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Review

Current techniques in protein glycosylation analysis A guide to their application

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The importance of glycosylation in biological events and the role it plays in glycoprotein function and structure is an area in which there is growing interest. In order to understand how glycosylation affects the shape or function of a protein it is however important to have suitable techniques available to obtain structural information on the oligosaccharides attached to the protein. For many years the complexity of the structures required sophisticated analytical techniques only available to a few specialist laboratories. In many cases these techniques were not available or required a large amount of material and therefore the number of glycoproteins which were fully characterised were relatively few. In recent years there have been substantial developments in the analysis of glycosylation which has significantly changed the capability to fully characterise molecules of biological interest. A number of different techniques are available which differ in terms of their complexity, the amount of information which is available from them, the skill needed to perform them and their cost. It is now possible for many laboratories who do not specialise in glycosylation analysis to obtain some information although this may be incomplete. These developments do, however, also make complete characterisation of a glycoprotein a much less daunting task and in many cases this can be performed more easily and with less starting material than was previously required. In this review a summary will be given of current techniques and their suitability for different types of analysis will be considered.

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Abbreviations: 2-AA, 2-amino benzoic acid; 2-AB, 2-amino benzamide; AMAC, 2-aminoacridone; ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; ES-MS, electro-spray mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; HPAEC-PAD, high pH anion exchange chromatography pulsed amperometric detection; MALDI-TOF, matrix assisted laser desorbtion mass spectrometry-time of flight; NMR, nuclear magnetic resonance spectroscopy; PA, pyridylamino; PNGase, peptide *N*-glycosidase.

The classical approach to glycosylation analysis has required a high degree of skill and taken a considerable length of time, and until recently the complete analysis of glycosylation of a protein was a major research project in itself. Although this may still be the case where a protein or other type of glycoconjugate has unusual glycosylation or has not been described previously, in those cases where the structures or ones closely related to them have been described previously then the process of analysis can be much more rapid [1]. Another very important development has been an increase in the level of sensitivity of analysis [2]. This means that it is now feasible to perform analysis on many naturally occurring glycoproteins of biological interest but which are only available in low amounts of 1–5 μg. Where glycoproteins of interest are difficult to purify in addition to being present in low amounts the development of techniques which make possible the analysis of material directly from polyacrylamide gels are especially of significance [3].

It is useful to consider the analysis of glycosylation in various stages of increasing complexity of the analysis and the information derived. This is important as in addition to showing that a protein is glycosylated and giving an idea of the degree and type of glycosylation, the preliminary analyses may suggest the most appropriate techniques for further characterisation. The techniques current available include the following:

At the glycoprotein level

- SDS Gel electrophoresis 1 or 2 dimensional
  - Glycan detection
  - N-glycan removal (PNGase F or endo-glycosidases)
  - Lectin blotting, Western blotting with antibody
- Lectin binding assays – microtitre plate based
- Lectin chromatography
  - Glycoproteins
  - Free glycans
- Mass spectrometry of glycopeptides
- Capillary electrophoresis of glycoproteins (glycoform separation)

On released glycans

- Carbohydrate electrophoresis on polyacrylamide gels
- Capillary electrophoresis
- HPLC
  - Weak anion exchange
  - Normal phase (amide)
  - Reversed phase
  - Graphitised carbon
  - High pH anion exchange chromatography pulsed amperometric detection (HPAEC-PAD)
- Gas liquid chromatography
  - Monosaccharide compositional analysis
  - Linkage analysis
- Mass spectrometry
  - Matrix assisted laser desorbtion Mass spectrometry – time of flight (MALDI-TOF)
  - Electrospray mass spectrometry (ES-MS)
  - Fast atom bombardment mass spectrometry (FAB-MS)
- Nuclear magnetic resonance spectroscopy (NMR)

In many cases the techniques may provide complementary information and the choice of technique is dictated to such considerations as accessibility to the technology and the amount of time and money available. In other cases the amount or type of material restricts the choice of technique for a particular application. In general the simpler and less costly techniques such as gel electrophoresis or lectin blotting provide less information or are more restricted in the type of structures which can be analysed. The information on the pattern of glycosylation can however be useful in comparing samples where there is a

disease related change in glycosylation or to monitor glycosylation consistence in recombinant glycoproteins [4]. This can later be supplemented with more sophisticated techniques such as HPLC or mass spectrometry where more detailed knowledge of the structures is required. The different techniques will now be described.

INTACT GLYCOPROTEIN ANALYSIS

SDS/PAGE

A strategy for protein glycosylation analysis is illustrated diagrammatically in Fig 1. Initially information on the type and degree of glycosylation on the intact glycoproteins can often be obtained through use of SDS/PAGE. Protein glycosylation may be detected by the mild oxidation of adjacent hydroxyl groups present on the monosaccharides by periodate oxidation. The resulting aldehyde groups can be detected either chemically or at much higher sensitivity by reaction with a reagent such as a biotin hydrazide, which can then be detected with a streptavidin conjugate linked to a colourimetric detection system [5]. Such techniques are relatively simple to perform [6] and there are also commercial kits available. It should be noted that both false negative and false positive reactions can occur so suitable controls with a known glycoprotein and non-glycosylated protein should accompany any analysis of this type.

Use of enzymes

Further information regarding glycosylation may be obtained by using glycosidase enzymes in conjunction with SDS/PAGE. The asparagine aminohydrolase enzymes (peptide *N*-glycosidases) PNGase F and PNGase A cleave the linkage of *N*-acetylglucosamine to asparagine and thus will remove many N-linked glycans [7, 8]. Since PNGase A has more limited peptide specificity [9]. PNGase F is generally used for this purpose when studying glycoproteins. If the migration of a band changes following incubation of the protein with this enzyme the presence of N-linked glycosylation is indicated. It should be noted that the accessibility of glycan chains to this enzyme can vary and therefore the rate of removal may be very different between different proteins. It is advisable to analyse the incubation after different incubation times to check for complete removal. PNGase F is able to removal all N-linked glycans with the exception of those with an α-1-3-linked fucose on the reducing terminal *N*-acetylgalactosamine [10] whereas these may be cleaved from glycopeptides by PNGase A [9]. It is also possible to use endo-glycosidase enzymes such as endo-F but in this case the *N*-acetylglucosamine residue is left attached to the asparagine on the peptide [11]. The shift in relative molecular mass following glycan removal may give an indication of the contribution to the M_r of the protein but since glycosylated proteins may have abnormal mobilities on SDS/PAGE the value may not be reliable. A review on the use of enzymes for deglycosylation by O'Neill [8] gives further information and examples. Unfortunately there is no comparable enzyme for removal of O-linked glycans as the currently available enzymes such as that from *Streptococcus pneumoniae* only appear to cleave a restricted type of glycan of the type Gal β1,3-GalNAc with no sialic acid substitution [12] although some report a broader specificity [13].

Lectins

Additional information on the type of glycan may be obtained by use of appropriate lectins or antibodies directed against carbohydrate structures. These may be used on Western blots from SDS/PAGE gels [14] or in microplate systems when the protein has been immobilised onto the plate [15]. Detection by lectins does require careful optimisation and use of controls to ensure that the result is due

to a specific interaction of the lectin and structures identified in this way should be confirmed by use of other techniques.

Mass Spectrometry

Recent advances in instrumentation and techniques in mass spectrometry have made it possible, in some instances, to ionise intact glycopeptides or indeed some glycoproteins [16, 17]. This has been reviewed by Carr *et al.* [18] who give several examples and explains the principles of a variety of techniques in mass spectrometry. Electrospray MS (ES-MS) gives good spectra but microheterogeneity can complicate interpretation [19]. It may be possible to obtain information on the glycoforms present when the pattern is not too complex. Matrix Assisted Laser Desorbtion Ionisation with Time of Flight detection (MALDI-TOF) has also been used although the resolution is not as good as electrospray [20]. Further information can be obtained by use of endoglycosidase enzymes to distinguish glycosylated peptides [21]. Fragmentation of the glycopeptide can also be induced in ES-MS and this may allow detection and some characterisation on peptide glycosylation [22] providing the data can be interpreted. At the present time however the complexity and size of most glycoproteins prevents detailed characterisation of glycan structure in the intact molecule.

Capillary electrophoresis

Another technique which is capable of providing information about various glycoforms is that of capillary electrophoresis [23]. The glycoforms of such glycoproteins as ribonuclease B have been resolved and identified [24]. The proteins must be first purified to a high level and there may be problems with selective loss of some glycoforms during this process. This technique is however increasingly being used especially for monitoring recombinant glycoproteins [25] and has been combined with mass spectrometric detection [26] which enables much more information to be gained by the technique. For those looking for more detail on use of capillary electrophoresis in glycoprotein analysis this subject has been recently reviewed [27–29].

Release of glycans

At the present time most studies on the detailed nature of glycosylation and the sequence analysis of glycans is carried out following release of glycans from the glycoprotein. The release of N-glycans may be carried out either by use of enzymatic [8] or by chemical means [30]. In many cases comparable results may be obtained by use of either technique however it is important to optimise the reaction conditions used for enzymatic release. As discussed above the most commonly used enzyme is PNGase F and although there may be steric considerations which can lead to incomplete or selective release many have been successfully deglycosylated by use of this enzyme. In some cases it may be necessary to denature the protein in order to ensure efficient removal [31] but SDS should not be used in the buffer as this will inhibit PNGase F. Non-ionic detergents such as Triton may be used instead. Details of suitable buffer systems are given in [32]. Denaturation of the protein prior to use of the enzyme is often employed. Recently it has been demonstrated that it is possible to use this enzyme to release glycans from proteins in SDS/PAGE gels [3] and this enables glycosylation analysis to be carried out with purification of the protein and can be especially useful where proteins are difficult to purify or are only available in low amounts.

Hydrazinolysis has the advantage over other chemical techniques used for release, such as beta-elimination, of leaving the released glycan with a free reducing terminus which is available for derivatisation to enable high detection at high sensitivity (see below). Reli-

able procedures for release of N-glycans by hydrazinolyis are now well established [33] and the technique has also been automated [34]. The technique may also be used to release O-linked glycans which are more labile and therefore released under milder conditions [33]. Care must be taken however to prevent 'peeling' which can occur if water is present during the hydrazinolysis reaction and leads to degradation of the released glycans. As mentioned above the endo-*N*-acetylgalactosaminidase generally used has a very limited specificity for O-glycan structures [12] and thus at present there is no good alternative to chemical release.

Labelling

Most glycans are difficult to detect in chromatographic or electrophoretic techniques as they do not have chromogenic groups they generally require derivatisation prior to such analysis as detection by physical properties, e.g. refractive index is not sufficiently sensitive for analytical purposes. Although the labelling may be accomplished by introduction of radioactive [35] or chromogenic groups it is now generally carried out with fluorescently active groups which enable detection at high sensitivity (in low pmole range). There are a range of different fluorophores which have been described and those most commonly used are aminopyridine [36], 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) [37], 2-aminoacridone (AMAC) [38], 2-amino benzamide (2-AB) and 2-amino benzoic acid (2-AA) [39]. These are all introduced using a relatively simple and efficient reaction of reductive amination where the free aldehyde group at the reducing terminal C1 of the glycan is reacted with the amino group of the dye and subsequently converted to a stable conjugate by reduction. The use and properties of a large number of tags which may be introduced into the glycan has been reviewed by Honda [40] A number of considerations need to be taken into account when labelling the glycan such as stability of the glycan during labelling (e.g. loss of sialic acid) and minimisation of side reactions (such as epimerisation or β-elimination) and the efficiency and non-specificity of labelling. The changes the dye may make to properties such as the hydrophilicity or size of the glycan can also effect separation. The dyes 2-AB and the closely related 2-AA have been investigated for all these properties [39] and provide convenient means of derivatisation under simple reaction conditions. Derivatives with 2-AB are suited for many HPLC separations whilst 2-AA provides a negative charge for separation by electrophoresis.

Profiling – PAGE

After release it is possible to get a profile of the glycans that are present and here a number of alternative or complementary techniques can frequently be used. PAGE analysis is very convenient for comparative analysis and is compatible with further analysis [41, 42]. In order to introduce a charge for electrophoretic separation the glycans are first derivatised. ANTS labelling has been widely used for separations and provides high mobility and sensitive detection. However the high charge may prevent separation of some neutral glycans and 2-AA labelling has been used to separate and identify closely related bi-antennary structures as found in IgG. In order to separate glycans a higher concentration of acrylamide has to be used than for protein electrophoresis. The buffer counter-ions used also affect the separations and the best resolution of neutral glycans is achieved in a Tris/glycine buffer [43]. This technique is well suited for comparison of different samples as in protein glycosylation in normal or disease states or in routine screening for consistency of glycosylation as in production of recombinant glycoproteins. It also has the advantage that it is compatible with separation by normal phase HPLC on amide-bonded silica columns (see below).

Profiling – high pH anion exchange chromatography (HPAEC)

The technique of HPAEC in which free glycans are separated on the basis of either acidic charged groups or the ions of hydroxyl groups formed at high pH in the case of neutral glycans [44]. The technique is generally combined with pulsed amperometric detection (PAD) which has the advantage of not requiring derivatisation and is fairly sensitive [45]. It is commonly used in profiling but due to the use of high salt gradients it is not as compatible with further analysis as other HPLC techniques. The basis of the separation is complex and can be more difficult to interpret and prediction of the elution position of oligosaccharides and thus identification of peaks can be uncertain. This technique has been reviewed by Lee [46] and this reference contains further information and examples of separations.

Other HPLC separations

HPLC techniques for glycan analysis are now very sensitive and can be readily combined with further analysis by a number of techniques. In order to produce reliable gradients and good resolution however, good quality pumping systems with mixing at high pressure are required. A number of different column systems which rely on different properties of the glycan for separation are currently used and have been comprehensively reviewed in a recent issue of the *Journal of Chromatography* [47]. Where the glycans have been labelled with 2-AB they can be separated on an amide-silica column in normal phase chromatography as described by Guile *et al.* [48]. Glycans labelled with 2-amino anthranilic acid (2-AA) [49] or aminoacridone [50] may also be analysed on this column system. This gives separation based on hydrophilic interactions so the number of hydroxyl groups and therefore the size predominates but other features can contribute and closely related structures can be resolved. Standardisation with a ladder of glucose oligomers allows glucose unit values to be calculated for each peak which can be predictive of the type of glycan as the residue, linkage and position in the glycan can all contribute to the glucose unit value [51]. It is possible to combine data either on a different system such as reversed phase HPLC or by obtaining accurate values by mass spectrometry which can be helpful in characterising peaks. This may be performed by MALDI-TOF but it will require sample clean up. The major means of peak identification however relies on the use of specific exoglycosidases to progressively digest structures. From knowledge of likely structures based on their elution positions and from their behaviour with carefully chosen glycosidase digests a full sequence can in many cases be determined. [48]. Digestions with mixtures of exoglycosidases can be carried out on a pool of glycans [52] and the data interpreted to show most of the structures present, which considerably reduces the number of steps which have to be performed and also requires less glycoprotein.

An alternative system is to use pyridylamino (PA) derivatives have been characterised on a number of different types of HPLC column including anion exchange reverse phase on octadecylsilica and normal phase on amide-silica columns to give a multidimensional separation which can allow identification of a particular glycan as described by Takahashi [53].

Mass spectrometry techniques

Mass spectrometry is increasing being used in analysis of released glycans due to both the advances in performance and ease of use and reduction of cost of the instrumentation The technique still however requires optimised sample preparation techniques to remove contaminants such as salts which may affect ionisation of the glycans and a degree of skill is required to get reliable spectra. A recent review is available which gives complete details

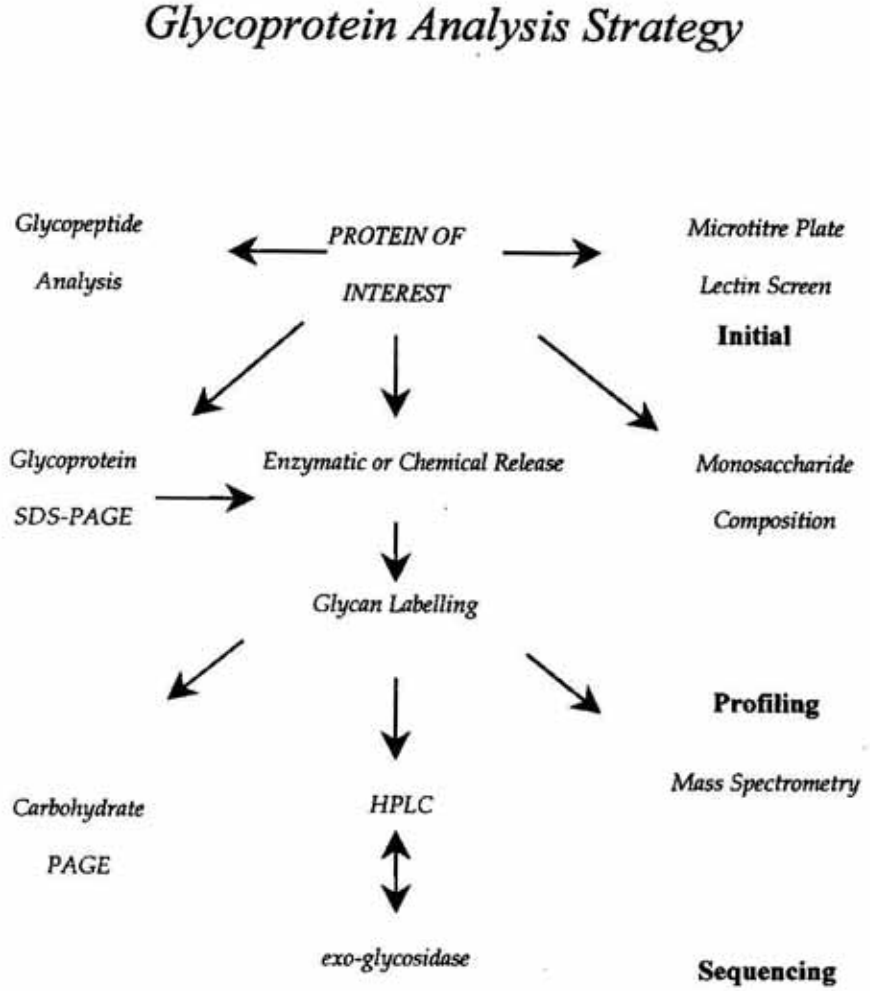

Figure 1. An outline of the general strategy for glycoprotein analysis.

The different techniques which can be used are shown. In any analysis it is likely that some but not all of these will be used to obtain structural data. The choice of technique will be dictated by their availability and the type and amount of sample.

of the various techniques and applications [54]. Currently the most widely used technique for analysis of released glycans is MALDI-TOF and there have been considerable improvements in the instrumentation in the past few years which have helped to make the technique easier to use and more reliable. There has also been development of matrices which are particularly suited to glycan analysis such as α-cyano-4-hydroxycinnaminic acid or 2,5-dihydroxybenzoic acid [55]. Following sample cleanup on micro-columns of ion exchange C-18 or proprietary columns, the sample is mixed with an excess of the matrix and recrystalised before analysis. Ionisation of the sample is accomplished by absorption of the laser energy by the matrix which is then detected as a positive ion usually in a time-of-flight detector. The masses of the molecular ion peaks are then measured and compared with calculated values for glycans composed of different monosaccharides. Although the technique gives only the mass and not the identity of the glycan by combining mass spectrometry with exoglycosidase digestions or by analysing fractions prepared by HPLC from such digestions structures may be confirmed. On line detection from microbore HPLC in liquid chromatography coupled with mass spectrometry (LC/MS) is also being developed [56]. For information on the linkage between the monosaccharides it is possible to

Table 1. Comparison of different techniques and typical applications

Technique	Requirements	Complexity	Typical applications
Glycoprotein level			
SDS/PAGE analysis glycan detection	Identifiable band on gel	Low	Initial determination of glycosylation
Enzymatic deglycosylation (endoglycosidase F/H or PNGase F or A)	Suitable enzyme and optimisation	Low	Degree and possibly information on type of glycosylation
Lectin analysis e.g. ConA, SNA, GNA	Standardised lectin	Low	Preliminary identification of type and amount of glycan present
Capillary electrophoresis	CE system and protocol	Medium	Glycoform analysis
Mass spectrometry	Instrumentation and expertise for interpretation	High	Mass and site attachment of glycan
Glycan level			
Enzymatic release	Optimised procedure	Low	N-glycan release
Hydrazinolysis	Reagents, safety considerations	Medium	N- and O-glycan release
Labelling e.g. by reductive amination	Glycan with reducing terminus	Low	Preparation for chromatographic or electrophoretic analysis
HPAEC-PAD	Chromatographic and detection system	Medium	Profiling glycans
SDS/PAGE	Gel system and labelled glycans	Low	Screening, profiling
HPLC	HPLC system fluorescent detection	Medium	Quantitative profiling, sequencing, glycan separation
Mass spectrometry	Instruments and expertise	High	Mass profiling, glycan identification
NMR	Instrument expertise sufficient sample	High	Full structural analysis conformational studies

permethylate free hydroxyl groups on the glycan, hydrolyse to give monosaccharides these are then acetylated to form partially methylated alditol acetates which can be separated by gas liquid chromatography coupled to mass spectrometry (GC/MS) as reviewed by Hellerqvist [57]. It is probably true to say that mass spectrometric techniques are still not at the stage of routine analysis but are now approaching this level.

Capillary electrophoresis

This technique is also used in analysis of released oligosaccharides. If reproducible separations can be achieved capillary electrophoresis is an attractive technique as it is capable of high resolution, is rapid, requires small amount of material and in some cases can be used without derivatisation. However it is not yet in widespread use and requires optimisation for particular types of analysis. In the technique of micellar electrokinetic capillary chromatography (MEKC) surfactants such as SDS are used which form micelles and this technique has been applied to the separation of glycans from recombinant tissue plasminogen activator [58]. It is however generally used with fluorescent labels such as 2-aminoacridone [59] used in capillary zone electrophoresis. Recent advances have been made in the electrophoresis of derivatised glycans [60]

and the technique can also be compatible with mass spectrometric analysis with specialised instrumentation. Developments are continuing in this area which may make the technique more widely used [61].

Nuclear magnetic resonance (NMR)

Finally NMR techniques are capable of assigning a structure unambiguously even for novel structures [62]. It is a non-destructive technique but does require relatively large amounts of sample (milligram range) and requires the instrumentation and expertise to interpret the data. Structural analysis can involve matching against regions of spectra from known glycans in fingerprinting or can be used in sequencing [63]. The monosaccharides, linkage positions and anomericity can all be identified by various techniques and for more complex structures multidimensional studies are now employed. It may be used to give conformational information on most oligosaccharide structures providing sufficient material is available for analysis.

Thus there are a number of techniques which are now used in analysis of protein glycosylation. The most appropriate technique for a particular study depends on a number of factors which are summarised in Table 1. The variety of options for analysis means that many laboratories can now apply some level of glycosylation analysis and thus more information which can relate this to the structure and function of glycoproteins involved in a number of different roles.

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