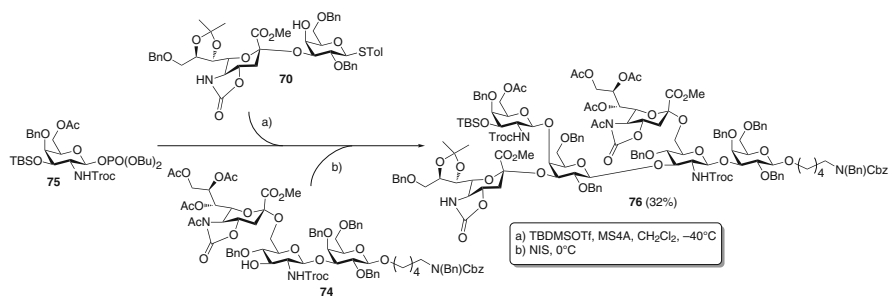


Scheme 11 One-pot synthesis of tetramannose and subsequent synthesis of *Mtb* lipomannan



Scheme 12 Regioselective synthesis of the disaccharide donor/acceptor and one-pot synthesis of the trisaccharide acceptor

Together with the hexasaccharide **76**, the authors also synthesized a panel of fragments of RM2 antigen. The deprotection of these compounds followed by the conjugation to a carrier protein (nontoxic cross-reactive material of diphtheria toxin, CRM₁₉₇) allowed to study the activity as vaccine. The biological tests were performed by immunization of mice with 2 µg of glycoconjugate together with 2 µg



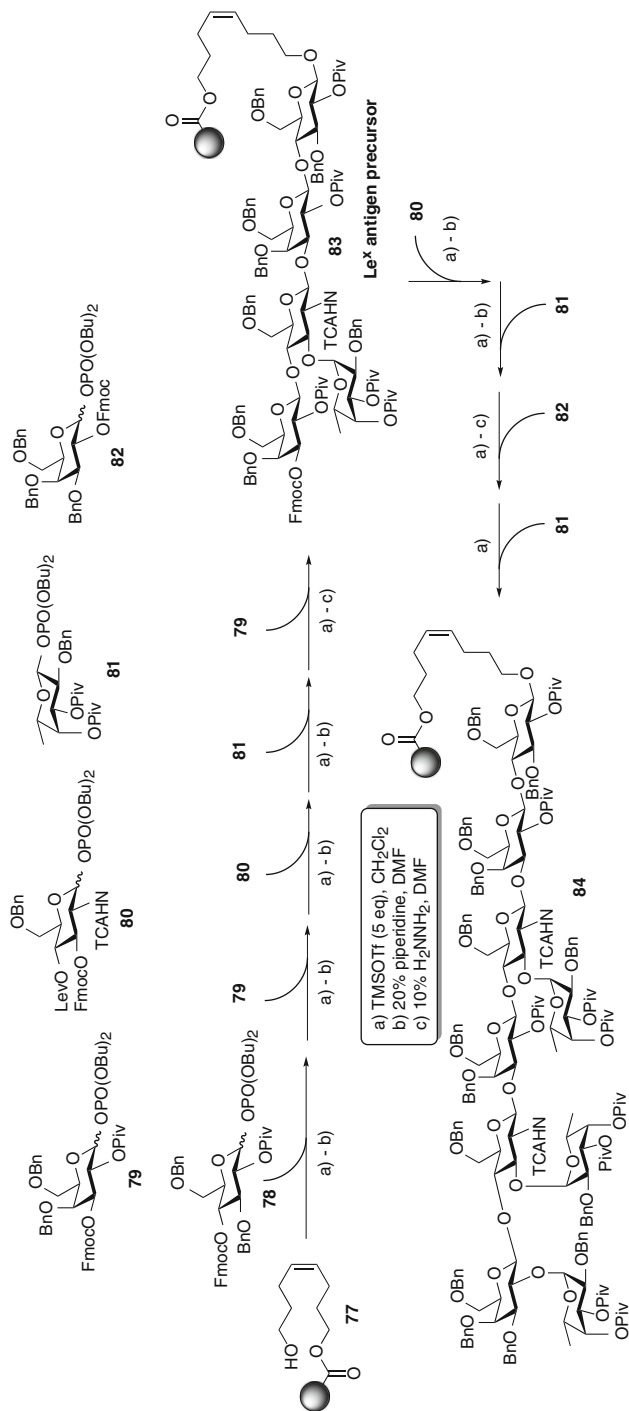
Scheme 13 One-pot [1 + 2 + 3] synthesis of hexasaccharide precursor of RM2 antigen

of adjuvant: the vaccination was carried out by giving three doses at 2-week intervals. After 2 weeks from the last injection, the sera were collected and analyzed with glycan microarray. The authors observed that the anti-RM2 IgG titers increased as vaccination proceeded: in particular the best results were obtained when glycolipid C34 (an α -galactosylceramide derivative) was used as adjuvant. In addition, the strongest anti-RM2 antigen titers were obtained with an antigen/protein ratio of 4.7 (CRM₁₉₇-RM_{4.7}), using the hexasaccharide RM2 as antigen (Chuang et al. 2013).

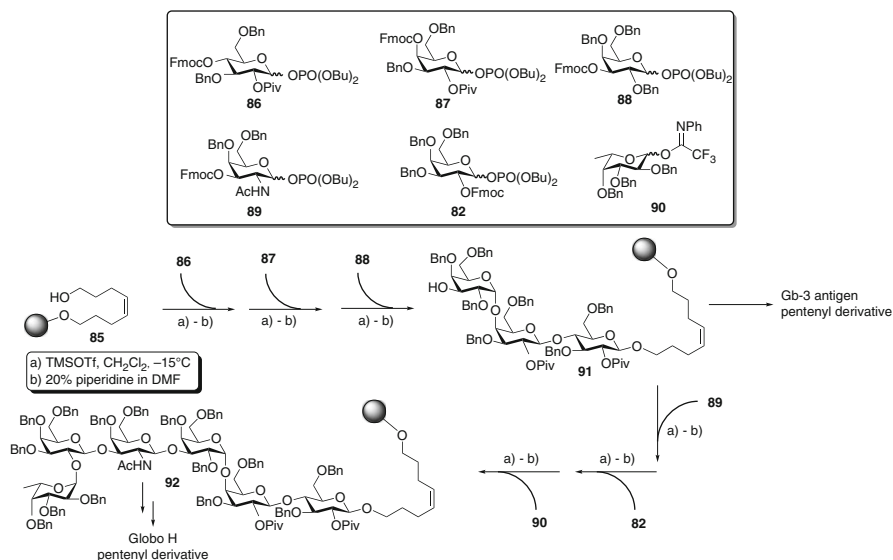
2.3 Solid-Phase Oligosaccharide Synthesis

Together with the programmable one-pot synthesis and other methodologies for the preparation of oligosaccharides (like fluoros-tag assisted solution-phase synthesis (Jaipuri and Pohl 2008)), the solid-phase oligosaccharide synthesis (SPOS) is another approach developed during the last years. SPOS offers two main advantages: (1) only a single purification by chromatography is needed in most cases at the end of the synthesis and (2) unwanted reagents and side products can be easily removed by washing and filtering. Due to the significant improvements in carbohydrate chemistry, during the past decade, there has been a great development of solid-phase oligosaccharide synthesis, confirmed by the numerous contributions reported in the literature (Seeberger and Haase 2000; Seeberger 2001; Danishefsky et al. 1993; Randolph et al. 1995; Rademann and Schmidt 1996; Roussel et al. 2000; Jonke et al. 2006; Nicolaou et al. 1997, 1998). In particular, an automated oligosaccharide synthesizer was developed by Seeberger and coworkers by modification of a peptide synthesizer (Plante et al. 2001; Seeberger 2008) and employed for the synthesis of the nonasaccharide of Le^y-Le^x (KH-1) antigen derivative **84** (Scheme 14) (Love and Seeberger 2004).

With a careful choice of the leaving group (glycosyl phosphates) and orthogonal protecting groups (permanent and temporary like Fmoc and Lev), the authors synthesized the nonasaccharide precursor **84**. The general strategy can be rationalized as follows:



Scheme 14 Automated solid-phase synthesis of Le^x- and Le^x-Le^x precursors



Scheme 15 Automated solid-phase synthesis of Gb-3 and Globo H antigens

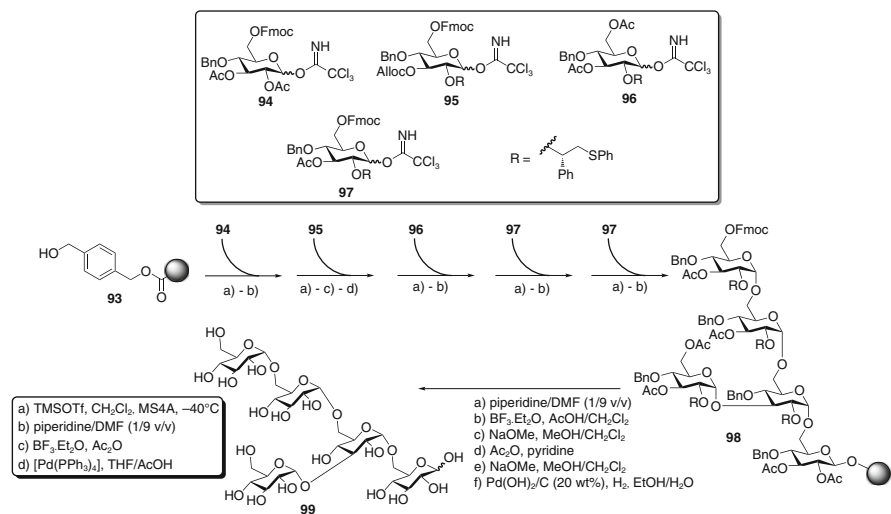
1. Coupling using TMSOTf (5 eq) as a promoter: the glycosyl phosphate was added twice to drive the reaction to completion.
2. Deprotection of the temporary group: Fmoc was removed by treatment with 20 % piperidine in DMF, while Lev was selectively removed using 10 % hydrazine in DMF.
3. Release of the oligosaccharide from the resin using sodium methoxide.

After purification by HPLC, the nonasaccharide was obtained in 6.5 % overall yield in a total time of 23 h. Using this strategy it is also possible to synthesize the Le^x antigen **83**.

Another application of the automated solid-phase oligosaccharide synthesis is the preparation of the tumor-associated carbohydrate antigens Gb-3 and Globo H by Seeberger group (Scheme 15) (Werz et al. 2007b).

In a total time of 12 h, the fully protected Gb-3 antigen was obtained in 46 % overall yield (after cleavage from the solid support and purification by chromatography), while the synthesis of the fully protected Globo H antigen required 23 h (30 % overall yield).

A significant challenge in SPOS is the formation of 1,2-*cis*-glycosides. Different progresses have been achieved in the synthesis of α -galactosidic (Gb-3 and Globo H SPOS) and β -mannosidic linkages (Codee et al. 2008). In 2010 Boons and coworkers developed a 1,2-*cis*-glycosylation strategy driven by the protection at C-2 with a chiral auxiliary (Scheme 16) (Boltje et al. 2010). Using (*S*)-(phenylthiomethyl)benzyl chiral auxiliary as a protecting group at position 2 of the glycosyl donor, the glycosylation proceeds via a trans decalin ring



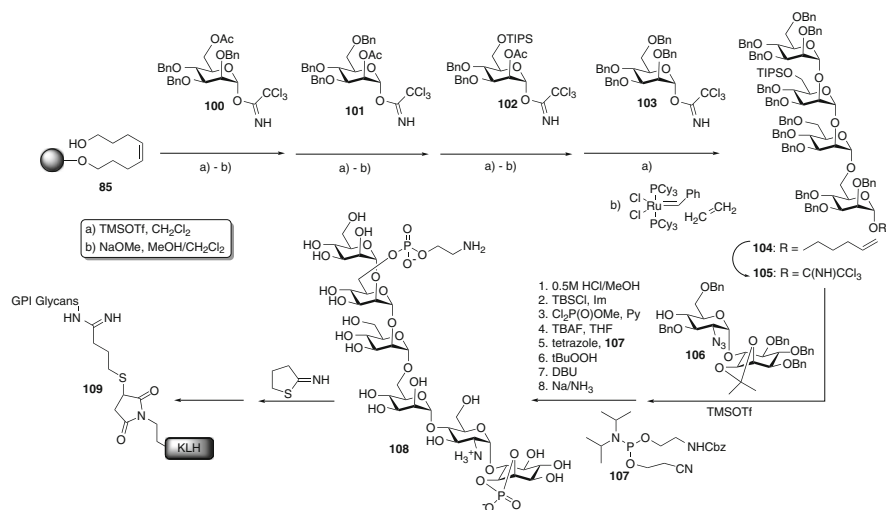
Scheme 16 Stereoselective solid-phase synthesis of tetraglucose residue

intermediate (equatorial sulfonium ion) allowing the stereoselective formation of 1,2-*cis*-glycosidic bond. In particular, with this procedure, the authors were able to synthesize a branched pentasaccharide **99** with different 1,2-*cis*-glycosidic linkages in 13 % overall yield (corresponding to 86 % yield per step). Another advantage of the chiral protecting group is that it can be converted into an acyl group on the solid support under conditions compatible with the removal of the temporary groups.

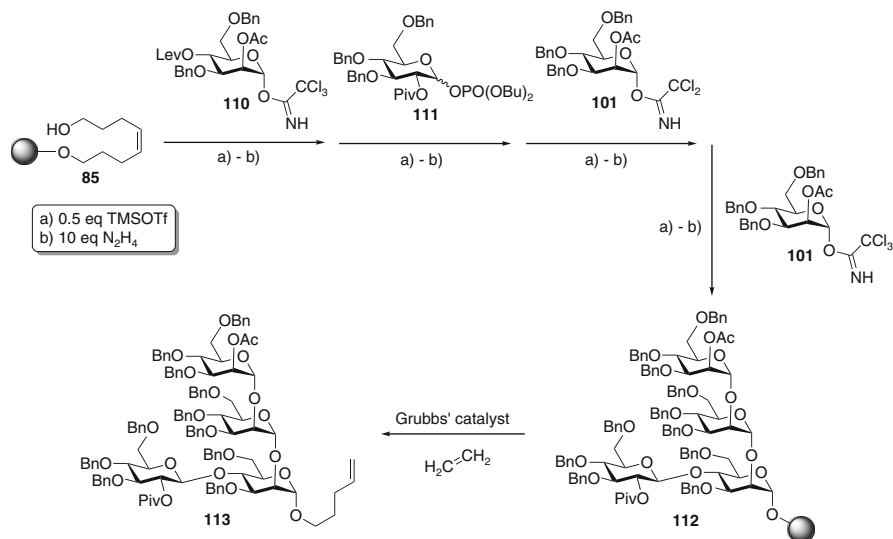
The solid-phase oligosaccharide synthesis was also employed for the synthesis of the *Plasmodium falciparum* glycosylphosphatidylinositol (GPI). This protozoan parasite is responsible for 95 % of the malaria deaths worldwide. Seeberger and coworkers synthesized the tetramannose **104** precursor of the hexasaccharide **108** (Scheme 17), which was then converted into the conjugate **109** by the treatment with maleimide-activated keyhole limpet hemocyanin (KLH) (Schofield et al. 2002).

The conjugate **109** was administrated to mice. The authors observed significant protection against the parasite-induced pathology (malarial acidosis, pulmonary edema, cerebral syndrome).

In 2001 Seeberger and coworkers reported the automated solid-phase synthesis of the branched *Leishmania* cap tetrasaccharide (Scheme 18) (Hewitt and Seeberger 2001). *Leishmania* parasites cause the leishmaniasis, a tropical disease affecting over 12 million people worldwide. The lipophosphoglycan (LPG) antigen of the parasite is composed of a GPI anchor, a repeating phosphorylated disaccharide, and an oligosaccharide cap. Previous promising immunological studies indicated the cap tetrasaccharide as an attractive vaccine target. The automated SPOS was performed using both glycosyl trichloroacetimidates (**110** and **101**) and the glycosyl phosphate **111** as donors with different esters as temporary protecting groups. The desired tetrasaccharide **112** was synthesized using three monosaccharide building



Scheme 17 Automated solid-phase synthesis of tetramannose residue and subsequent solution-phase synthesis of *P. falciparum* GPI-base malaria conjugate vaccine



Scheme 18 Automated solid-phase synthesis of *Leishmania* Cap tetrasaccharide pentenyl precursor

blocks (**101**, **110**, **111**) in a modified ABI 433A peptide synthesizer. After cleavage with the Grubbs catalyst and ethylene, the cap oligosaccharide **113** was obtained in 50 % yield (HPLC) in a total time of 9 h in the synthesizer and less than 4 days starting from the monosaccharide precursors.

3 Carbohydrate-Protein Conjugate Vaccines

As mentioned before, carbohydrate antigens could induce a strong, long-lasting, and protective immune response only after their transformation into T cell-dependent immunogens by conjugation of multiple copies of the saccharide antigen to the surface of a carrier protein (Lesinski and Westerink 2001; Weintraub 2003).

Carrier proteins must be nontoxic and non-reactogenic and should not share T cell epitopes with relevant capsular pathogens. Diphtheria toxoid (DT), CRM₁₉₇, keyhole limpet hemocyanin (KLH), tetanus toxoid (TT), recombinant exoprotein from *Pseudomonas aeruginosa* (rEPA), outer membrane protein from *N. meningitidis* serogroup B (OMP), and more recently protein D derived from non-typable *H. influenzae* are among the most used proteins.

Commonly employed conjugation methods make use of chemoselective reactions between a suitable functional group available on the protein and another one on the carbohydrate moiety. Anchoring sites on the protein can be lateral amines (lysine residues), carboxylic acids (aspartic or glutamic acid), or sulfhydryls (cysteine). On the contrary, attachment sites on the carbohydrate portion can be the intrinsic carbonyl group at the reducing end or aldehyde groups inserted by random periodate oxidation of oligo- or polysaccharides. These random strategies however do not allow an accurate control of the conjugation reaction. Moreover, the precise structure of the carbohydrate portion eliciting the immune response cannot be established a priori, leading to low batch-to-batch reproducibility and difficult characterization of the products. The use of synthetic carbohydrates provided with a suitable linker ending with amino, carboxylic, sulfhydrylic, or olefinic groups ensures to preserve the structural integrity of the saccharide moiety. Examples of chemoselective conjugation of these functional groups are shown in Fig. 2 (Adamo et al. 2013). However, care must be taken in the choice of the linker. In particular, it has been shown that these moieties could be highly antigenic and therefore suppress antibody responses to weakly immunogenic saccharide antigens such as self-antigens (Buskas et al. 2004; Costantino et al. 2011).

Many well-known glycoconjugates employed for antibacterial vaccine formulation were synthesized, exploiting these approaches. For instance, vaccines against *H. influenzae* type b (PRP-TT, Sanofi-Pasteur) and *N. meningitidis* serogroup A (MenafrivacTM) (Frasch et al. 2012) are approved and available on the market, whereas a 15-valent-CRM₁₉₇ conjugate against *S. pneumoniae* from Merck and a 3-valent-CRM₁₉₇ conjugate targeting Group B *Streptococcus* from Novartis are in advanced clinical trials. Other recent examples of glycoconjugates currently under study as vaccine candidates include the CRM₁₉₇ conjugate of synthetic fragments of *Clostridium difficile* polysaccharide (Danieli et al. 2011; Adamo et al. 2012) and carba-analogues of *N. meningitidis* A capsular polysaccharide (Gao et al. 2012, 2013).

However, the conjugation chemistry still remains difficult to control, with random regio- and stereochemistry and unpredictable stoichiometry. Different methods have been recently developed in order to obtain well-defined

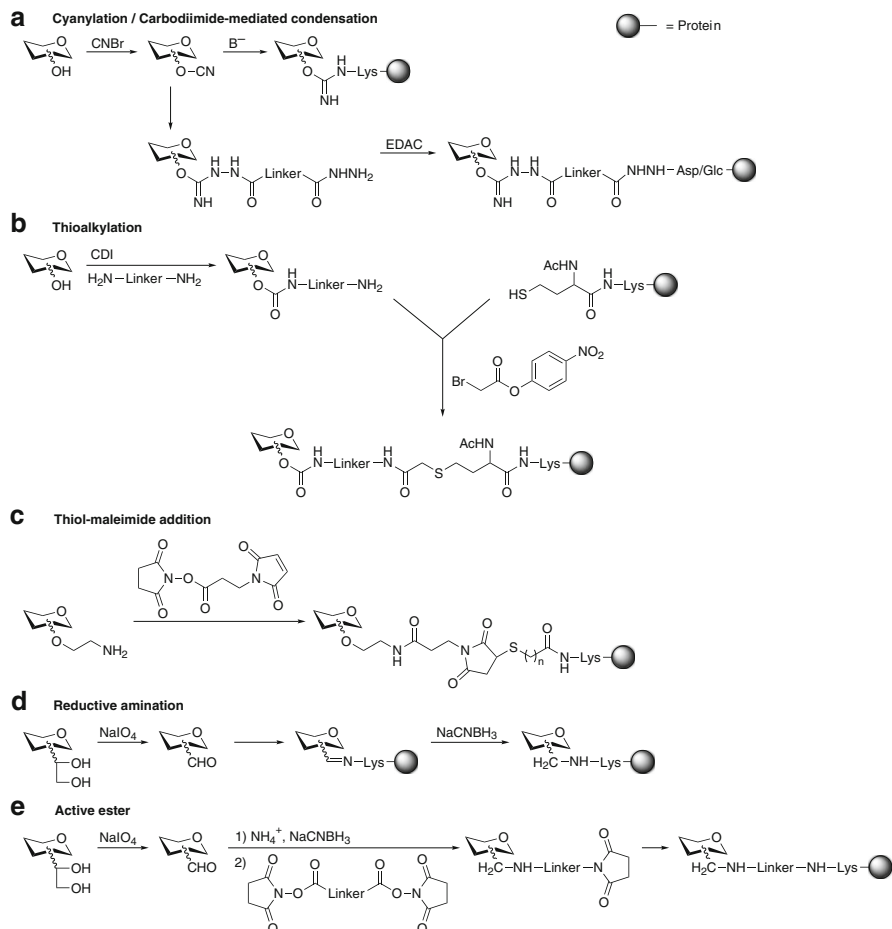
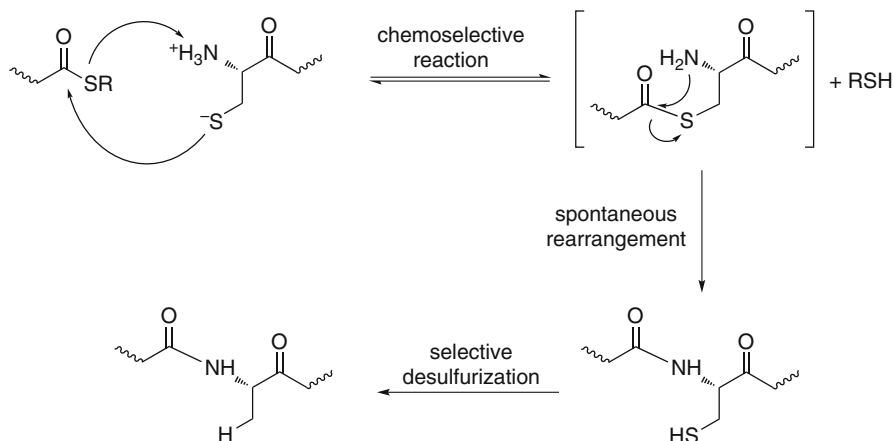


Fig. 2 Commonly employed strategies for the conjugation of carbohydrate antigens to carrier proteins

protein-carbohydrate conjugates. Among them, native chemical ligation, site-selective protein modification, and chemoenzymatic approaches are worthy of note (Adamo et al. 2013).

3.1 Native Chemical Ligation

Native chemical ligation (NCL) is a methodology that permits the construction of small proteins through the convergent chemoselective linkage of two unprotected peptide fragments: the first one containing a thioester as C-terminal residue and the



Scheme 19 Mechanism of the native chemical ligation

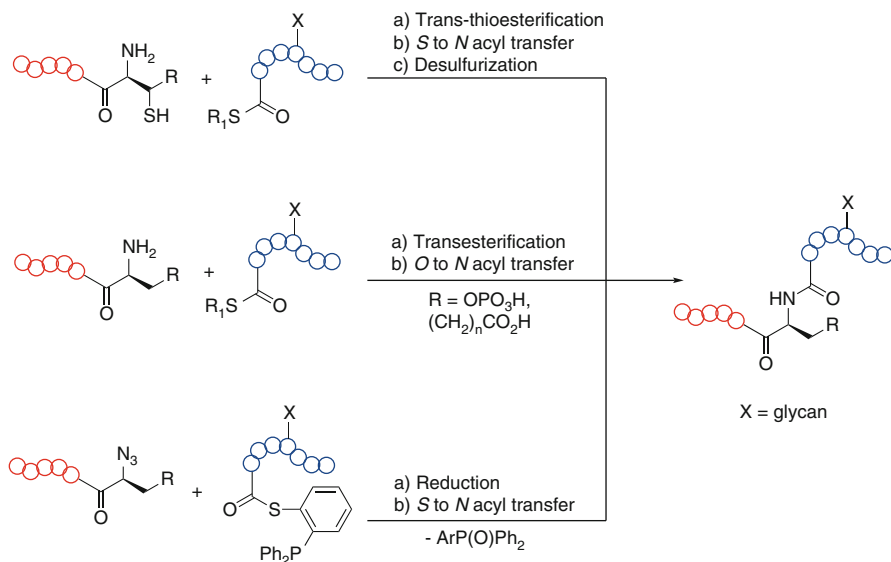
other one bearing a free cysteine at the *N*-terminus (Dawson et al. 1994; Schnolzer and Kent 1992). The reaction mechanism is shown in Scheme 19.

Unlike conventional strategies based on automated solid-phase peptide synthesis (SPPS), which usually rely on long linear reaction sequences and protecting group manipulations, the benefits arising from a convergent total synthesis approach that uses unprotected (glyco-)peptide fragments make NCL a leading methodology for the preparation of glycoconjugates.

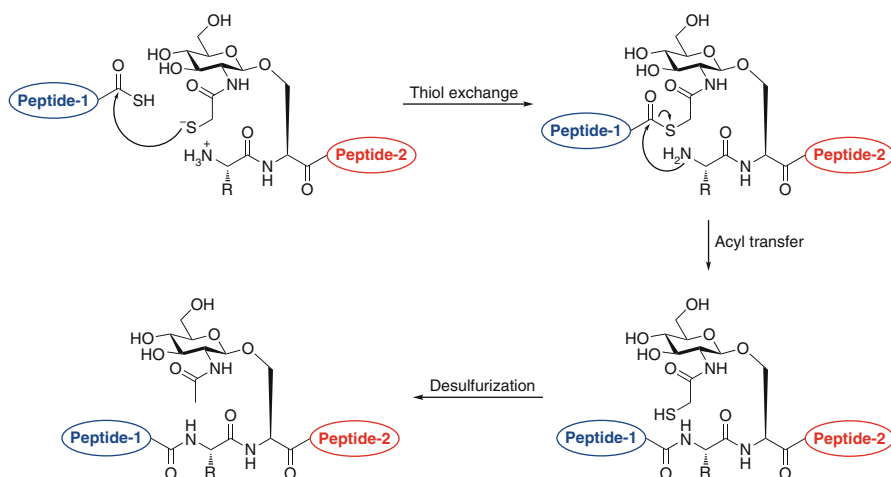
The first examples of NCL-mediated glycoprotein synthesis were reported in 2008 (Yamamoto et al. 2008; Becker et al. 2008). Firstly, a glycan was linked to an amino acid, followed by incorporation into a polypeptide by SPPS. Finally, the resulting glycopeptide chain was coupled via NCL with another peptide sequence, allowing for a fast and chemoselective chain elongation.

This protocol has been studied in detail, and many other types of chemical ligations have been reported in the literature (Payne and Wong 2010). Relevant examples are ligation involving thiol-free moieties such as phosphates (of phosphoserine or phosphothreonine), aspartate or glutamate residues (Thomas et al. 2011), and azides (Scheme 20) (Nilsson et al. 2003).

In addition, a protocol developed by Wong and coworkers, the so-called sugar-assisted chemical ligation, allows for a traceless ligation using thiol-containing glycosyl auxiliaries, which is particularly relevant for the preparation of glycopeptide fragments. It is based on the ligation promoted by β -2-(thioacetamide)-glucosamine-*O*-serine, β -2-(thioacetamide)-glucosamine-*O*-threonine, and β -2-(thioacetamide)-glucosamine-*N*-asparagine residues, which represent favored handles for subsequent (chemo)enzymatic attachment of more complex glycan epitopes for vaccine preparation (Scheme 21) (Brik et al. 2006).



Scheme 20 Different chemical ligation strategies



Scheme 21 Mechanism of the sugar-assisted chemical ligation

3.2 Site-Selective Protein Modification

A complementary protocol to the stepwise insertion of glycosylated amino acid building blocks in the early stages of NCL-like approaches is the site-selective protein modification.

The key aspect is the presence of a bioorthogonal functionality, commonly named “tag,” at a specific point in the protein, that allows for a late-stage homogeneous glycosylation (Chalker et al. 2011a; Gamblin et al. 2009). The tagging can be obtained either by site-selective protein modification or by selective protein tagging. The former approach involves site-specific chemical modification under mild conditions of natural residues, such as cysteine, its elimination product, and dehydroalanine, which are well suited for thiol-ene reaction and Michael addition (Bernardes et al. 2008; Lin et al. 2008; Chalker et al. 2011b). Even more interesting is the selective protein-tagging approach. This methodology relies on the insertion of nonnatural amino acids (UAAs) into proteins by genetically reprogramming the translation cascade in *E. coli*. Translation has a great intrinsic capacity to be adapted to accommodate new building blocks, since the relationship between the template (mRNA) and the product (polypeptide) is defined by the genetic code, which utilizes aminoacyl-tRNA adapters to establish the map between mRNA and the protein sequence.

The use of modified aminoacyl-tRNA thus led to the incorporation of UAAs in the expressed protein with excellent consistency (Liu and Schultz 2010). Some of the most representative examples include homoallylglycine for thiol-ene reactions (Floyd et al. 2009); *S*-allyl- or *Se*-allylselenocysteine for cross-metathesis reactions (Lin et al. 2008); azidohomoalanine, suitable for the well-known Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (Boutureira et al. 2010); Staudinger and traceless Staudinger ligations (Loka et al. 2010; Bernardes et al. 2011); and also recent strained alkyne-based Cu-free cycloaddition protocols (Agard et al. 2004). Other important transformations include the use of carbonyl handles, typically employed in oxime bond formation (Hudak et al. 2011); homopropargylglycine and other alkyne handles for CuAAC, thiol-yne reactions (Li et al. 2013); and Sonogashira as well as Suzuki cross-couplings with *p*-halophenylalanine tags (Fig. 3) (Spicer and Davis 2013).

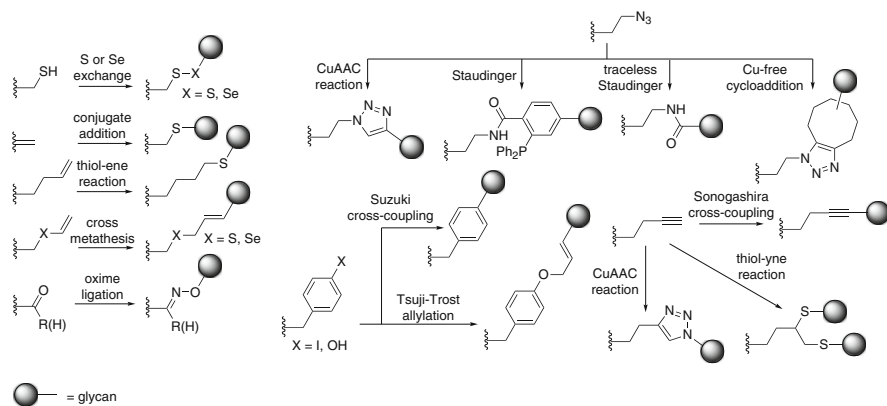


Fig. 3 Significant examples of selective protein-tagging techniques

Excellent orthogonality of different tags permits differentiation of the conjugation sites for multiple presentations of different antigens (van Kasteren et al. 2007).

If needed, the glycan pattern can be extended by sequential addition of further monosaccharide units using a chemoenzymatic approach based on endoglycosidases (Fernandez-Gonzalez et al. 2010).

3.3 Protein Glycan Coupling Technology

The discovery that some bacteria, such as *Campylobacter jejuni*, possess a *N*- or *O*-glycosylation machinery that can modify several proteins opened a new way towards selective glycoconjugation (Nothhaft and Szymanski 2010). The key enzyme of this pathway was found to be an oligosaccharyltransferase (OST) named PglB, which demonstrated relaxed glycan specificity and the ability to transfer a number of polysaccharides to acceptor proteins containing the extended glycosylation sequence recognition pattern D/EYNXS/T (where X and Y are any amino acids except proline). When *E. coli* was genetically modified to express the general glycosylation locus, it was established, for the first time, that recombinant glycoproteins could be obtained (Wacker et al. 2002).

These findings were recently translated into a novel approach, named protein glycan coupling technology (PGCT), where selected glycans are coupled to target proteins using an *E. coli* recombinant system to produce an unlimited and readily purified supply of vast combinations of glycoconjugate vaccine candidates. Moreover, in a more advanced vision, both the glycan and protein syntheses could be triggered inside the *E. coli* host, avoiding the long multistep synthesis of the glycan and the need of manipulating pathogenic bacteria for protein isolation (Terra et al. 2012). To this end, the loci encoding the glycan and the target carrier protein (containing the appropriate consensus sequon; otherwise it can be directly engineered (Ihssen et al. 2010)) have to be cloned and expressed in *E. coli*, usually on a suitable plasmid. Finally, the coupling enzyme has to recognize the reducing end of the target glycan in order to transfer it to the protein (Fig. 4).

Although still in its infancy, PGCT is however a very promising technique. Striking examples concern glycoconjugate vaccine candidates obtained by conjugation of *Shigella dysenteriae* O antigen 1 to two different carrier proteins (Ihssen et al. 2010).

3.4 Peptides as T Cell Epitopes

In addition to the abovementioned problems, another important aspect should also be taken into account when designing glycoconjugate vaccines. Due to the presence of diverse T cell epitopes, carrier proteins are highly immunogenic and therefore elicit strong T cell responses, which can overcome that of the saccharide moiety (especially in the case of very short oligosaccharide fragments) causing hapten suppression (Herzenberg and Tokuhisa 1980). The use of a single peptide as a T cell

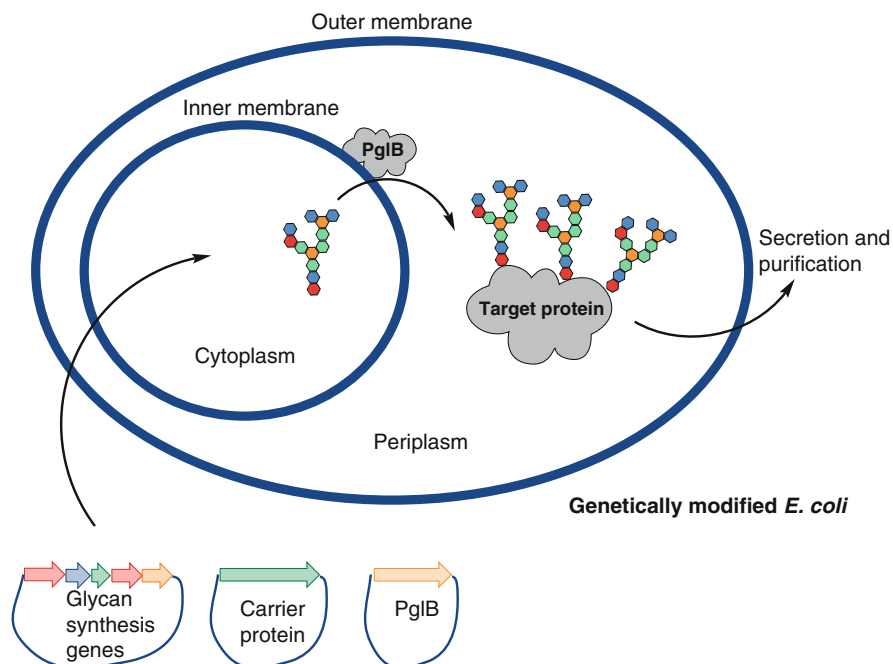
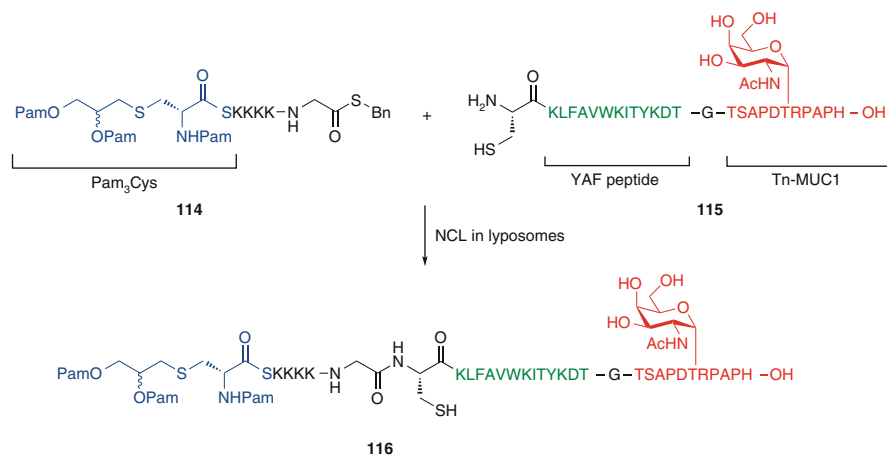


Fig. 4 Protein glycan coupling technology mechanism

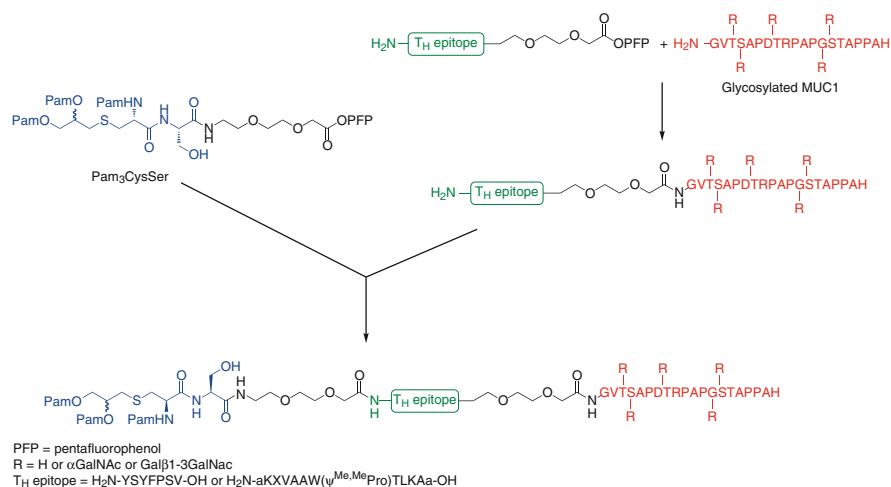
epitope should give rise to a more robust immune response, more specifically directed towards the carbohydrate antigen. To address this issue, over the last decade, a number of computer algorithms that map the locations of MHC-restricted T cell epitopes within proteins of various origins have been developed. As a result, it has been found that some sequence regions of protein antigens might sensitize CD4+ cells (Weber et al. 2009). This finding led to the identification of small and simple T_H peptide epitopes that are chemically defined, easily synthesized by SPPS, and able to generate effective helper T cell responses in humans. Among them, the Pan HLA DR-binding Epitope (PADRE), OVA_{323–339} and YAF peptides, TT heptapeptide, and tridecapeptide from poliovirus are widely used (Panina-Bordignon et al. 1989; Diethelm-Okita et al. 1997, 2000).

Boons recently developed a tricomponent synthetic anticancer vaccine **116** incorporating the Tn antigen (α GalNAc) linked to YAF peptide and also containing the immunoadjuvant Pam₃Cys (Scheme 22) (Ingale et al. 2009).

Other two examples were reported by the Payne group (Wilkinson et al. 2011, 2012). Both are anticancer vaccines containing the Tn and TF (Gal β 1-3GalNAc) antigens as carbohydrate epitopes, commonly overexpressed in cancer cells. In both vaccines the B cell epitope is linked to the T cell epitope, tetanus toxoid peptide (YSYFPSV) or PADRE, via an ethylene glycol spacer. The peptide *N*-terminus is connected via the same spacer to the Pam₃Cys moiety terminated with a serine residue. The three building blocks were assembled by means of a pentafluorophenyl



Scheme 22 Synthesis of tricomponent synthetic anticancer vaccine by NCL

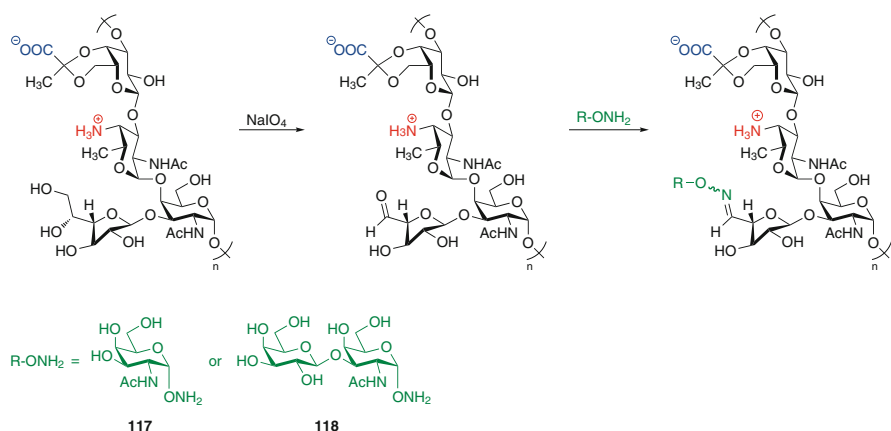


Scheme 23 Synthesis of the three-component anticancer vaccine candidates

ester-mediated condensation (Scheme 23). Mice immunization confirmed the strong adjuvant role of the Pam₃CysSer and the superior immunogenicity of tricomponent vaccines compared to others lacking the T cell epitope.

3.5 Zwitterionic Polysaccharides

Zwitterionic polysaccharides (ZPS) are a class of structurally distinct bacterial polysaccharides that possess a zwitterionic charge motif distributed along the



Scheme 24 Synthesis of the TACA-PSA1 conjugates

chain, within the repeating unit. Typical examples are the Sp1 capsular polysaccharide from *S. pneumoniae* type 1 and capsular polysaccharides A1, A2, and B from *Bacteroides fragilis*. A number of recent reports described the ability of such compounds to behave like traditional T cell-dependent antigens, activating CD4+ T cells both in vitro and in vivo through the traditional MHC-dependent mechanism, without protein conjugation (Cobb and Kasper 2008; Surana and Kasper 2012; Avci et al. 2013; Bloem et al. 2013; Berti and Adamo 2013).

Due to their unique behavior, ZPSs are gaining attention as potential scaffolds for vaccine assembly, acting both as carrier and adjuvant. Andreana and coworkers recently reported the first two examples of entirely carbohydrate vaccine constructs containing the α -aminoxy derivatives of Tn and TF tumor-associated cancer antigens attached to PSA1 polysaccharide (**117** and **118**, Scheme 24) (De Silva et al. 2009, 2012; Bourgault et al. 2014). Immunization studies conducted on the Tn-PSA1 conjugate **117** showed the production of high titers of specific IgG antibodies, even in the absence of any external adjuvants.

4 Fully Synthetic Vaccines

The use of sugars as ligands for protein targeting (such as antibodies) is a challenging task because carbohydrate-protein interactions are typically weak, with dissociation constants in the mM range. In living cells, this issue is circumvented by multivalent receptor-ligand interactions, where multiple ligands interact simultaneously with multimeric receptors inducing an increase of the binding affinities of several orders of magnitude (multivalency effect). When this phenomenon is referred to carbohydrate-receptor interactions, it is commonly named “cluster glycoside effect” (Lundquist and Toone 2002).

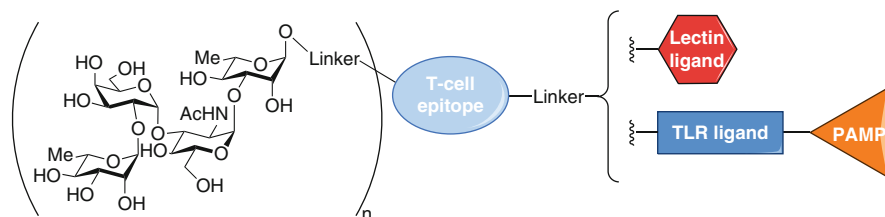


Fig. 5 Composition of fully synthetic vaccines

A major goal pursued by the vaccinology research is to reproduce the multivalency effect into fully synthetic systems using polyfunctional scaffolds capable of displaying multiple copies of carbohydrate antigens and that can be univocally characterized from a chemical point of view (mass and NMR spectroscopy). Under this point of view, the traditional glycoconjugate vaccines can be considered as an example of replication of the cluster glycoside effect, since they contain a large number of carbohydrate antigens exposed on the protein surface that are collectively much more immunogenic than the corresponding monovalent forms.

Fully synthetic vaccines are based on a modular architecture and incorporate into the same molecule different functional units: a synthetic glycan or a multivalent glycocluster, a synthetic T cell epitope, and an adjuvant, such as a ligand of TLR or C-type lectin receptor onto dendritic cell surface. Each of them is synthesized independently and then chemically conjugated in a convergent way, providing total control on the chemical structure (Fig. 5).

During the last years, a wide range of synthetic clustered glycosides has been designed to interfere in an array of biological processes. Among them, glycopeptides (Lee and Lee 1994; Krauss et al. 2007; Ohta et al. 2003; Pujol et al. 2011), glycodendrimers (Chabre and Roy 2010; Andre et al. 2001; Heidecke and Lindhorst 2007; Mintzer et al. 2012; Touaibia and Roy 2007; Turnbull and Stoddart 2002), glycopolymers (Kiessling et al. 2006; Bes et al. 2003; Otsuka et al. 2010; Ponader et al. 2012; Rieger et al. 2007), glyconanoparticles (Marradi et al. 2010; Wang et al. 2011), glycofullerenes (Cecioni et al. 2011; Nierengarten et al. 2010; Compain et al. 2010), glycoalexarenes (Dondoni and Marra 2010; Sansone et al. 2011; Baldini et al. 2007; Cecioni et al. 2009; Andre et al. 2011), and sugar-functionalized carbon nanotubes (Hong et al. 2010) and quantum dots (Kikkeri et al. 2010; Robinson et al. 2005; Yang et al. 2010) were shown to be efficient biomimetics of natural glycoclusters, often reaching low nM activities. This continuously growing topic is well covered by some recently published reviews (Bernardi et al. 2013; Galan et al. 2013; Marradi et al. 2013; Peri 2013; Shiao and Roy 2012; Blanco et al. 2013; Sansone and Casnati 2013). In the following sections, we will focus on systems with different architectures, specifically designed for vaccine developments.

4.1 Dendrimers

Dendrimers are repetitively branched molecules. The name comes from the Greek word δένδρον (dendron), which translates to “tree” (Astruc et al. 2010). Dendritic molecules are characterized by structural perfection: they are monodisperse and usually highly symmetric around the core, adopting a globular three-dimensional morphology. Dendritic molecules can be categorized into low-molecular weight and high-molecular weight species. The first class includes dendrimers and dendrons, while the latter comprises dendronized polymers, hyperbranched polymers, and polymer brushes. Dendrimers can be considered to have three major portions: a core, an inner shell, and an outer shell. In principle, a dendrimer can bear different functionalities in each of these portions to control properties such as solubility, thermal stability, and attachment of compounds for specific applications. Synthetic processes can also precisely control the size and number of branches on the dendrimer. Such molecules can be assembled following two different approaches. In the divergent method, the dendrimer is assembled from a multifunctional core, which is extended outward by a series of reactions. However, each step must be driven to completion in order to avoid mistakes in the dendrimer, which can cause trailing generations (some branches are shorter than others, Fig. 6a). On the other hand, in the convergent methods, dendrimers are built from

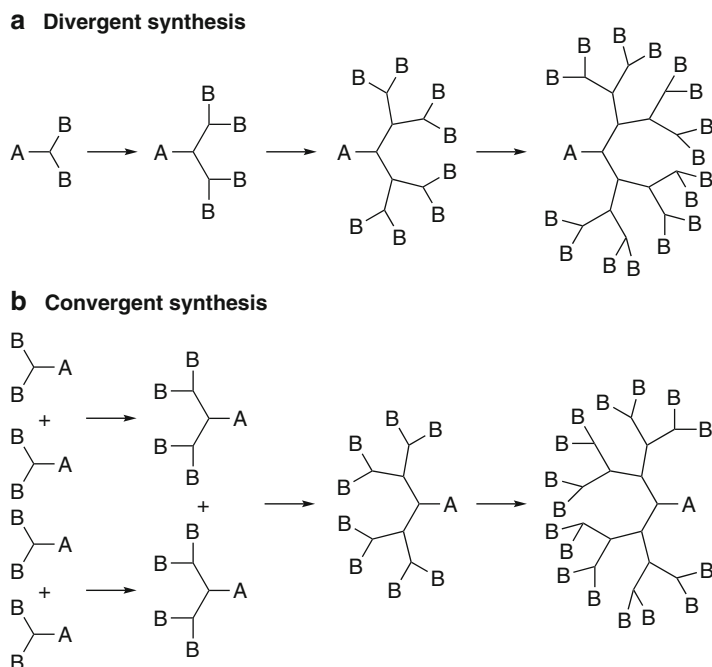
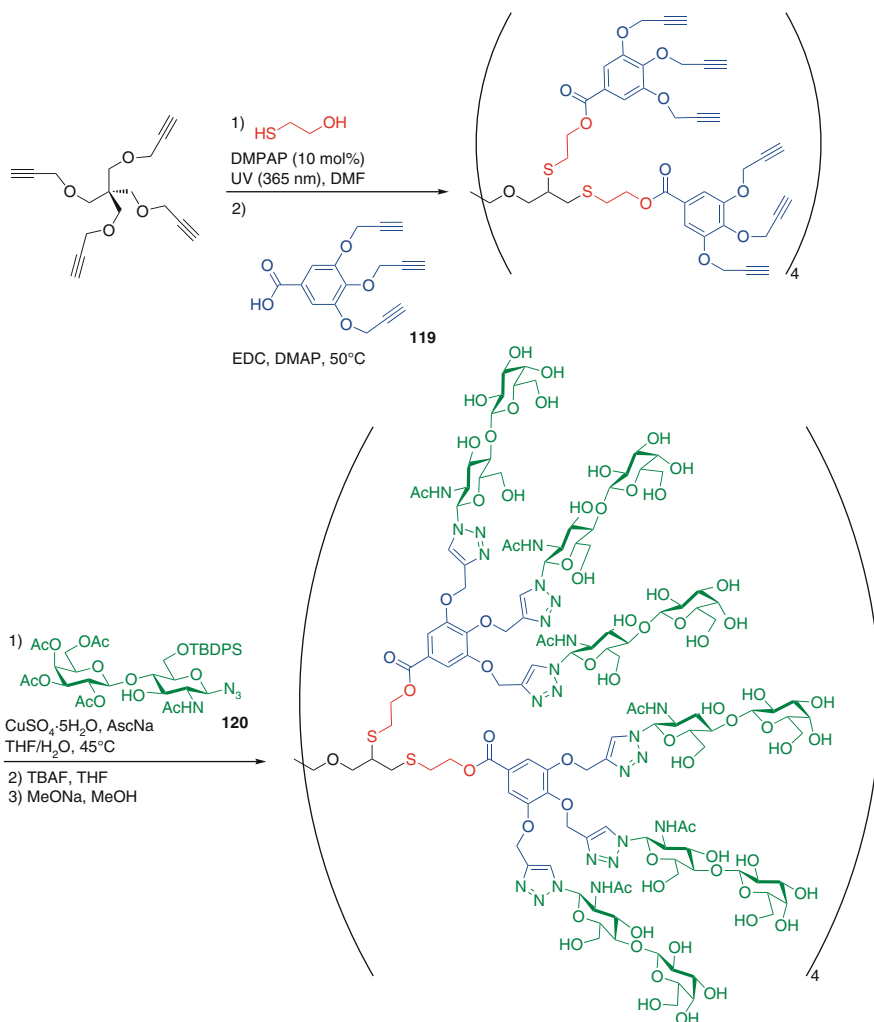


Fig. 6 Outline of convergent and divergent syntheses of dendrimers

small molecules that end up at the surface of the sphere, and reactions proceed inward to the center core (Fig. 6b). This method makes it much easier to remove impurities and shorter branches. However dendrimers made this way cannot be as large as those made by divergent methods because of crowding due to steric effects around the core.

The possibility to incorporate a huge variety of building blocks containing orthogonal functional groups at each layer allows for the construction of original dendritic architectures that differ in terms of constitution, valency, and peripheral functionalities, exemplified by Roy et al. in a recent paper (Sharma et al. 2014). They proposed a new family of model glycoclusters and glycodendrimers, decorated with *N*-acetylglucosamine (LacNAc) termini, that can be assembled through a divergent original “onion peel strategy.” High chemo- and regioselective photolytic thiol-ene coupling (TEC) and thiol-yne coupling (TYC) were used to rapidly and sequentially grow the dendritic architectures and to install a high degree of branching (Dondoni and Marra 2012; Gingras et al. 2013). Further branching (up to 36 valences) was added by EDC-mediated esterifications (or amidations) of polypropargylated dendrons (such as **119**), to which the LacNAc azide derivative **120** was attached by “click chemistry” (Scheme 25). As far as the biological relevance is concerned, LacNAc represents a part of the Lewis^x and Lewis^y tumor-associated carbohydrate antigens (Heimburg-Molinario et al. 2011), and it is known to possess strong binding affinities towards galectins, a cancer-associated family of proteins (Zhou 2003; Liu and Rabinovich 2005; Salatino et al. 2008; Rabinovich and Toscano 2009). Therefore, a protein binding study was carried out with a model leguminous lectin from *Erythrina cristagalli* agglutinin (ECA). Interestingly, the glycodendrimers exhibited high relative potencies, with an up to 216-fold enhancement in global affinity with respect to the reference monomer.

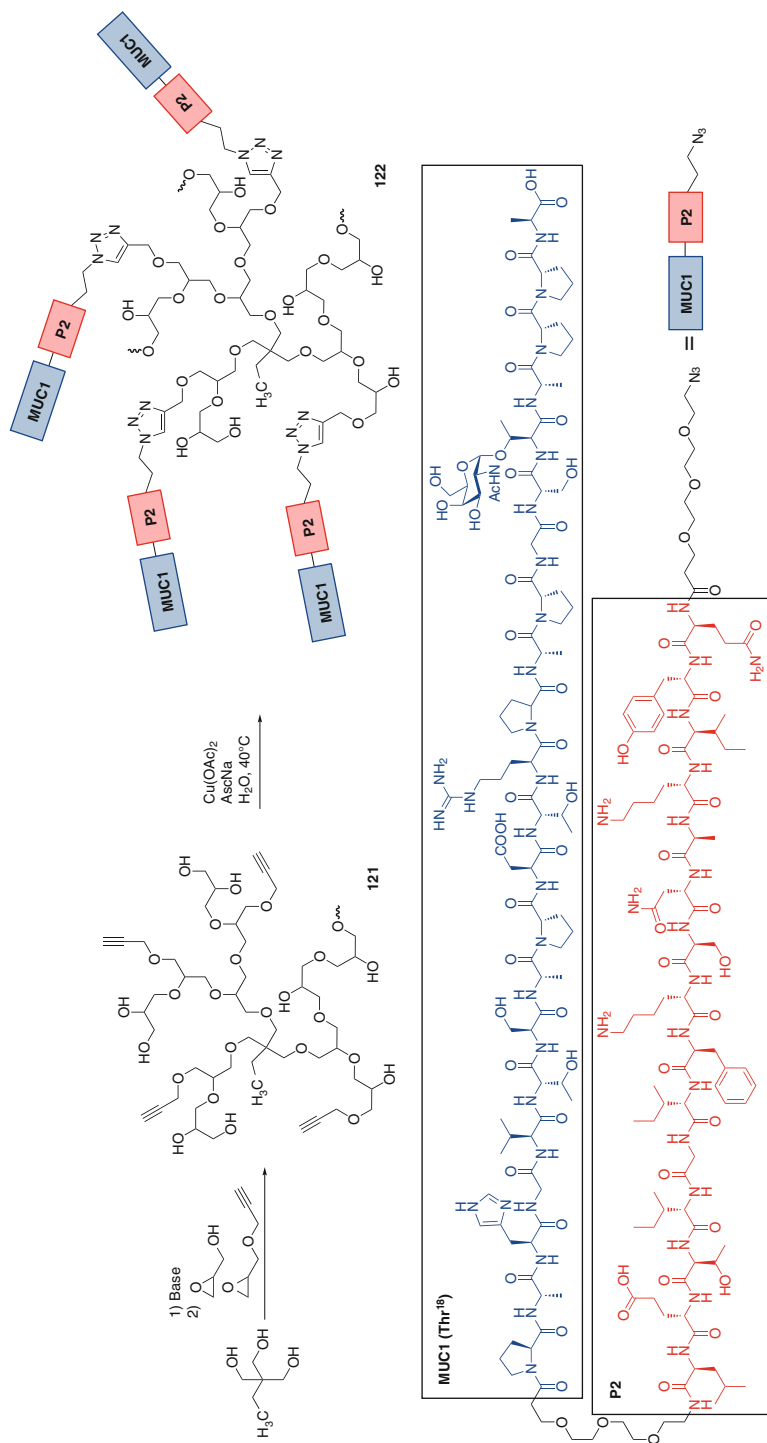
An example of the use of high-molecular weight species was reported by Kunz (Glaffig et al. 2014). The authors employed a water-soluble and readily available polymer, which is the multi-alkyne-functionalized hyperbranched polyglycerol (hbPG) **121**, obtained in a one-step copolymerization of glycidol and its propargyl ether. The scaffold presents a globular shape and a flexible dendrimer-like structure, which allows an optimal antigen presentation to the immune system. Moreover, polyglycerols are suitable as inert carriers for biomedical applications, as they are considered biocompatible and non-immunogenic. A tumor-associated MUC1 glycopeptide combined with the immunostimulating T cell epitope P2 from tetanus toxoid was coupled to the terminal alkyne groups by “click chemistry” (Scheme 26). MUC1 is a tumor-associated glycoprotein member of the mucins family, densely glycosylated high molecular weight proteins implicated in a variety of epithelial cancers. Typical TACAs of mucins include Tn, sTn (NeuAc α 2-6GalNAc), and TF, α -*O*-glycosylated to serine/threonine residues in the peptide backbone (Heimburg-Molinario et al. 2011; Pashov et al. 2011). The resulting construct **122** was shown to induce a strong immune response in mice, raising IgG antibodies able to recognize human breast cancer cells.



Scheme 25 Representative synthesis of LacNAc decorated “onion peel” dendritic structures via an accelerated divergent strategy

4.2 (Cyclo)peptide Scaffolds

Membrane-bound tumor-associated glycoproteins like MUC1 are known to be highly overexpressed on tumor cells. Therefore, they represent an attractive target for anticancer vaccine formulation (Pashov et al. 2011; Beatson et al. 2010). Over the years, considerable efforts have been devoted to the synthesis of glycopeptide assemblies that include either a carrier protein or T cell epitopes in order to induce stronger immunological responses.



Scheme 26 Synthesis of hyperbranched polyglycerol-based antitumor vaccine

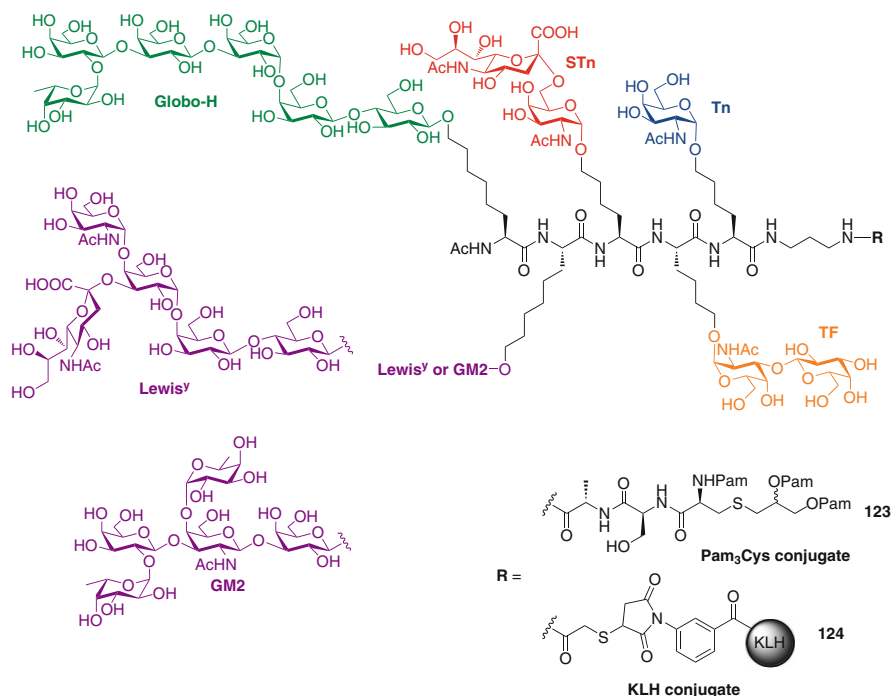


Fig. 7 Structure of the pentavalent vaccine candidates **123** and **124**

The major contribution to this field came from Danishefsky and coworkers. They reported the synthesis and immunological evaluation of several multivalent, fully synthetic, glycopeptide-based antitumor vaccines (Ragupathi et al. 2006). The authors speculated that the combination of several carbohydrate antigens closely associated with a particular cancer type could induce a more robust immune response, decreasing the percentage of tumor cells that can elude an immunological response. The constructs were assembled following a unimolecular multivalent approach, where several distinct TACAs are displayed on a single peptide backbone (Kim and Varki 1997). A sequential assembly of the glycopeptide in order of carbohydrate size, from the smallest (Tn) to the largest and most complex one (Globo H), was performed. The functionalized carbohydrate antigens were prepared either by manipulation of glycol epoxides or sulfonylaziridines, whereas the spacers were built through Horner-Emmons reactions followed by asymmetric hydrogenation or by cross-metathesis (Fig. 7). The results coming from mice immunization point out that the immunological properties of the individual antigens are preserved in the multivalent structures. However, it seems also that the immunogenicity is influenced by the position of the antigen on the peptide backbone. Overall, the antibodies showed significant reactivity with all three cell lines tested (MCF-7, LSC, DU-145). These vaccine candidates are now at the stage of evaluation in clinical trials.

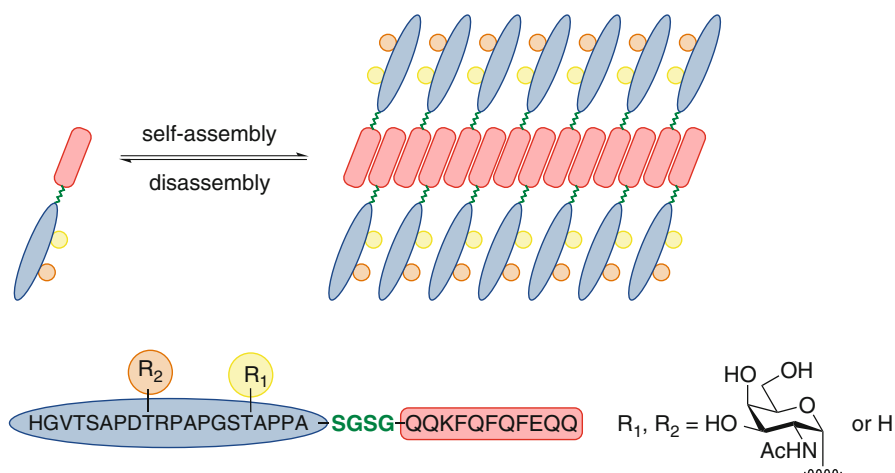
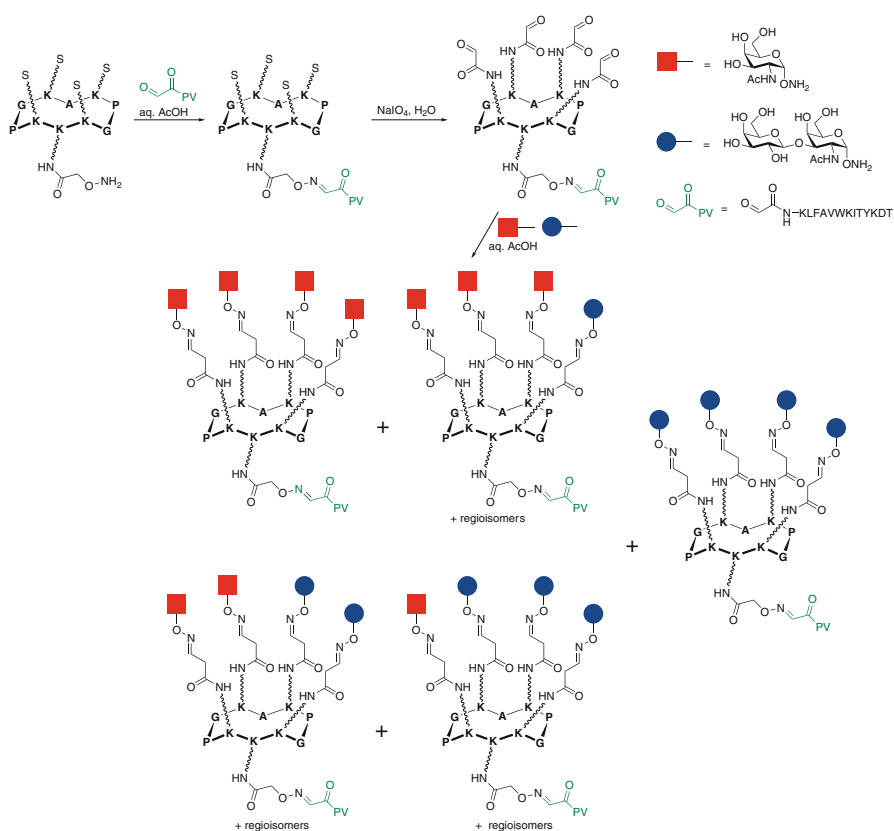


Fig. 8 Structure of self-adjuvanting, self-assembling vaccine candidates with different MUC1 glycosylation pattern

Another exciting approach was reported by the Li team (Huang et al. 2012). They synthesized self-adjuvanting vaccine candidates containing Tn-glycosylated full-length MUC1 VNTR (variable number of tandem repeats) domains (Pashov et al. 2011) conjugated to a self-assembled peptide sequence (Q11 domain, Fig. 8) (Jung et al. 2009). The Q11 domain could aggregate in solution, under mild conditions, into fibers over 200 nm long, thus displaying multivalent B cell epitopes on the fiber surface, and it served both as adjuvant and vaccine carrier (Rudra et al. 2010). Immunological studies revealed the production of high titers of antibodies capable of recognizing human breast tumor cells.

Conversely, the major advantage in the use of small cyclopeptides over linear ones is certainly their locked and rigid conformations that allows for a well-defined spatial orientation of functional groups, thus preventing hindrances between the assembled elements. Up to now, the most widely used architecture is Mutter's Regioselectively Addressable Functionalized Templates (RAFTs), extensively explored by Dumy and Renaudet (Dumy et al. 1995; Renaudet and Dumy 2003; Grigalevicius et al. 2005; Renaudet and Dumy 2008). RAFTs are cyclic decapeptides constrained in an antiparallel β -sheet by two L-proline-glycine β -turns and stabilized by intramolecular hydrogen bonds in solution. They display two functional faces that can be independently functionalized.

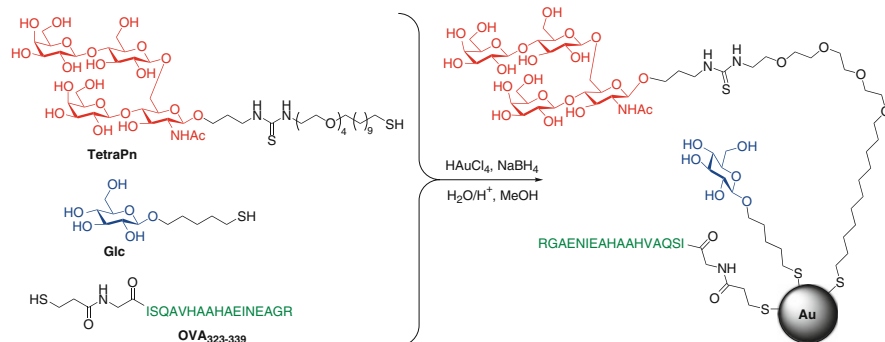
One of the most recent applications of RAFTs was the combination of various TACAs into a modular synthetic vaccine candidate (Fiore et al. 2013). One face of the scaffold was decorated with a mixture of the Tn and the TF carbohydrate antigens, by means of a randomized chemoselective ligation of aminoxy carbohydrates to glyoxaldehydes anchoring sites under mild conditions. The bottom side was provided with an immunostimulating T cell peptide sequence derived from polio virus type 1 (PV, Scheme 27).



Scheme 27 Chemoselective synthesis of RAFT-based anticancer vaccine candidates

4.3 Gold Nanoparticles

In 2001 the Penadès group reported for the first time the use of gold nanoparticles (GNPs) as scaffolds for the multivalent presentation of biologically relevant oligosaccharides through self-assembled monolayers (SAMs) (de la Fuente et al. 2001). Since then, comprehensive studies have been carried out on the influence of loading, presentation of the ligands (i.e., type and length of the linker), and core size on the interaction with the specific receptor (Marradi et al. 2013; Fallarini et al. 2013; Reynolds et al. 2013; de la Fuente and Penades 2006; Manea et al. 2008). In 2012 the same group reported the first example of a fully synthetic carbohydrate vaccine based on GNPs (Safari et al. 2012). By using the “direct” synthesis, they were able to prepare gold nanoparticles (2 nm average diameter) coated with thiol-ended linkers bearing, respectively, a synthetic tetrasaccharide (the single repeating unit of the *Streptococcus pneumoniae* type 14 capsular polysaccharide), the T cell epitope OVA peptide, and β -D-glucopyranoside in variable



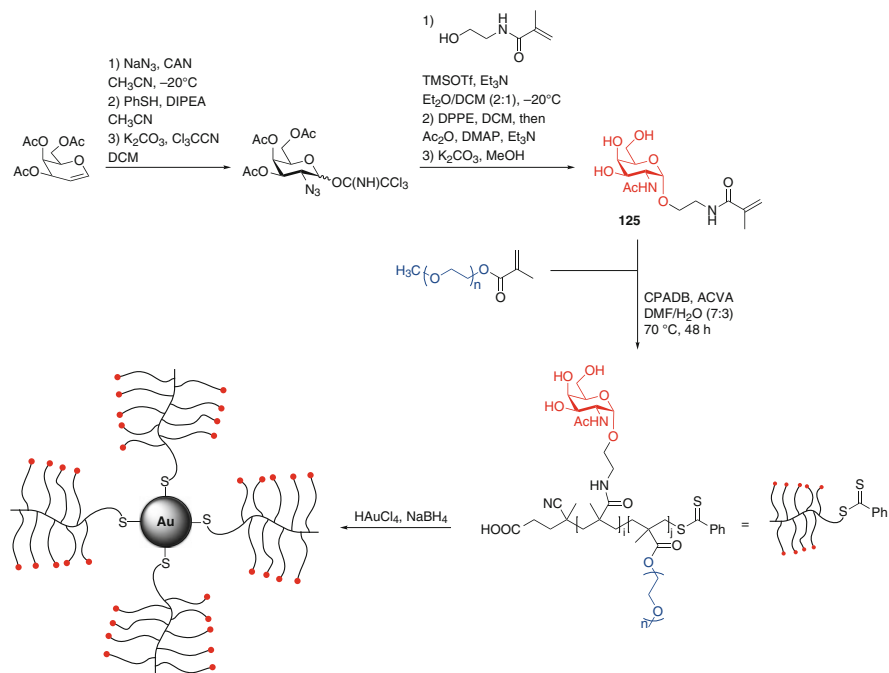
Scheme 28 “Direct” synthesis of sugar-decorated GNPs

ratio (Scheme 28). The authors found out that the molar ratio between the three components and the simultaneous presence of the OVA peptide and the tetrasaccharide antigen were critical for the induction of high titers of specific and functional IgG antibodies.

Another interesting example was reported by Davis group (Parry et al. 2013). The aim of the work was to closely mimic, on gold nanoparticles surface, the overexpression of truncated core 1 mucin-type glycans such as the Tn antigen occurring on the surface of cancer cells. To this end, a glycopolymer displaying multiple copies of the Tn antigen was used to coat and stabilize the surface of GNPs. First of all, a Tn-antigen glycan bearing a polymerizable linker **125** was prepared and then converted by Reversible Addition Fragmentation chain Transfer polymerization technique (RAFT) into various PEG block copolymers with different glycan densities and chain lengths (Scheme 29). Sodium borohydride was finally used to reduce the dithioester end groups of the polymers to thiol and to simultaneously produce GNPs in situ. Immunological studies showed a strong and long-lasting production of antibodies that are selective for the Tn antigen and cross-reactive towards mucin proteins displaying Tn.

4.4 Calixarenes

Calixarenes are a family of cyclic oligomers obtained by the condensation of phenols and formaldehyde (Sansone and Casnati 2013). The even-numbered macrocycles ($n = 4, 6, 8$) are easily obtained using cheap reagents through well-consolidated synthetic procedures and are also commercially available. The odd-numbered homologues ($n = 5, 7, 9$), although known in the literature, are rarely used, as they require more complicated syntheses. The considerable success met by calixarenes, especially calix[4]arenes, as scaffolds for the construction of multivalent ligands can be ascribed to a combination of different factors.



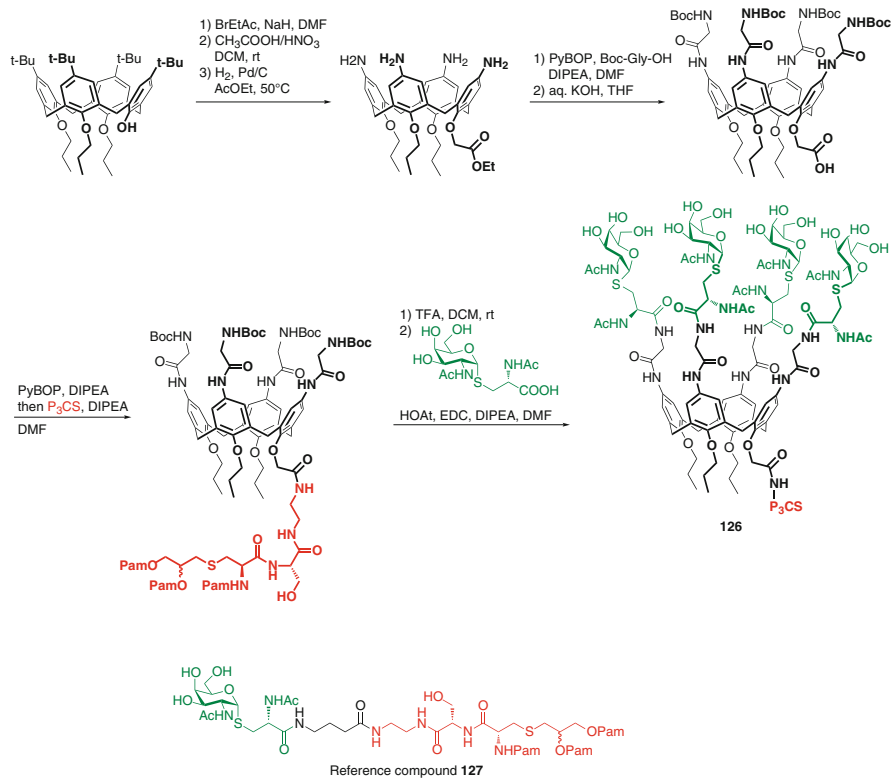
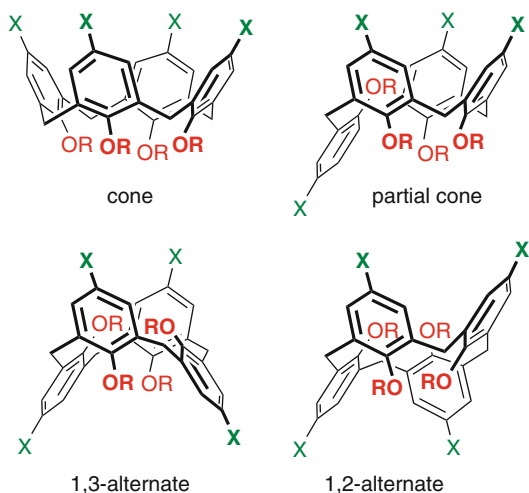
Scheme 29 Preparation of glycopolymer-stabilized GNPs

Among them, the ease to modulate valency, size, structure, and topology of the binding group presentation, together with the possibility to keep them conformationally mobile to exploit an induced-fit binding, or, in contrast, to lock them in a more preorganized specific structure. In fact, while the tetramethoxy and tetraethoxy derivatives are conformationally mobile, the selective introduction of larger groups can lock the macrocycle into one of the four possible conformations. These structures were named by Gutsche as cone, partial cone, 1,3-alternate, and 1,2-alternate (Fig. 9) and are characterized by a remarkably different spatial orientation of the phenolic units.

Functionalization of the calixarene scaffold can be addressed both at the phenolic oxygen atoms (lower rim) and at the para position (with respect to the OH group) on the aromatic nuclei (upper rim), and chains of variable length and containing most of the functional groups used for the glycoconjugation can be introduced.

An interesting example of such an approach is represented by an anticancer vaccine candidate **126** (Geraci et al. 2008). A cone calix[4]arene bearing four Cys-linked tumor-associated Tn antigens at the upper rim was functionalized at the opposite lower rim with the immunoadjuvant Pam₃Cys moiety (Scheme 30). When mice were immunized with the tetravalent calixarene, a fourfold increase in antibody production was observed with respect to a monovalent analogue **127** exposing the same immunoadjuvant but with a single antigen.

Fig. 9 Stable conformations of calix[4]arenes



Scheme 30 Synthesis of Tetra-S-TnAg-Gly-CX-P₃CS

5 Conclusions

Infectious diseases have still strong impact on public health, both in industrialized and developing countries, due to their significant health-related costs for clinical treatment. According to the WHO, the most cost-effective strategy for controlling infections caused by pathogenic microorganisms is the vaccination practice, which should confer long-term protective immunity on the population. Carbohydrate-based vaccines, specifically designed to target pathogen-specific cell surface saccharide structures, showed enormous potential benefits for human health. In particular, it has been ascertained that optimal protection of the population, including newborns and young children, elderly, and immunocompromised patients, may be achieved with the administration of glycoconjugate vaccines that convert the T cell-independent immune response typical of plain polysaccharides into a T cell-dependent response, thereby establishing immunological memory.

However, the manufacture of glycoconjugate vaccines based on carbohydrate antigens isolated from their natural sources is a great challenge that may lead to heterogeneous compositions and batch-to-batch variability, thus raising severe issues of quality assurance and potential difficulties for their approval by the licensing authority.

Over the recent years, the synthesis of complex glycans has made significant progress and is becoming a relevant way to provide oligosaccharide antigens with well-defined chemical structure and devoid of bacterial contaminations which could derive from purification of biological materials. This could be a crucial feature to improve batch-to-batch consistency in vaccine manufacturing and to confer a better safety profile. In addition, synthetic oligosaccharides can be valuable tools for epitope recognition, i.e., to identify within the polysaccharide antigen the minimal structural requirements needed to elicit a protective immune response.

However, glycoconjugate constructs containing synthetic oligosaccharides may encounter a problem of low immunogenicity, as the multi-epitope protein carrier could overlook and weaken the immune response against the saccharide antigens. Some of the most innovative approaches that emerged during the last years in the field of vaccinology are therefore based on the concept that a fully competent immune response targeting poorly immunogenic synthetic carbohydrate antigens may be achieved by including in the vaccine setting only those elements strictly required to elicit a robust carbohydrate-specific immune response, devoid of any unnecessary component. Accordingly, there is a growing interest towards new kind of vaccines where the protein carrier is replaced by a synthetic short peptide able to act as a T cell epitope and to enhance the immunogenicity of the carbohydrate antigen. These new constructs include also fully synthetic vaccines exploiting the multivalency effect, where a significant enhancement of the immunogenicity is achieved by display of multiple copies of the oligosaccharide antigen on a suitable polyfunctional scaffold. A variety of scaffolds with different molecular architectures and chemical properties have been developed and applied during the last years, giving rise to new and very promising vaccine candidates that could open a new front line in vaccinology.

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