3.23 Neoglycoproteins

M. Monsigny, A.-C. Roche, É. Duverger, and O. Srinivas, CNRS & Université d'Orléans, Orléans, France

3.23.1 Introduction

The presence of both lectins and complex carbohydrates in the biological fluids, on the surface as well as inside cells, is well documented (see Refs.: 1 and 2 for reviews). Specific interactions between lectins and complex carbohydrates (glycoproteins, glycolipids, polysaccharides, or proteoglycans) are involved in several basic phenomena such as phagocytosis, endocytosis, intracellular traffic, signal transduction, cell–cell recognition, inflammation processes, cell matrix adhesion, opsonization, cell growth control, cell regulation and differentiation, acrosome reaction, cellular trafficking, cancer cell metastasis, etc. The physicochemical aspects as well as the biological functions of lectin–glycoconjugate interactions depend, to some extent, on the density of the lectins and on the organization of the sugar moieties of glycoconjugates.

The recognition of an individual simple sugar by a lectin is usually in a low-affinity range (about $10^3 1 mol^{-1}$).[3] Conversely, complex oligosaccharides or saccharide clusters, as well as neoglycoproteins, bind lectins in a high-affinity range (up to $10^8 1 mol^{-1}$). Multivalency is a strategy used by both the binding partners to circumvent the intrinsic low affinity of carbohydrate–protein interactions. On the one hand, the carbohydrate binding proteins (lectins) often exist as multimeric and/or possess multiple carbohydrate recognition domains and on the other hand, carbohydrate moieties are often numerous in glycoconjugates. Multivalency leads to the possibility of establishing multiple separate connections resulting in a strong attractive binding force.

Numerous reviews and books dealing with the preparation and the use of neoglycoproteins are available.[4–14] The reader of this chapter will find in these materials a considerable numbers of references. Various methods are available for constructing multivalent structures. Some of them frequently include conjugation of carbohydrate ligands with proteins or other polymers:[9,10,15] these glycoconjugates are called 'neoglycoproteins' (glycosylated proteins) and glycopolymers, respectively. These approaches have been successful, although the products are ambiguous in composition and structure. Alternatively, high-affinity ligands of low molecular weight (complex oligosaccharides from natural or synthetic sources and small oligosaccharide clusters such as glycodendrimers[16,17] or glycoclusters[18] have been developed and can be used as tools to evidence and to study the functions of endogenous lectins as well as devices to target molecules of interest (see Refs.: 9, 10, 19, and 20 for reviews). Such oligosaccharides or glycoclusters may also be bound to a protein, conferring to the neoglycoprotein a very high apparent affinity.

3.23.2 Historical Aspects

Avery and Goebel,[21] in 1929, prepared neoglycoproteins referred to gluco-globulin, galacto-globulin, gluco-albumin, and galacto-albumin: horse serum globulin and crystalline egg albumin substituted with glucosides and galactosides, respectively. They used those neoglycoproteins as antigens to induce the production of immune sera. They demonstrated that the sera contained two separate types of antibodies: the anticarbohydrate antibodies present in sera prepared with the neoglycoproteins which were specific of the sugar borne by the neoglycoprotein used as immunogen and the antiprotein antibodies. The antiprotein antibodies exhibit specificity linked to the original protein (globulin or albumin) and the antibodies reactive with the conjugated sugar-proteins are specific for unrelated proteins containing the same carbohydrate (galactosides or glucosides). Their results demonstrated that the carbohydrate moiety and not the protein molecule determine the serological specificity of the conjugated antigen.[21–23] Then, Goebel and Avery[23] prepared a neoglycoprotein based on the capsular polysaccharide (CS) of type 3 *Pneumococcus*, a polymer of aldobionic acid.[24] This work was initiated on the basis that one of the free hydroxyl groups of the polysaccharide could be replaced by a nitrobenzyl group without incurring a loss in specificity of the polysaccharide. The nitroderivative was then reduced to the amino compound which in turn was coupled through its diazonium derivative to a protein, yielding a neoglycoprotein made with 'complex' oligosaccharides. Such a conjugate had a single constituent common to the *Pneumococcus* cell, namely, the CS and the conjugates should behave as a 'synthetic antigen', inducing specific

antibodies upon immunization, which share similar specificity as those produced by immunization with intact bacterial cells.

Later, Goebel[25] showed that a neoglycoprotein containing the repeating unit of this polysaccharide cellobiuronic acid – but not the neoglycoproteins containing cellobioside, glucoside, or glucuronide – gives rise in rabbits to antibodies which are specific and characteristic of the saccharide constituent of the CS of type 3 *Pneumococcus*. It was concluded that the antiserum to the synthetic antigen containing cellobiuronic acid conveys passive protection on mice to infection with virulent *Pneumococcus* types 2, 3, and 8.

Several years later, Iyer and Goldstein[26] prepared neoglycoproteins in order to study, in a quantitative approach, the interaction between glycoconjugates and concanavalin A, a plant lectin specific for mannosides and glucosides. Similarly, Privat and co-workers[27] prepared neoglycoproteins bearing chitin oligomers (GlcNAcβ-(4GlcNAcβ-)$_n$ with $n=0$–3) and showed that those neoglycoproteins interact with another plant lectin: wheat germ agglutinin, inducing the precipitation of the complex in a concentration-dependent manner, analogous to an immunoprecipitation.

3.23.3 Neoglycoproteins

Neoglycoproteins have been prepared from various proteins (bovine and human serum albumin (HSA), ribonuclease, ferritin, diphtheria toxin, streptavidin, etc.) as well as glycoproteins (ovalbumin, horse radish peroxidase, serum globulin, etc.). It is obvious that the most interesting neoglycoproteins are those prepared from sugar-free proteins and serum albumin is the most popular protein selected by many researchers to prepare well-defined neoglycoproteins. Serum albumin is highly soluble in neutral or alkaline medium and its molecular weight is large enough (roughly M_r: 67000) to allow a heavy sugar substitution.

3.23.3.1 Serum Albumin

The properties of serum albumin are very nicely presented in reviews by Peters,[28] and Carter and Ho.[29] Mammalian serum albumin contains about 60 lysines (**Table 1**). The three-dimensional structure of HSA has been determined by crystallography to a resolution of 0.28 nm.[30,31] It comprises three homologous domains that assemble to form a heart-shaped molecule. Each domain is a product of two subdomains that possess common structural motifs (**Figures 1** and **2**). The principal regions of ligand binding to HSA are located in hydrophobic cavities in the first subdomains II and III, which exhibit similar chemical properties. The structure explains numerous physical phenomena and should provide insight into future pharmacokinetic and genetically engineered therapeutic applications of serum albumin. As shown in **Figure 3** from the primary sequence, 60 lysines of bovine serum albumin (BSA) are dispersed throughout the molecule. Serum albumin is also very rich in cysteine, as shown in **Figure 2** accounting for the exceptional stability of

Table 1 Amino acid composition of BSA[272]

Ala	48	Arg	26	Asn	14	Asp	41	Cys	35
Gln	21	Glu	58	Gly	17	His	16	Ile	15
Leu	65	Lys	60	Met	5	Phe	30	Pro	28
Ser	32	Thr	34	Trp	3	Tyr	21	Val	38

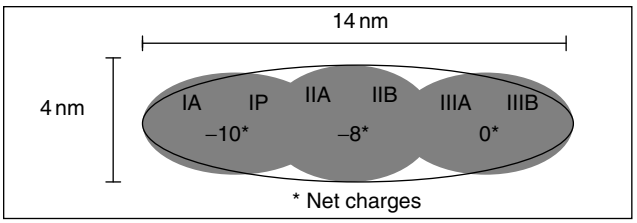

Figure 1 Schematic structure of serum albumin, adapted from Peters, 1985:[28] the molecule is made of three domains (I, II, III) containing 10, 8, and 0 negative net charges, respectively. Each domain is divided into subdomains A and B.

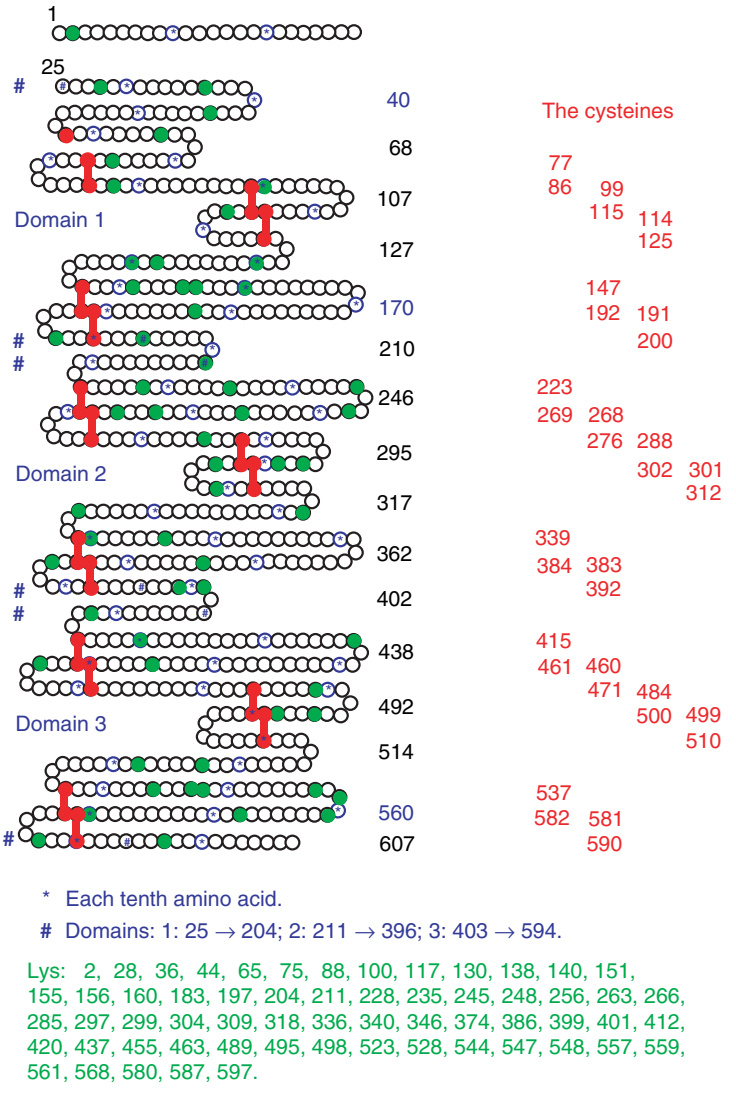

Figure 2 Structure of BSA showing both lysine and disulfide bridges present in the three domains (I, II, III), according to Peters.[28]

the mature protein. The albumin molecule is not uniformly charged within the primary structure. At neutral pH, Peters[28] calculated a net charge of −10, −8, and 0 for domains, I, II, and III for BSA (**Figure 1**). Interestingly, the lysines are well dispersed on the surface of the protein as shown in **Figure 4**.

3.23.3.2 Neoglycoprotein Synthesis

The coupling of saccharide residues to the surface of proteins has long been used as a straightforward strategy for the creation of high-valence neoglycoproteins; such approaches are still in use.[11] During the last two decades, advances in the area of bioconjugate synthesis methods have led to the development of mild methods for the preparation of neoglycoproteins suitable for vaccination purposes.[11,20,32–34] The maintenance of three-dimensional conformation or tertiary structures of the protein carriers, especially the bacterial toxins, necessitates the use of mild coupling procedures. In fact, the choice of coupling procedures is often limited by the propensity of carrier protein to undergo changes in the tertiary structure or denaturation under the reaction conditions. The synthesis of neoglycoproteins primarily involves random or defined coupling sites on the surface of the protein carrier and their covalent modification with oligosaccharides at their reducing end or functionalization of the oligosaccharides bearing a spacer arm.

MKWVTFISLL LLFSSAYSRG VFRR

DTHKSE IAHRFKDLGE EHFKGLVLIA

FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL

HTLFGDELCK VASLRETYGD MADCCEKQEP ERNECFLSHK

DDSPDLPKLK PDPNTLCDEF KADEKKFWGK YLYEIARRHP

YFYAPELLYY ANKYNGVFQE CCQAEDKGAC LLPKIETMRE

KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE

FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN

QDTISSKLKE

CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK

NYQEAKDAFL GSFLYEYSRR HPEYAVSVLL RLAKEYEATL

EECCAKDDPH ACYSTVFDKL KHLVDEPQNL IKQNCDQFEK

LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS RSLGKVGTRC

CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC

TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP

DTEKQIKKQT ALVELLKHKP KATEEQLKTV MENFVAFVDK

CCAADDKEAC FAVEGPKLVV

STQTALA

Figure 3 Primary sequence of mature BSA.[272] One lysine is in the N-terminal peptide of the preproalbumin and is lost upon processing to the mature albumin, $M_r = 66430$.

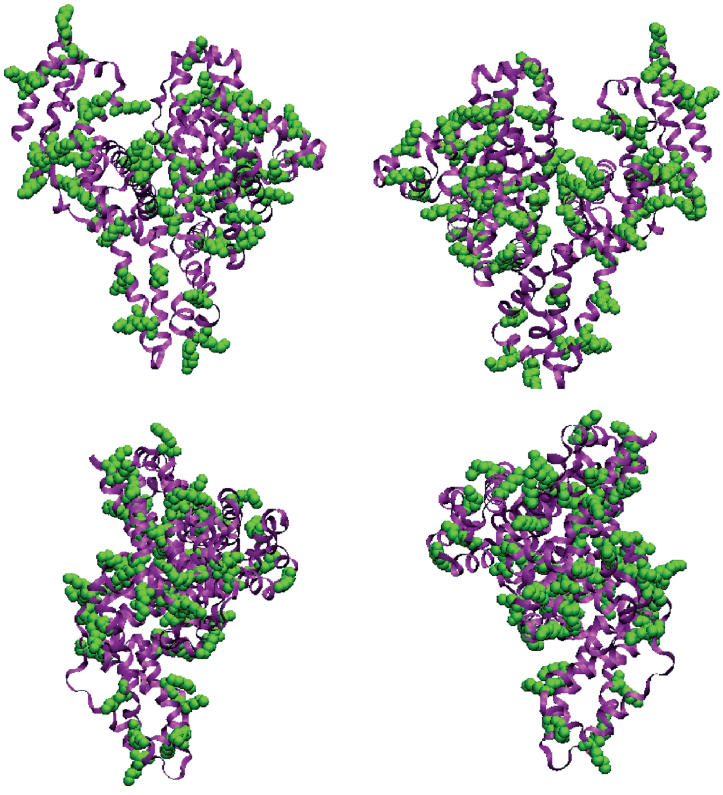

Figure 4 Four views of lysyl residues (green) in HSA. Credit:[284] http://www.ks.uiuc.edu/Research/vmd/.

A variety of chemical conjugation methods have been employed for the attachment of antigenic carbohydrate epitopes by targeting specific amino acid residues present on the carrier protein surface. Although these methods are not site specific and lead to a variable loading of the protein carrier, they have nevertheless been the most effective means to prepare neoglycoproteins so far. Some of the popular approaches are discussed briefly below: for comprehensive reviews on neoglycoproteins and artificial glycoprotein synthesis, see Refs.: 35 and 36 and on structural and synthetic aspects of neoglycoprotein vaccines, see Ref.: 37 and Section 3.23.7.

3.23.3.2.1 Conjugation to lysine

As shown in **Figure 5**, several chemical reactions lead to neoglycoproteins in which lysyl residues are substituted with a glycopolyol or with a carbohydrate moiety through a spacer arm.

3.23.3.2.1.1 Reductive amination

The conjugation of the reducing end of the oligosaccharides with the ε-amino group of lysine leading to the formation of the Schiff's base is one of the most frequently employed methods. Since the formation of a Schiff's base is a reversible equilibrium-driven process, the *in situ* reduction of the imine formed using sodium cyanoborohydride ($NaBH_3CN$) drives the reaction toward the formation of stable amine adducts.[38] The ε-amino groups of lysines are useful for direct coupling not only to the reducing terminal of poly- or oligosaccharides but also to aldehyde functionalities generated on short spacer arms linked to the saccharide. The reactive aldehyde functionalities can be easily generated from spacer arms by ozonolysis of unsaturated alkyl spacers or hydrolysis of acetals. Periodate cleavage of saccharide residues to produce reactive aldehydes followed by reductive amination in the presence of cyanoborohydride has also been extensively utilized. Borch and co-workers[39] showed that sodium cyanoborohydride ($NaBH_3CN$) reduces a wide variety of organic functional groups with a remarkable selectivity. The reduction of aldehydes and ketones is pH dependent, the reaction proceeds readily at pH 3–4. Reaction of an aldehyde or ketone with ammonia, primary amine, or secondary amine at pH 7 in the presence of BH_3CN^- leads to primary, secondary, or tertiary amines, respectively, via reductive amination of the carbonyl group. Then, Gray[38] successfully applied this new method to transform reducing sugars to glycopolyol-amines in a good yield. Knowing that carbonyl groups are not reduced at pH above 5, while the Schiff bases are, Gray and co-workers[40] showed that a disaccharide such as cellobiose

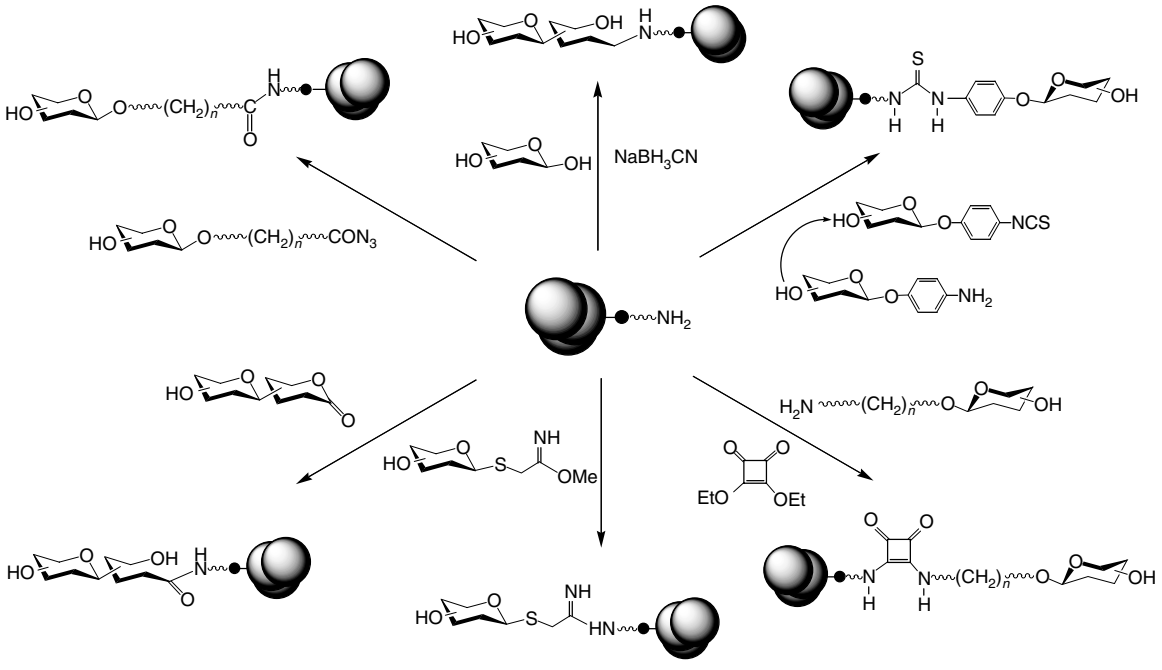

Figure 5 Neoglycoprotein synthesis by conjugation of oligosaccharides to lysyl residues.

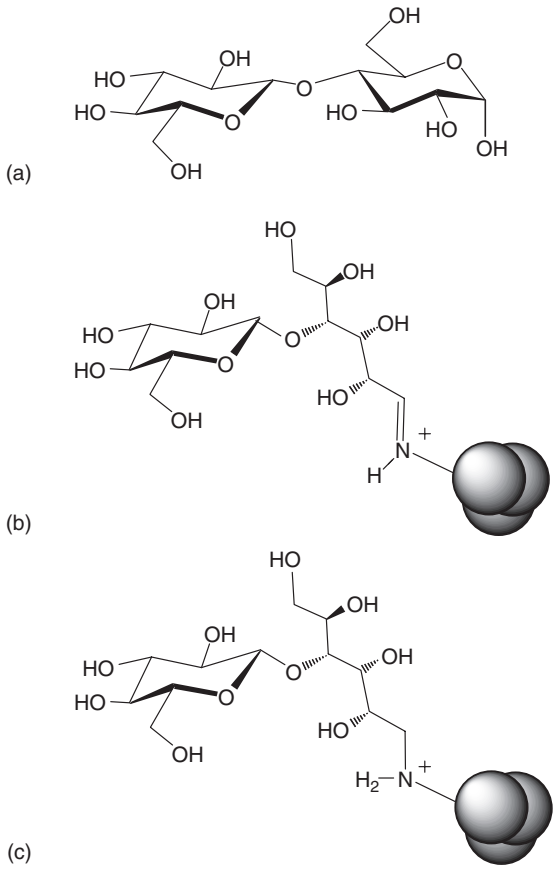

Figure 6 Preparation of glycosylated serum albumin according to the reductive amination procedure developed by Gray.[38] Cellobiose a, (292 μmol) reacted at 37°C for about 10 days with 1 μmol of BSA at pH 8.0 in the presence of 1590 μmol of sodium cyanoborohydride, allowing to reduce the Schiff base b, to a secondary amine c, the neoglycoprotein contained about 26 residues of glucose.

reacted with the amino groups of serum albumin at pH 8.0 leading to a protein substituted with up to 26 sugar moieties according to the reaction shown in **Figure 6**. The sugar linked to an amino group of the protein is transformed during the reduction step into a linear polyol-amine. For instance, lactose[41] has been coupled to the cross-linked dimer of bovine pancreatic ribonuclease A as well as serum albumin; in the last case, up to 20 mol of lactose/mol of protein was coupled at the end of a 5-day reaction; while the glycosamination reaction can be carried out at pH 7, the coupling reaction was 2–3 times faster at pH 9, in agreement with Baues and Gray's data.[42] When derivatives of ribonuclease dimer that contained up to eight *N*-ε-1-(1-deoxylactitolyl)-lysine residues per molecule were injected into mice, 69% of this neoglycoprotein was found in the liver after 10 min; while with the nonglycosylated enzyme, the liver uptake was only 4%, the majority of the neoglycoprotein being found in the kidneys.

Some years later, Lee and co-workers prepared thioglycosides ended with a diacetal derivative (see **Figure 7**) allowing upon deprotection to ω-aldehydo-alkyl 1-thio-glycopyranosides.[43] The length of the spacers between the sulfur atom and the amino groups of BSA may be modulated in order to give more freedom to the presentation of sugar moieties.

3.23.3.2.1.2 Use of O- and S-glycosides with a spacer arm

3.23.3.2.1.2.1 Formation of thiourea linkages Phenylisothiocyanate glycosides (see Ref.: 44 for a review) derived from *p*-amino-aryl glycosides using thiophosgene[45] or thiocarbonyldiimidazole[46,47] have been extensively used for conjugation of simple sugars or complex oligosaccharides onto lysines (**Figure 8**). Oligosaccharyl *p*-nitro-anilide (*p*NA) pyroglutamyl glycosynthons derived from unprotected oligosaccharides provide an easy approach to synthesize phenylisothiocyanate derivatives of complex oligosaccharides.[47]

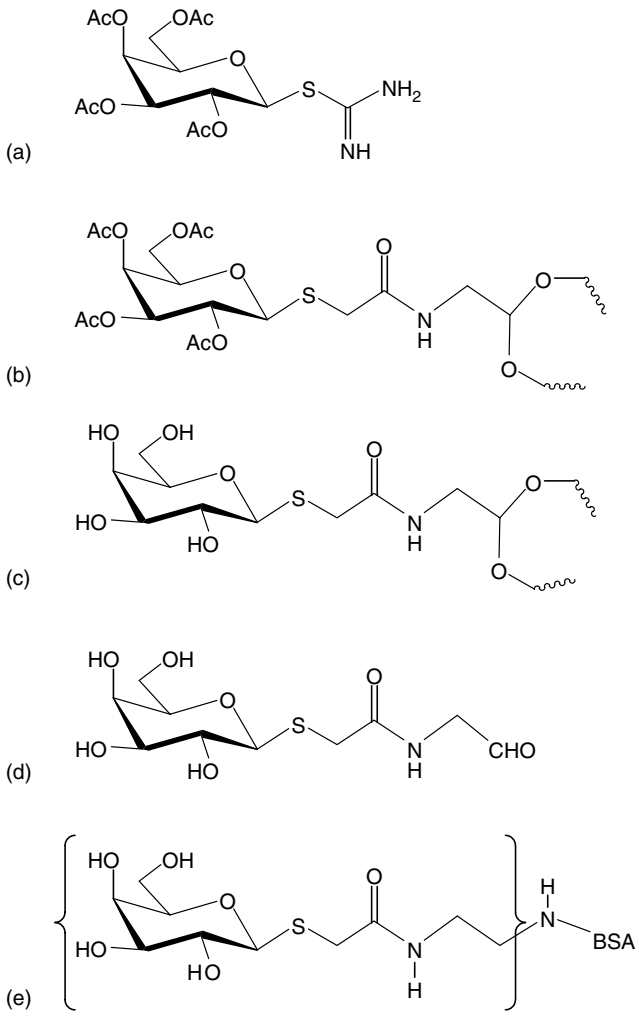

Figure 7 The tetra-*O*-acetylgalactosyl-β-pseudothiouronium a, was reacted with chloro-acetylamido-acetaldehyde dimethyl acetal Cl-CH_2-CO-NH-CH_2-CH(O-CH_3)$_2$ in the presence of K_2CO_3 and $NaHSO_3$ leading to tetra-*O*-acetylgalactosyl-β-thio-acetylamidoacetaldehyde dimethyl acetal b, and converted in the presence of $NaOCH_3$ (sodium methoxide) in methanol to galactosyl-β-thio-acetylamidoacetaldehyde dimethyl acetal c, and then in mild acid medium to galactosyl-β-thio-acetylamidoacetaldehyde d; finally, d, was allowed to react in the presence of sodium cyanoborohydride ($NaCNBH_3$) with BSA at neutral pH (close to 7), leading to galactosylated BSA, a neoglycoprotein e; as much as 40 thiogalactosides (one of which is shown inside the brackets) were linked to the protein-amino groups (one of which is shown outside the brackets).[43,285]

The allyl trisaccharide GalNAcβ-4[Fucα-3]GlcNAcβ-glycoside was transformed in 96% yield into the 3-(2-aminoethylthio)propyl spacer by radical addition of cysteamine hydrochloride under UV irradiation, then activated with thiophosgene and coupled to BSA[48] The ligand/protein ratio of the neoglycoprotein, based on protein and amino-sugar analyses, was 6:1 mol/mol.

3.23.3.2.1.2.2 Formation of amidine linkages δ-Alkylimidate glycosides (2-iminomethoxymethyl thioglycosides) were synthesized[49,50] and used to prepare neoglycoproteins (**Figure 9**). Such neoglycoproteins retain the number of positive charges of the native protein because the linkage is an amidine.

3.23.3.2.1.2.3 Formation of amide linkages Other approaches to prepare neoglycoproteins via coupling to lysines involve condensation of oligosaccharides with spacer arms (carbohydrate or non-carbohydrate-based) such as (1) oligosaccharide containing sugar lactones;[51] (2) activated acyl azides derived from oligosaccharyl-acyl hydrazides;[52] (3) oligosaccharides that contain an amino spacer with diethyl squarate;[53] (4) oligosaccharides with a spacer arm ended with a carboxylic group activated as *N*-hydroxysuccinimide (NHS)[54] or sulfo-NHS esters; (5) a thioglycoside

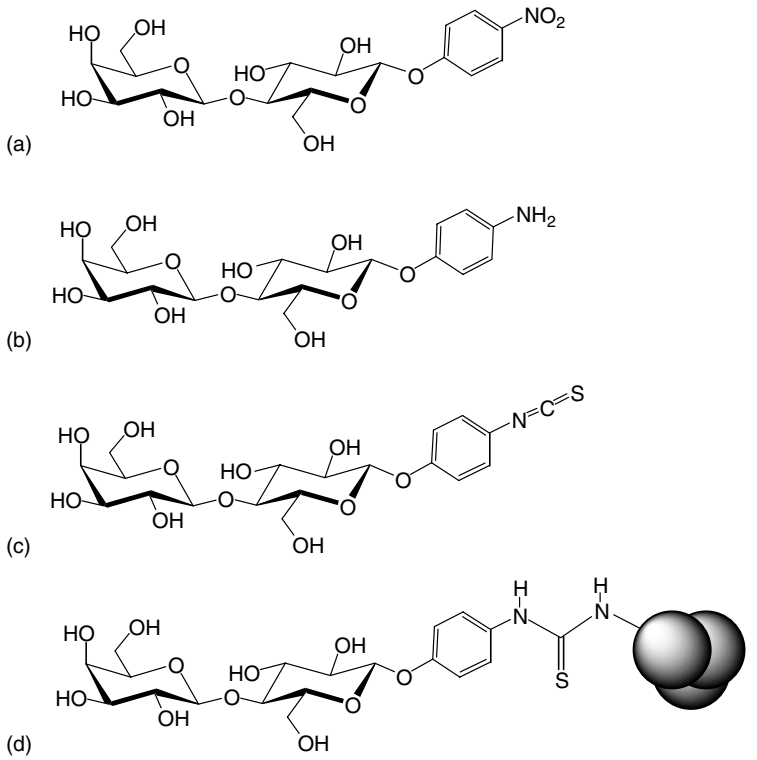

Figure 8 Synthesis of a neoglycoprotein d, containing lactosyl-β-phenylthiocarbamyl moieties.[5,127,128] A glyco-*para*-nitrophenyl conjugate a, was reduced in the presence of hydrogen and platinium oxide[45] or palladium on charcoal[127] to a glyco-*para*-aniline derivative b, then converted into a glyco-phenylisothiocyanate c, by using thiophosgene[45] or thiocarbonyl-bisimidazole[46] as in Quétard and co-workers.[47] Finally, the last compound c, reacted with BSA at pH 9.0 leading to a neoglycoprotein d, containing up to 54 sugar moieties.

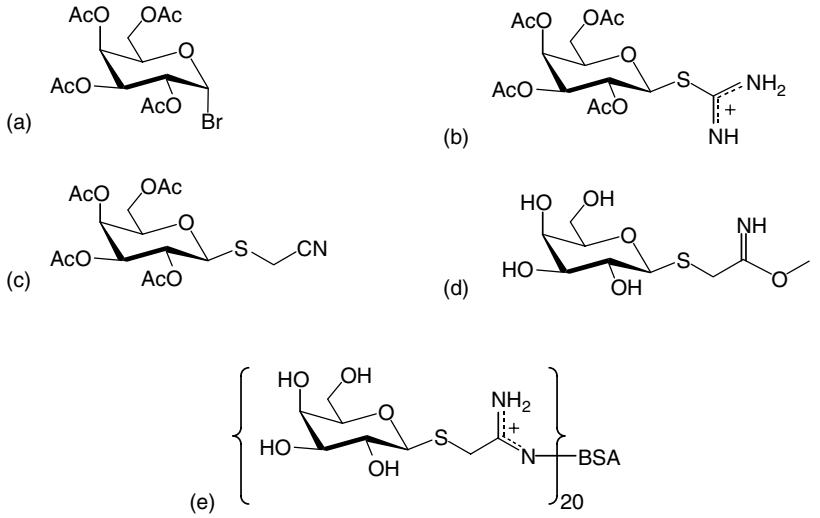

Figure 9 Synthesis of neoglycoproteins according to Lee and co-workers.[50] Acetobromogalactose a, was converted in the presence of thiourea $CS(NH_2)_2$ into tetra-*O*-acetylgalactosyl-β-pseudothiouronium b; b, was reacted with chloro-acetonitrile in the presence of K_2CO_3 and $NaHSO_3$ leading to tetra-*O*-acetylgalactosyl-β-thio-acetonitrile c, and converted in the presence of $NaOCH_3$ (sodium methoxide) in methanol into galactosyl-β-thio-imidate d; the imidate was allowed to react with BSA at pH close to 9, leading to galactosylated BSA, a neoglycoprotein.

corresponding to galactosyl-globoside (SSEA-3), Galβ-3GalNAcβ-3Galα-4Galβ-4Glcβ-S-CH_2-CH_2-COOH activated with *N*,*N*,*N*′,*N*′-tetramethyl(succinimido)uronium tetrafluoroborate (TSTU) and coupled to BSA to give the corresponding neoglycoprotein (11.6 mol of saccharide/mol of BSA);[55] (6) the preparation of neoglycoproteins containing Fucα-2Gal and Galβ-4GlcNAc glycosides, achieved by transforming 2-bromoethyl glycosides into a methoxycarbonylethylthioethyl derivative which was used to substitute both BSA and keyhole limpet hemocyanin (KLH).[56]

3.23.3.2.1.3 Use of N-glycosides with a spacer arm

3.23.3.2.1.3.1 Glycosylamines as starting material Glycosylamines (see Refs.: 57 and 58 for reviews) are easily prepared in the presence of an excess of ammonia and can be isolated from such a medium, but they are not stable in neutral or slightly acidic media; conversely, they become stable upon acylation (**Figure 10**). Glycosylamines made from D-sugars have usually a β-configuration, the α-configuration being less stable than the β-configuration. Upon N-acylation, the *N*-acylglycosylamides are stable and it is therefore possible to isolate them. *N*-acylglycosylamides may also be obtained directly by action of ketene on D-glycosylamine. Glycosylamines may undergo Amadori rearrangement: a transformation of glycosylamine into 1-amino-1-deoxy-2-keto derivative was shown to occur with *N*-arylglycosylamines when they were heated for a few hours in ethanol solution with a weak acid as a catalyst. The Amadori rearrangement occurs especially in the presence of both a compound having an activated methylene group and a catalytic amount of a secondary amine or in the presence of glacial acetic acid. The mechanism involves the addition of a proton to the nitrogen atom of the glycosylamine; therefore, when the amine is further substituted by acylation as in *N*-acetylglycosylamide, the protonation is inhibited and the Amadori rearrangement is limited.

Various glycosylamines were obtained, more than a century ago, by Lobry de Bruyn[59] in 1895 by dissolving a reducing sugar in warm water and adding anhydrous methanol saturated with ammonia. Within 10 days, the glycosylamine crystallized. A β-glycosylamine can also be obtained by dissolution of the sugar in liquid ammonia followed by evaporation of the solvent.[60] More recently, 2-acetamido-2-deoxy-β-D-glucopyranosylamine[61] was prepared with a 70±10% yield by dissolving *N*-acetylglucosamine in saturated aqueous ammonium hydrogen carbonate; the solution was kept at 20°C for 45 days or at 30°C for 6 days. The main compound, however, was not the expected glycosylamine but rather a glycosylcarbamate. However, when the crude product was purified by cation exchange chromatography, the expected glycosylamine was eluted with 2M ammonia in a methanol–water mixture.

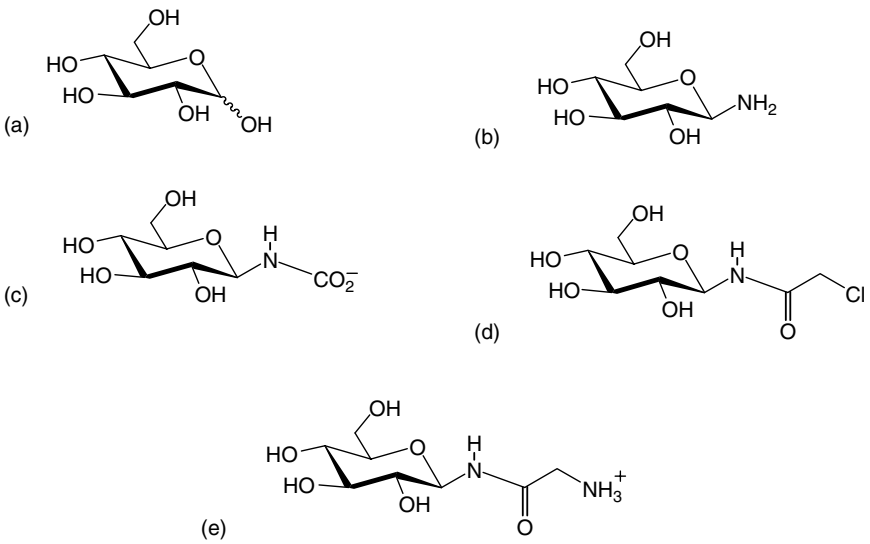

Figure 10 Preparation of glycosylamide derivatives. A reducing sugar such as glucose a, may be converted to a glycosylamine derivative b, (mainly as a β-anomer) upon incubation in methanol ammonia mixture,[59] liquid ammonia,[60] ammonium hydrogen bicarbonate,[61] for several days (up to 10 days) at room temperature. With a solution of ammonium hydrogen bicarbonate, byproducts can be obtained such as an *N*-glycosylcarbamate[62] c. A glycosylamine is easily converted back to the initial sugar if it is not acylated. Many activated acids may be used to obtain a stable glycosylamide such as the chloro-acetyl glucosylamide shown d. The last compound may be then converted to a glycyl-glycosylamide e.[63]

Glycosylamines were also obtained[62] by treating reducing sugars with 16M commercial aqueous ammonia in the presence of NH_4HCO_3. The solution was heated at 42°C for 36h. The yield in the expected glycosylamine was almost quantitative, the presence of glycosylcarbamate being rather low (<8%). Manger and co-workers[63,64] prepared *N*-(glycyl)-*β*-glycosylamides from oligosaccharides ending with a reducing *N*-acetylglycosylamine. Glycosylamines were acylated with chloroacetic anhydride in 1M sodium bicarbonate, the pH being kept above 7. The *N*-chloroacetylglycosylamides obtained were then ammonolyzed by action of saturated ammonium bicarbonate at 50°C for 8h in a sealed tube. The *N*-(glycyl)-glycosylamide was purified by a cation exchange chromatography on a carboxymethyl gel. Alternatively, *N*-(glycyl)-glycosylamides were prepared by reaction of Fmoc-glycine in dimethylformamide (DMF) with *β*-glycosylamine dissolved in a mixture of dimethylsulfoxide (DMSO) and DMF *N*-ethyldiisopropylamine, HBTU, and HOBT in DMF at room temperature for about 2h. The overall yield of *N*-(glycyl)-*β*-glycosylamide upon deprotection was 55% based on the starting sugar.[65]

Glycosylamine derivatives of oligosaccharides may also be obtained by enzymatic hydrolysis of *N*-acetylglucosaminyl-asparagine of glyco-amino acids, glycopeptides, or glycoproteins containing N-glycan moities (see above). The enzyme, called PNGase F or *N*-glycanase, cleaves the linkage between the amino group of the *β*-glycosylamine and the *β*-carboxylic group of the aspartyl residue, with an optimal activity in slightly alkaline medium pH 8.5 or 9.0.[66] The glycosylamine derivatives of the oligosaccharides released from glycopeptides are stable in slightly alkaline medium, but conversely they lose rapidly the amine group when they are kept in slightly acidic medium. On these bases, Tarentino and co-workers[67] showed that such *β*-glycosylamine derivatives of oligosaccharides released from glycoproteins with PNGase F were very efficiently substituted at pH 8.8.

On the purpose of preparing oligosaccharide derivatives easily detected by absorption in ultraviolet light (280nm), easily labeled by radioiodination, and easily usable for further condensation onto various molecules or matrices, glycosylamines have been selectively acylated by reaction with an N-protected tyrosine, *N*-(tyrosyl)-glycosylamides were prepared.[68] The glycosylamine derivatives of oligosaccharides, isolated from glycoproteins upon enzymatic hydrolysis and treated with ammonium bicarbonate, were reacted with Boc-Tyr-OSu (*N-tert*-butyloxycarbonyl-tyrosyl-succinimidyl ester) in DMF at 50°C for 3h. Then, the conjugate was purified by gel filtration. Finally, the Boc group was released by treating the dry oligosaccharide with trifluoroacetic acid at room temperature for 10min.

Similiarly, a *β*-aspartyl-glycosylamide was prepared by Otvos and co-workers.[69] In this case, the 2-acetamido-2-deoxy-1-(*N*′-Fmoc-*β*-aspartyl)-*β*D-glucopyranosylamide[69] was obtained by coupling Fmoc-Asp-OtBu in DMF in the presence of diisopropylcarbodiimide with the *β*-glycosylamine of *N*-acetyl-D-glucosamine dissolved in water; this synthon was then used to prepare various glycopeptides. Various other synthons were also obtained in a similar way. For instance, the *β*-glycosylamine of an heptasaccharide containing five mannoses and two *N*-acetylglucosamines (Man_5)-GlcNAc*β*-4GlcNAc*β*-NH_2 was coupled with a pentapeptide Ac-Tyr-Asp-Leu-Thr-Ser-NH_2 in dimethylsulfoxide in the presence of HOBT, DIEA, and HBTU, leading to the expected glycopeptide.

3.23.3.2.1.3.2 *N*-Glycosylamino acid derivatives as starting material An oligosaccharide, ended with a reducing sugar, was converted into *N*-(*β*-glycosyl)-glycyl-*p*-nitroanilide: the oligosaccharide was dissolved in an organic solvent such as dimethylformamide, 1-methyl-2-pyrrolidone, or dimethylsulfoxide and allowed to react with the amino group of glycyl-*p*-nitroanilide, at 50°C for 5 days. As expected, this glycosylamino acid derivative was stable in alkaline conditions, even in aqueous solution, as in the case of glycosylamines (see above), but it was not stable in neutral or acidic conditions. Upon addition of an acylating agent, *N*-acetyl-imidazole, the conjugate was selectively N-acetylated within 30min at room temperature.[70] The N_1-acetyl-N_1-glycosyl-glycyl-*p*-nitroanilide was purified by gel filtration and was found to be quite stable in a large range of pH. The anomeric configuration of the glucose residue linked to the amino acid was found to be *β* on the basis of the proton NMR analysis.

Another example is given (**Figure 11**) with the preparation of *N*-glycosyl-pyroglutamyl derivatives also called 'glycosynthons'. The incubation of a sugar with an α-glutamyl derivative in the presence of imidazole led to a quantitative coupling[47,71] within 6h at 50°C. In the absence of imidazole the reaction was slower and more side reactions occurred. As in the case of *N*-glycosylglycine derivative, the *N*-glycosyl pyroglutamyl derivatives were not stable in aqueous medium, except in alkaline conditions. The conjugate was readily stabilized by adding into the solution of an *N*-glycosyl-pyroglutamyl derivative, BOP and imidazole; the intramolecular acylation was complete within 30min at room temperature. This compound was found to be stable at room temperature at any pH between 3.6 and 9, at room temperature as well as at 90°C in either slightly acidic or neutral conditions. Various *N*-glycosyl-pyroglutamyl derivatives were prepared by this procedure, starting with either monosaccharides or oligosaccharides. In all cases, the yield of the expected glycoamino acid derivatives was very high. Various *N*-glycopyroglutamyl-amido-ethyldithiopyridines were prepared with a high yield (up to 94%). In all cases the conjugates appeared to have a *β*-anomeric configuration with an *H*-1 resonance of the glycosylamide around 5.15ppm and a $J_{1,2}$ coupling constant