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Review

Recent advances in the analysis of carbohydrates for biomedical use

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ABSTRACT

Glycans are widely distributed in biological systems in free state as well as conjugated forms as parts of glycoproteins, glycolipids, and proteoglycans. Because glycans are not synthesized directly by the corresponding genes but a combination of the related enzymes and substrates, the structures of glycans are quite diverse and sensitive with the changes of physiological conditions. Due to the extremely complex heterogeneities of glycans, it has been a big challenging target to analyze comprehensive glycan profiles (i.e. glycome) and determine characteristic glycan(s) for clinical use. Recent advances in separation sciences such as capillary/microchip electrophoresis and mass spectrometry have made it possible to analyze glycans for various practical uses. New emerging technologies of microarray and bioinformatics have also been applied to glycome/glycomics studies. In this review, recent topics on glycan analysis in clinical use are described with their historical background. Some results obtained by our studies are also shown.

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Contents

1. Introduction 703
2. Guide for selection of methods for glycan analysis 704
3. Releasing of glycans from glycoconjugates 704
4. Labeling of glycans for their highly sensitive detection 704
5. Analysis of glycans by MS 706
6. HPLC analysis 708
7. Capillary electrophoresis and microchip electrophoresis 713
8. Micro arrays for glycan analysis 717
9. Analysis of glycopeptides by MS after enrichment procedure 718
10. Bioinformatics tools and databases for glycans 718
11. Future perspectives 721
References 721

Abbreviations: 2AA, 2-aminobenzoic acid; 2AB, 2-aminobenzamide; ABEE, 4-aminobenzoic acid ethyl ester; AGC, AutoGlycoCutter; AMAC, 2-aminoacridone; APTS, 8-aminopyrene-1,3,6,-trisulfonic acid; BCSDB, Bacterial Carbohydrate Structure DataBase; CAE, capillary affinity electrophoresis; CCSD, Complex Carbohydrate Structure Database; CE:, capillary electrophoresis; CEC, capillary electrochromatography; CFG, consortium for functional glycomics; CGE, capillary gel electrophoresis; ESI, electrospray ionization; Fmoc-Cl, 9-fluorenylmethyl chloroformate; FT-ICR, Fourier transform ion cyclotron resonance; Fuc, L-fucose; GAG, glycosaminoglycan; Gal, D-galactose; Gal*N*Ac, *N*-acetyl-D-galactosamine; GlcA, D-glucuronic acid; Glc*N*Ac, *N*-acetyl-D-glucosamine; HILIC, hydrophilic interaction liquid chromatography; KEGG, Kyoto Encyclopedia of Genes and Genomes; LTQ, linear ion trap; MALDI, matrix-assisted laser desorption/ionization; Man, D-mannose; MS, mass spectrometry; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; NP, normal phase; PA, 2-aminopyridine; PGC, porous graphitized carbon; QIT, quadrupole ion-trap; RP, reversed phase; SNA, *Sambucus nigra* agglutinin; TOF, time-of-flight; Xyl, D-xylose.

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1. Introduction

Glycans in glycoconjugates such as glycoproteins, proteoglycans and glycolipids participate in various biological events such as cell recognition, cell–cell interaction, inflammation, and disease progression. Glycosylation is one of the important posttranslational modifications of proteins, and most of the glycans attached to glycoproteins are classified into *N*-glycans and *O*-glycans. Chitobiose (Glc*N*Acβ1-4Glc*N*Ac) residue at the reducing end of *N*-glycans is attached to Asn residue through the amide-linkage. *N*-glycans are classified into high-mannose, hybrid- and complex-type glycans, and are widely distributed in biological systems. *N*-Acetylgalactosamine (GalNAc) of the *O*-glycans is attached to Ser/Thr residues through the *O*-glycosidic linkage. This type of glycans is usually referred to "mucin-type" glycans due to their predominant occurrence on mucus glycoproteins, and has eight core structures from core 1 to core 8. Mammals including human synthesize core 1, 2, 3 and 4 structures [1]. Proteoglycans composed from a protein core substituted with glycosaminoglycans (GAG) are ubiquitously found in the extracellular matrix, and are also one of the major glycoconjugates. GAGs are linear polymers, and have repeating disaccharide units of hexosamine and hexuronic acid, and are generally sulfated except for hyaluronic acid (hyaluronan, HA), while keratan sulfate (KS) consists of poly-N-acetyllactosamine backbone. GAGs except for HA are covalently attached to serine residues of the core protein in the *O*-linked manner through the core tetrasaccharide structure of GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser [2,3]. Condroitin sulfate/dermatan sulfate (CS and DS respectively) are synthesized once Gal*N*Ac is transferred to the core tetrasaccharide, while heparin and heparan sulfate (HP and HS, respectively) are formed when Glc*N*Ac is initially added.

Biosynthesis of these glycans is regulated by a number of factors such as: (i) the expression of related glycosyltransferases or glycosidases (responsible for either maturation or catabolism process), (ii) proper locations (endoplasmic reticulum, Golgi, cytosol or lysosome) of these enzymes, and (iii) the functional machinery of sugar nucleotides. Thus, micro environmental changes greatly affect the synthetic efficiency of the glycans. For example, complex-type *N*-glycans are predominantly present when cells are tumorized [4]. It was also reported that cancer-associated *O*-glycans were highly sialylated but less sulfated [5–7] and were often truncated.

Tn (Gal*N*Ac-) and T (Galβ1-3Gal*N*Ac) antigens as well as their sialylated analogues (sialyl-Tn and sialyl-T) became predominant with progression of cancer [8]. Both Tn and T antigens have been exploited to develop cancer vaccines. Their increased occurrence was reported to be associated with advanced cancer, invasive and highly proliferative tumors, metastasis and a poor clinical outcome [9]. IgG species containing fucosylated or agalactosylated glycans became predominant in serum samples of patients suffered from rheumatoid arthritis [10]. Lindahl et al. determined KS in brain samples of the patients suffering from Alzheimer disease, and found decrease of KS having highly sulfated species [11]. Rossi et al. reported the relationship between excessive synthesis of low-sulfated chondroitin sulfate and chondrodystrophy [12]. Changes in GAG structures with tumorigenesis were also reported [13,14]. As described above, many important findings on glycans as useful clinical markers have been accumulated.

It has been challenging to analyze glycans in biological samples from the following reasons: (1) only trace amount of glycans are found, and we can not amplify them as nucleic acids; (2) large number of possible structural combinations should be considered due to varieties of monosaccharide species, linkage types, and anomeric configurations. Accordingly, automatic analysis of glycans is especially difficult to realize, and the methods generally used are still less convenient and cumbersome than those used for the analysis of nucleic acids or proteins. For detailed structural studies on glycans, NMR analysis is the most powerful technique when we can use large amount of glycan samples. However, only trace amount of glycans are available when we handle the glycan samples derived from clinical samples, and we must perform high-sensitive analysis in most cases. In addition, analysis of a large number of samples is especially required to establish clinical application after finding the possible bio-markers based on glycans. Therefore, rapidness and easy operation are also important for practical use.

Currently, glycans from clinical samples are analyzed using high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and newly developed techniques in mass spectrometry such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization mass spectrometry (ESI-MS), and lectin arrays [15–17]. The strategies for the analysis of glycans have their own strong/weak points, and appropriate methods should be selected for each glycan sample. In this review, characteristics of the current methods for the analysis of glycans, and their applications to

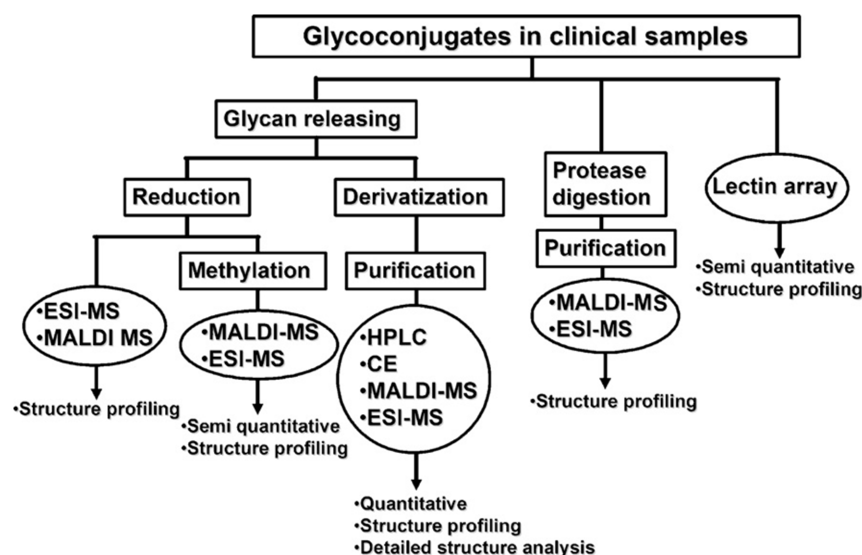


Fig. 1. Analysis of glycans from glycoconjugates.

biological/clinical samples are shown. Some discussions on bioinformatics for glycans are also shown.

2. Guide for selection of methods for glycan analysis

General scheme for the analysis of glycans derived from glycoprotein samples is shown in Fig. 1.

Because of the extremely complex structures and heterogeneity of glycans derived from glycoconjugates in clinical samples, glycans have to be analyzed after releasing from the core protein. The released glycans are often labeled with fluorescent tags [18] or fully methylated [19,20], and analyzed using HPLC, CE, MALDI-TOF MS, and ESI-MS [15,16]. These techniques are useful both for quantitative determination and structural analysis. MS/MS technique makes it possible to obtain mass profiling of glycans using the mixture of glycopeptides obtained from glycoproteins after digestion with proteases [21]. The approach has been utilized for confirmation of glycosylation sites [22,23]. However, it should be noticed that mass profiling of glycans using mass spectrometry is not appropriate for quantitative analysis, unless specific standard samples having deuterium labeled glycans are employed [24]. Recent advances of microarray technologies in glycome should be noticed. The prototype glycan arrays presented at the Consortium for Functional Glycomics (CFG) [25] and UK Glycoarray Consortiun [26] provided information on various glycan-binding proteins (GBPs) and basic concept on glycan array technologies. Kuno et al. developed a machine for high-throughput glycome using Evanescent-field fluorescence-assisted lectin microarray [27,28].

3. Releasing of glycans from glycoconjugates

Releasing of *N*-linked glycans using anhydrous hydrazine (hydrazinolysis) has been widely employed as the chemical method [29,30]. Although hydrazinolysis requires relatively long reaction time, it releases *N*- and *O*-glycans in high yield. However, the reaction requires cautions for handling highly toxic and hazardous anhydrous hydrazine reagent. Therefore, *N*-glycoamidases such as *N*-glycanase F and glycoamidase A having broad specificity are generally used to release *N*-glycans from the peptide backbone [31]. For the release of *N*-glycans attached to Asn residues through *N*-glycosidic linkage, treatment with peptide N^4-(N-acetyl-β-glucosaminyl) asparagine amidase (PNGase F or *N*-glycanase F; EC 3.5.1.52) is most frequently employed. The glycans are released as the *N*-glycosylamine form, and the sequence, Asp-X-Ser/Thr (X is any amino acid other than proline) thus formed is used for the determination of glycosylation sites [24]. In the release of *N*-glycans by enzymatic method, it should be noticed that three-dimensional structure of the glycoprotein often inhibits the action of *N*-glycoamidases, and denaturation of the proteins followed by protease digestion (typically trypsin digestion) is required prior to enzymatic releasing reaction [16]. Sheeley and Reinhold developed a facile method for the release of N-glycans: glycoprotein samples were reduced and denatured with SDS and mercaptoethanol, and directly digested with N-glycoamidase in the presence of non-ionic surfactants such as NP-40 and Triton-X100 [32].

The bands/spots observed on SDS–PAGE and 2D-PAGE were digested with N-glycoamidase F and the released glycans were analyzed [33–35]. Royle et al. developed the in-gel digestion method and succeeded in one-pot releasing and purification of glycans in the 96-well plate for the analysis of *N*-glycans in serum samples [36].

There is no *O*-glycan releasing enzyme (i.e. *O*-glycanase) capable of releasing a wide variety of *O*-glycans, and *O*-glycans are still being released from the core proteins by chemical methods, typically by β-elimination with mild alkali [37] or mild hydrazinolysis [38,39]. NaOH-catalyzed β-elimination in the presence of sodium borohydride under mild conditions is the common method for routine analysis of *O*-linked glycans [40–42]. However, the released *O*-glycans are reduced to alditol forms to prevent degradation (i.e. peeling reaction). Consequently, the original reducing terminal is no longer available for modification via its original carbonyl groups, which is often essential for sensitive and high-resolution analysis of glycans.

Releasing of *O*-glycans with the intact reducing group has been reported by several groups. Royle and coworkers employed mild hydrazinolysis to obtain *O*-glycans from microgram quantities of glycoproteins [38,39]. Huang et al. developed a method for releasing *O*-glycans in the presence of ammonia [43]. Karlsson and Packer reported an in-line flow releasing system for alkaline β-elimination to release the *O*-glycans with the reducing ends from the core protein immobilized on the column [44]. Recently, Yamada et al. reported an automatic glycan releasing apparatus for the release of *O*-glycans from mucin-type glycoproteins and named the apparatus "AutoGlycoCutter" (Fig. 2: AGC) [45].

Although releasing reaction of *O*-glycans from the core protein is generally performed under mild conditions for a long time (typically16–48 h), the auto glycan releasing system is carried out at high temperature but for an extremely short reaction time (~3 min). After the reaction, the reaction mixture is immediately deionized by passing through a column packed with cation-exchange resin, and *O*-glycans are obtained as reducing form without significant degradation. The procedure for *O*-glycan releasing reaction using the apparatus is as follows (Fig. 2b). The sample solution of glycoprotein or glycopeptide is introduced by an automatic injector to the flow of an aqueous alkali solution (0.5 M LiOH) at 1.0 ml/min. *O*-Glycans are released during passing through the reaction coil (0.25 mm i.d., 10 m length) thermostated at the elevated temperature. The alkaline solution containing the released *O*-glycans is immediately cooled and neutralized by a cartridge packed with cation-exchange resin (1 ml). After releasing reaction, the cartridge is regenerated with 0.25 M H_2SO_4. Because the total time required for the release of *O*-glycans for one sample is ca. 15 min (including regeneration of the ion-exchange cartridge), more than 50 samples can be treated in a day. It should be noted that the system can be applied to the analysis of GAGs [46], that are also released in high efficiency from the core protein by β-elimination in the presence of alkali [47]. Although enzymes such as endo-β-xylosidase and cellulase that exhibit endo-β-xylosidase activity have been reported for releasing glycosaminoglycans (GAGs) from the core protein [48,49], specificity of the enzymes is a big problem for routine analysis of GAGs. The system was connected to an auto spotter (AccuSpot from Shimadzu) for direct MALDI MS measurement for routine analysis of *O*-glycans [50]. To achieve this objective, the second version of the system (AGC-2) was developed, which affords glycan releasing reaction in micro/semi micro scale. In this system, a portion of the released glycans (ca. 10%) is introduced into the AccuSpot by a splitter, and the glycan solutions are spotted on the MALDI sample plate. For high-sensitive analysis in MALDI MS, the authors carried out on-plate labeling of the released *O*-glycans with phenylhydrazine [51,52]. The system allowed MS analysis of *O*-glycans of bovine submaxillary mucin at 1 μg level as protein, and was applied to the analysis of the *O*-glycans expressed on cancer cells and total glycoproteins in human serum samples.

4. Labeling of glycans for their highly sensitive detection

The released glycans obtained as described above do not have distinct absorption at wavelengths above 200 nm, and labeling with chromophores or fluorophores is essential for their sensitive detection. In addition, most carbohydrates other than sialic acids and

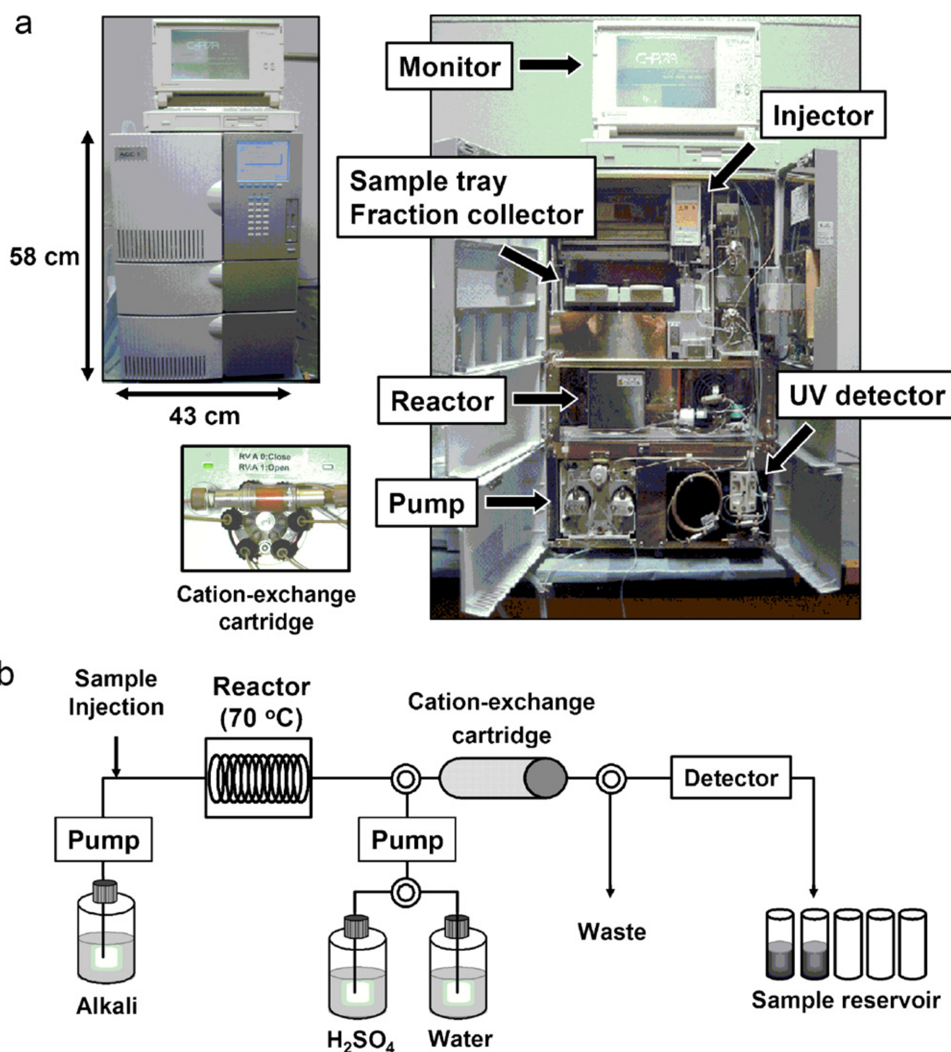


Fig. 2. Auto glycan releasing system (AutoGlycoCutter: AGC) for releasing *O*-glycans from the core protein (a) Apparatus. (b) Diagram of AGC for releasing *O*-glycans. Ref. [47] with permission from the publisher.

uronic acids have no electric charges. For the separation by CE, tags having charges are advantageous for their high resolution analysis. The use of appropriate tags improves detection sensitivity and separation efficiency in the analysis by HPLC, CE and MS.

Glycans can be labeled at their reducing ends by reductive amination. Primary amino group of the tag molecule reacts with the aldehyde group of the glycan to form Schiff base, which is reduced by a reducing agent such as sodium cyanoborohydride [53] or borane-dimethyl amine [54]. A number of amine reagent have been developed for labeling of glycans by reductive amination [18]. 2-Aminopyridine (PA), 2-aminobenzamide (2AB), 2-aminobenzoic acid (2AA) and 4-aminobenzoic acid ethyl ester (ABEE) are the most frequently used as the labeling reagents. Labeling with PA allows highly sensitive detection of glycans by fluorometry, and has been used as one of the standard glycan analyzing methods [55,56]. Takahashi et al. analyzed PA-glycans by HPLC using a series of separation modes of ion exchange, reverse phase and normal phase, and realized 3D-mapping for glycan analysis [57,58]. PA-glycans are detected with high sensitivity using positive ion mode by mass spectrometery [18,59,60]. Rudd et al. analyzed 2AB-labled glycans by normal-phase HPLC–MS [39,61–63], and constructed a glycan database [36,64]. 2AA-labeled glycans developed by Anumula have been analyzed by HPLC [65]. 2AA-labeled glycans are advantageous both for HPLC and CE analyses due to carboxylic acid residue (i.e. negative charge) of 2-aminobenzoic acid. Neutral and acidic glycans were successfully analyzed [66], and detected with high sensitivity in negative ion mode by mass spectrometry. Sialo- or sulfated glycans are also successfully detected [67]. It should be noted that 2AA labeling reaction can be performed in aqueous solution [45,67]. Labeling with ABEE is especially useful for ESI/MS [18]. Cheng et al. developed a method for sequencing of ABEE-labeled glycans by ESI-MS [68].

Amino-aromatic compounds having sulfonic acid residues such as 8-aminopyrene-1,3,6-trisulfonic acid (APTS) [69,70], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [71] and 5-aminonaphtalene-2-sulfonic acid (ANSA) [72] are highly negatively charged, and available for CE analysis. Because glycan analysis kit using APTS for CE is commercially available, a number of reports have been published. Recently, Gennaro et al. reported CE-MS system for APTS-labeled glycans [73].

2-Aminoacridone (AMAC) is useful for fluorescent labeling and separation of acidic glycans based on its high hydrophobicity, and was applied to the analysis of the constituent disaccharides of GAGs by LC–MS [74].

Reductive amination usually requires large excess amount of reagents, and a number of clean-up procedures to remove the excess reagents have been reported. Size exclusion chromatography using Sephadex LH-20 or Sephadex G-25 has been widely used for purification of the labeled glycans [45,67,75], although relatively long time is required. Extraction with organic solvents

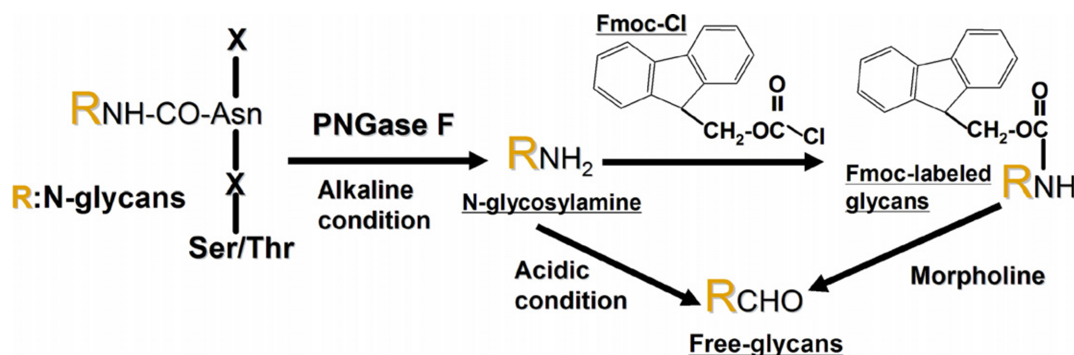


Fig. 3. Fmoc-labeled *N*-glycans after the release of *N*-glycans from glycoprotein samples. *N*-Glycans are released from core proteins by digestion with PNGaseF as *N*-glycosylamine form. Although *N*-glycosylamine is hydrolyzed to yield free *N*-glycans under acidic conditions, *N*-glycosylamine is stable, and easily labeled with Fmoc under weakly basic conditions (~pH 8.0). *N*-Glycans labeled with Fmoc are easily converted to the free *N*-glycans by incubation with morpholine.

[18,76], purification using solid phase such as graphite carbon column [77] and Oasis HLB [78] have been reported. Hydrophilic interaction liquid chromatography (HILIC) is also an alternative method [65,79]. Pabst et al. reported the clean-up method using a cyano-SEP cartridge [18]. Royle et al. purified 2AB-labeled glycans using an solid phase extraction (SPE) microelution plate [36]. These methods often accompany residual reagents, and make it difficult to analyze quite minute amount of glycans.

Aryl hydrazones, formed by the reaction between carbonyl compounds and aryl hydrazines, are the most classical derivatives for chemical identification of carbohydrates. 2,4-Dinitorophenlyhydrazine and dansyl hydrazine have been used for labeling of neutral monosaccharides, and applied to the analysis by HPLC [80,81]. Recently, Lattova et al. realized high-throughput MS analysis of glycans labeled with phenylhydrazine [82,83]. Derivatization was performed on the plate for MALDI-TOF MS measurement. They reported that hydrazone formation proceeded quite easily under mild conditions, and the reaction products could be directly used for MS measurement without clean-up procedures [51,52]. Kamoda et al. developed a fluorescent derivatization of *N*-glycans using 9-fluorenylmethyl chloroformate (F-moc-Cl), which is widely used as a protecting reagent for amino groups during peptide synthesis, and also used for derivatizaiton of primary and secondary amines [84,85] (Fig. 3). When *N*-glycans attached to Asn residues are digested with PNGase F, the glycans are released as *N*-glycosylamine, and easily labeled with Fmoc-Cl. The Fmoc-labeling method has some strong points: (1) simple procedure; Fmoc reagent is just added to the reaction solution after digestion with PNGase F, and it is easy to remove excess reagent by extraction with chloroform; (2) high-speed analysis (within 4 h) including the enzymatic glycan releasing reaction and the labeling reaction; (3) high-sensitivity (5 and 30 times higher sensitivities than those for 2AA and PA-labeled *N*-glycans, respectively); and (4) easy recovery of free-form glycans by incubation with morpholine in dimethylformamide solution at room temperature [84]. Furukawa et al. reported that glycans can be rapidly and efficiently purified and labeled by employing glycan-specific chemical ligation onto aminooxy-functionalized polymers, which were termed "glycoblotting" [86,87]. They achieved highly sensitive MALDI-TOF MS analysis of *N*-glycans in serum samples [86].

In permethylation, hydrogens on hydroxyl groups, amino groups, and carboxyl groups are replaced by methyl groups, yielding a hydrophobic derivative [88]. This is an advantageous derivatization method, because it enhances the glycans' signal strength by MS measurement with both ESI and MALDI [89]. Permethylation facilitates MS spectral interpretation since both acidic and neutral structures can be measured in the positive ion mode [90]. Sialic acid residues are stabilized by methylation [91]. Moreover, tandem MS of sodium adducts of permethylated glycans provides detailed information on linkage positions [92]. For structural characterization of glycans, permethylation is often preceded by reduction of the reducing end with sodium borohydride to form an open-ring alditol, thereby providing a mass tag which facilitates spectral interpretation [92]. Ciucanu and Kerek introduced the basic permethylation protocol [93] which has become a standard procedure for permeyhylation, the method was applied to the analysis of *N*- as well as *O*-glycans [90,94,95]. Morelle and Michalski provided a detailed step-by-step protocol for permethylation with summaries of advantages and disadvantages [91]. Kang et al. proposed a new permethylation method using cartridges packed with powdered or meshed sodium hydroxide to allow effective and quantitative microscale permethylaiton of glycans for a short time [96], and applied this miniaturized device to a high-throughput method and combined it with an isotope labeling strategy [20,97]. An elegant approach for sequential double permethylation method for the analysis of sulfated glycans was introduced. Briefly, sulfated glycan samples are permethylated prior to the methanolysis to cleave of their sulfate groups. These glycans are then subjected to the second permethylation using deuteromethyl iodide to label the hydroxyl groups formed by methanolysis. This method allows the detection and structural characterization of sulfated glycans using MS, avoiding the problem of signal suppression due to the presence of sulfate groups [98]. Carboxyl groups of monosaccharides such as sialic acids were derivatized to methyl esters to stabilize sialic acid linkages for subsequent mass-spectrometric analysis [99]. Toyoda et al. found that methylation of α2,3-linked sialic acids proceeds less efficiently than those having α2,6-linkages. The authors suggested that this was a major problem in terms of glycan biomarker discovery using MALDI MS, and developed a novel amidation method using acetohydrazide which can completely modify both types of linkages of sialoglycans [100].

5. Analysis of glycans by MS

Two types of soft ionization techniques, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), are generally used for the analysis of glycans. MALDI-MS data reduce complexity, because multiple charged ion species are not generated and only single charged ions are observed. Moreover, highly sensitive analysis (pmol-fmol order) can be achieved. However, sulfate and sialic acid residues are often cleaved from the analyte during MS measurement. In contrast, fragmentation of glycans can be reduced, and sensitive detection of sialylated and sulfated glycans is achieved using ESI MS. ESI MS often affords complex spectra, because multiple charged ions are easily generated. Four mass analyzer systems, time-of-flight (TOF), ion trap (IT), Fourier transform ion cyclotron resonance (FT-ICR), and

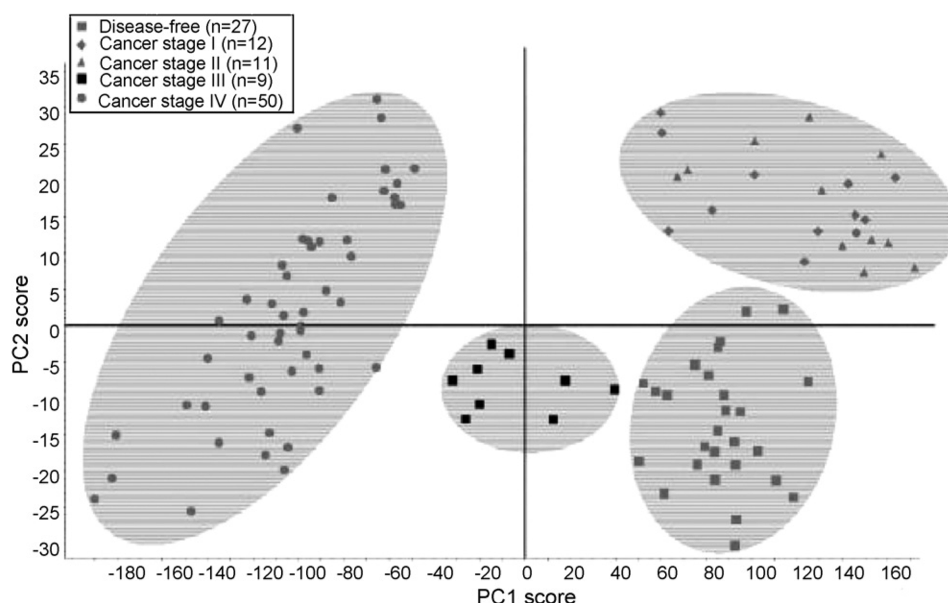


Fig. 4. PCA score plot of *N*-glycan profiles derived from the sera of healthy individuals and breast cancer patients at different stages of the disease. PC1, principal component 1; PC2, principal component 2. Ref. [111] with permission from the publisher.

Orbitrap are used in the analysis of glycans. In addition, tandem MS methods such as TOF/TOF, QIT-TOF, LTQ FT-ICR, and LTQ-Orbitrap are used to analyze the sequence of glycans. Recently, ion mobility spectrometry (IMS) technology is used for glycan analysis [101], which discriminates structural isomers of glycans which are not confirmed by conventional MS methods [102]. MS analysis provides high throughput glycan structural analysis. However, quantification of glycans in clinical samples is difficult, because ionization efficiencies are significantly different among different types of glycans. Therefore, sample pretreatment and statistical analysis are essential for quantitative analysis of glycans by only using MS techniques.

MALDI-TOF MS is available for direct measurement of the mixtures of glycans derived from clinical samples. Although various matrix materials have been reported, 2,5-dihydroxybenzoic acid (DHB) is most frequently used [103]. However, α-cyano-4-hydroxycinnamic acid (CHCA) was employed for MS measurement of fully methylated glycans [90]. Sulfated glycans show extensive fragmentation of sulfate groups upon MALDI-MS measurement, but the use of liquid matrix such as 1-methylimidazolium α-cyano-4-hydroxycinnamate and tetrabutylammonium 2,5-dihydroxybenzoic acid improves the quality of MS data of sulfated glycosaminoglycans [104], and some applications for MS measurement of dermatan sulfate (DS), chondroitin sulfate (CS), heparin and heparan sulfate were reported [105,106].

Some important reports on mass profiling of glycans in clinical samples using MALDI-MS have appeared. Cooke et al. examined mass profile of mucin-type *O*-glycans released from gastric biopsy samples after infection with *Helicobater pylori*. The *O*-glycans were obtained by β-elimination in the presence of sodium borohydride, and analyzed by MALDI FT-ICR MS after clean-up procedure by graphitized carbon-solid phase extraction. In addition, ESI-MS was also used for characterization of acidic glycans containing sialic acids or sulfate groups. The authors found that acute *H. pylori* infection in *rhesus macaques* was accompanied by a dramatic but transient loss in *O*-glycans (especially core 1 and core 3 glycans having low molecular masses) [107,108]. The authors' group applied the technique to the analysis of *O*-glycans in serum samples of breast cancer patients and ovarian cancer patients [109,110].

It is often difficult to evaluate the expression of each glycan in complex mixtures obtained from clinical samples because of the release of sialic acids and sulfate groups, differences in ionization efficiencies, and fragmentation of glycosidic linkages during MS measurement. To overcome these problems and improve the sensitivity, they are often analyzed after permethylation [20]. Collaborative studies among multi laboratories on MS analyses of *N*- and *O*-glycans were performed, and the results were compared with those obtained by HPLC analysis [16,17]. Kyselova et al. employed a combination of permethylation and MALDI-TOF MS analysis of *N*-glycans in serum samples derived from 27 healthy subjects and 82 breast cancer patients from stage 1 to stage 4 [111,112]. They analyzed the data by principal component analysis (PCA), and achieved diagnosis of breast cancer (Fig. 4). It should be noted that N-glycan profiles can discriminate cancer stages.

They also carried out additional statistical analyses through nonparametric ROC (receiver operating characteristic) and ANOVA (analysis of variance) for approximately 50 individual *N*-glycans, and showed that seven complex-type sialoglycans and one hybrid-type sialoglycan gave significant difference between healthy subjects and cancer patients. The authors employed similar techniques to analyze glycan profiles from serum samples from different cancers and cancer cells [113–115].

In contrast to MALDI-MS which is used as a stand-alone technique, ESI-MS is connected with separation techniques such as LC and CE in the on-line manner. Especially, LC-ESI-MS methods are often used for glycan analysis in clinical samples. Detection sensitivity of ESI-MS depends on the spray solvent. In positive-ion mode, water/methanol or acetonitrile containing a volatile acid is generally used to enhance ionization of glycans [116]. When various ions such as H^+, Na^+ and Li^+ adduct ions are observed, Na salts are added in the spray solvent to suppress the formation of other ions [116]. In negative-ion mode, water/acetonitrile containing ammonia or ammonium salts (ammonium bicarbonate or ammonium acetate) is often used. Water/acetonitrile without salt is also used in negative ion mode analysis. Selections of the solvents give distinctive effect on fragmentations of glycans in MS/MS analysis [116,117].

Three separation modes of NP, RP and PGC (porous graphitized carbon) in HPLC are available in ESI-MS. Fluorescent labeled glycans such as with PA and 2AB were analyzed by NP and HILIC

HPLC-ESI-MS [38,39,118]. Free or alditol-type glycans are often analyzed by PGC-LC-ESI MS [41,42,119]. LC-ESI-MS in RP mode was used to the analysis of permethylated glycans and AMAC-labeled unsaturated disaccharides from GAGs [74,120]. Recently, Volpi et al. applied reversed-phase ion-pairing HPLC to ESI-MS analysis of GAG-derived glycans [121,122]. The suppression of sample diffusion and conditions for electronic spray are important to achieve sensitive detection in ESI-MS analysis, and nano-LC-ESI MS methods are primarily used in microanalysis. In addition, chip-ESI MS methods for glycan analysis are being increased [123,124]. The application of the chip-ESI MS concept for the analysis of *N*- and *O*-glycan mixtures was reported in urine samples from the patients suffering from congenital disorders of glycosylation (CDG) [125]. Flangea et al. also applied the chip-ESI MS to analyze GAGs derived from mouse brain [126].

To achieve separation of isomeric and isobaric structures in complex mixtures of glycans, *N*-glycans released from human serum samples were analyzed by a combination of MALDI-FT ICR MS and microchip LC MS [127]. *O*-Glycans were also analyzed in the similar manner [128]. An amide-silica chip is also available for the analysis of GAGs [129].

6. HPLC analysis

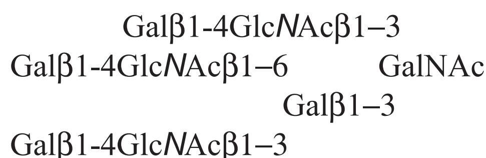
Various modes of HPLC separation have been developed for the analysis of glycans. Normal phase (or hydrophilic interaction), ion-exchange, reversed phase and adsorption (on porous graphitized carbon columns) are the typical ones.

Because glycans are highly hydrophilic and polar substances, their interaction with normal-phase matrices are enhanced in solvents containing organic solvent at high concentrations, and are preferentially eluted with solvent mixtures by increasing water content. Especially, amine-based, amide-based, and zwitterionic sulfobetaine HILIC (ZIC HILIC) columns have been extensively used to analyze glycans in clinical samples [130–133]. Normal phase chromatography achieves separation of glycans mainly based on hydrogen bondings and ionic interactions. Glycans having sulfate and sialic acid residues are strongly retarded. Especially, amino-based stationary phase column shows highly ionic interaction with glycans. HILIC is sometimes referred to size separation which correlates with glycan sizes. However, monosaccharides in glycans often affect the elution times: generally hexoses are more strongly kept on the stationary phase than N-acetylhexosamines and 6-deoxy hexoses [134]. HILIC-HPLC has been often employed for fluorescent labeled glycans [18,36,38,65,133–137]. Rudd and coworkers reported analyses of 2AB-labeled *N*- and *O*-glycans on a TSK-amide80 column. The elution times (as expressed by glucose units, GU) were compared with those of the standard dextran oligomers, and the structures were confirmed by digestion with a combination of specific exoglycosidases. And the results on the glycan structures and their retention characteristics were employed for construction of the database (Glycobase) [36,138]. The method was applied to the analysis of the glycans in serum and cell samples [61,63]. They also confirmed the aberrant galactosylation of IgG in rheumatoid arthritis patients as compared with healthy controls and significant correlation between the levels of aberrant IgG galactosylation and disease activity [139]. Hitchcock et al. reported profiles of chondroitin sulfate epitopes expressed on different joint tissues as a function of age and osteoarthritis. Glycosaminoglycans were extracted from joint tissues (cartilage, tendon, ligament, muscle, and synovium), and depolymerized with chondroitinases. The oligosaccharide products were differentially isotope labeled with deuterated 2AA, and subjected to amide column-online LC/MS [140].

Retentions of sialylated PA-labeled glycans were examined with a ZIC stationary phase [133]. The separation was done using a solvent at low-salt concentrations under weak anion exchange effect, for connection with ESI-MS [133,141,142]. Anumula employed an amine-bonded polymer column (Polymer-NH_2) with a volatile solvent to analyze 2AA-labeled glycans [65]. The separation showed similar profiles to those obtained by HPAEC-PAD [143]. Kakehi's group achieved comprehensive analysis of *N*-glycans as well as *O*-glycans as 2AA derivatises in cultured cancer cells using an amine-bobded polymer column [45,144]. An example for the analysis of *O*-glycans in a few leukemia cancer cell lines is shown in Fig. 5 [67].

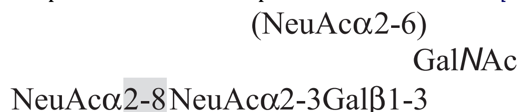
All the cells other than HL-60 cells showed relatively simple chromatograms (Fig. 5A). The peaks observed on each chromatogram were collected and analyzed by MALDI-TOF MS and MALDI QIT-TOF MS^n techniques. All leukemia cell lines commonly contained sialyl-T and disialyl-T as major *O*-glycans. While disialo core-2 type glycans were mainly observed in HL-60 cells, the cells also expressed polylactosamine-type and monofucosylated polylactosamine-type *O*-glycans (Fig. 5B).

Porous graphitized carbon as the stationary phase is often used for the analysis of aliditol-type glycans. Separation is achieved based on hydrophobic, polar and ionic interactions using water-organic solvent (typically acetonitrile) [145]. Trifluoroacetic acid or formic acid were also used as an acidic modifier [94,128]. Highly sialylated glycans are retarded strongly, and eluted with the basic eluent containing a salt such as ammonium formate [42,146–148]. Eluent having wide pH range is available for the glycan analysis, and advantageous for the measurement of alditol-type *O*-glycans by LC-ESI-MS with high sensitivity [119]. Schulz et al. applied PGC LC-ESI MS to the analysis of *O*-glycans in mucin-type proteins separated by SDS-polyacrylamide/agarose composite gel (SDS-AgPAGE) [41]. And the technique was used for the profiling of *O*-glycans of mucins secreted in saliva [149,150]. They found *O*-glycans of which C-3 branch consisted of branched I-antigen type structural epitopes (Glc*N*Acβ1-3(Glc*N*Acβ1-6)Galβ1-). The branch point was initiated on the galactose residue of core 1 and core 2 structure.



These glycans were often modified with fucose and/or sialic acid residues to form sialyl LewisX epitope [151]. The authors applied the technique to the analysis of the *O*-glycosylation of MUC1 in breast, prostate and gastric cancer [42]. Stadlmann et al. analyzed *N*-glycans of IgG species produced from different cell lines, and found that *N*-glycan profiles showed distinctive variations with cell lines and some IgG products contained *N*-glycans having α-Gal epitope [152].

Weak anion exchange (WAX) chromatography has been generally used for glycan analysis [39,153–155]. Storr et al. analyzed 2AB-labeled *O*-glycans from MUC1 in serum samples of breast cancer patients by a combination of WAX and NP-HPLC, and showed that highly sialylated glycans were abundantly present in the breast cancer patients at advanced stages. In addition, α2-8 linked sialic acids were present in these samples as indicated below [62].



WAX HPLC is important in the initial analysis of glycans by multidimensional HPLC [156,157]. Volpi et al. used strong anion exchange chromatography for the analysis of unsaturated disac-

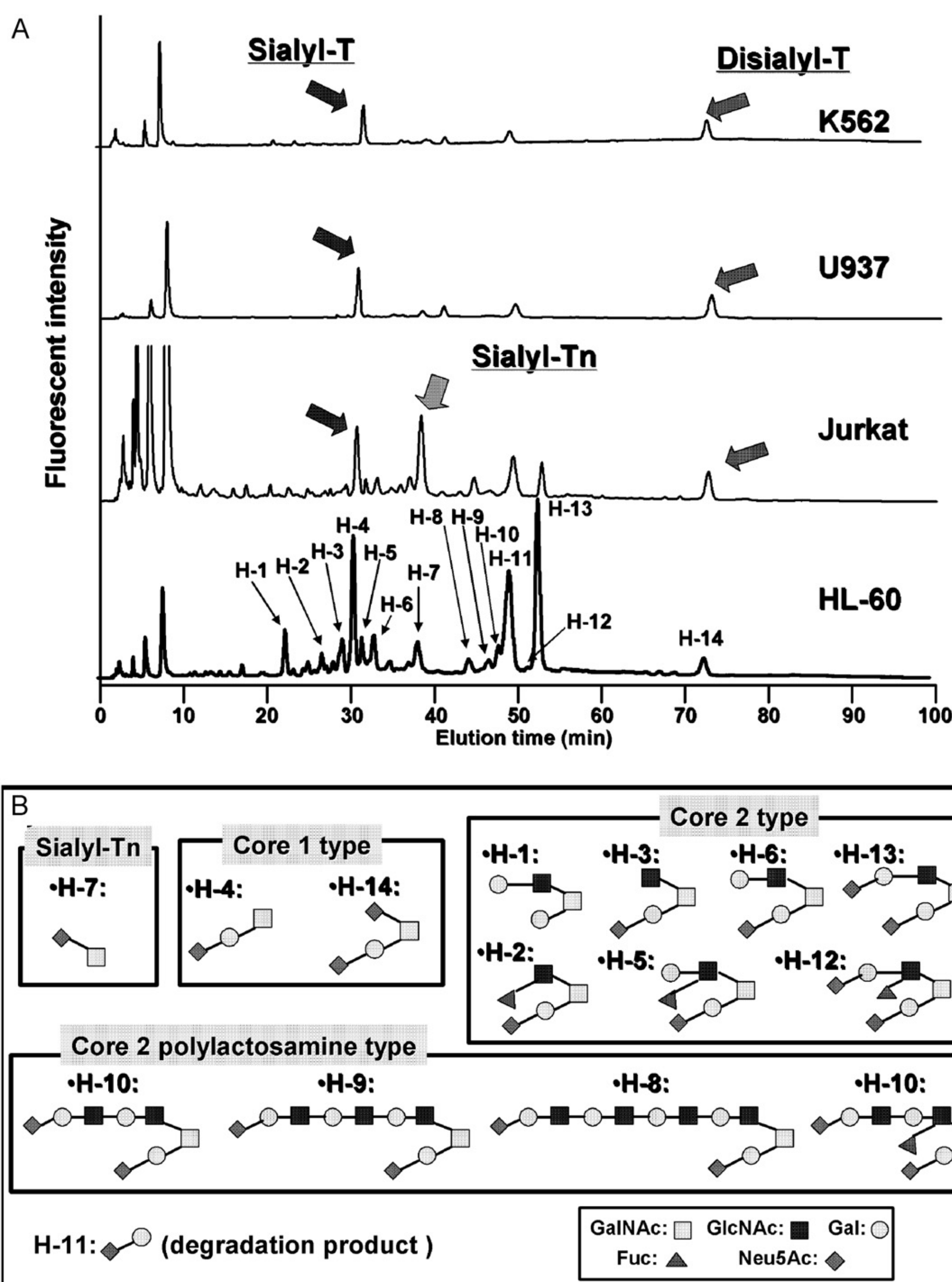


Fig. 5. NP-HPLC analysis of *O*-glycans derived from leukemia cancer cell lines. *O*-Glycans derived from K562 (chronic myelogenous leukemia), U937 (histiocytic lymphoma), Jurkat (acute T cell leukemia) and HL-60 (acute promyelocytic leukemia) cells were released by the auto glycan releasing system followed by labeling with 2AA. (A) Analytical conditions for HPLC: column, Asahi Shodex NH2P-50 4E (4.6 mm × 250 mm); eluent, solvent A, 2% CH_3COOH in acetonitrile; solvent B, 5% CH_3COOH/3% triethylamine in water; gradient condition, linear gradient (30–95% solvent B) from 2 to 82 min, maintained for 20 min. *O*-Glycans observed in HL-60 (B). Ref. [67] with permission from the publisher.

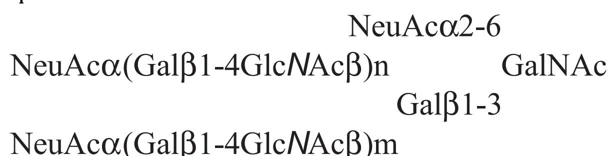
charides derived from glycosaminoglycans (GAGs) in skin samples from a patient with pseudoxanthoma elasticum and normal subjects [158,159]. They also analyzed GAGs in urine samples of patients suffered from mucopolysaccharidoses, and assessed the efficiency in enzyme-replacement therapy [160].

The molecular basis of the interaction between serotonin and *N*-acetyylneuraminic acid was examined by proton NMR [161], and the principle was applied to purification of some glycoproteins [162]. El Rassi et al. applied the technique to the analysis of sialoglycans and gangliosides using a serotonin-immobilized column [163]. Naka et al. employed serotonin-affinity HPLC for group separation of 2AA-labeled *N*-glycans prepared from various cancer cell lines using gradient elution with ammonium acetate, and achieved separations of *N*-glycans based on the number of sialic acid residues [144]. Mass profiling of each group of asialo-/neutral, mono-, di-, tri- and tetra-sialo glycans were obtained by MALDI-TOF MS after collection of the eluent.

The similar technique was applied to the analysis of *O*-glycans in cultured cancer cell lines [67] (Fig. 6). 2AA-labeled O-glycans derived from 6 epithelial cancer cells showed characteristic pro-

Fig. 6. Analysis of *O*-glycan pool released from some cancer cells by serotonin affinity chromatography. *O*-Glycans derived from gastric cancer cell lines (MKN7 and MKN45), colonic cancer cell lines (HCT-15 and LS174T), and pancreatic cancer cell lines (BxPC3 and PANC1) were analyzed by serotonin affinity chromatography (A). Analytical conditions; solvent A, water. Solvent B, 40 mM ammonium acetate; gradient condition, linear gradient (5–75% solvent B) from 2 to 37 min and 75–100% solvent B from 37 to 45 min. The absolute amounts of asialo-, monosialo-, disialo-, and tirisalo-glycans were determined by the fluorescent intensities of the collected fractions (B). Ref. [67] with permission from the publisher.

files (Fig. 6A). In addition, the expression levels of *O*-glycans were varied with the cell lines, but leukemia cells expressed relatively small amount of *O*-glycans (Fig. 6B). The fractions collected by serotonin affinity chromatography were examined by MALDI-TOF MS and MALDI-QIT-TOF MS, and the results revealed the presence of 80 fucosylated, sulfated, or polylatosamine-type *O*-glycans. It should be noticed that MKN45 cells (gastric adenocarcinoma) expressed large amount of highly extended trisialo-polylactosamine type glycans having molecular masses higher than 6000. The glycan has a unique structure as shown below.



O-Glycans of MKN45 and MKN7 cells (both from gastric adenocarcinoma cell lines) at different differentiated stages were also analyzed (Fig. 7). As clearly indicated in Fig. 7, poorly differentiated MKN45 cells expressed a large amount of polylactosamine-type glycans (left column). In contrast, highly differentiated MKN7 cells did not produce such glycans, but expressed relatively simple glycans (right column). These results may indicate that *O*-glycan profiles can be a marker for the malignancy of the tumors.

Takahashi et al. reported three dimensional (3-D) sugar-mapping technique for the analysis of pyridylaminated neutral and sialyl glycans [156,164,165]. Naka et al. employed a combination of serotonin affinity chromatography and an amide-based column for the analysis of *N*-glycans derived from cultured cancer cells [144]. The results are shown in Fig. 8, and summarized in Fig. 9.

Asialo/neutral, monosialo-, disialo-, trisialo-, and tetrasialo-glycans collected by serotonin affinity chromatography were

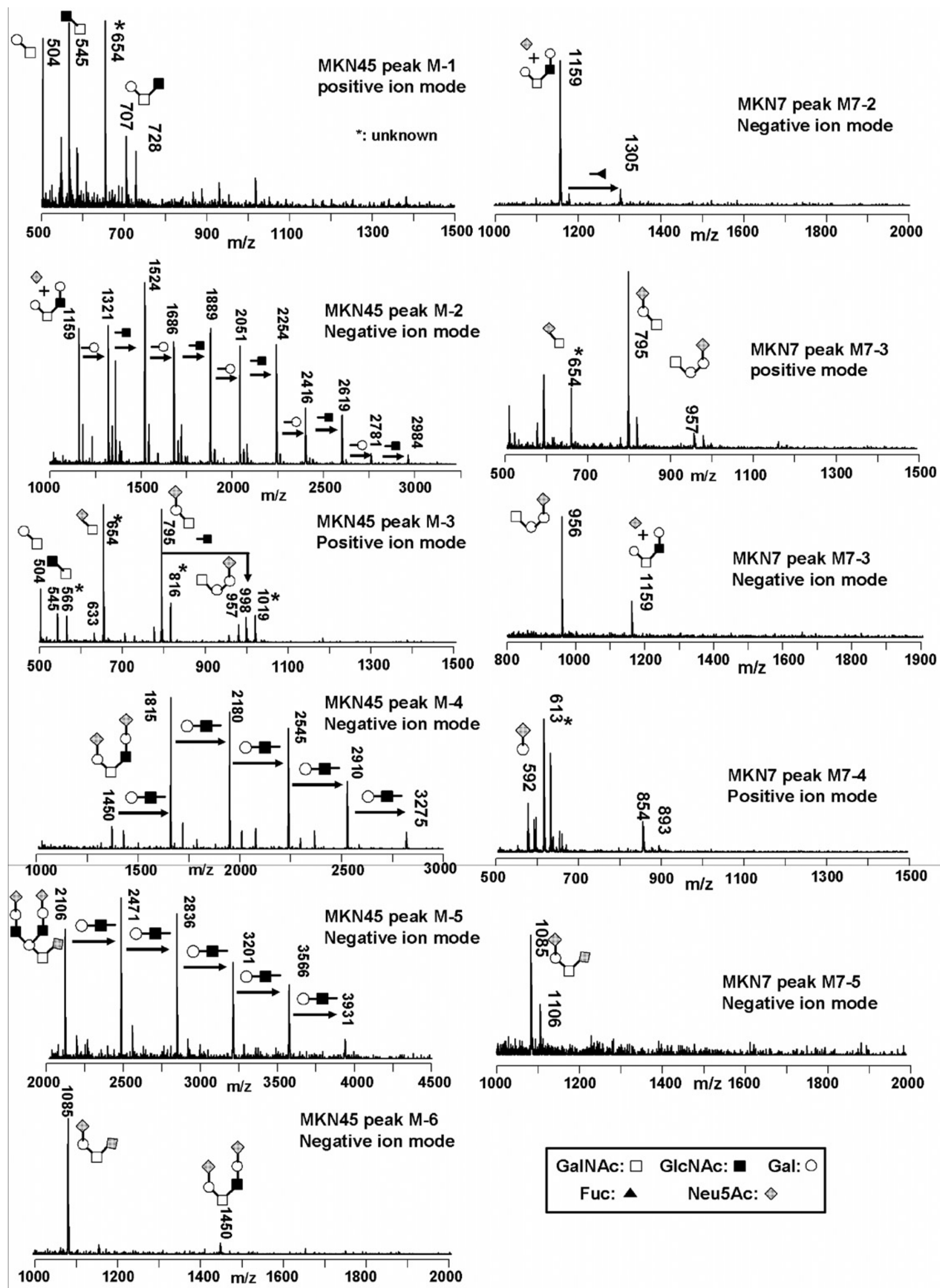


Fig. 7. MALDI-TOF MS analysis of *O*-glycan fractions derived from MKN45 (left column) and MKN7 cells (right column). The panels in the figure indicate the results on the fractions separated by serotonin affinity chromatography (Fig. 6). Ref. [67] with permission from the publisher.

analyzed by normal phase HPLC using a TSK Amide80 column after digestion with neuraminidase. Each fraction showed characteristic glycan profiles for each cell line, and 80 *N*-glycans in total were confirmed (Fig. 8). Fig. 9 summarizes the results. The column in the left side of Fig. 9 shows the list of complex-type asialoglycans found in A549, MKN45, ACHN, and U937 cells. The glycans are further categorized if they contain fucose residue(s). The glycans marked in circles are commonly found in all cancer cells, and those marked in squares are characteristic ones observed in specific cancer cells. As indicated in Fig. 9, polylactosamine-type glycans are possible markers for some tumors.

Comprehensive analysis of both *N*- and *O*-glycans reveals important implication on biosynthesis of N- and *O*-glycans having polylactosamine residues [67,144]. As shown in Fig. 10, MKN45 cells express large amount of polylactosamine-type *O*-glycans which have molecular masses of higher than 6000.

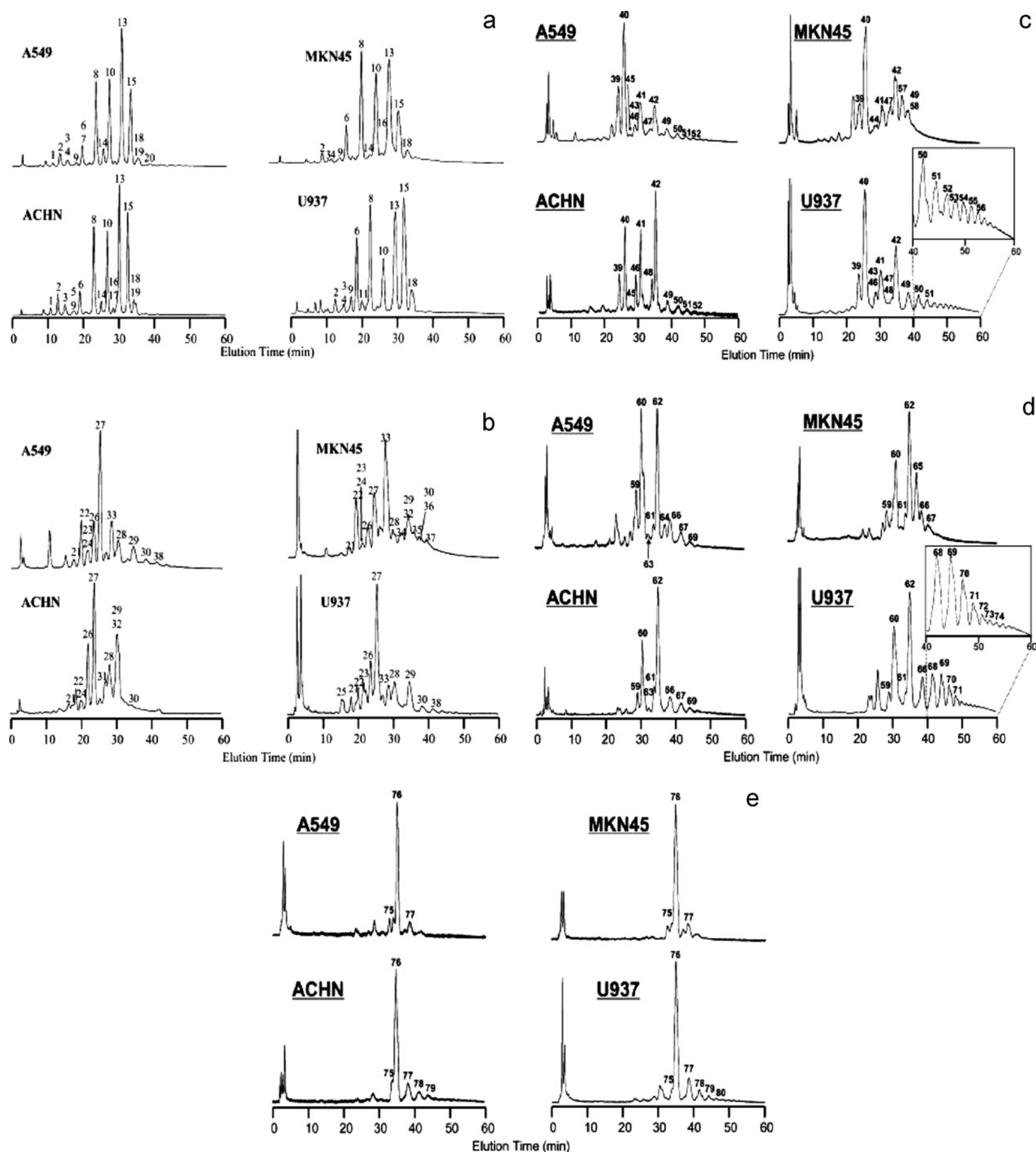


Fig. 8. Analysis of asialo/high-mannose (a), monosialo- (b), disialo- (c), trisialo- (d) and tetrasialo- (e) glycan fractions obtained from some cancer cell lines by serotonin affinity chromatography. Analytical conditions for HPLC: column, TSK Amide80 (4.6 mm × 250 mm); eluent, solvent A, 0.2% CH_3COOH in acetonitrile; solvent B, 0.5% CH_3COOH/0.3% triethylamine in water; gradient condition, linear gradient (30–95% solvent B) from 2 to 82 min, maintained for 20 min. The glycan mixture was analyzed after digestion with neuraminidase. Ref. [144] with permission from the publisher.

In contrast, *N*-glycans observed in MKN45 cells did not contain glycans having extended polylactosamine residues, but were highly fucosylated (Fig. 10A). As indicated in Fig. 10B, fucosylation apparently controls the length of polylactosamine residues. These results may indicate that elongation of polylactosamine residues in *N*- and *O*-glycans have different mechanisms, but whichever outstanding possibility on diagnosis of tumors by comprehensive analysis of glycans in biological samples is exemplified in Fig. 10A.

Ishizuka et al. employed the similar technique to analyze free glycans in cultured cancer cell lines [166], and found that a significant amount of unusual, complex-type free *N*-glycans were accumulated in the stomach cancer-derived cell lines, MKN7 and MKN45. The most abundant and characteristic glycan found in these cells was determined to be NeuAcα2-6Galβ1-4Glc*N*Acβ1-2Manα1-3Manβ1-4Glc*N*Ac. Biochemical analyses indicated that the free glycans were cytosolic ones derived from lysosomes due to low integrity of the lysosomal membrane. Since the accumulation of these glycans were specific to only these two cell lines among the various cancer cell lines examined, these cytosolic N-glycans may serve as a specific biomarker for diagnosis of specific tumors.

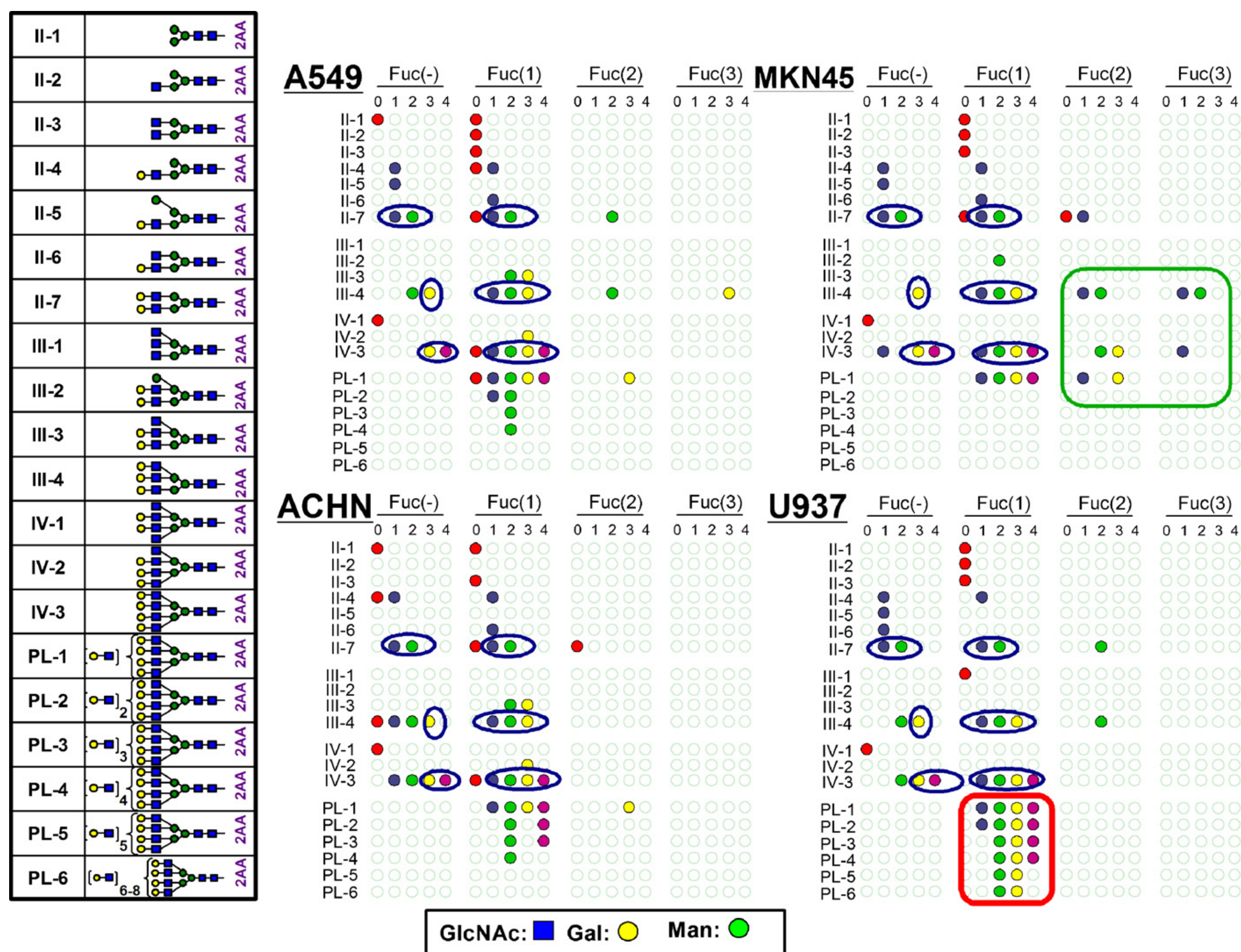


Fig. 9. Profiles of *N*-glycans in cell membrane fractions of A549, MKN45, ACHN and U937 cells. List of *N*-glycans found in membrane fractions of four cancer cells (A). Asialo/high-mannose, monosialo, disialo, trisialo, and tetrasialo-oligosaccharides are shown in the order. Glycans observed in each cell line (B). The *N*-glycans marked in circle were commonly found in all cancer cells, those marked in squares are characteristically found in MKN45cells and U937 cells, respectively. Ref. [144] with permission from the publisher.

7. Capillary electrophoresis and microchip electrophoresis

Capillary electrophoresis (CE) allows high-speed and high resolution analysis for glycan analysis [167–170]. Because CE dose not require long time for pre-conditioning prior to the analysis, throughput of CE analysis is generally higher than HPLC analysis. Highly sensitive detection is achieved by connecting the system with a laser-induced fluorescence (LIF) or an MS detector. Due to extremely high-resolving power of CE, it is practical and useful to compare migration times with those of standard samples, if available, for structural studies. Although separation is achieved theoretically based on charge to mass ratios of the analyte ions, we can use various separation modes in glycan analysis. Free zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), gel electrophoresis (CGE), affinity electrophoresis (CAE), and electrochromatography (CEC) are the typical ones.

CZE and MEKC were applied to analyze both acidic (sialylated glycans and GAGs) and neutral glycans [171–173]. Fused silica capillary tube is generally used to analyze the neutral glycans under neutral or basic conditions. Acidic glycans and the glycans labeled with highly negatively charged fluorescent tags are analyzed under acidic conditions to minimize electro endoosmotic flow [174,175]. In MEKC mode, negatively charged surfactants such as sodium dodecy sulfate (SDS) and taurodeoxycholic acid are added to the electrolyte to form micelles having negative charge. These micelles interact with the analyte ions in the hydrophobic manner, and afford excellent resolution of glycans labeled with hydrophobic reagents such as AMAC [171,172].

CGE has been employed to analyze the glycans derived from GAGs or APTS- and 2AA-labeled glycans, which have negative charges. Capillary tubes of which inner surface are modified with a neutral polymer to suppress the electro endoosmotic flow (EOF) are used. Polymers such as polyethylene glycol (PEG), polyethylene oxide (PEO) and polyacrylamide are added in the electrolyte to separate the glycans by molecular sieving effect [47,66,176–178]. Separation is performed based on the negative charges of the analyte ions. Generally, the analyte ions of smaller molecular sizes are passed through the capillary faster than the larger molecules.

CEC is a fusion of the techniques of CE and LC. Capillary columns packed with stationary phases for LC are used, and EOF generated by applying voltage is employed as the driving force. CEC allows higher resolution than LC, because sample diffusion is minimized [179,180].

CAE can observe the interaction between carbohydrates and proteins in solution state. When the electrophoretic mobility of the conjugate of carbohydrate and carbohydrate-binding protein

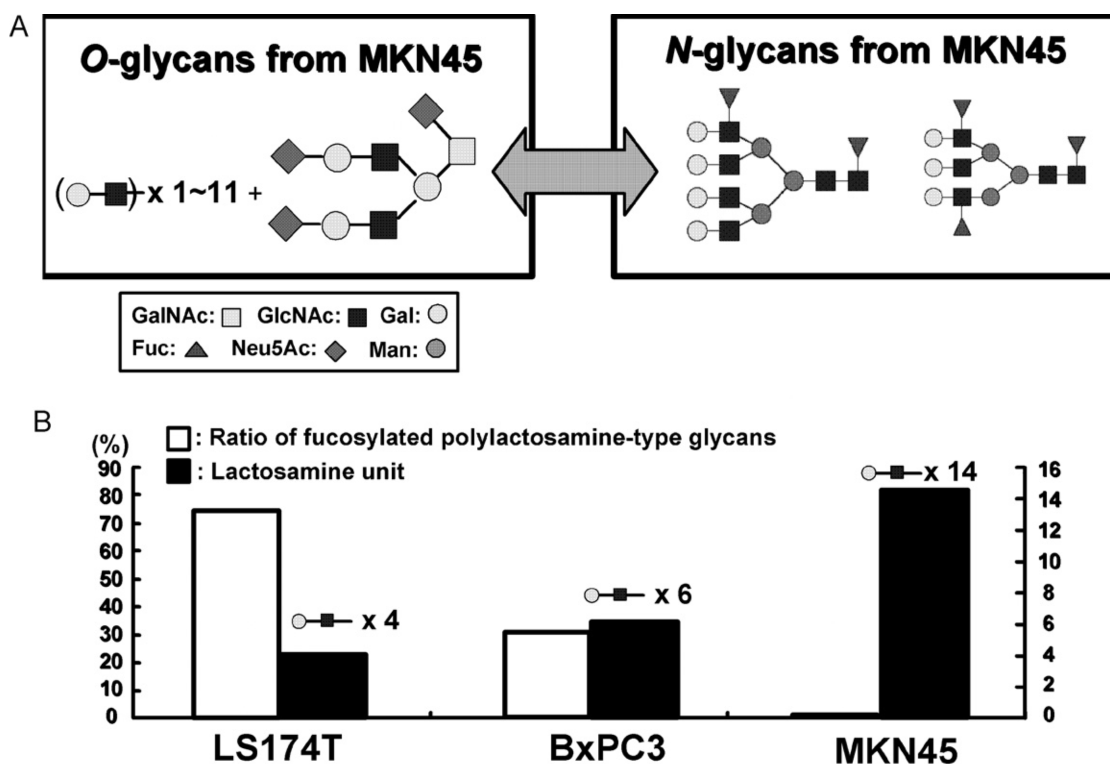


Fig. 10. Relationship between degree of fucosylations and the number of lactosamine units. *O*-Glycans in MKN45 cells are extensively elongated with lactosamine units and not substituted with fucose residues (A). Comparison of fucosylation and the number of lactosamine units of *O*-glycans among three cancer cell lines. (B) In LS174T cells, 75% of *O*-glycans are fucosylated and the number of lactosamine units is four or less. In BxPC3, *O*-glycans have six lactosamine units in average, and 35% of *O*-glycans are fucosylated.

is different from those of carbohydrates and carbohydrate-binding proteins (i.e., lectins), analysis of the interaction between carbohydrates and carbohydrate-binding proteins is a useful tool for acquiring structural information of carbohydrates [181]. Nakajima et al. developed CAE technique for high throughput structural analysis of glycans as shown in Fig. 11 [75]. At the initial step, a mixture of fluorescent-labeled carbohydrates is analyzed by CE in the absence of lectin in the electrolyte (Fig. 11a). Then, the same sample is analyzed in the presence of a lectin whose specificity is well established. When the lectin recognizes carbohydrate A, the peak A is observed later due to the equilibrium formation between the conjugate and carbohydrate A. On the contrary, carbohydrate C,

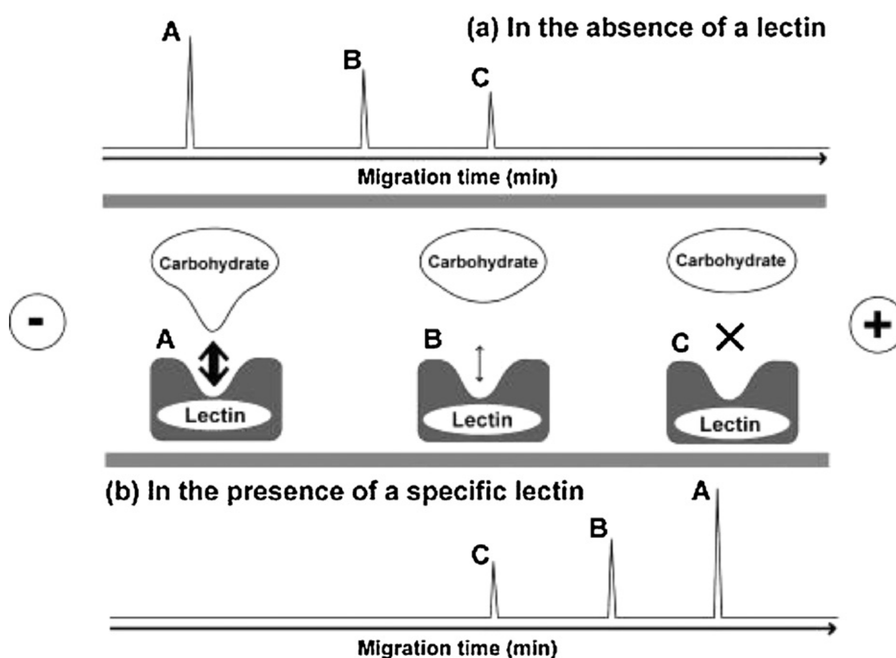


Fig. 11. Principle of capillary affinity electrophoresis (CAE). Ref. [75] with permission from the publisher.

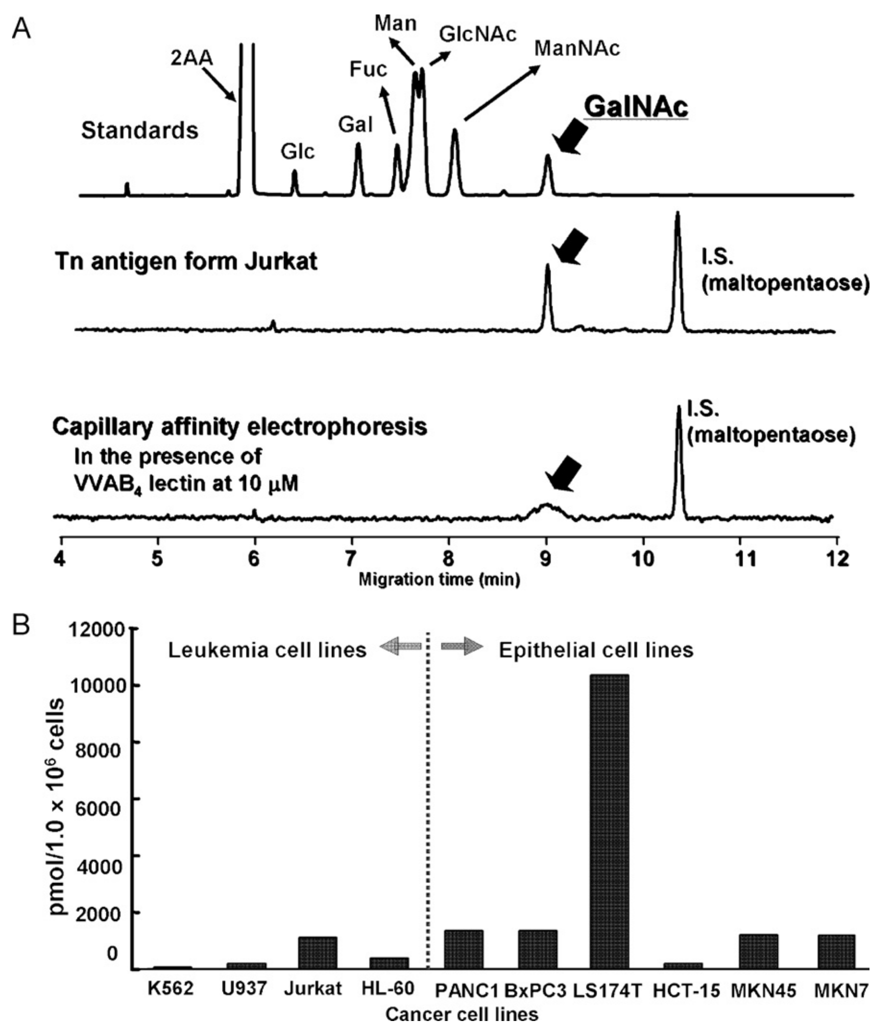


Fig. 12. Determination of Tn antigen in some cancer cells by CE. CE and CAE analysis of 2-AA-labeled Tn antigen form Jurkat cells (A). Analytical conditions for CE and CAE. Capillary, DB-1 capillary (100 µm i.d. × 40 cm); running buffer, 100 mM Tris–borate buffer (pH 8.3) containing 5% PEG70000; applied voltage, 25 kV; injection, pressure method (1.0 psi for 10 s); temperature, 25 °C; detection, helium–cadmium laser-induced fluorescence (excitation 325 nm, emission 405 nm). CAE of the Tn antigen from Jurkat cells was performed in the presence of GalNAc-specific lectin ($VVAB_4$). The peak observed at 10.3 min was due to maltopentaose labeled with 2-AA, which was used as the internal standard for migration times. The migration time of 2-AA-labeled maltopentaose was not changed even in the presence of VVAB4. Expression of Tn antigen in 10 cancer cell lines (C). The amount of Tn antigen was calculated by peak area observed by CE. Ref. [195] with permission from the publisher.

which does not show affinity to the lectin, is observed at the same migration time as that in the absence of the lectin. Carbohydrate B shows weak affinity to the lectin and is observed slightly later. Thus, the migration order of the carbohydrates A–C, changes as shown in Fig. 11b. By repeating the procedures using an appropriate set of lectins, carbohydrate chains are categorized based on the affinities towards the selected set of lectins. Nakajima et al. reported profiling of N-glycans and milk oligosaccharides by CAE [75,182]. They also applied CAE to screening of carbohydrate binding proteins in biological sources [183]. Ishizuka et al. revealed the linkage position of sialic acid of free oligosaccharides observed in gastric cancer cell lines by CAE [166]. The proposed method is an alternative to the microarray, and has the advantages in the assessment of the interaction in solution state. This is especially important for considering the interaction between carbohydrate and carbohydrate-binding protein in biological systems.

CE employs a few nanoliters of a sample solution for injection to a narrow capillary. Therefore, relatively high concentration of the samples solution should be used. This is one of the important disadvantages, when biological samples are analyzed by CE. For this reason, various on-line concentration techniques have been developed for ultra sensitive detection by CE analysis [184–186]. Kamoda et al. developed an on-line concentration method using head-column field-amplified sample stacking technique for CE-LIF analysis of N-glycans [186]. They achieved 300-fold higher sensitive detection than conventional methods, and succeeded in the analysis of N-glycans derived from glycoproteins after separation on a 2D-gel [35].

Callewaert et al. developed a high-throughput method for the analysis of *N*-glycans using a multicapillary DNA sequencer and applied the method to clinical samples for profiling of total *N*-glycans in serum glycoproteins. They labeled *N*-glycans with APTS and used CGE mode for their analysis [177]. All steps for sample preparation were done in a 96 well plate, and 96 samples were simultaneously determined in half an hour [176]. In addition, they confirmed partial structures of some glycans in complex mixtures by sequential digestions with a combination of some exoglycosidases (sialidase, β1-4-galactosidase, fucosidase, and *N*-acetylhexosaminidase) [187]. In CE analysis, the injection volume to the capillary is quite small. Therefore, a few microliters of the sample solution are sufficient for each digestion. This is an important strong point for sequential enzyme digestions for structural studies on glycans. The method was applied to diagnosis of hepatic disease [187]. They reported that the method was useful for diag-

nosis of cirrhosis and hepatocellular carcinoma and progression of liver fibrosis by comparing relative abundances of four complex-type *N*-glycans [188].

Constituent monosaccharides afford fundamental information on glycoconjugates in biological samples. CE has been applied to analyze monosaccharides [189]. Borate buffer is often used for the separation of monosaccharides by utilizing formation of borate complexes [190–192]. Kodama et al. achieved chiral separation of monosaccharide enantiomers by adding (*S*)-3-amino-1,2-propanediol in borate buffer as a chiral selector [193,194].

Incomplete elongation of *O*-glycans in mucins (i.e. overexpression of Tn antigen) has been found in many tumors. And Tn is one of the most specific human cancer-associated structures and a possible early biomarker of cancer [8]. Recently, we developed a method for the determination of Tn antigen (Gal*N*Ac-*O*-Ser/Thr) by CE [195]. Briefly, after releasing Tn antigen (Gal*N*Ac) from the core proteins by AGC (Fig. 2), the released Gal*N*Ac was labeled with 2AA, and analyzed by CE after collecting the fractions containing 2AA-labeled Gal*N*Ac by RP HPLC. CE analysis of Tn antigen derived from Jurkat cells is shown in Fig. 12.

The authors achieved excellent resolution of Gal*N*Ac from other monosaccharides by CE (Fig. 12A; upper panel). The peak appeared at 9 min was confirmed as Tn antigen by CAE using the electrolyte containing VVAB4 lectin at 10 μM concentration (Fig. 12A; middle and lower panel) [196]. The authors applied the method to the determination of Tn antigen in some cancer cells (Fig. 12B). All cancer cell lines examined expressed significantly different amounts of Tn antigen. Of the examined leukemia cell lines, Jurkat cells expressed Tn antigen most abundantly. Because Jurkat cells lack a molecular chaperone (cosmc) which is necessary for expression of core 1 beta 3 galactosyl transferase activity [197], Jurkat cells cannot establish the core 1 structure, and the truncated *O*-glycan (i.e., Tn antigen) which is a precursor of core 1 type glycans was abundantly observed. Epithelial cancer cells other than HCT-15 cells expressed Tn antigen more abundantly than Jurkat cells. Especially, LS174T cells expressed about 10 times larger amounts of Tn antigen than other epithelial cancer cells. It was reported that LS174T cells are highly metastasis cells [198]. Therefore, expression level of Tn antigen may correlate with the degree of metastasis.

A number of glycoprotein pharmaceuticals have been developed and employed for clinical use. Most of them are produced by recombinant technologies using living cells, and have intrinsic structural heterogeneity in glycan parts (i.e. glycoform). It should be noticed that each glycoform has different biological activities. Therefore, evaluation of glycoforms is required for the assessment of quality of glycoprotein pharmaceuticals.

CE is employed to analyze glycoforms of recombinant human erythropoietin (rhEPO). hEPO has three *N*- and one *O*-glycosylation sites and the glycans have variable numbers of sialic acid residues [199]. Because sialylation level of EPO is concerned with their half-life [199], various methods were developed to evaluate the sialylation of rhEPO [200–202]. Kinoshita et al. successfully separated rhEPO glycoforms using commercially available surface-modified capillaries in the running buffer containing hydroxypropylmethylcellulose [202]. CE analysis is also useful for profiling of *N*-glycans expressed on recombinant monoclonal antibody pharmaceuticals (rmAb). Recombinant mAbs contain a conserved *N*-glycosylation site in the CH2 region of the heavy chain. It is well known that *N*-glycosylation of rmAb concerns with the biological, pharmacological and physicochemical functions of rmAbs, such as resistance to proteases, binding to monocyte Fc receptors, interaction with complement component C1q, circulatory half-life *in vivo*, and activity of antibody-dependent cellular cytotoxity (ADCC) [203–206]. Kamoda et al. established a method for the analysis of N-glycans derived from mAb using 2AA labeling methods. They applied this method to comparative analysis of *N*-glycans expressed on three rmAb products, rituximab, trastuzumab, and palivizumab (Fig. 13) [66].

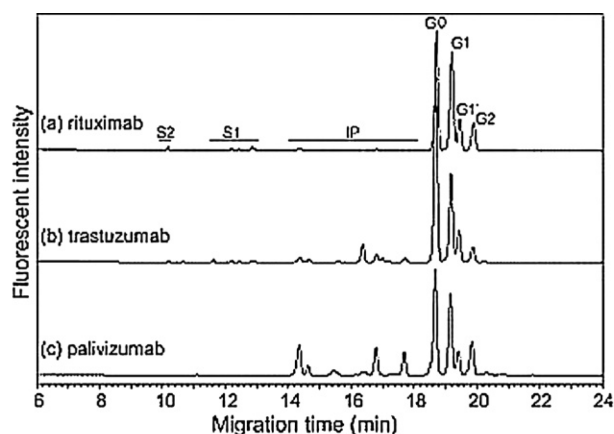


Fig. 13. N-glycans from some rmAb pharmaceuticals by CE after 2-AA derivatization. Analytical conditions for CE. Capillary, DB-1 capillary (100 μm i.d. × 40 cm); running buffer, 100 mM Tris–borate buffer (pH 8.3) containing 5% PEG70000; applied voltage, 25 kV; injection, pressure method (1.0 psi for 10 s); temperature, 25 °C; detection, helium–cadmium laser-induced fluorescence (excitation 325 nm, emission 405 nm). Ref. [66] with permission from the publisher.

Although fucosylated complex-type glycans (G0, G1, G1' and G2) were commonly observed in these rmAbs, minor glycans such as sialo complex-type glycans (S1 and S2), and high mannose and agalacto complex glycans (incompletely processed-species: IP) were significantly different among rmAbs. Especially, palivizumab showed expression of large amount of glycans at IP region, but did not express sialo complex-type glycans. Differences of the amount of sialo- and high-mannose type glycans in rmAbs possibly affects clearance or biological activity of the pharmaceutical preparations [207]. Therefore, it is important to evaluate these glycans for pharmaceutical development of rmAb.

In CE analysis, APTS has been extensively employed for fluorescent labeling of glycans [69,70,77]. Because 2-AA derivatives have less negative charges than APTS derivatives, the analysis generally requires longer time, but the 2-AA labeling method has some advantages: (1) good resolution among sialo, high-mannose-type and asialo complex-type *N*-glycans with good reproducibility; (2) compatibility with HPLC analysis; (3) stability of 2-AA labeled glycans. The isolated glycans by HPLC are available for further structural studies using such as MS^n techniques.

Recently, oversulfated condroitin sulfate (OSCS) in certain lots of heparin preparations was identified as the toxic contaminant responsible for severe side effects following intravenous heparin administration. The United States Pharmacopoeia (USP) and European Pharmacopoeia (Eur. Ph.) announced immediate revision of their monographs for heparin sodium by adding two tests for OSCS based on NMR and CE according to the recommendation by US Food and Drugs Administration (FDA). In addition, these heparin preparations were often contaminated with dermatan sulfate (DC) due to starting biological sources. And various methods for assessment of heparin preparations were developed [208–211]. CE method recommended by FDA employs a bare fused silica capillary and 36 mM sodium phosphate (pH3.5) as the separation buffer. However, the method afforded poor resolution among OSCS, heparin and DS. Somsen et al. modified the FDA method using the high-concentration electrolyte (850 mM Tris phosphate pH 3.0) and gave good resolution of these materials [211].

Combinations of CE with MALDI-MS with an automatic spotting device were reported [212]. Tegeler et al. connected CEC with MALDI MS, and analyzed *N*- and *O*-glycans derived from human bile salts stimulated lipase [179]. For the hyphenation of CE to ESI-MS, a number of interfaces have been described as reviewed by several

authors [213–215]. Most of the commercially available interfaces consist of a coaxial triple-tube sprayer, the inner-most tube being the separation capillary, the middle tube the sheath-flow delivering capillary and the outermost the spray gas tube. Non-volatile running buffers containing polymers of high-molecular masses are not available, but volatile buffers (typically formic acid–ammonia buffer) have to be employed, although high-resolution separation of glycans is often compromised. CE-ESI-MS to glycan profiling in the biomedical filed has recently been highlighted [169,216]. Lynn et al. applied CE-ESI MS to the analysis of *N*-glycans from rmAb. They achieved high resolution analysis of *N*-glycans using volatile buffers, and connected ESI-MS with CE-LIF in the online manner [73]. Nakano et al. applied CE-ESI MS to the analysis of the *N*-glycans labeled with Fmoc, and succeeded in rapid mapping of *N*-glycans from rmAb and α-fetoprotein of different biological sources. The total analysis time including an enzymatic glycan releasing reaction was within 5h [85]. The proposed method will be useful for routine work for validation of glycoprotein pharmaceuticals and disease biomarkers.

Over the past years, miniaturization of analytical devices has been the focus of attention and has led to development of microchip electrophoresis (ME). Glass, quartz, and polymethylmetacrylate (PMMA) are used as the material for microchip, and electrophoresis is performed in microchannels fabricated on chips. ME has extremely high throughput ability for the analysis of glycans. Dang et al. succeeded in separation of N-glycans from α1-acid glycoprotein and immunoglobulin within 80 s by ME [217]. Zhuang et al. achieved profiling of *N*-glycans from total serum proteins in 3 min [218]. Callewaert also developed a profiling method for *N*-glycans from total serum proteins by ME [219]. They compared the *N*-glycan profiles between chronic hepatitis and chirrhosis patients. Matsuno et al. applied ME to analyze GAGs, and also GAG-derived glycans and unsaturated disaccharides in 3 min by ME [220,221].

8. Micro arrays for glycan analysis

Glycomics, studies on glycans and glycan-binding proteins in various biological systems, is an emerging field in the post genome and post proteomics era. To understand glycan alterations of proteins, we have to consider (1) total glycan profiles and (2) expression of specific glycans.

Lectin array is one of the technologies for the finding of specific glycans in specific disease and disease state, and a newly developed technique which enables ultrasensitive glycan profiling in a high throughput format. Fukui et al. proposed a fundamental concept for glycan arrays [26]. Lectins have been one of the most important tools in carbohydrate analysis and their utilization in microarray-based technologies has created new opportunities to profile glycan variations [222,223]. A microarray consists of discrete probes immobilized a high-density spot onto a solid support such as glass or nitrocellulose. When a fluorescent analyte was challenged, multiple binding events can be observed simultaneously. High-throughput feature of microarrays combined with a miniaturized format allows parallel analysis of multiple analytes with minimal sample consumption. This is an important issue for precious clinical samples and makes these analytical methods well suited for glycomics [224].

Since the introduction of lectin microarrays in 2005 [28,225,226], the technology has been used for the glycomic analysis of various biological samples including glycoproteins, bacteria, and mammalian cell-surfaces [227–229]. Interesting studies on glycomic comparison of HIV-1 and T-cell microvesicles demonstrated that the overall glycosylation showed clear similarities [230]. Lectin microarrays have been used to profile glycosylaiton of cancer cells by Hirabayashi et al. [231]. Using the Evanescent detection system which is extremely highly sensitive than traditional microarray scanners, they succeeded in observation of fluorescently labeled glycoproteins in formalin-embedded tissue sections of normal and cancer patients. They found that variations in glycan profiles will be used to differentiate between normal and malignant tissues. Because the binding specificities of a number of lectins have been extensively characterized using carbohydrate arrays [25,232] and frontal affinity chromatography (FAC) [233], linkage-specific information on glycome is directly obtained.

A number of profiling methods using array technologies were reported. Pilobello et al. analyzed glycosylation profiles on cell surfaces. Samples differentially labeled with Cy3- and Cy5-dyes were mixed in equal amounts, and used for the analysis by lectin microarray. Competitive binding between the two analytes gives ratiometric data. The use of a common reference addresses issues arising from differences in sample labeling and inherent differences in the lectin activity that can arise from minor alterations in the print conditions and variations observed with different lectin preparations [229]. They analyzed glycans expressed on cell membrane fraction and evaluated the alternation of glycans during differentiation of HL-60 cells [229]. Zheng et al. developed the lectin arrays in microscope detection manner. Fluorescent labeling was not used in their methods, but whole cells were used to observe the interaction against gold plates on which several different lectins were immobilized. After washing, the gold plates were analyzed by microscope [234]. They achieved relative quantification among the spots using imaging software, and applied to glycan profiling of cell surface expressed on normal and human breast tumor. *Sambucus nigra* agglutinin (SNA) interacted with the metastatic sublines more obviously than the parental cell lines. Because SNA shows specificity toward α2,6-linked sialic acid, these results suggest that α2,6-linked sialic acid has some important roles in metastasis. They also found that wheat germ agglutinin (WGA), a Glc*N*Ac specific agglutinin, shows strong affinity toward the bone-metastatic cell lines [235]. These results indicate that tissue-specific homing behavior of cancer cells are correlated with glycan profiling on cancer cells.

Kuno et al. developed a new system for lectin microarray which does not require the washing procedure [28]. The system is based on a unique principle, that is, the Evanescent-field fluorescence-detection principle. An electromagnetic wave, termed an Evanescent field, is propagated only within a wavelength distance from the sensor surface (∼100–200 nm), which means that the Evanescent wave is effective in only 0.02% of the sample solution, providing very low background detection (Fig. 14).

The Evanescent-field method has advantage to analyze relatively weak interactions, because it permits *in situ* detection of ongoing interactions under equilibrium conditions. It means that the procedure does not require washing after a probing reaction. Uchiyama et al. optimized Evanescent-field fluorescence-assisted lectin microarray and achieved high sensitive detection (less than 1 ng/ml of glycoproteins). They also applied the method to profile the glycans on cell surface [236]. Patwa et al. analyzed glycans of serum proteins between normal subjects and pancreatic cancer patients [237,238]. The glycoproteins from serum samples were isolated by lectin affinity chromatography and then fractionated by HPLC. These fractions are spotted in array format and examined the interactions with various lectins to identify potential glycan changes. In addition, they confirmed the proteins on the array spot by MS/MS analysis.

Antibody-lectin sandwich method was reported. In this method, specific glycoproteins in samples were caught using antibody arrays. Then, their glycosylation profiles were screened by addition of lectins. Chen et al. applied this method to detect the biomarkers for pancreatic cancer, and found that alpha-1-β-glycoprotein

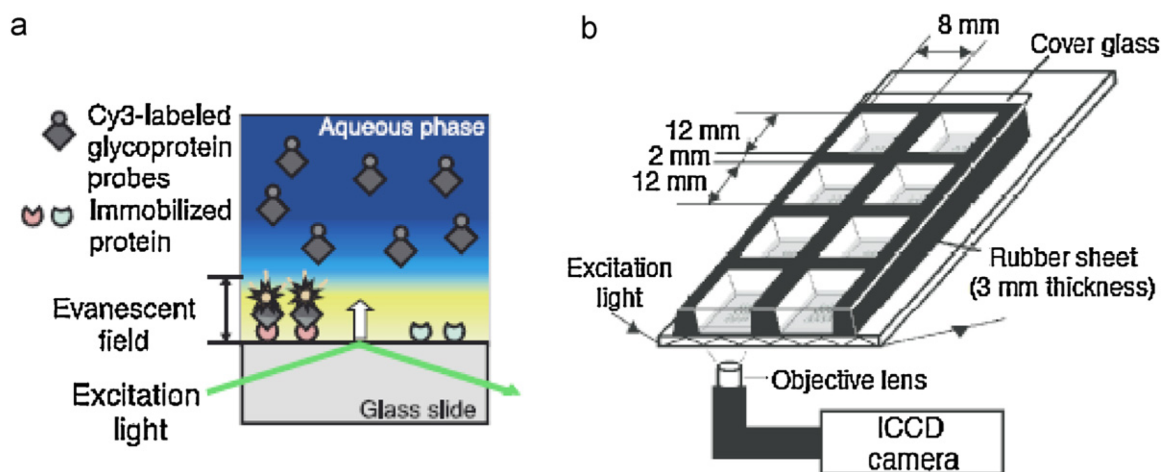


Fig. 14. Evanescent-field fluorescence-detection system. (a) An Evanescent wave is created at the surface of the glass slide by internal reflection of the excitation light, and excites the probes selectively only when they bind to the immobilized lectin on the surface. This surface-confined excitation, which has a limited penetration depth of about 200 nm into the adjacent probe solution, allows specific detection of bound probes under equilibrium conditions without washing steps. (b) Cutting model illustration of an optical scheme for the detection system: Excitation light is irradiated into the edge of the glass slide and propagates by total internal reflection. Eight wells on the slide, which was fabricated by a rubber sheet, allow interaction with 8 different probes simultaneously. Ref. [28] with permission from the publisher.

in pancreatic cancer patient's serum strongly interacted with SNA [239]. These results suggest that lectin glyco-antibody microarrays are a promising approach for the early detection of pancreatic cancer. Kuno et al. reported antibody-overlay lectin microarray. In this method, non-labeled glycoproteins react with the lectin arrays [227]. After the reactions, a target glycoprotein is detected with a biotinylated specific antibody followed by detection with the fluorescently labeled streptavidin. They also developed a method termed as antibody-assisted lectin profiling (ALP) for the analysis of glycosylation of specific glycoproteins expressed on clinical samples as follows: (i) a target protein is enriched from clinical samples by immunoprecipitation with a specific antibody recognizing a core protein moiety; (ii) the target glycoprotein is quantified by immunoblotting using the same antibody used in (i); and (iii) glycosylation difference is analyzed by means of antibody-overlay lectin microarray. They analyzed glycosylation patterns of prostate-specific antigen (PSA) and podoplanin derived from various cell lines by ALP [227].

9. Analysis of glycopeptides by MS after enrichment procedure

The amount of glycans in biological samples is often too minute to determine, and should be enriched from the large amount of biological samples such as serum, biological fluids and tissues.

Glycopeptides enriched from biological samples are analyzed by MS/MS techniques using CID (collision-induced dissociation), IRMPD (infrared multi photon dissociation), ECD (electron-capture dissociation), and ETD (electron-transfer dissociation) [240].Briefly, glycoproteins in samples were digested with proteases such as trypsin, and the glycopeptides from the digested mixture are enriched by lectin affinity chromatography. Concanavalin A (Con A) is often used for enrichment of *N*-glycosylated glycopeptides [22,241,242]. Although Con A has relatively broad specificity, but can not retain triantennary, teraantenary complex-type glycans, and sialoglycans. To collect glycopeptides modified with these glycans, WGA, which interacts with complex-type glycans and sialoglycans is employed [243]. WGA and Jacalin, which bind to Gal*N*Ac-Ser/Thr that is not substituted at C6 are used for enrichment of *O*-glycoproteins [244–247]. SNA is also used for enrichment of sialo-glycopeptides. In addition, to achieve comparative analysis of glycopeptides, multi-dimensional lectin chromatography is used [247,248].

Physical and chemical methods are also useful for enrichment of glycopeptides. Glycopeptides are purified from peptide mixture using size exclusion chromatography, because molecular sizes of glycopeptide are generally lager than simple peptides [249]. Hydrophilicity of the glycans also is used to separate the glycopeptides from the mixture of peptides. Wada et al. enriched glycopeptides based on hydrophilic interaction between glycopeptides and Sepharose [250], and succeeded in differential analysis of glycans on plasma and cellular fibronectins [251]. They also analyzed *O*-glycosylaiton of hinge region of IgA [17] and revealed that *O*-glycosylation was reduced in IgA from the patients suffered from rheumatoid arthritis [252]. On-line HILIC-ESI MS methods using ZIC-HILIC column and amide-based column were applied to analyze glycopeptides [133,253]. Kurogochi et al. reported a unique method to enrich the sialylated glycopeptides [254]. Their method involves selective oxidation of sialic acid residues of glycopeptides to elaborate terminal aldehyde group and subsequent enrichment by chemical ligation with a polymer reagent, namely reverse glycoblotting technique [255].

Structural analysis of clustered glycans such as mucin-type glycoproteins is still difficult even if using the techniques described above. It is highly desirable to develop a method to be able to analyze these glycans.

10. Bioinformatics tools and databases for glycans

Informatics tools and databases for glycobiology and glycomics research have dramatically increased. Three nice reviews on bioinformatics tools are available for structural and biological analysis of glycans [256–258].

Major databases of glycan structures are shown in Table 1. Databases for structures of glycans were initially established at Complex Carbohydrate Structure Database (CCSD) [259,260]. The most important databases are the Kyoto Encyclopedia of Genes and Genomes GLYCAN (KEGG GLYCAN) [261], Consortium for Functional Glycomics (CFG) [262], GLYCOSCIENCES.de [263], Bacterial Carbohydrate Structure DataBase (BCSDB) [264], and EuroCarbDB [265].

CCSD called as CarbBank was developed by Complex Carbohydrate Research Center (CCRC) at the University of Georgia. The main aim of CarbBank was to find all publications in which specific carbohydrate structures were reported [259,264]. However, since the funding stopped during the second half of the

Table 1
Glycan structural data base.

Name	URL	Refs.	Characteristics
Complex Carbohydrate Structure Database (CCSD, CarbBank)	http://www.boc.chem.uu.nl/sugabase/carbbank.html	[259,260]	• Update stopped
Encyclopedia of Genes and Genomes GLYCAN (KEGG GLYCAN)	http://www.genome.jp/kegg/glycan/	[261]	• Enzyme database • Pathway map • CBP database
Consortium for Functional Glycomics (CFG)	http://www.functionalglycomics.org/static/index.shtml	[262]	• Mammalian glycan database • CBP database • MS and microarray database • Enzyme database
GLYCOSCIENCES.de	http://www.glycosciences.de/	[263]	• 3D modeling tools • Statistically analysis tools • NMR and MS database
Bacterial Carbohydrate Structure DataBase (BCSDB)	http://www.glyco.ac.ru/bcsdb3/index.html	[264]	• Bacterial glycan database.
Eurocarb DB	http://www.ebi.ac.uk/eurocarb/home.action	[265]	• Tools for MS, HPLC and NMR analysis • MS, HPLC, and NMR data base
Glycome DB	http://www.glycome-db.org/About.action	[268,269]	• Meta data base • Defining glycan structure format based on XML (GlycCT)
Japan Consortium for Glycobiology and Glycotechnology DataBase (JCGGDB)	http://jcggdb.jp/	–	• Comprehensive glycan database established in Japan (meta data base)
GlycoSuiteDB	http://glycosuitedb.expasy.org/glycosuite/glycodb	[279,280]	• Glycoprotein glycan structures

1990s, CarbBank was no longer updated. KEGG GLYCAN provides the database of not only glycan structures but also the glycan-related enzymes and carbohydrate binding proteins (CBP). The biosynthetic and metabolic pathway maps for glycans are also available. These data in KEGG GLYCAN is linked to KEGG's various other genomic data, particularly genes or enzymes which catalyze reactions for synthesizing the corresponding glycan [261]. GLYCOSCIENCES.de was established by the German Cancer Research Center (DKEZ). The glycan structures in this database can be retrieved according to the structure, mass, and chemical shift of NMR spectra. A special feature of GLYCOSCIENCES.de is that it contains the information on not only the two dimensional structure but also the three dimensional structure of glycans. This database also provides the statistically analyzing tools and three dimensional modeling tools for glycans and glycoproteins [263]. The aim of the BCSDB is to provide a database of structural, bibliographic and related information on bacterial carbohydrate structures. The search criteria can be fragments of the structures, chemical sift of NMR, and indexed tags, including microorganisms, bibliography and keywords [264]. CFG database was built starting from mammalian structures in CarbBank and structures obtained from a private database (developed by Glycominds Ltd., Lod, Israel). CFG also provides the database of carbohydrate binding proteins (CBP) and glycan-related enzymes. In addition, MS spectra and microarray profiling of glycans expressed in cells and tissues can be seen in this database [262]. EuroCarbDB provides the software and standards for the systematic collection of carbohydrate structures and their experimental data [266,267].

These databases are developed independently, and different manners of structure expression are employed. Data exchanges among the databases are still at the infant stages, and routine data exchanges as in nucleotide and protein databases are not available. Recently, Glycome DB, meta-database for public glycan database, was developed by German Cancer Research Center Heidelberg [268,269]. Glycome DB analyzes all the existing public databases and define a sequence format based on Extensible Markup Language (XML, GlycoCT) capable of storing all structural information of carbohydrate sequences [265]. Thus, it is possible to get an overview of all glycan structures in the different databases and to crosslink common structures in the different databases by using Glycome DB. CCRC developed GLYDE-II, in which glycan structures are defined with XML [270]. GLYDE-II is being accepted as the standard format for the exchange of glycan structure data [271].

Bioinformatics tools for structural analysis of glycans are shown in Table 2. Glycobase and GALAXY express retention times of glycans on HPLC, and they are expressed as glucose units (GU) that are calculated by comparing retention times of dextran standard. GU values of the objective glycans in samples are compared with those of the standard glycans in these databases. We have to pay attention in derivatives of glycans, because retention times are significantly changed depending on the glycan derivatives. GALAXY was established to analyze PA (2-aminopyridine) labeled glycans. In contrast, 2AB (2-aminobenzamide) labeled glycans are used in Glycobase. Royle et al. developed high throughput method for the analysis of N-glycans from sera by using Glycobase [36]. CASPER and NMR database in GLYCOSCIENCES.de is useful for interpretation of NMR spectra [263,272].

Table 2
Bioinformatics tools for analysis of glycans.

Tool name	URL	Refs.	Characteristic of tools
Tools for MS analysis			
Glyco-peakfinder	http://www.ebi.ac.uk/eurocarb/gpf/Introduction.action	[266]	• Peak Annotation
GlycoWorkbench	http://www.ebi.ac.uk/eurocarb/gwb/home.action (Software download page)	[267,282]	• Peak annotation • In silico fragmentation
Glycofragment	http://www.glycosciences.de/tools/GlycoFragments/fragment.php4	[283]	• In silico fragmentation
GlycoMod	http://www.expasy.org/tools/glycomod/	[278]	• Peak annotation
GlycanMass	http://www.expasy.org/tools/glycomod/glycanmass.html	-	• Molecular weight calculation
GlycoPep DB	http://hexose.chem.ku.edu/sugar.php	[284]	• Peak annotation • Focusing glycopeptides
Cartoonist	http://www.parc.com/work/focus-area/mass-spectra-analysis/	[281]	• Peak annotation • Focusing N-glycans
Tools for HPLC analysis			
GlycoBase	http://glycobase.univ-lille1.fr/base/	[36]	• Peak annotation • GU unit value • Focusing 2AB labeled glycans
GALAXY	http://www.glycoanalysis.info/ENG/index.html	[156]	• GU units value • Focusing PA labeled glycan • 3D Map
Tools for NMR analysis			
CASPER	http://www.ebi.ac.uk/eurocarb/casper.action	[272]	• Signal annotation
NMR data base in Glycosciences.de	http://www.glycosciences.de/sweetdb/start.php?action=form_shift_estimation/	[263]	• Chemical sift (1H,13C-NMR) search
Tools for analysis of glycosylation sites			
GlySeq	http://www.glycosciences.de/tools/glyseq/	[273]	• Statistical analysis of glycoprotein sequences
NetNglyc	http://www.cbs.dtu.dk/services/NetNGlyc/	[276]	• Prediction of N-glycosylation sites
NetOglyc	http://www.cbs.dtu.dk/services/NetOGlyc/	[274,275]	• Prediction of O-glycosylation sites
GlyProt	http://www.glycosciences.de/modeling/glyprot/php/main.php	[277]	• In silico glycosylation of proteins

Glyseq, NetNGlyc, NetOGlyc and Glyprot support analyzing glycopeptides and glycoproteins. Glyseq can find the frequency of glycosylation on potential sites by statistical analysis of the amino acids surrounding the glycosylation sites based on protein sequence from SwissProt and Protein Data Bank (PDB) [273]. NetNGlyc and NetOGlyc predict the glycosylation sites of glycoproteins and glycopeptides using neural network model system [274–276]. Prediction tools for various kinds of glycans are provided by The Center for Biological Sequence Analysis at the Technical of Denmark University (CBS). GlyProt evaluates whether a potential *N*-glycosylation site is spatially accessible, and generates reasonable three-dimensional models of glycoproteins with user-definable glycan moieties. In addition, this tool indicates how the physicochemical parameters such as molecular weight, radius of gyration and polar and charge of solvent accessible surface area are changed among glycoforms of a protein [277].

GlycoMod, Cartoonist, Glyco-peakfinder and Glycoworkbench are useful for interpretation of MS spectra. GlycoMod is the web-based free access to compositional analysis of glycans [278], and is designed to calculate all possible compositions of a glycan structure from the experimentally determined molecular ions. Several derivatizations and reducing-end modifications can be taken into account. Moreover, this tool is able to compute glycopeptide compositions. The derived compositions are used to search for matching structures in GlycoSuiteDB [279,280]. Cartoonist was developed for the automated annotation of MS spectra of *N*-glycans [281]. This tool only considers the glycans that are possibly synthesized by mammalian organisms. The web-based tool "Glyco-Peakfinder" is programmed for assignment of glycan compositions, which is intended to be entirely a *de novo* platform for compositional analysis, and is used for assignment of all types of fragment ions including monosaccharide cross-ring cleavage products and multiple charged ions. It also accepts computation of derivatized carbohydrate molecules by permethylation, peracetylation, perdeuteromethylation, acetylation and reducing-end modifications [266]. Moreover, reduced glycans and glycoconjugate such as glycopeptide and glycolipid can be handled. The derived compositions are used to search for matching structures in GLYCOSCIENCES.de.

GlycoWorkbench was developed for annotation of MS spectra and simulation of fragmentation in MS/MS analysis (In silico fragmentation) as shown in Fig. 15 [267,282]. The main component of

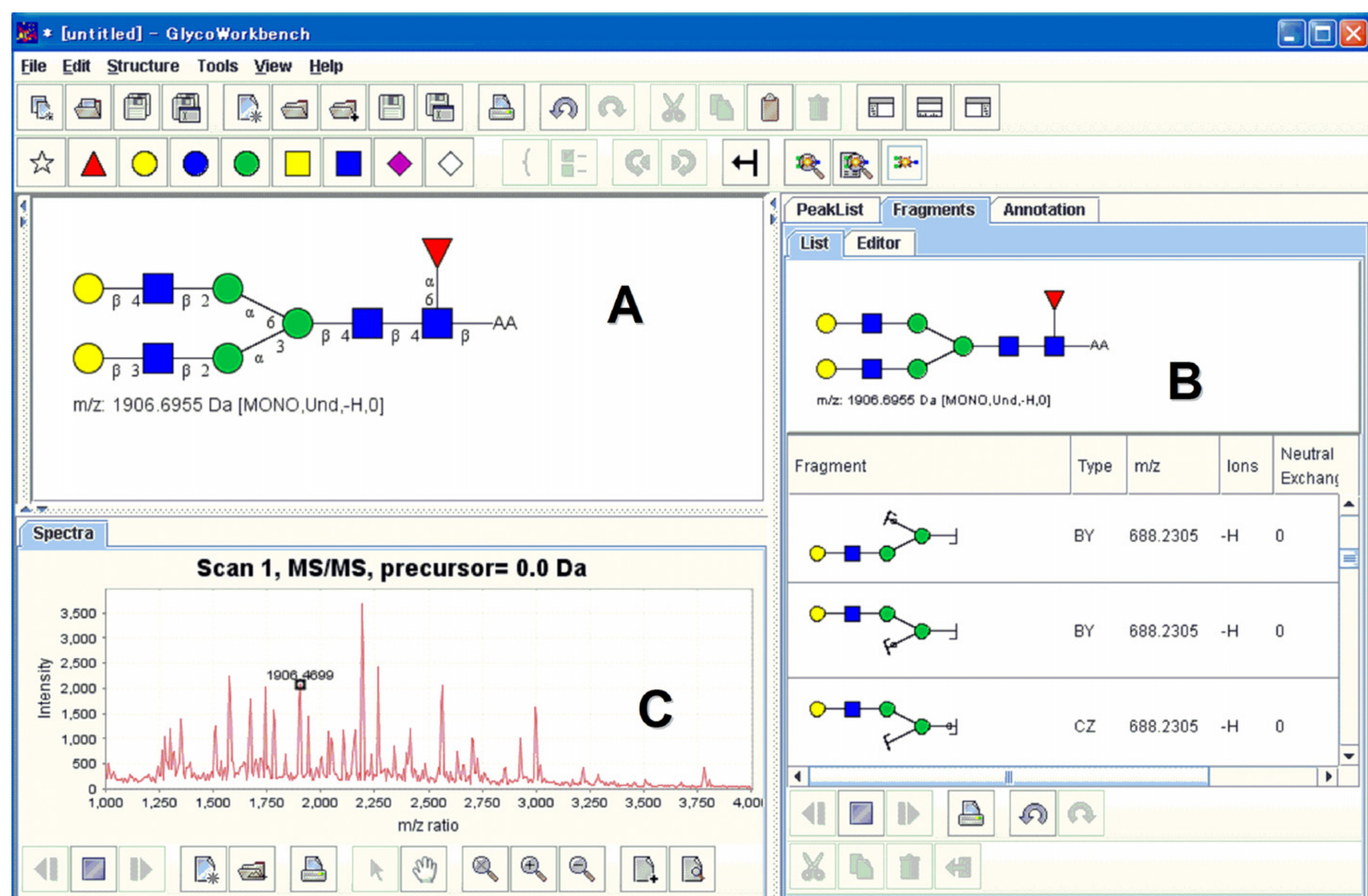


Fig. 15. 1Analysis of MS spectra by GlycoWorkbench The glycan drawing canvas (A), the peak list and fragmentation list panel (B), and the spectra panel (C) are shown.

GlycoWorkbench is GlycanBuilder, which is a flexible visual editor especially designed for a rapid input of glycan structures (Fig. 15A). Users can make the arbitrary glycan structures in this space and simulate the MS/MS fragmentation (Fig. 15B). This software can simulate all topologically possible fragmentations of the precursor molecular ion, applying both multiple glycosidic cleavages and cross-ring fragmentations, to cover the broadest possible range of conditions. In addition, experimental MS and MS/MS spectra can be imported to this software (Fig. 15C) and assigned the glycan structure and fragment ions. The derived compositions and observed molecular ion peaks are used to search for matching structures in CFG, Carbbank, Glycosciences.db and GlycomeDB.

Systems of annotation in these softwares and tools still largely depend on user's expertise. These softwares and tools are being improved to achieve the high throughput and automatic annotation.

11. Future perspectives

In this review, brief history and recent advances in the strategies for glycan analysis in biomedical and clinical researches are reviewed. The most important techniques for glycome and glycomics are a combination of separation techniques with high resolving ability and MS technologies. Especially, development in MS has greatly improved to investigate glycan mass profiling in clinical samples. In addition, lectin/glycan microarray techniques reveal biological significances of glycans in physiological conditions and disease states.

Glycans in glycoproteins and proteoglycans are extremely heterogeneous. For practical clinical use of characteristic glycan(s) in specific glycoconjugate(s) at the early stage of diseases, we have to determine them at extremely low concentration level (maybe less than 10^{-18} mol/ml). To realize identifying such minute amount of glycans, we have to enhance 10^2–10^3 times enhancement in sensitivity. However, development in the newly developing technologies will solve this problem in the near future.

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