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RESEARCH PAPER

Analysis of native and APTS-labeled N-glycans by capillary electrophoresis/time-of-flight mass spectrometry

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Abstract The glycosylation of proteins is of particular interest in biopharmaceutical applications. The detailed characterization of glycosylation based on the released carbohydrates is mandatory since the protein stability, folding, and efficacy are strongly dependent on the structural diversity inherent in the glycan moieties of a glycoprotein. For glycan pattern analysis, capillary electrophoresis with laser-induced fluorescence using 8-aminopyrene-1,3,6-trisulfonic acid (APTS)-labeled glycans is used frequently. In this paper, a robust capillary electrophoresis–mass spectroscopy method both for the analysis of APTS-labeled glycans and unlabeled charged glycans is presented. The background electrolyte consists of 0.7 M ammonia and 0.1 M ε-aminocaproic acid in water/methanol 30:70 (*v*/*v*). High separation efficiency including separation of structural isomers was obtained. The method was validated in terms of reproducibility and linearity. Submicromolar sensitivity is achieved with linearity up to 24 μM. The ability to analyze APTS-labeled, as well as unlabeled, charged glycans enables the determination of labeling and ionization efficiency: APTS-labeled glycans show a factor of three better ionization efficiency compared to non-labeled native glycans. The presented method is applied to the analysis of pharmaceutical products. Furthermore, the system can be applied to the analysis of 2-ANSA-labeled glycans, though separation efficiency is limited.

Keywords APTS · 2-ANSA · CE-MS · Glycan · Glycosylation

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Abbreviations

AGP	α_1-Acid glycoprotein
2-ANSA	2-Aminonaphthylsulfonic acid
APTS	8-Aminopyrene-1,3,6-trisulfonic acid
EACA	8-Aminocaproic acid
CFG	Consortium for Functional Glycomics
CSE	Capillary sieving electrophoresis
SL	Sheath liquid

Introduction

The development of protein-based pharmaceutical products has increased in recent decades, due to their high potential to selectively treat diseases. Many pharmaceutically relevant proteins including antibodies are glycosylated. The study of glycosylation can be performed on different principles. Next to intact protein and glycopeptide characterization, the detailed characterization of glycosylation based on released carbohydrates is mandatory since the protein stability, folding, and efficacy are strongly dependent on specific glycosylation [1–6]. The analysis of N-glycans based on capillary electrophoresis with laser-induced fluorescence (CE-LIF) is widespread and a fast tool in the pharmaceutical industry [7–9]. In this context, derivatization is mandatory for glycans with respect to separation (permanent charge) and detection (fluorescence) [10]. The most abundant derivatization reactions in this field are based on a reductive amination using the reducing glycan termini; thereof, 8-aminopyrene-1,3,6-trisulfonic acid (APTS) is one of the most popular labeling agents [11–13]. Furthermore, commercially available analysis kits promote widespread application. CE-LIF for APTS-labeled glycans often uses pseudogels, which are high-viscosity compounds that achieve sieving effects (i.e., separation due to size) [7, 14]. These capillary sieving electrophoresis (CSE—term is used

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according to IUPAC nomenclature [15]) methods with LIF detection provide high resolving power, i.e., separation of structural isoforms and short analysis time combined with highly sensitive detection. However, since the direct confirmation and characterization of glycans is not possible by LIF detection, an alternative detection technique is required. The combination of CE and mass spectrometry is a powerful tool to identify unknown glycan species [13, 16–20]. However, the common CSE-LIF methods are not compatible with mass spectroscopy (MS) detection, since applied pseudogels and electrolytes as phosphates are not volatile. Few CE-MS methods using APTS-labeled glycans have been presented [11, 20–23]. However, the separation efficiencies are either low or difficult to compare as different glycans have been analyzed. The acidic CE-MS system by Gennaro and Salas-Solano and Liu et al. [11, 22] presents high separation efficiency (i.e., G1F and G1F′ can be separated, see structure in Fig. 1) comparable to the here-proposed method. The presented CE-MS methods use acidic background electrolyte (BGE) systems, e.g., based on formic acid [21, 23] or ε-aminocaproic acid (EACA) [11, 20, 22]. Acidic CE-MS systems with negligible electroosmotic flow (EOF) might be sensitive to pressure influences (i.e., siphoning effects, nebulizer gas flow, etc.) and bear the risk of air or SL intake or creation of a moving ion boundary from outlet to inlet. CE-MS systems creating an EOF in MS direction as basic BGE systems can prevent these effects. However, CE-MS methods using basic BGEs have been developed for the analysis of native glycans [24–26].

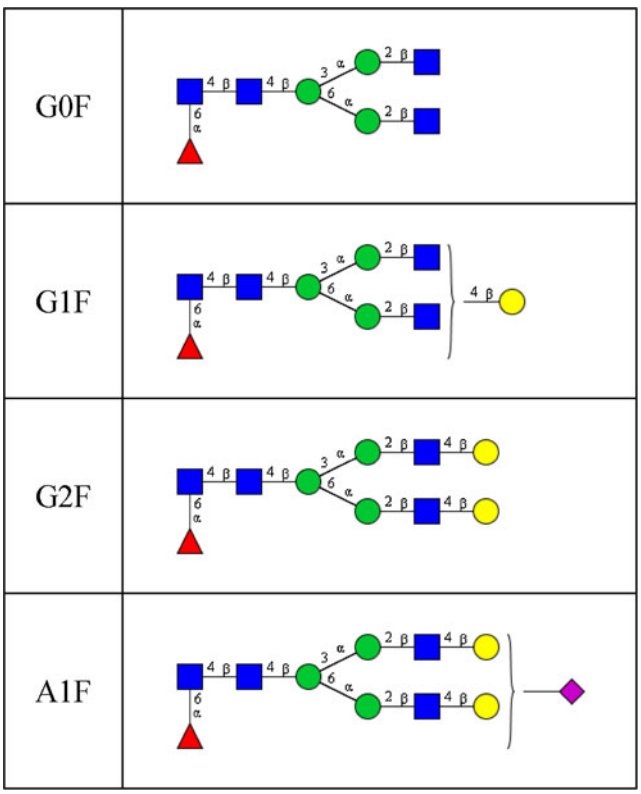


Fig. 1 Structures of all glycan standards used in this work. The glycan structures are displayed according to the CFG nomenclature: *blue squares*, *N*-acetylgalactosamine; *green circles*, mannose; *yellow circles*, galactose; *red triangles*, fucose; *violet diamond*, *N*-acetylneuraminic acid

Here, a robust CE-MS method based on a basic electrolyte with cathodic detection for APTS-labeled glycans as well as charged native glycans was developed and validated. The basic BGE creates a strong EOF towards the MS, thus, carries the negatively charged native glycans (bearing sialic acids, sulfonic acids, or phosphates) as well as APTS-labeled glycans to the MS, resulting in high resolution power. As indicator for acceptable separation efficiency, the resolution of the two isomeric glycans of G1F is chosen, as these glycans are often abundant in antibodies. G1F-glycans are asialo-, biantennary complex-type N-glycans with one galactose attached to either end of the antennae (Fig. 1). Additionally to APTS-labeled glycans, the 2-ANSA label was applied on this CE-MS method. As shown for 5-ANSA, this type of label is applicable to HPLC, CE, and—in contrast to APTS—also to matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [27, 28]. However to our knowledge, no CE-MS method has been presented.

Materials and methods

All solutions were prepared using ultrapure water (18 MΩ cm at 25 °C, SG Ultra Clear UV from Siemens Water Technologies, USA). 2-Propanol, methanol (each LC-MS grade), acetic acid (p.a.), formic acid (p.a.), sodium hydroxide (p.a.), and aqueous ammonia (30 % p.a.) were obtained from Carl Roth GmbH und Co. KG (Karlsruhe, Germany). Ammonium acetate (p.a.), ammonium carbonate (p.a.), EACA, sodium cyanoborohydride ($NaCNBH_3$) in 1.0 M tetrahydrofuran, and citric acid monohydrate (p.a.) were obtained from Sigma-Aldrich (Steinheim, Germany). "ES Tuning mix" solution was obtained from Agilent Technologies (Waldbronn, Germany). Native glycan standards, APTS-labeled, and 2-ANSA-labeled IgG glycans were obtained from Ludger (Abingdon, UK). APTS-labeled glycans of pharmaceutical products were provided by Merck Serono (Rome, Italy). APTS-labeled glycans from bovine α1-acid glycoprotein (AGP) were obtained from Solvias (Basel, Switzerland). APTS-labeled A1F standard was partially labeled as follows: 5 μg of glycan standard was labeled with 1.5 μL APTS solution (20 mM APTS in 0.6 M citric acid), 1.5 μL 0.6 M citric acid, and 1.0 μL 1.0 M $NaCNBH_3$ in tetrahydrofuran for 1 h at 37 °C. After incubation, 21 μL water was added resulting in a final concentration of 96 μM.

CE

CE experiments were performed in fused silica capillaries of 90, 60, or 43 cm length (Polymicro Technologies, AZ, USA) at +30 kV (inlet) on a CE 7100 instrument (Agilent, Waldbronn, Germany). Background electrolytes were based on ammonia and EACA, ammonium acetate or ammonium carbonate, each containing a high percentage of methanol as described in the text. After optimization, the final BGE contained 0.1 M EACA and 0.7 M ammonia in 30:70 water/methanol with an apparent pH of 10. The capillary was flushed with methanol, 1 M sodium hydroxide, and water before the first use or after storage. The sample was injected hydrodynamically by the application of a pressure of usually 50 mbar for 12 s. The injection was followed by dipping the capillary into a water vial in order to prevent contamination of the BGE. For CZE-MS coupling, a coaxial sheath–liquid interface was used (Agilent Technologies). Sheath liquid of 2-propanol/water 1:1 (*v*/*v*) is supplied by syringe pump at a flow rate of 4 μL/min.

MS

MS experiments were performed on a Q-TOF instrument (microTOFQ, Bruker Daltronik, Bremen, Germany). Electrospray ionization was used in negative ion mode applying 4.0 kV on the MS inlet (sprayer on ground). The flow rate of dry gas (nitrogen) was 4.0 L/min at a temperature of 170 °C. The nebulizer gas pressure was set to 0.2 bar. Spectra were acquired in a mass range of *m*/*z* 300–2,500 at a rate of 1 Hz (summation of 5,000 single spectra). An external calibration was performed by infusing tuning mix solution at 4 μL/min every morning. As post-processing, the data were internally calibrated based on observed sodium–EACA clusters. The identification of glycans was based on the accurate mass of doubly and triply charged glycans (mass deviation <5 ppm). Data analysis was performed using DataAnalysis Version 4.0 from Bruker Daltronik (Bremen, Germany). The identification could be verified by comparison of the isotopic distribution, i.e., overlaying the simulated mass spectra with the measured mass spectra. All glycan structures are displayed using the simplified Consortium for Functional Glycomics (CFG) nomenclature: square, *N*-acetylhexosamine; circle, hexose; trangle, fucose; diamond, sialic acid.

Results and discussion

Method development

Starting point of the development was a method for the analysis of native glycans using a BGE of 0.1 M EACA and 0.9 M ammonia with methanol in a ratio of 30:70 [24]. Since the application of this method to APTS-labeled glycans resulted in broad peaks and reduced separation, an appropriate method was developed. The BGE contained ammonia, EACA, and methanol at varying concentration. The decrease of ammonia concentration in BGE from 0.9 to 0.7 M improves the resolution of the APTS-labeled glycans, while peaks got sharper and the intensities increase. Further reduction of ammonia results in longer analysis time, with hardly any improvement in separation efficiency. In a range of 50–70 % methanol, a higher percentage of methanol enhances the peak resolution and intensities, while increasing the analysis time. In the proposed method, a concentration of 70 % of methanol was chosen in order to separate isomeric glycans. Lower methanol content can be applied if shorter analysis time is required. Reduction to 50 % methanol shortens the analysis by a factor of 1.5.

Alternative background electrolytes as 100 and 50 mM ammonium acetate and 50 mM ammonium carbonate each in 50 % methanol/water (*v*/*v*) were tested. However, no acceptable separation (i.e., low resolving power and broad peaks) could be achieved. Finally, the separation was performed using a BGE of 0.7 M ammonia and 0.1 M EACA in 70 % aqueous methanol in bare fused silica capillaries with an apparent pH of 10 (measured with water-based calibration of pH electrode).

The presented method offers the possibility to analyze APTS-labeled in parallel with charged, unlabeled glycans as sialylated glycans (negatively charged in alkaline BGE). Figure 2 shows the base peak electropherogram of α1-acid glycoprotein glycans (AGP, bovine), which were partially labeled by APTS. The charged native glycans (22–31 min) are clearly separated from the APTS-labeled glycans (31–45 min). The main peaks are biantennary N-glycans of complex type with different amounts of sialic acids as annotated in Fig. 2 being detected both as labeled and unlabeled species. The non-labeled biantennary glycan without sialic acid is uncharged, thus migrates with the EOF and was not detected by ESI-MS. This example presents nicely the ability of the new method for parallel separation of charged native and APTS-labeled glycans.

Separation efficiency

The CSE-LIF methods provide high separation efficiency, which is important for glycan analysis in the biopharmaceutical sector. Therefore, an important method criterion for CE-MS is high separation power, e.g., the ability to separate structural isomeric glycans similar to CSE-LIF. The separation of the two isoforms of G1F, as abundant structural isomers in protein-based biopharmaceuticals, was examined as marker for efficient structural isomer separation. The isomers were separated due to their different effective size (charge due to dissociation of APTS is expected to be

identical). The 1,3-linked isoform showed shorter migration time than the 1,6-linked glycan. A resolution of 1.2 was obtained for G1F of a recombinant fusion protein using the proposed CE-MS system, though in CSE-LIF a resolution of 1.8 could be achieved using the method of Ruhaak et al. [29] (data not shown). The differences in CE-MS and CE-LIF were probably caused by sieving effects of the pseudogel electrolyte in CSE-LIF as well as different BGE compositions (e.g., pH, solvation of the analytes, viscosity).

The overall separation efficiency is demonstrated for a complex glycan mixture of a therapeutic antibody. The 15 glycans are baseline separated (compare Fig. 5, depicted later), including further pairs of structural isomers beside G1F.

Validation

Reproducibility experiments based on APTS-labeled glycans from a recombinant fusion protein were conducted in 12 runs over 5 months. The measurements were analyzed in terms of variance in migration time, relative peak ratio, and separation efficiency based on the four main glycan species G0F, G1F, G1F′, and G2F (see structures Fig. 1). Different capillaries with same capillary lengths (90 cm) were applied. The relative standard deviations of migration time, relative peak ratio (intensity) for the four main glycans (G0F, G1F/G1F′, and G2F) and the resolution based on the FWHM of G1F/G1F′ were determined, as listed in Table 1. The three parameters show an acceptable precision for the characterization of glycans.

The linearity was determined using APTS-labeled A1F in a concentration range of 1.5–48 μM. A1F is a monosialylated, core-fucosylated biantennary complex-type N-glycan (Fig. 1). This naturally charged glycan was chosen since the actual concentration of APTS-labeled glycan (i.e., corrected for labeling efficiency) could be determined using the approach described in the following paragraph. A concentration of 48 μM APTS-labeled A1F in stock solution was calculated. Based on this stock solution, all calibration standards were diluted. Instead of a 90-cm capillary length as used for separation, here, a length of 43 cm is used, since there is no need of high separation efficiency in this purpose and analysis time can be reduced. However, a comparison of peak width and intensity in 43 to 90-cm-long capillaries was performed to ensure the applicability of 43 cm capillary length in this context. Furthermore, about the same amount of analyte was injected (10 nL of sample, injection 6 s instead of 12 s at 50 mbar) to ease the transfer to standard separation conditions. Each concentration was analyzed twice; the calibration plot is displayed in Fig. 3.

The calibration regression factor of 0.9988 was calculated in a range of 1.5–24 μM. At a level of 48 μM, the calibration curve is no longer linear, as shown in Fig. 3. A limit of detection of 0.2 μM (1.9 fmol injected) and a limit of quantitation of 0.7 μM (6.7 fmol injected) was calculated based on signal-to-noise-ratio of 3 and 10, respectively. Thereof, a total amount of about 1 pmol of glycan arises from the required sample amount of about 5 μL in CE. The dynamic range of almost 2 orders of magnitude is appropriate for the application to glycan identification of biopharmaceutical products as concentrations are frequently in the nanomolar to low micromolar range and quantitation is usually not performed by CE-MS but rather by CE-LIF.

Ionization and labeling efficiency of APTS-labeled glycans

The presented method enabled the determination of labeling efficiency for APTS-labeled sialylated glycans, since the unlabeled glycans were detectable in parallel. Also, the different ionization behavior of APTS-labeled to unlabeled glycans could be determined. Therefore, a native A1F glycan sample was measured first. Afterwards, the same sample solution was partially APTS-labeled (at 37 °C for 1 h, see

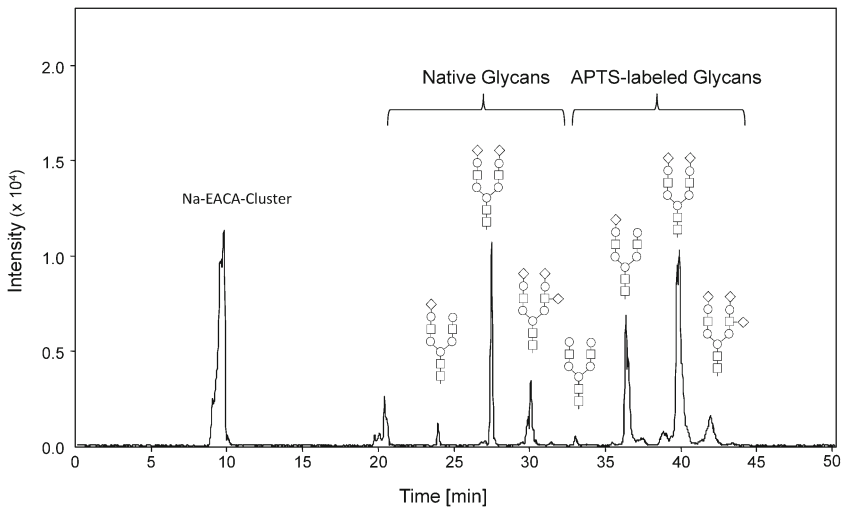


Fig. 2 Base peak electropherogram of APTS-labeled glycans from bovine AGP separated in fused silica capillary of 86 cm length applying the proposed basic BGE system (0.1 M EACA and 0.7 M ammonia in 30:70 water/methanol). The main glycans are annotated: *squares*, *N*-acetylhexosamine; *circles*, hexose; *triangles*, fucose; *diamonds*, sialic acid

Table 1 Precision based on the main APTS-labeled glycans (G0F, G1F, and G2F) of a recombinant fusion protein. Twelve analyses over 5 months were performed according to the proposed method (see Fig. 2) using capillaries of 90 cm length

	Mean	RSD [%]
Relative peak ratios		
G0F/G1F	1.4	5.9
G1F/G1F'	4.3	7.9
G1F/G2F	2.7	11.2
G0F/G2F	1.9	8.3
Migration time		
G0F	1,575 s	3.1
G1F	1,629 s	3.8
G1F'	1,651 s	4.0
G2F	1,704 s	2.6
Resolution		
G1F/G1F'	1.2	15.4

"Materials and methods") to obtain a solution with native and labeled glycans. The unlabeled amount of A1F in the labeled sample was quantified based on the first analyzed native sample. Assuming the rest to be labeled, the ionization efficiency could be calculated. Figure 4 shows the electropherograms of the analyzed native and labeled A1F sample. About 4 % of the A1F standard was found to be desialylated (A1F → A0F) during the labeling process (peak 3). Two percent desialylated A1F was observed at the peak of A1F obviously related to a desialylation in the mass spectrometer. The labeled peak area was calculated by summing up all labeled peak areas, sialylated and desialylated A1F, assuming that the error caused by differences in ionization efficiency is negligible. The native A1F sample and the APTS-labeled solution of A1F were analyzed twice within some weeks. Table 2 shows the areas of unlabeled, expected labeled (assuming the same response as unlabeled), and detected labeled peak area related each to the unlabeled native A1F sample. The ionization of the APTS-labeled glycan (here, A1F) was a factor of 2.9 enhanced compared to the non-labeled glycan.

The calculated labeling efficiency was about 44 % for the A1F standard using the described labeling procedure. For pharmaceutical products, the labeling procedure should be adjusted dependent on the containing glycans. Highly sialylated glycan mixtures are usually treated carefully (at 37 °C) to avoid loss of sialic acids [12]. Hence, for provided pharmaceutical products with sialylated glycans, a labeling efficiency of around 50 % was determined. For glycan mixtures containing no sialic acids, the labeling reaction time could be expanded and the reaction temperature decreased (60 °C); thus, a labeling efficiency of 80–90 % was determined.

Application to pharmaceutical products

The method was successfully applied to identify and characterize the APTS-labeled glycans of three different pharmaceutical products: a therapeutic antibody, a recombinant fusion protein, and a cytokine-based product. The identification of glycans was based on accurate mass of doubly and triply charged glycans with an acceptable mass deviation (<5 ppm). In this way, TOF/MS enabled the calculation of possible glycan compositions; however, neither the differentiation between isobars as different hexoses or hexosamines nor linkage determination was possible. MS/MS experiments might allow the determination of glycan sequence by cleavage of glycosidic bonds. However, for APTS-labeled glycans, cross-ring cleavages are rare; hence, information about linkage position are missing [19]. For all three applied

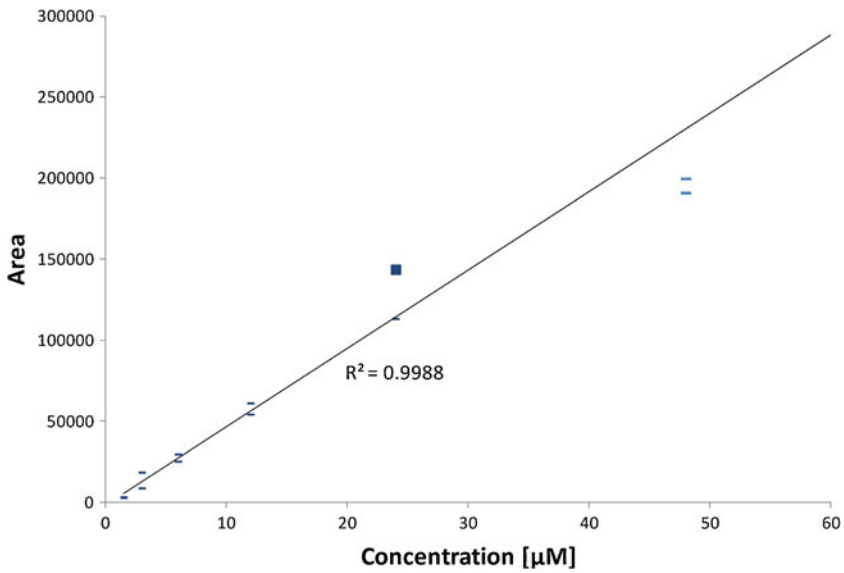


Fig. 3 Calibration plot of APTS-labeled A1F in a range of 1.5–48 µM. The regression curve is calculated up to 24 µM. The second value of 24 µM (*filled square*) is defined as outlier

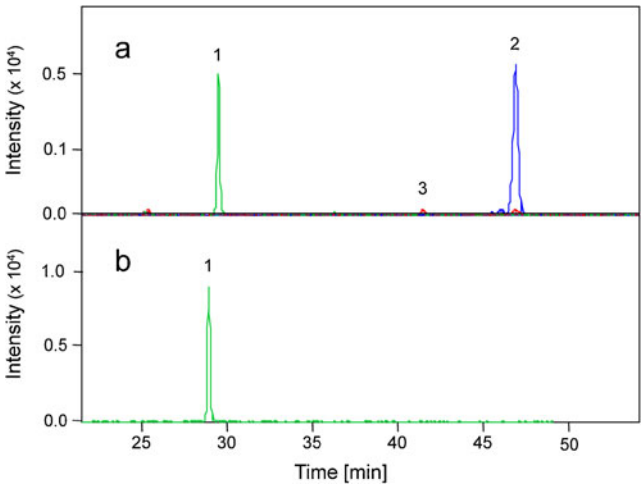


Fig. 4 Extracted ion electropherogram (EIE) of unlabeled A1F (*1*), APTS-A1F (*2*), and desialylated APTS-A1F (*3*) (using the doubly, triply, and fourfold charged glycans). a EIE of an APTS-labeled A1F sample; b EIE of unlabeled A1F sample (48 μM A1F each). Experiments were performed applying the proposed CE-MS method using a capillary length of 90 cm (BGE as given in Fig. 2)

biopharmaceutical products, the released APTS-labeled glycan pool was analyzed. Thirteen glycans were derived from the recombinant fusion protein and seven glycans from the cytokine product. The applied therapeutic antibody bears the most complex glycan pool with 15 different glycan structures including three structural isomer pairs. Therefore, as an example, the analysis of the antibody-derived glycans is presented in Fig. 5. The overlaid, extracted ion electropherograms of all characterized glycans demonstrates the high resolving power of the proposed method for complex glycan compositions of biopharmaceutical samples. The APTS-labeled glycan pool was in parallel analyzed by CSE-LIF (not shown). The migration order was generally reversed due to the cathodic instead of anodic detection. All glycans determined by CSE-LIF (method by Ruhaak et al. [29]) were found also by CE-MS as annotated in Fig. 5. Three so far not identified glycans could be characterized by CE-MS.

Table 2 The relative peak areas of unlabeled glycan standard in two labeled samples are normalized to the separately analyzed native sample (line 1). Assuming the rest of A1F labeled, the relative labeled peak areas are calculated (line 2). The detected peak area of APTS-labeled A1F related to the native sample (line 3). The resulting ionization enhancement factor for APTS-labeled glycan is shown in line 4. Experiments were performed applying the proposed CE-MS method using a capillary length of 90 cm (BGE as given in Fig. 2)

	Labeled sample 1	Labeled sample 2
Area of unlabeledA1F	54	59
Expected area of labeled A1F	46	41
Detected area of labeled A1F	134	121
Ionization enhancement factor	2.9	2.9

Application to 2-ANSA-labeled samples

As an alternative labeling reagent, 2-ANSA was tested for IgG glycans. As shown for 5-ANSA, this type of label is applicable to HPLC, CE-LIF, and—in contrast to APTS—also to MALDI mass spectrometry [27]. Comparing 2-ANSA and APTS, the proposed CE-MS method with normal polarity was employed on an IgG glycan sample in bare fused silica capillaries of 60 cm length. The capillary length was reduced to decrease the analysis time, while the differences in separation efficiency were still determinable. The analysis of APTS- and 2-ANSA-labeled glycans applying the here-presented CE-MS method is shown in Fig. 6. The comparison of these two labels demonstrates the high potential of APTS in terms of separation efficiency. The ion electropherograms of G0F, G1F, and G2F glycans of IgG were extracted to ease the comparison. Anticipated reduced separation efficiency was demonstrated for ANSA-labeled glycans. Since, the smaller number of sulfonic acids of 2-ANSA decreases the mobility against the EOF and, hence, the resulting resolving power due to a shorter migration time. Modifying the BGE in a way that the EOF is reduced (increase of methanol (90 %) and decrease of ammonia to 0.23 and 0.35 M in the BGE) did not improve the separation to an extent as obtained for APTS-labeled glycans. Alternatively, 2-ANSA-labeled samples might be

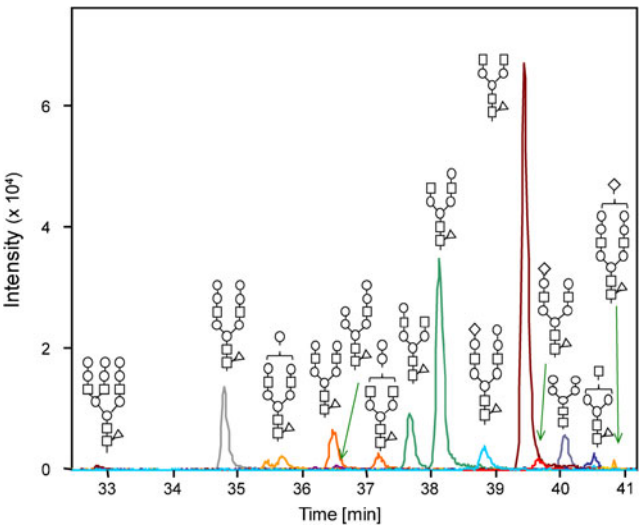


Fig. 5 EIE of the main glycans of a therapeutic antibody (using the doubly, triply, and fourfold charged glycans). Separation was performed with the proposed BGE system (as given in Fig. 2) using a fused silica capillary (90 cm). *Squares*, *N*-acetylhexosamine; *circles*, hexose; *triangles*, fucose; *diamonds*, sialic acid

separated in acidic background electrolytes. Using an acidic separation buffer in a CSE-LIF approach, Briggs et al. [27] obtained an increased separation efficiency of glycans modified with single sulfonated label (5-ANSA) compared to the application of trisulfonated APTS label.

Here, the glycan intensities were increased using the 2-ANSA-label, explicable by higher hydrophobicity of 2-ANSA compared to APTS increasing the ionization efficiency, as described by Walker for different cationic glycan labels [30].

Conclusion

This paper presents a novel CE-TOF-MS method with basic background electrolyte for the analysis of APTS-labeled glycans. The basic background electrolyte enabled a robust CE-MS application due to its strong EOF, minimizing pressure and flow effects at the capillary outlet. Good separation efficiency, i.e., separation of G1F/G1F′ was obtained for biopharmaceutical samples. The sensitivity in the low femtomole range (picomole of sample required) is appropriate for this application. The identification of unknown glycans for different types of biopharmaceutically relevant proteins supports this finding. A detailed and systematic study on the identification of glycans analyzed by different CSE-LIF methods using this proposed as well as an acidic CE-MS method will be following soon.

The possibility to analyze APTS-labeled and charged, native glycans simultaneously with high separation efficiency is unique in this field. The proposed method enables the determination of labeling and ionization efficiency. Although the APTS label does not introduce hydrophobicity, the ionization efficiency is a factor 3 higher than for native glycans. In comparison to APTS-labeled glycans, the separation efficiency for 2-ANSA-labeled glycans applying the presented method is reduced, while the ionization efficiency was increased. Nevertheless, the proposed method is applicable to glycans derivatized by other acidic labels such as 2-ANSA enabling a complementary analysis in addition to, e.g., MALDI-TOF-MS.

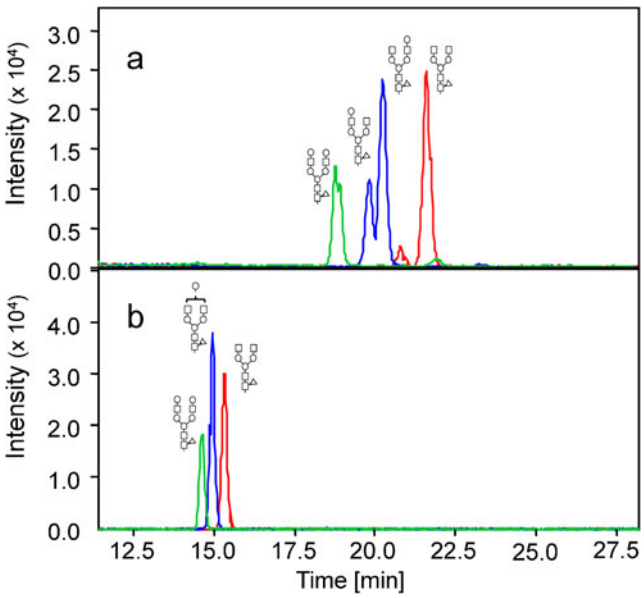


Fig. 6 EIE of G0F, G1F, and G2F (using the doubly, triply, and fourfold charged glycans). a EIE of APTS-labeled IgG glycan sample; b EIE of 2-ANSA-labeled IgG glycan sample. Separation was performed applying the proposed BGE system (see Fig. 2) using a fused silica capillary of 60 cm length. *Squares*, *N*-acetylhexosamine; *circles*, hexose; *triangles*, fucose; *diamonds*, sialic acid

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Conflict of interest The authors declare no conflict of interest.

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