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Journal of Chromatography A, 705 (1995) 89–103

JOURNAL OF CHROMATOGRAPHY A

Review

Fluorophore-assisted carbohydrate electrophoresis Technology and applications

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Abstract

Carbohydrates, in particular the complex carbohydrates conjugated to proteins and lipids, have important functions in a variety of biological systems. Their isolation and structural determination–prerequisites for elucidation of their biological functions–have been technical challenges for many decades. Almost all available chromatographic and electrophoretic methods as well as NMR and MS have been applied to carbohydrate analysis but none has proved satisfactory in terms of simplicity, sensitivity, reproducibility, cost and requirement for materials. Recently, a technique called fluorophore-assisted carbohydrate electrophoresis was developed which is very promising. It separates fluorescently-labeled carbohydrates on polyacrylamide gels and uses a charge-coupled device camera to detect and quantitate the products. This review describes the principles of the method and its applications to several aspects of research on carbohydrate-containing biological biomolecules.

Contents

1. Introduction

Carbohydrates are ubiquitous in nature and found in all living species. They are central to energy generation and storage, on the one hand, and provide mechanical support for cells, on the other. Thus, the oxidation of carbohydrates, $nO_2 + (CH_2O)_n \rightarrow nCO_2 + nH_2O$, is the primary metabolic energy-generating process throughout the biosphere, and the cellulose of

0021-9673/95/$29.00
SSDI 0021-9673(94)001252-0

woody plants, the cell walls of bacteria, and the exoskeletons of insects and crustacea are all carbohydrates. Indeed, cellulose and chitin are the two most abundant biopolymers on earth. Complex carbohydrates have been shown to be bioactive and to participate in cellular and biochemical interactions in a wide variety of biological systems [1]. For example, the carbohydrate moieties of glycoproteins affect both their physicochemical and biological properties including folding and three-dimensional structure, solubility and stability, circulatory life time [2,3], susceptibility to proteases [4], biological activity and its modulation [5], molecular and cellular recognition [6], immunogenicity [7], cell adhesion [8], egg fertilization [9], lymphocyte homing [10] and bacterial and viral interactions with host tissues [11,12]. Furthermore, carbohydrates in recombinant proteins confer important activities on, as well as influence the stability and bioavailability of, genetically engineered glycoprotein drugs [13–17]. More than a dozen glycoproteins of therapeutic interest have been produced by recombinant DNA techniques [18]. Impressive examples are glycocerebrosidase for the treatment of Gaucher's disease [19,20], erythropoietin for the treatment of anaemia in hemodialysis patients [21] and tissue plasminogen activator as a thrombolytic agent [22]. More recently, the recruitment of leukocytes to injured tissue was found to occur via interaction of its cell surface carbohydrate Sialyl-Lewis [Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc] with endothelial cell selectins which are expressed in response to cytokines released during the inflammatory response [23–25]. This discovery has created intense interest in the development of sugar-based anti-inflammatory and anti-tumor drugs [26,27]. In addition, changes in protein glycosylation have been shown to be useful molecular markers in the diagnosis of several human diseases [28–34]. With the increased awareness of the biological significance of protein glycosylation, there has been a growing demand for rapid, convenient and cost-effective analytical methods to characterize both the carbohydrate content and chemical structure of the carbohydrate moieties of glycoproteins.

2. Current methodology

The complete characterization of a glycoprotein requires the analysis of the primary structure and conformation of both the carbohydrate side chains and the protein to which they are attached; and identification of the glycosylation site(s), the anomeric specificity of the linkage and the pattern of carbohydrate heterogeneity at each glycosylation site. The characterization of the protein part is relatively easy owing to the availability of well-established methods, techniques and equipment, whereas full elucidation of carbohydrate structures —historically tedious and laborious— continues to be a challenge. Numerous sugar chain variations can be formed from a small number of monosaccharide units. For example, two amino acids can form only two dipeptides, but two monosaccharides can form as many as 32 disaccharides since the linkage can occur at any of the four hydroxyl groups per monosaccharide, exist in either of two anomeric forms and involve either furanose or pyranose rings. The number of isomers increases geometrically as the number of constituents increases since branching becomes a possibility. In addition, because of the lack of template in their biosynthesis, sugar chains are microheterogeneous even at a single glycosylation site, and very often glycoproteins have more than one glycosylation site. In addition to single glucose and N-acetyl glucosamine moieties which are linked to Lys and Ser/Thr residues, respectively, complex carbohydrates can be attached to a protein in either of two linkages. In N-linked oligosaccharides, N-acetylglucosamine (GlcNAc) is the reducing terminal monosaccharide linked to the amide group of an asparagine side chain in an Asn–X–Ser(Thr) sequence. In O-linked oligosaccharides, N-acetylgalactosamine usually is the reducing terminal sugar linked to the hydroxyl group of a Ser or Thr residue in the polypeptide backbone. O-linked sugar chains are usually short and formed by the stepwise transfer of a monosaccharide to the Ser and Thr residue from its nucleotide derivative. In contrast, N-linked sugar chains are synthesized by a series of additions and removals that include lipid-linked

intermediates [35]. They are usually larger than O-linked sugar chains and can be divided into three groups: complex-type sugar chains, high mannose-type sugar chains and hybrid-type sugar chains [5]. All three types contain a common pentasaccharide core structure, Manα1-6(Man-α1-3)Manβ1-4GlcNAcβ1-4GlcNAc.

The first step in the structural characterization of a carbohydrate chain is the release of the oligosaccharides from the glycoprotein to generate an intact pool of oligosaccharides that are subsequently separated from one another. These are difficult tasks. Both enzymatic and chemical methods have been used to cleave oligosaccharides from glycoproteins. Two classes of enzymes have been identified that can release asparagine-linked oligosaccharide chains, and both are now commercially available. *endo*-β-N-Acetylglucosaminidases (EC 3.2.1.96) hydrolyse the glycosidic bond between two N-acetylglucosamine residues [36,37], thus leaving one GlcNAc residue on the protein. Peptide-N-(N-acetyl-β-glucosaminyl)-asparagine amidase (EC 3.2.2.18) hydrolyses GlcNAc–Asn linkages. The oligosaccharides cleaved by this enzyme are thus intact and possess a reducing terminal. It has proved to be the most efficient enzyme for releasing N-linked oligosaccharides from glycoproteins [38]. Enzymatic release of O-linked carbohydrate chains from glycoproteins by endo-α-N-acetylgalactosaminidase (EC 3.2.1.97) is not used widely since its substrate specificity is quite strict [39]. It only recognizes the disaccharide Galβ1-3GalNAc.

Chemical release of oligosaccharides has been applied successfully to both N-linked and O-linked sugars [40]. For the latter, β-elimination in the presence of an alkaline solution of $NaBH_4$ is often used [41]. Hydrazine and trifluoromethanesulfonic acid (TFMS) can be used to cleave both N-linked and O-linked sugar chains [42,43]. Hydrazinolysis has increasingly received attention since it can discriminate between N-linked and O-linked sugars [44]. It also has the advantage that it generates oligosaccharides with reducing termini which allow subsequent labelling and derivatization for easy downstream separation and detection. Hydrazine unavoidably degrades the protein part of the glycoprotein. If the protein part must be kept intact, enzymatic cleavage or hydrolysis with TFMS should be used.

Separation of heterogenous glycoforms from one another to yield homogenous oligosaccharides is yet another very difficult step in carbohydrate analysis, since the monosaccharide constituents of complex carbohydrates usually have very similar chemical structures. Often the compositions of different oligosaccharides differ by only one or two monosaccharides, and in some cases the oligosaccharides have the same composition but differ only in one or two anomeric linkages. Chromatographic techniques such as GLC, TLC, low-pressure LC and HPLC have all been used for carbohydrate separation and purification [44–55]. Methods for separation have been developed based on both size and charge. Anionic oligosaccharides containing sialic acid, uronic acid, phosphate or sulfate groups can be separated by ion-exchange chromatography. For uncharged oligosaccharides, gel permeation chromatography on Bio-Gel P-4 usually is a good choice. Neutral sugars can also be separated by ion-exchange chromatography in borate buffer if they have *cis*-hydroxyl groups which allow formation of a charged borate complex. In addition, affinity chromatography on immobilized lectin columns is used widely to separate oligosaccharides and differentiate different sugar structures. Since the specificity of this method is very high, it not only allows the separation of structurally similar oligosaccharides, but also provides information about their terminal structures [56]. Recently, NMR and MS have gained prominence and are now the most important methods for detailed elucidation of carbohydrate structure [57–59]. By a combination of the currently available techniques, several thousand complex carbohydrates have now been characterized and reported. However, these methods are still not ideal: they are time-consuming, involve expensive equipment and require expert interpretation of the data. Also, a relatively large amount of starting material is required which sometimes is very difficult to obtain from biological samples. Clearly, the

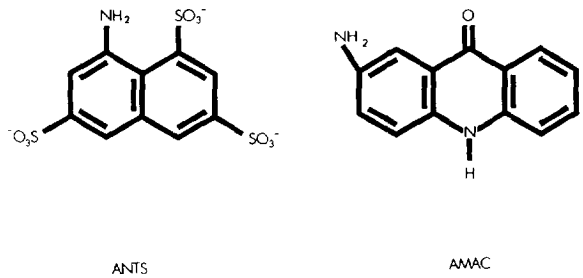
existence (or introduction) of a rapid, simple, sensitive, cost-effective method would fill a critical niche in carbohydrate analysis.

For this reason, a method based on electrophoretic separation of carbohydrates fluorescently-labeled at the aldehydic reducing termini shows promise for the structural characterization of oligosaccharides, and may be particularly useful for multiple sample analysis and comparison [60–67]. The method has been termed fluorophore-assisted carbohydrate electrophoresis (FACE) or the less euphonic polyacrylamide gel electrophoresis of fluorophore-labeled saccharides (PAGEFS). This review describes the principles of FACE and its application to several aspects of carbohydrate analysis.

3. Fluorophore-assisted carbohydrate electrophoresis

FACE technology was first described by Jackson in 1990 [63]. It combines the high resolution and simplicity of polyacrylamide gel electrophoresis (PAGE) with the sensitivity and visibility of fluorescence. In principle, carbohydrates that have a reducing terminus (aldehydic carbon) are reacted with a fluorophore that has a primary amino group. The resultant Schiff base is stabilized by reductive amination with sodium cyanoborohydride to yield the final stable fluorescently labeled derivatives which are separated by PAGE under appropriate conditions and followed by detection and quantification of the fluorescence of the bands under UV illumination.

The fluorophores that have been used most often are 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [62–65] and aminoacridone (AMAC) [60,61] whose chemical structures are shown in Fig. 1. ANTS has three sulfonic acid groups, an excitation maximum at 365 nm and an emission that peaks at 515 nm. ANTS labeling not only confers fluorescence on the carbohydrate but also adds three negative charges that enable the previously neutral molecule to migrate in an electric field. Both acidic and neutral



ANTS AMAC

Fig. 1. Chemical structures of ANTS and AMAC.

saccharides can be labeled with ANTS. Separation of ANTS-labeled carbohydrate derivatives is based both on charge and size. The mobility of neutral saccharide derivatives depends mostly on their size as all possess the same charge contributed by ANTS. However, the conformation of the saccharides and the configuration of their hydroxyl groups may also contribute to their migration, presumably by perturbing their effective size and interaction with the gel matrix. Thus, glucose, galactose and mannose are resolved readily, as are maltose (Glcα1-4Glc), isomaltose (Glcα1-6Glc) and cellobiose (Glcβ1-4Glc) [63].

Several electrophoretic buffer systems have been tested for the separation of fluorescently labeled saccharides [61,63,64]. For ANTS-labeled saccharides, the Tris–glycine system of Laemmli [68], commonly used for sodium dodecyl sulfate (SDS)-PAGE, gives satisfactory results. SDS is not required for separation to occur and is therefore omitted throughout the procedure. The concentration of the gel can be varied and optimized for specific purposes. Generally, 30% acrylamide is adequate for most of the N-linked oligosaccharides. A gradient gel may be necessary to separate saccharides that have a wide range of size and charge. A ladder of glucose polymers ranging from glucose to maltooligosaccharides of approximately 30 glucose units have been well separated in a 20 to 40% gradient gel [63].

The other fluorophore, AMAC, has a 425 nm excitation wavelength and an emission maximum at 520 nm. It is uncharged at neutral and alkaline conditions and is thus useful for the analysis of

small acidic saccharides. AMAC derivatives of neutral saccharides do not migrate in the Tris–glycine buffer system. They can migrate into the gel and be separated in a borate-containing buffer owing to the formation of a charged borate complex with the *cis*-hydroxyl groups of the saccharides. Therefore, AMAC serves to differentiate neutral and acidic saccharides by electrophoresis in borate and non-borate buffer systems. Another feature of AMAC is that it undergoes a significant reaction with sialic acid by an unknown mechanism [61]. All commonly occurring monosaccharides (Glc, Gal, Man, Fuc, SA, GlcNAc and GalNAc) can be labeled with AMAC and separated subsequently from one another by PAGE in a borate buffer. AMAC is the reagent most often used for monosaccharide analysis.

The chemical reaction of ANTS with a reducing sugar is shown in Fig. 2. The derivatization of carbohydrates with either ANTS or AMAC should have 1:1 stoichiometry and complete modification has been obtained with up to 100 nmol glucose, lactose, maltopentaose [63] and Gal-6-SO_3^- under the conditions described in the legends to Figs. 3 and 4 for ANTS and AMAC labeling, respectively. The emission wavelength of the fluorophores for both ANTS and AMAC derivatives of saccharides produces yellow bands under UV illumination after appropriate filtration. A 10 pmol band is easily visualized. One simple way to view and record FACE gels is with a standard long wavelength UV light box and the type of arrangements commonly employed for photographing ethidium bromide-stained DNA gels. Greater sensitivity can be achieved by recording a digitized image of the FACE gel with a cooled, charge-coupled device (CCD) camera. With both ANTS and AMAC-labeled saccharides, as little as 0.1 pmol per band can be detected with the Glycoscan system of Millipore (Bedford, MA, USA) or the FACE imaging system of Glyko (Novato, CA, USA). Since the derivatization is stoichiometric and quantitative, the intrinsic fluorescence of the polyacrylamide gel and the buffers is low, and the linear range of the CCD camera is wide, quantitative carbohydrate analysis can be achieved easily by direct comparison of the fluorescence intensity of a given band with that of a known amount of internal standard.

4. Oligosaccharide profiling by FACE

In studies involving glycoproteins, it is often necessary to compare the degree of glycosylation of two or more glycoproteins. For example, the glycosylation of a recombinant glycoprotein must be compared to that of its native counterpart. This comparison is usually performed either "indirectly" by monosaccharide composition analysis or "directly" by oligosaccharide profile analysis. Experimentally, composition analysis is easier, and for this reason has commonly been preferred. However, like peptide mapping and fingerprint analysis of a protein, profiling analysis of the oligosaccharides released from glycoproteins is more informative and gives the properties of the oligosaccharides such as charge, size and linkage.

N-Linked oligosaccharide analysis by FACE starts with enzymatic release of glycans from the glycoproteins. Peptide N-glycosidase F (PNGF) has proved to be an ideal glycosidase for N-linked glycan release. It is, in fact, an amidase

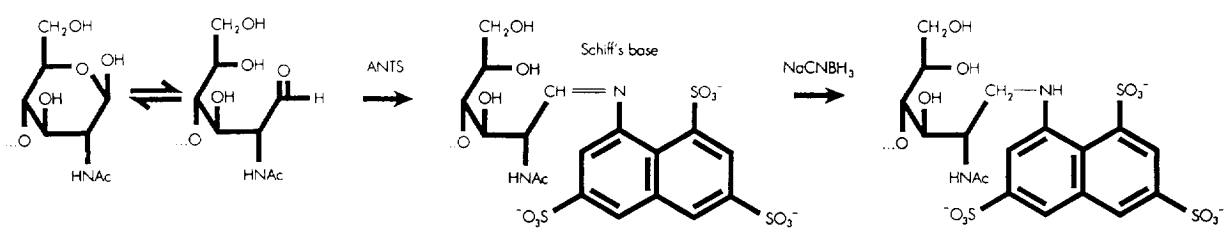


Fig. 2. Derivatization of a reducing sugar by ANTS.

rather than a glycosidase since it cleaves the GlcNAc–Asn linkage. All three types of N-linked glycans can be released by PNGF under appropriate conditions. The liberated oligosaccharides have a reducing terminus which is ideal for subsequent derivatization with ANTS. The amount of glycoprotein required to obtain a clear and informative profile of N-linked oligosaccharide varies with both the carbohydrate content of the glycoprotein and the microheterogeneity of the sugar chains. In most cases, 50 μg of glycoprotein are adequate. Conditions for the release of N-linked oligosaccharides by PNGF and the subsequent derivatization of the glycans with ANTS from various amounts of glycoproteins have been defined [65]. The N-linked oligosaccharide profiles of six glycoproteins are shown in Fig. 3. Most of the N-linked saccharides migrate somewhere between G_5 and G_{12}.

N-Linked oligosaccharide profiling is very useful for comparison of the glycosylation patterns of glycoproteins. Multiple samples can be analyzed side-by-side on the same gel and, hence, the method is ideal for examining of the oligosaccharide chains of a glycoprotein isolated from different species or at different stages during development or cellular differentiation. It can also be used to identify changes in glycosylation that might accompany certain physiological or pathological conditions. For recombinant glycoproteins, FACE oligosaccharide profiling promises to be a simple, convenient means for quality control to monitor batch-to-batch consistency. This is particularly important when the carbohydrate moiety participates in the biological function of the glycoprotein.

Profiling of O-linked oligosaccharides of glycorpoteins is essentially the same as for N-linked oligosaccharides except for two changes. Instead of PNGF, hydrazinolysis is used to release the oligosaccharide from the proteins, and a higher percentage of acrylamide gel must be used because O-linked oligosaccharides are usually small and contain neuraminic acid. As an example, the O-linked oligosaccharides obtained from the glycoprotein fetuin are shown in Fig. 4.

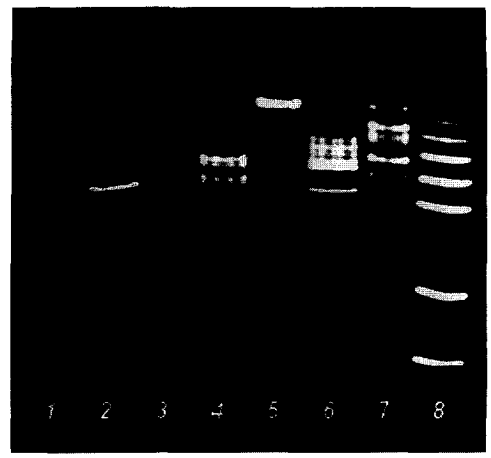


Fig. 3. N-Linked oligosaccharide profiles of glycoproteins. A 50-μl aliquot of a 1 mg/ml solution of a glycoprotein in water was combined with 50 μl of 40 m*M* sodium phosphate buffer, pH 8.6. SDS and β-mercaptoethanol were added to final concentrations of 0.1% and 50 m*M*, respectively, and the mixture was boiled for 5 min to denature the protein. It was cooled on ice and Nondient P40 (NP-40) was added to a final concentration of 0.5%. (If necessary, 0.2% SDS can be used to denature the protein, but the concentration of NP-40 must then be increased to give a final concentration that is five times higher than SDS so that PNGF will be fully active.) PNGF, 5 mU, was added to cleave the oligosaccharides from the glycoprotein and the mixture was incubated at 37°C. After 2 h, 3 volumes of ice-cold ethanol were added to precipitate the protein. The supernatant, containing released oligosaccharides, was dried in a centrifugal vacuum evaporator at a temperature ≤45°C to avoid oligosaccharide degradation. The residue was resuspended in 5 μl of 0.15 *M* ANTS in 15% (v/v) acetic acid. Then 5 μl of 1.0 *M* sodium cyanoborohydride in dimethyl sulfoxide (DMSO) were added, and the suspension was mixed, briefly centrifuged to bring the reactants to the tip of the tube, and incubated at 45°C for 3 h. After drying in a centrifugal vacuum evaporator the fluorescent-labeled oligosaccharides were redissolved in 20 μl of 20% glycerol and 4 μl of the sample was used for electrophoresis. Each lane corresponds to the oligosaccharides released from 10 μg glycoprotein. A 30% acrylamide gel was used with the Laemmli [68] buffer system but with SDS omitted. Electrophoresis was carried out at 5°C, 15 mA with an upper limit setting of 800 V and 60 W. A fluorescent image of the gel was recorded with a CCD camera under UV illumination. Lanes: 1 = control, no glycoprotein; 2 = transferrin; 3 = carboxypeptidase Y; 4 = fetuin; 5 = asialofetuin; 6 = α_1-acid glycoprotein; 7 = α_1-antitrypsin; 8 = a standard ladder of glucose polymers in which the lowest band is G_2.

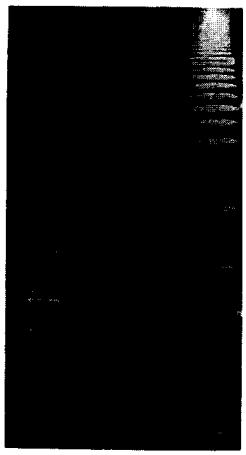


Fig. 4. O-Linked oligosaccharides of fetuin. A 50-μl volume of 1 mg/ml fetuin in a conical reaction tube was dried in a centrifugal vacuum evaporator and placed in a vacuum dessicator over P_2O_5 to remove all traces of water. The sample was dried overnight, 50 μl of anhydrous hydrazine were added and the tube was vortexed, overlayed with dry nitrogen and capped tightly. After 3 h incubation in a sand bath at 60°C, the sample was again dried in the centrifugal vacuum evaporator and resuspended in 30 μl of 0.2 *M* $NaHCO_3$, pH 10.7. Acetic anhydride, 3 μl, was added to re-N-acetylate the primary amino groups generated during hydrazinolysis. The sample was incubated on ice for 15 min, dried in the centrifugal vacuum evaporator and labeled with ANTS as described in Fig. 3. The labeled oligosaccharide mixture was electrophoresed in a 40% acrylamide gel and the products were visualized with the FACE system. Lanes: 1 = the migration pattern of some known oligosaccharides: maltoheptaose, maltohexaose, maltopentaose, maltotetraose, maltotriose, cellotriose, galactobiose, maltose, lactose, galactosylgalactose, N-acetylgalactosamine, galactose, glucose and 6-deoxyglucose, from top to bottom, respectively; 2, 3 = the O-linked oligosaccharides from 10 μg fetuin; 4 = a standard ladder of glucose polymers in which the lowest band is glucose itself.

The conditions for hydrazinolysis of O-linked oligosaccharides have been described by Patel et al. [44].

Both N-linked and O-linked oligosaccharide profiling can be carried out either analytically or preparatively. Individual oligosaccharide bands can be cut from a preparative gel, eluted into water and subjected to subsequent analysis for monosaccharide composition and oligosaccharide sequence.

5. Monosaccharide composition analysis by FACE

Most glycoproteins contain up to seven different kinds of common monosaccharides: mannose (Man), fucose (Fuc), galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and N-acetylneuraminic acid (Neu5Ac). Such monosaccharides can be divided into three groups: neutral sugars comprising Glc, Gal, Man and Fuc; amino sugars including GlcNAc and GalNAc; and acidic sugars containing Neu5Ac. Amino sugars and neutral sugars react with both ANTS and AMAC. Neu5Ac lacks an aldehydic reducing group and thus does not react with ANTS. It can, however, form a fluorescent derivative with AMAC by an as yet unknown mechanism. Monosaccharide composition analysis is therefore usually carried out with AMAC. As shown in Fig. 5, AMAC derivatives of the seven monosaccharides can be separated by PAGE in a buffer that contains borate. Monosaccharide composition analysis can be done with both an intact glycoprotein and purified oligosaccharides such as those eluted from a preparative oligosaccharide profiling gel. Since the thermal and acidic stabilities among the three groups of monosaccharides differ, three different hydrolysis conditions have been developed to achieve optimal recovery of neutral, amino and acidic sugars, respectively. Table 1 lists conditions for hydrolysis of the three types of sugars from both intact glycoproteins and isolated oligosaccharides. For neutral sugars in an intact glycoprotein, more than 90% of the sugars can be recovered by hydrolysis in 2 *M* TFA at 100°C for 5 h. For amino sugars, 4 *M* HCl at 100°C for 3 h is usually adequate for the purpose. After hydrolysis, re-N-acetylation of the amino sugars must be performed to reverse whatever deacetylation has occurred during this process. This can be accomplished by incubating the samples on ice with 1 *M* acetic anhydride in 0.2 *M* $NaHCO_3$, pH 10.7, for 15 min. For neutral and amino sugars from a purified oligosaccharide, the hydrolysis time can be reduced to 1 h

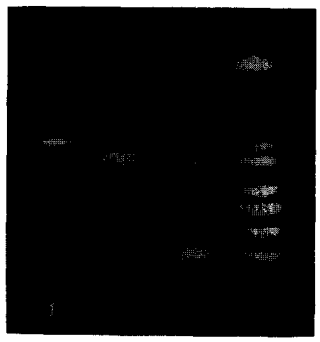


Fig. 5. Monosaccharide composition analysis of human transferrin. Three tubes containing 50 μg human transferrin each were dried in a centrifugal vacuum evaporator. A 100-μl volume of 0.1 *M* trifluoroacetic acid (TFA) was added to tube 1 and it was incubated at 80°C for 30 min to hydrolyze neuraminic acid. A 100-μl volume of 2 *M* TFA was added to tube 2 and it was incubated at 100°C for 5 h to hydrolyze neutral sugars. 100 μl of 4 *M* HCl was added to tube 3 and it was incubated at 100°C for 3 h to hydrolyze amine sugars. After incubation, the samples were again dried in a centrifugal vacuum evaporator. Tube 3 was subjected to re-N-acetylation as described in the legend to Fig. 4. For AMAC labeling, the samples were resuspended in 2.5 μl of 15% (v/v) acetic acid, mixed with 2.5 μl of 0.2 *M* AMAC in DMSO, followed by 5 μl of sodium cyanoborohydride, mixed and incubated at 45°C for 3 h. AMAC-labeled monosaccharides were dried, redissolved in 5 μl of DMSO, and diluted with 20% glycerol to a final volume of 20 μl. A 4-μl aliquot of this solution was then applied to a monosaccharide composition gel. Concentrations of acrylamide in the separating and stacking gels are 20 and 4%, respectively, and the electrophoresis buffer is 0.1 *M* Tris base–borate/boric acid, pH 8.3. Electrophoresis was carried out at 25 mA per gel for 2.5 h. Lanes: 1 = neuraminic acid; 2 = neutral sugars; 3 = amine sugars; 4 = monosaccharide standards: GalNAc, Neu5Ac, Man, Fuc, Gal and GlcNAc, from top to bottom, respectively.

and 30 min, respectively. Neu5Ac, which is very labile, requires special attention. Appropriate conditions for Neu5Ac analysis from glycoproteins and purified oligosaccharides are 0.1 *M* TFA or 0.1 *M* HCl at 80°C for 30min, respectively. Moreover, the labeling efficiency of Neu5Ac by AMAC is much lower than that of neutral and amino sugars, presumably because of a different reaction mechanism. Therefore, a Neu5Ac labeling control should be used every time. N-Acetyllactosamine is here recommended as a monocomposition control to monitor the efficiency of the hydrolysis, re-N-acetylation and fluorophore labeling reactions.

It should be noted that determination of monosaccharide composition by FACE has been thought not to compare favorably with other methods available for monosaccharide analysis. Thus, ion-exchange chromatography at high pH with electrochemical detection [69] or reversed-phase chromatography of precolumn derivatized sugars [70,71] have been shown to provide an accurate determination of monosaccharide composition. However, when combined with other applications of FACE such as oligosaccharide profiling and sequencing, monosaccharide analysis by the same method becomes a convenient extension of this technology.

6. Oligosaccharide sequencing by FACE

Oligosaccharide sequence analysis is the most difficult step in the complete structural determination of a glycoprotein. Unlike protein and DNA sequencing, it requires identification of (i) the particular carbon atom involved in each linkage, (ii) the anomeric specificity of the linkage and (iii) the identity and order of each monomer. Several physical, chemical and enzymatic methods have been developed and refined for oligosaccharide sequencing [72–75].

Table 1
Conditions for hydrolysis of intact glycoproteins and isolated oligosaccharides

Analysis	Intact glycoproteins	Isolated oligosaccharides
Neuraminic acids	0.1 *M* TFA, 80°C, 30 min	0.1 *M* HCl, 80°C, 30 min
Neutral sugars	2 *M* TFA, 100°C, 5 h	2 *M* TFA, 100°C, 1 h
Amino sugars	4 *M* HCl, 100°C, 3 h	4 *M* HCl, 100°C, 30 min

Sequential digestion with exoglycosidases of known specificities is the one most commonly used for this purpose [75].

Oligosaccharide sequencing is a third major potential application of FACE technology. Oligosaccharide profiling and monosaccharide composition analyses are prerequisites for sequence determination. Even more importantly, the oligosaccharide must be available in a homogeneous form. This can be achieved by oligosaccharide preparative electrophoresis. The oligosaccharide isolated in this way would have been fluorescently labeled already with ANTS which remains at the reducing end throughout the procedure. The digestion products can thus be analyzed for a shift in mobility without additional treatment. Further, the composition analysis of the isolated oligosaccharide provides preliminary information about the oligosaccharide such as content of neuraminic acid and mannose. This information is very useful in determining the appropriate exoglycosidases to apply. Moreover, the selectivity of the releasing enzymes is also helpful in oligosaccharide sequencing. PNGF releases all three types of N-linked oligosaccharides from glycoproteins, whereas endoglycosidase H only releases high-mannose and hybrid oligosaccharides. Therefore, if an oligosaccharide isolated from a PNGF-released profile does not appear on the endoglycosidase H-released profile, it must be a complex-type oligosaccharide. Based on the Neu5Ac content from monosaccharide composition analysis, it is easy to decide whether neuraminidase or β-galactosidase should be used to cleave the outermost monosaccharide. Subsequent to the first exoglycosidase digestion, an aliquot of the product mixture can be taken for gel analysis to determine the mobilities of the remaining oligosaccharides. The next exoglycosidase digestion can be carried out with either the digestion mixture or the gel-purified product from the previous digestion. Repetitive treatment of the same digestion mixture with different exoglycosidases will generate the final core structure, Manβ1-4GlcNAcβ1-4GlcNAc or Manβ1-4GlcNAcβ1-4(Fucα1-6) GlcNAc. Hence, the oligosaccharide sequence can be deduced from knowledge of the general structural features of N-linked oligosaccharides, the degradation patterns resulting from digestion with exoglycosidases of known specificity, and the pre-determined migration patterns of known sequence. Table 2 lists the mobility of ANTS-labeled N-linked oligosaccharide standards as defined by Glyko. The migration values are expressed as a degree of polymerization, DP, which describes the mobility of an oligosaccharide relative to a mixture of glucose polymers. For example, a band that migrates in a position corresponding to maltotetraose (G_4) has a DP of 4, and to maltopentaose (G_5) a DP of 5. An oligosaccharide that migrates halfway between G_4 and G_5 has a DP of 4.5. Table 3 shows the shift in mobility of an ANTS-labeled oligosaccharide that would occur on removal of a single monosaccharide. These values can serve to correlate mobility shifts measured in the sequencing gel with the number of monosaccharides released sequentially from an oligosaccharide. It should be kept in mind that photobleaching of a fluorophore-labeled oligosaccharide will inevitably occur during UV-assisted excision from a preparative gel, and this precludes accurate quantitation of isolated oligosaccharides. Quantitation of exoglycosidase-released monosaccharides by monosaccharide composition gel analysis is likely to be less than satisfactory since both the exoglycosidase and the buffer may interfere with the efficiency of derivatization.

The position and anomeric character of a glycosidic linkage can be determined by the specificity of the exoglycosidase employed. Three neuraminidases with distinct specificities are commercially available. Neuraminidase I releases only α2-3 linked neuraminic acid, neuraminidase II cleaves both α2-3 and α2-6 linkages, whereas neuraminidase III is non-specific and removes all neuraminic acids (α2-3,-6,-8 linkages) from glycoproteins. This allows the linkage of a neuraminic acid in a glycoprotein to be determined readily by appropriate use of these enzymes.

Recently, a new strategy for sequencing oligosaccharides has been developed [76]. This so-called reagent-array method of analysis involves

Table 2
Mobility of ANTS-labeled N-linked oligosaccharides

Oligosaccharides	DP
Oligomannose 9	8.8
Oligomannose 8	8.1
Oligomannose 7	7.4
Oligomannose 6	6.5
Oligomannose 5	5.7
Disialylated (2α2-6), galactosylated bi-antennary	5.4
Disialylated (2α2-6), galactosylated bi-antennary, core-fucosylated	5.7
Disialylated (2α2-3), galactosylated bi-antennary	6.4
Disialylated (2α2-3), galactosylated bi-antennary, core-fucosylated	6.7
Trisialylated (2α2-6, 1α2-3), galactosylated bi-antennary	6.2
Trisialylated (1α2-6, 2α2-3), galactosylated bi-antennary	6.7
Asialo-, galactosylated bi-antennary	7.8
Asialo-, galactosylated bi-antennary, core-fucosylated	8.5
Asialo-, galactosylated tri-antennary	9.6
Asialo-, galactosylated tri-antennary, core-fucosylated	10.5
Asialo-, galactosylated tetra-antennary	11.5
Asialo-, galactosylated tetra-antennary, core-fucosylated	12.1
Asialo-, degalacto, bi-antennary	5.6
Asialo-, degalacto, bi-antennary, core-fucosylated	6.2
Asialo-, degalacto, tri-antennary	6.4
Asialo-, degalacto, tri-antennary, core-fucosylated	7.1
Asialo-, degalacto, tetra-antennary	7.3
Asialo-, degalacto, tetra-antennary, core-fucosylated	7.9
Conserved tri-mannosyl core	4.2
Conserved tri-mannosyl core, core-fucosylated	4.8
Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc	3.3
Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc	4.0
Manβ1-4GlcNAcβ1-4GlcNAc	2.5
Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc	3.2

incubating each of nine aliquots of an oligosaccharide to be sequenced with a set of defined, preprepared mixtures of exoglycosidases, i.e. the reagent array. Each sample glycan will be digested from the non-reducing end until only a limit glycan fragment remains. Then, the products from each of the nine digestion mixtures are combined and subjected to gel permeation chromatography on Bio-Gel P-4. This generates a signature of the hydrodynamic volume/relative molar ratio of the stop-point fragments. By comparing this signature with the theoretical signatures of oligosaccharides with known sequence, the structure of the glycan can be deduced by a best-fit computer program. The reagent-array method has been modified by Oxford Glycosystems and a carbohydrate sequencer (RAAM 1000 GlycoSequencer; Oxford Glycosystems, Abingdon, UK) based on this technology has been developed and is now commercially available.

Table 3
Monosaccharide contribution to oligosaccharide DP[a]

Monosaccharide	Mobility shift, DP units
Neu5Ac	-1
Gal	+1
GlcNAc	+0.75
Man	+0.75
Fuc	+0.6

[a] Defined by Glyko, Novato, CA, USA.

FACE oligosaccharide sequencing uses the

same principle [75]. In this case, the digestion products need not be combined, but can be analyzed side-by-side in different lanes of the same gel. Fig. 6 shows an example of this method applied to an oligosaccharide isolated from human serum glycoproteins. Typically a FACE sequencing experiment would involve setting up five separate enzyme digests. About 50–100 pmol of ANTS-labeled oligosaccharide would be used in each of the five tubes. The first tube does not receive any enzyme and is used as a size marker for the location of the starting material on the gel. The second sample is digested with neuraminidase III, which releases all the neuraminic acids. The third tube contains neuraminidase III and β-galactosidase which will release both neuraminic acids and galactose. The fourth tube contains neuraminidase III, β-galactosidase and β-N-acetylhexosaminidase which will release neuraminic acids, galactose and N-acetylglucosamine. The fifth tube contains all of the above enzymes plus α-mannosidase which will degrade the residual oligosaccharide to its core structure.

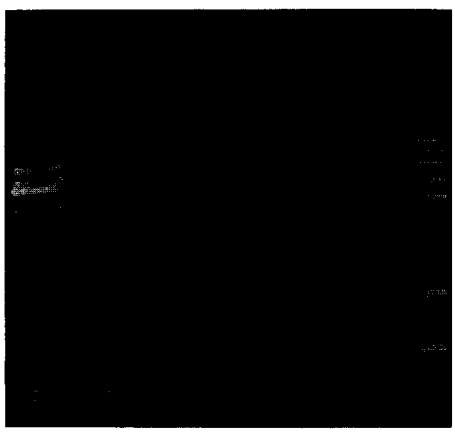


Fig. 6. Sequence analysis of an oligosaccharide from human serum glycoproteins. N-Linked oligosaccharides were released from 50 μl of normal human serum and labeled with ANTS under the conditions described in the legend to Fig. 3. After preparative N-linked oligosaccharide gel electrophoresis the oligosaccharide with a DP value of 5.3 was isolated by aqueous extraction of the gel slice. Lane 1 shows the migration pattern of all the ANTS-labeled oligosaccharides released from serum glycoproteins. The isolated DP 5.3 oligosaccharide was divided into five equal fractions, each of which was incubated with the indicated combinations of glycosidases in 50 m*M* sodium phosphate buffer, pH 6.0, at 37°C overnight. The glycosidase digestion products were subjected to N-linked oligosaccharide profiling gel electrophoresis for mobility shift analysis. Other lanes: 2 = oligosaccharide DP 5.3; 3 = neuraminidase (4 mU) digestion product; 4 = neuraminidase (4 mU) and β-galactosidase (10 mU) digestion product; 5 = neuraminidase (4 mU) + β-galactosidase (10 mU) + N-acetyl β-glucosaminidase (80 mU) digestion product; 6 = neuraminaidase (4 mU) + β-galactosidase (10 mU) + N-acetyl β-glucosaminidase (80 mU) + α-mannosidase (20 mU) digestion product; 7 = a standard ladder of glucose polymers in which the lowest band is G_2. A comparison of the mobility change of each glycosidase digestion and the DP value of each digestion product with the standards in Tables 2 and 3 identifies the sequence of oligosaccharide DP 5.3 as Neu5Acα2 - 6Galβ1 - 4GlcNAcβ1 - 2Manα1 - 6(Neu5Acα2 - 6Galβ1 - 4GlcNAcβ1 - 2Manα1 - 3)Manβ1 - 4GlcNAcβ1 - 4GlcNAc.

Sequencing of complex branched oligosaccharides by FACE is more difficult and is currently under development.

7. Interactions of proteins and carbohydrates

Binding of carbohydrates to proteins is vital for the function of many important biological systems. Lectins and selectins are two well-known examples of carbohydrate binding proteins. The former play critical roles in initiating viral, bacterial, mycoplasmal and parasite infections; cell–cell adhesion; fertilization and development; proliferation and differentiation; and metastasis [77–82]. The latter have been recently identified as endothelial-cell-surface carbohydrate binding proteins that function in the recruitment of leukocytes to injured tissues [23–25].

Historically, carbohydrate–protein interactions have been investigated by a variety of techniques including quantitative precipitation, hapten inhibition, equilibrium dialysis and affinity chromatography on immobilized ligands. Although each of these techniques provides some unique answer, all either require large amounts of material or must use radioactive carbohydrate derivatives. Consequently, we have developed a gel retardation assay using FACE technology [83] and demonstrated its feasibility both for screening and identifying carbohydrate ligands of

proteins and for the determination of apparent dissociation constants of native and ANTS-labeled carbohydrate ligands. The ANTS-labeled carbohydrate or carbohydrate mixture is incubated with its binding protein(s), subjected to gel electrophoresis, visualized and quantified by the FACE system. A side-by-side comparison between protein-present and protein-absent samples easily identifies the carbohydrate ligand of the protein. Protein-bound carbohydrate remains at the top of the gel, whereas free carbohydrate migrates into the gel and is thereby separated. Fig. 7 illustrates a gel retardation assay of lectin–carbohydrate binding. Three ANTS-labeled oligosaccharides are separated from each other by gel electrophoresis. Each is seen to be retarded by its specific lectin. By this method, the

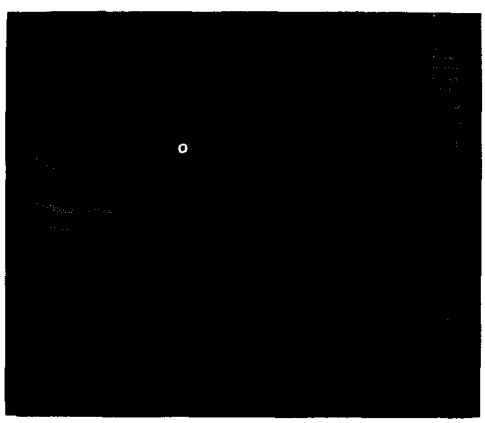


Fig. 7. Gel retardation assay of lectin-carbohydrate binding (from [83]). A mixture of three ANTS-labeled oligosaccharides, each 10 μM, was incubated with 20 μM *Sambucus nigra* agglutinin (SNA, lane 2), 20 μM *Galanthus nivalis* agglutinin (GNA, lane 3), 20 μM *Maackia amurensis* agglutinin (MAA, lane 4), 20 μM SNA + 20 μM GNA (lane 5), 20 μM SNA + 20 μM MAA (lane 6), and 20 μM GNA + 20 μM MAA (lane 7), respectively, in 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ at room temperature for 30 min at a final volume of 10 μl. Lane 1 is the oligosaccharides control incubated without any of the lectins. The three bands in lane 1 are, from top to bottom, Neu5Acα2 - 6Galβ1 - 4GlcNAcβ1 - 2Manα1 - 6(Neu5Acα2 - 6Galβ1 - 4GlcNAcβ - 2Manα1 - 3)Manβ1 - 4GlcNAcβ1 - 4GlcNAc, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc and Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc, respectively. Lane 8 is a standard ladder of glucose polymers in which the lowest band is G_2.

apparent dissociation constants for binding of the ANTS-labeled and unlabeled oligosaccharide Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3)-Manβ1-4GlcNAcβ1-4GlcNAc to the lectin *Sambucus nigra* agglutinin (SNA) have been determined to be 0.74 and 0.22 μM, respectively [83]. This is apparently the first example in which dissociation constants for labeled and unlabeled ligand have been measured separately. Usually, in order to measure the dissociation constant, it must be assumed that the binding of labeled and unlabeled ligand to a protein are the same. This is not always valid since labeling can frequently alter the structure of the ligand. This assumption is not required in the determination of binding affinities by gel retardation/FACE, constituting a unique advantage of this technique.

The gel retardation/FACE assay can also be used to study the interaction between proteins and glycosaminoglycans. The latter are negatively charged polysaccharides composed of repeating disaccharide units [84]. They play important biological roles in a variety of cell processes including cell-cell interactions, cell adhesion on the extracellular matrix, ligand and receptor binding, etc. [85]. All types of glycosaminoglycans can be fluorescently labeled by ANTS and appear as smeared bands on oligosaccharide profiling gels. Fig. 8 depicts how this method can detect the binding of ANTS-labeled N-acetylheparin to angiogenin, a potent inducer of neovascularization [86].

8. Potential applications of FACE in clinical diagnosis

It is known that changes in glycosylation of some glycoproteins are associated with various pathological conditions [31]. Thus, liver disease can be accompanied by reduced sialylation and increased glycan branching whereas cancer can be accompanied by increased fucosylation [87]. Table 4 lists some examples of carbohydrate diagnostic targets. Among them, FACE diagnosis may benefit the lysosomal storage diseases, a large group of genetically determined or ac-

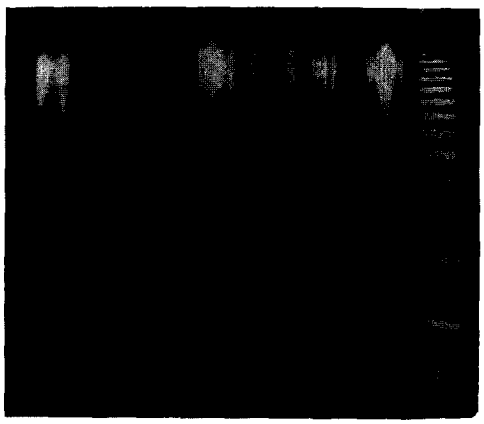


Fig. 8. Binding of proteins to glycosaminoglycans. N-Acetylheparin was labeled by ANTS under the conditions described in the legend to Fig. 3. ANTS-labeled N-acetylheparin, 500 pmol, was incubated with 1 nmol of protein in 10 μl PBS at room temperature, and after 30 min, the mixture was applied to an N-linked oligosaccharide profiling gel. Lane 1, control; lane 8, a standard ladder of glucose polymers in which the lowest band is G_2. The low fluroescence intensities clearly indicate that bovine and human angiogenins (lanes 2 and 3) bind to N-acetylheparin, whereas bovine angiogenin-like protein, ribonuclease A, lysozyme and actin (lanes 4–7) do not.

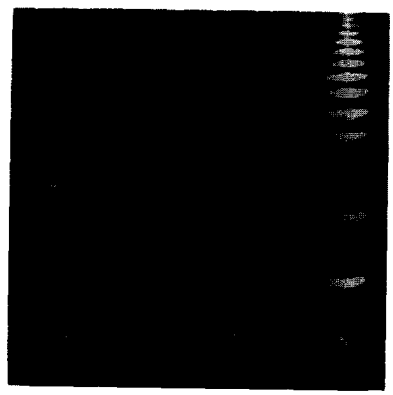


Fig. 9. FACE analysis of glycolipids. A 10-μg sample of monosialoganglioside(s) was incubated with 0.2 ml ceramide glycanase in 0.5 *M* NaAc, pH 5.0, and 75 μg/ml cholate in a final volume of 50 μl at room temperature overnight. After ethanol precipitation, the supernatant—which contains the glycans released from the glycolipid—was dried in a centrifugal vacuum evaporator and labeled with ANTS as described in Fig. 3. Lanes: 1–3 = the glycans released from monosialogangliosides GM_1, GM_2 and GM_3, respectively; 4 = a combination of the samples from lanes 1–3; 5 = the glycan of glycolipid in 5 μl normal human serum; 6 = a standard ladder of glucose polymers in which the lowest band is G_2.

quired glycolipid metabolic disorders [88,89], since glycolipids are less heterogeneous than glycoproteins and therefore easier to identify as diagnosis target(s). In addition, the carbohydrates or glycolipids that accumulate in the diseases are often present in urine so that sampling is convenient. For instance, patients with GM_1 gangliosidosis excrete GM_1 ganglioside in their urine. Fig. 9 shows that the glycans from

Table 4
Potential carbohydrate diagnostic targets

Pathological conditions	Potential carbohydrate markers
Bacterial infection	Bacterial polysaccharides
Cartilage tunor and sarcoma	Keratan and chondroitin sulfate
Choriocarcinoma	Chorionic gonadotropin glycosylation
Colorectal cancer	Glycosaminoglycans
Coronary heart disease	Apolipoprotein glycosylation
Hepatoblastoma	Sialic acids
Leprosy	Phenolic glycolipids
Lymphocytic leukemia	GM_1 glycolipids
Lysosomal storage diseases	Oligosaccharides, glycosaminoglycans, glycolopids
Melanoma	GD_3, GD_1 and GM_2 glycolipids
Mesothelioma	hyaluronic acid
Pancreas and urinary bladder tumor	Lewis antigens
Parasitic diseases	Protozoa-specific carbohydrates
Rheumatoid arthritis	Galactose, N-acetyl glucosamine in IgG

GM_1, GM_2 and GM_3 gangliosides can be separated and identified readily after ceramide glycanase digestion and ANTS labeling. Therefore, the abnormal appearance of GM_1 or GM_2 bands on a FACE gel analysis of urine or serum could be a marker of GM_1 gangliosidosis or Tay–Sach's disease, respectively. There is a large area of unexplored application of FACE in clinical diagnosis but the potential is clear.

9. Conclusions

FACE has been developed to fulfill the need for a rapid, simple and cost-effective method of carbohydrate analysis. The basic advantages of this technique are its simplicity and sensitivity. It separates carbohydrates by PAGE and thus utilizes the high resolving power of this method which is technically familiar and accessible for most laboratories. The carbohydrates are fluorescently labeled and their fluorescence is readily observed and recorded which allows detection and quantification at the subpicomolar level. Moreover, multiple samples can be analyzed in parallel and the results are visible and easy to interpret. A series of applications based on the use of FACE technology has been developed. Various kits now available include monosaccharide composition analysis, N-linked and O-linked oligosaccharide profile analysis, oligosaccharide sequence analysis, glycosaminoglycan analysis and several others. FACE is a significant addition to the panoply of techniques currently available for research in glycobiology. Its full potential, including applications in clinical diagnosis, has yet to be exploited.

Acknowledgements

I thank Drs. Jack T. Johansen of Millipore Corp. for providing the FACE system, Christopher M. Starr of Glyko, Inc. for providing reagents and supplies, Drs. James F. Riordan and Bert L. Vallee for valuable discussions and advice. This work was supported by the Endowment for Research in Human Biology, Inc.

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