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Profiling of oligosaccharide-mediated microheterogeneity of a monoclonal antibody by capillary electrophoresis

Based on complex formation of borate with carbohydrates in alkaline solutions, the oligosaccharide microheterogeneity of a monoclonal antibody was studied using capillary zone electrophoresis. In borate buffers characteristic separation patterns were found that could be attributed to the same antibody by their UV spectra, while in a phosphate buffer, under otherwise the same conditions, only a single peak was observed. *N*- and *O*-glycans were chemically hydrolyzed by trifluoromethane sulfonic acid, resulting in a completely deglycosylated protein; alternatively, *N*-glycans were enzymatically cleaved by incubation with peptide *N*-glycosidase F (PNGase F). In both approaches a changed antibody pattern was detected, indicating that the separation is due to carbohydrate microheterogeneity of the protein. Deglycosylation of the antibody by treatment with PNGase F was investigated by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). A shift to lower molecular masses of approximately 1500 Da for the enzymatically treated protein, compared with the intact glycoprotein, was found. The separation method was validated for linearity and reproducibility of migration time and peak area and optimized in terms of buffer pH, capillary temperature and borate concentration. This technique is sensitive to analyze batch-to-batch consistency in production and to test the stability of galenical formulations. After antibody storage in glass vials for 3 months at 37°C, the separation profile changed distinctly due to degradation at the carbohydrate or sialic acid moiety of the antibody, as indicated by MALDI-TOF-MS.

1 Introduction

Glycoprotein glycans are known to play an important role in biological processes such as cell-cell recognition, activities of the immune system and protein folding and stability. Because the biosynthesis of glycoprotein glycans is not genetically controlled, oligosaccharide-mediated microheterogeneity is considered an inherent characteristic of glycoproteins. In the biotechnological production of glycoproteins, *e.g.* monoclonal antibodies (mAb), the assessment of oligosaccharide microheterogeneity and its batch-to-batch consistency are important analytical tasks. While a number of analytical techniques are available for the analysis of isolated oligosaccharides, such as gel permeation chromatography [1], ion exchange chromatography with pulsed amperometric detection [2] and gas chromatography [3], only a few techniques are suitable for the characterization of carbohydrate microheterogeneity in the intact glycoprotein. Polyacrylamide isoelectric focusing has been the method of choice for this purpose [4]; however, it often shows a number of disadvantages, *e.g.*, poor reproducibility and the lack of quantitation. A comprehensive description of techniques for the analysis of glycoprotein glycans can be found in [5].

Capillary electrophoresis (CE) has evolved into a powerful separation technique for the analysis of biopolymers such as nucleic acids and proteins [6]. In recent publications successful separations of mono-, oligo- and polysaccharides were reported [7–9]. The use of CE for the profiling of oligosaccharide microheterogeneity of proteins and peptides has been reported by Taverna *et al.* [10], Rush and co-workers [11] and Landers *et al.* [12]. The present study describes the use of capillary zone electrophoresis to monitor mAb carbohydrate microheterogeneity. This technique was successfully employed for testing batch-to-batch consistency and for stability testing of the antibody. Additional information is obtained by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) which ideally supplements CE in acquiring knowledge of the integrity of the glycoprotein.

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Nonstandard abbreviations: mAb, monoclonal antibody; **MALDI-TOF-MS,** matrix assisted laser desorption ionization time-of-flight mass spectrometry; **PNGase F,** peptide-*N*-glycosidase F

Keywords: Monoclonal antibodies / Microheterogeneity / Capillary electrophoresis

2 Materials and methods

2.1 Chemicals

All chemicals were of highest purity. Disodium tetraborate, 1 M sodium hydroxide solution, sodium-dihydrogenphosphate and disodium hydrogenphosphate were purchased from Fluka (Buchs, Switzerland). Sinapinic acid, 2,5-dihydroxybenzoic acid and the high molecular weight standard containing cytochrome c, myoglobin and bovine serum albumin were obtained from Hewlett-Packard (Waldbronn, Germany).

2.2 Instrumentation

All experiments were carried out using an HP ^{3D}CE capillary electrophoresis system (Hewlett-Packard). If not stated otherwise, the following experimental conditions were used. Electrophoresis was performed in untreated open-tube fused silica capillaries (Polymicro; Phoenix,

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AZ; and Hewlett-Packard, respectively), 75 μm ID and different lengths as indicated, applying a potential of 15 kV. Prior to analysis the capillary was first rinsed with 0.1 M NaOH for 5 min and then with the running buffer for 1 min. The temperature of the capillary and the autosampler tray was maintained constant at 20°C. The electrolyte system consisted of 150 mM sodium tetraborate, pH 9.4 (prepared fresh daily). All buffers were prepared with Milli-Q (Millipore) water and filtered through a 0.2 μm filter (Sartorius, Göttingen, Germany). The sample (concentration, 0.1% w/v) was injected by pressure for 2 s at 50 mbar; detection was by UV absorbance at 200 nm. Integration of the peaks was accomplished by automated or manual analysis using the Hewlett-Packard Chemstation. Small day-to-day variations in migration time were corrected by appropriate changes in the applied voltage.

2.3 Cleavage of the carbohydrate moiety

The mAb was deglycosylated following two strategies. *N*- and *O*-glycosylated glycans were cleaved from the polypeptide by nonselective chemical deglycosylation, using anhydrous trifluoromethanesulfonic acid according to the protocol of the deglycosylation kit "GlycoFree™" from Oxford GlycoSystems (Abingdon, Great Britain). The deglycosylated protein had to be redissolved in 8 M urea prior to CE analysis. *N*-glycans were enzymatically deglycosylated using PNGase F according to a modified protocol "GlycoShift™" from Oxford GlycoSystems. In brief, reduction of disulfide bonds by mercaptoethanol was omitted because analysis and peak detection in CE were disturbed under these conditions.

2.4 MALDI-TOF-MS

Analysis of mAb by MALDI-TOF-MS was performed with a Hewlett-Packard instrument. Sinapinic acid and 2,5-dihydroxybenzoic acid were used as matrix. A protein standard mixture consisting of cytochrome c, myoglobin, and bovine serum albumin served as internal standard for the calculation of the molecular masses. Solutions of the antibody were dialyzed against double distilled water prior to analysis. One μL of a mixture of sinapinic acid, protein standard solution, and dialyzed sample (1:1:1 v/v/v) were mixed, transferred to the sample holder and dried in vacuum. Data of approximately 100 shots were collected at a signal intensity near the threshold in the positive ion mode. In general the laser energy was adjusted to approximately 5–6 μJ. The molecular masses of the standard proteins were measured with a relative error of less than 0.1%.

3 Results and discussion

3.1 Scope

It is well known that borate ions form stable complexes with diols, in particular with 1,2- and 1,3-diols in alkaline solutions. In its general form the complexation reaction can be written as

$$[B(OH)_4]^- + (HO)_2R \rightleftharpoons [(HO)_2BO_2R]^- + 2\ H_2O \quad (1)$$

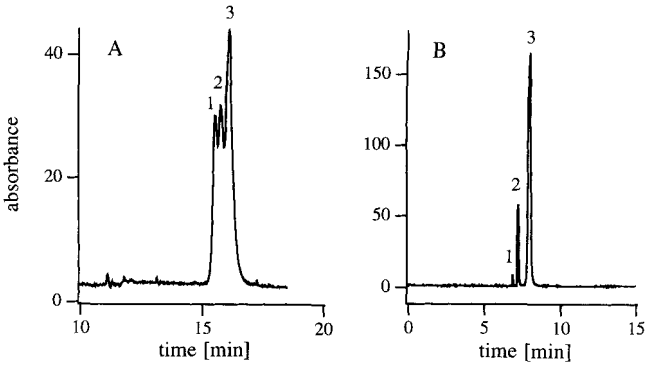


Figure 1. Separation of the monoclonal antibody (batch 1715) in (A) 150 mM sodium tetraborate, pH 9.4, and (B) 50 mM sodium phosphate, pH 9.4, by capillary zone electrophoresis. Capillary: 58 cm (50 cm eff. abbreviation?) × 75 μm ID. All other conditions as described in Section 2.2.

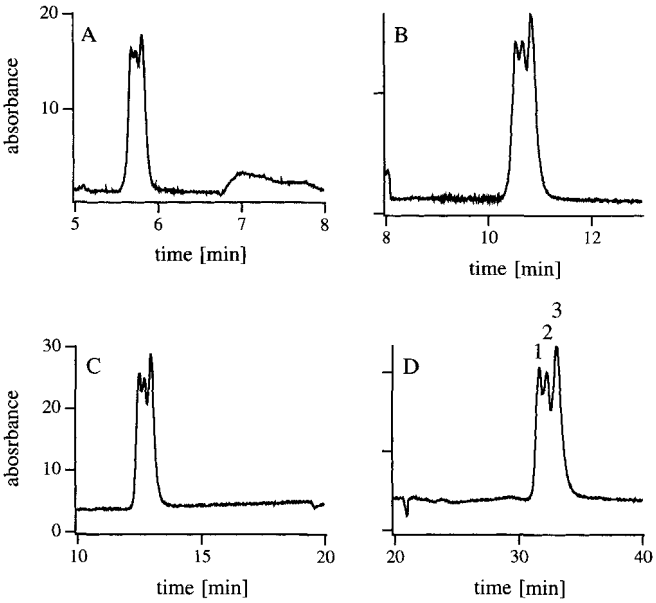


Figure 2. Influence of borate concentration on mAb separation profile (batch 1010). (A) 100 mM, (B) 150 mM, (C) 200 mM and (D) 250 mM sodium tetraborate, pH 9.4. The capillary length was 48 cm; other conditions as in Section 2.2.

$$[(HO)_2BO_2R]^- + (HO)_2R \rightleftharpoons [RO_2BO_2R]^- + 2\ H_2O \quad (2)$$

where $(HO)_2R$ is the diol and $[B(OH)_4]^-$ represents tetrahydroxyborate. Based on the different negative charges of the complexes even neutral sugars can be separated by CE. Hoffstetter-Kuhn and co-workers [13] have shown that the magnitude of complex formation depends on a number of different factors such as the number, arrangement and orientation of the hydroxyl groups of the sugar, borate concentration, buffer pH, *etc.* Upon considering these effects, it becomes obvious that predicting a migration profile of glycoproteins differing in their sugar moieties is impossible to date. On analysis of a biotechnologically produced mAb in a borate buffer, at pH 9.4, we detected three distinct peaks that showed the same UV spectra (Fig. 1A). Analysis of the same antibody in a phosphate buffer at the same pH revealed only a single peak that could be attributed to the protein (peak 3 in Fig. 1B). Peak 1 in Fig. 1B indicates the mobility of the electroosmotic flow and peak 2 repre-

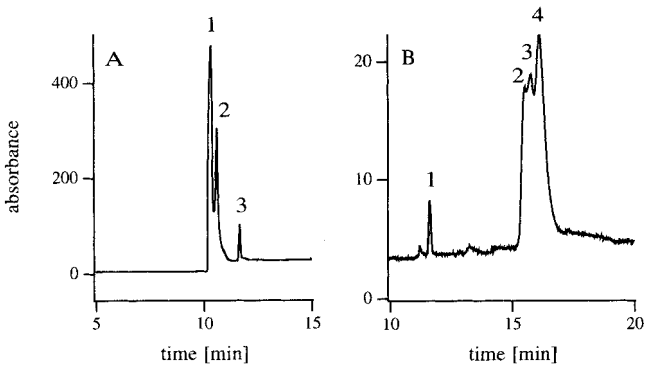


Figure 3. Capillary zone electrophoresis of mAb (batch 1010) in the borate system after cleavage of the carbohydrate moiety by (A) chemical hydrolysis in anhydrous trifluoromethanesulfonic acid and (B) incubation with PNGase F. (A) Peaks 1 and 2 originate from urea in the sample. Conditions as in Section 2.2, except capillary length, which was 48 cm.

sents glycine, which was added to the formulation to stabilize the antibody. This method is currently being used to assay glycine and antibodies in dosage forms.

3.2 Optimization of the separation method

The separation method using borate as complexing agent was optimized in terms of capillary temperature, borate concentration, and pH. The influence of the temperature was studied in the range of 20–40°C. It was found that lower temperatures improve the separation of the three peaks by stabilizing the complex as predicted by theory, with optimum resolution at 20°C. According to the law of mass action, higher borate concentrations and higher pH values also stabilize the complex. Depending on the magnitude of the complex formation of tetrahydroxyborate and the sugar moiety, the electrophoretic mobilities of the three peaks should change substantially, due to the additional negative charges caused by the complexation. The component that forms the strongest complex should be retained. Thus, the higher the borate concentration and the pH of the buffer, the better the separation should be. The influence of borate concentration on resolution (Fig. 2) was studied for the range of 50–250 mM tetraborate. Higher borate concentrations resulted in longer migration times of the three peaks; the resolution, however, did not further improve for concentrations higher than 150 mM tetraborate. Similar effects were found by Taverna *et al.* [9] for a recombinant plasminogen activator. In the range of pH 9.0–11.0, an optimum separation was found at pH 9.4. The addition of amines such as putrescine, to dynamically coat the capillary surface and to reduce the electroosmotic flow [12], did not further improve the separation.

Although resolution of the three peaks is moderate even under optimized conditions, the individual peaks could be quantified with fairly good precision with relative standard deviations of the peak area from 5.1% for peak 3, 5.5% for peak 2 and 7.3% for peak 1 ($n = 6$). Linearity of the peak areas was measured up to a protein concentration of 0.1% w/v, with correlation coefficients better than 0.999. Day-to-day and column-to-column reproducibilities were not determined so far. Replacement of the capillary with one from another supplier caused a significant change in migration time.

3.3 Cleavage of the carbohydrate moiety

We tried to confirm the hypothesis that the migration profile in the borate system is due to oligosaccharide-mediated heterogeneity of the antibody by cleaving the sugar moiety from the protein backbone. Any change in the carbohydrate moiety should have an impact on the separation profile in CE. Two strategies were applied for this purpose. First, antibody glycans were chemically hydrolyzed in anhydrous trifluoromethanesulfonic acid. This was considered to be the most successful technique in cleaving both *N*- and *O*-glycans, leaving the primary structure of the protein intact. Provided that hydrolysis of the carbohydrates is quantitative, only one peak should be found in the electropherogram. Figure 3A shows the separation of the deglycosylated protein in borate buffer. Only one protein peak (peak 3) could be identified by its UV spectrum, whereas the two major peaks were due to urea, added to redissolve the precipitated antibody after cleavage. As expected, the deglycosylated protein showed a lower negative mobility compared with the native glycoprotein. The step in the baseline is caused by urea or impurities thereof and was also found when analyzing a pure solution of 8 M urea.

In a second approach *N*-glycosylated glycans were enzymatically cleaved using PNGase F. However, some limitations in the use of PNGase F should be mentioned. First, hydrolysis rates of various *N*-glycans can differ significantly even after denaturation and reduction of the protein. Secondly, some *N*-glycans have been suggested to be completely resistant to enzymatic treatment, for instance those with core α-1,3 fucose. For this reason complete removal of all *N*-glycans could not be

Table 1. Ratio of peak areas of peaks 2 and 3 of the monoclonal antibody related to peak 4 as an internal standard after incubation with PNGase F under different conditions

Incubation conditions	Peak 2/Peak 4	Peak 3/Peak 4
1 Native protein (without incubation)	0.69	0.58
2 Incubation of the native protein with PNGase F, 15 h at 35°C followed by 33 h at room temperature	0.52	0.54
3 Incubation with PNGase F in a denaturing buffer, 15 h at 35°C	0.39	0.52
4 Incubation with PNGase F in a denaturing buffer, 15 h at 35°C followed by 33 h at room temperature	0.34	0.50

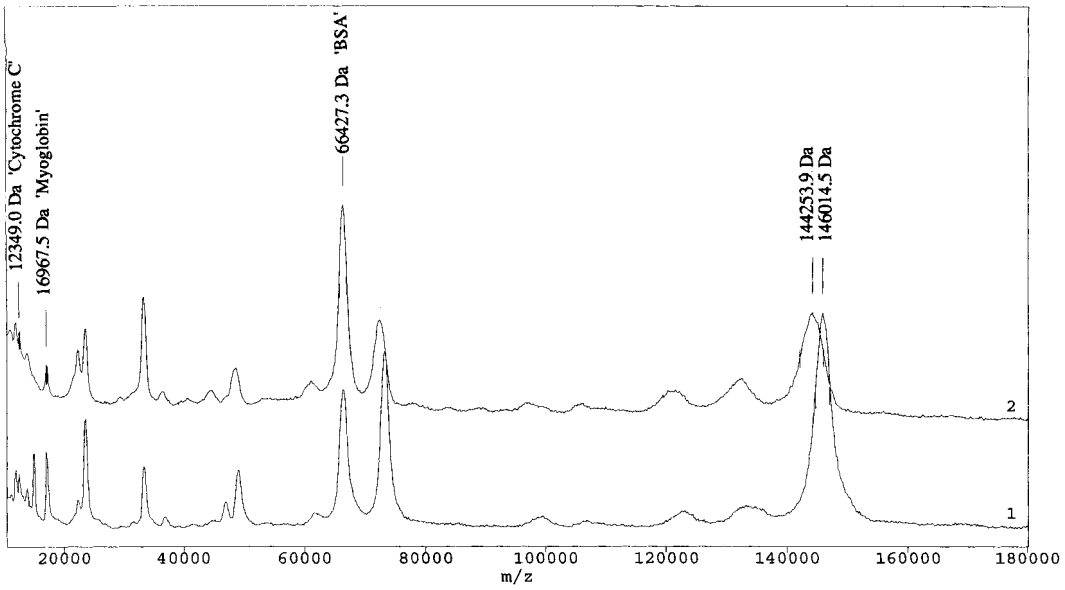


Figure 4. MALDI-FOF-MS profiles of (1) the intact mAb and (2) mAb incubated with PNGase F. A standard protein mixture consisting of cytochrome c (12349 Da), myoglobin (16967 Da) and bovine serum albumin (66427 Da) served as internal standard for the calculation of the molecular masses of the antibody. Peaks at approximately 73000 Da can be attributed to the double charged antibody, peaks at 23300 Da and 491000 Da correspond to the light and heavy antibody chains. Spectra accumulated from 100 single shots. Intensity is given in arbitrary units, molecular mass in Da (m/z).

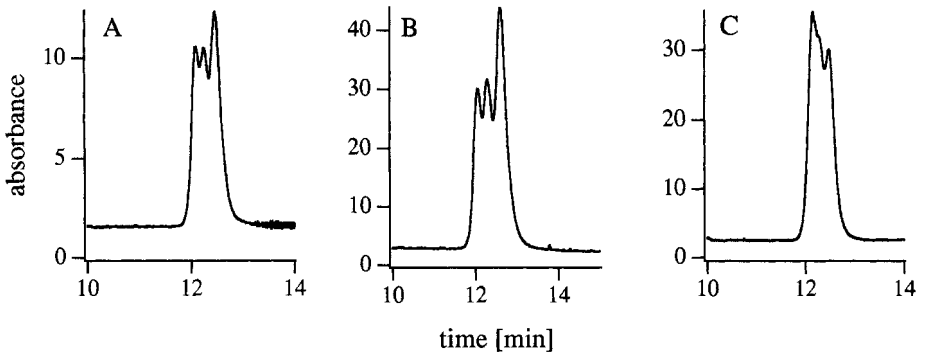


Figure 5. Capillary zone electrophoresis (borate system) of different batches. (A) Batch 1010, (B) batch 1715 and (C) batch 2070. Capillary length: 48 cm; other conditions as in Section 2.2.

expected. Figure 3B shows an mAb separation after incubation with PNGase F. Peak 2 of the profile was dramatically decreased, whereas a new peak at 11.7 min could be identified, which is assumed to be identical to the corresponding peak in Fig. 3A. Peak 4 of the profile was not measurably influenced by the enzyme. The extent of degradation strongly depended on the incubation conditions (shown in Table 1). Taking the area of peak 4 as an internal standard, different ratios of the peak area were calculated. The slowest rate of hydrolysis was measured for the native protein where the access of the enzyme to the binding sites is assumed to be hindered by native conformation. Significantly higher rates were measured after incubation of the antibody under denaturing conditions. Nevertheless, long incubation times were needed to obtain a measurable effect.

Samples incubated with PNGase F were analyzd by MALDI-TOF-MS for changes of the molecular mass of the antibody (Fig. 4). While an average molecular mass of 146014 Da ± 40 ($n = 4$) was found for the intact antibody, it decreased to 144473 ± 289 kDa ($n = 4$) after PNGase F treatment. This decrease of approximately 1500 Da in molecular mass can be attributed to the cleavage of an oligosaccharide unit from the protein. Analysis of *N*-glycans performed by Oxford GlycoSystems using the RAAM 2000 methodology revealed a tentative oligosaccharide structure belonging to the agalacto-, biantennary *N*-glycan group, core-substituted with fucose. Molecular masses of the simplest structures of this group range from approximately 1400 to 1700 Da [14]. Incubation of the antibody with PNGase F not only caused a shift to lower molecular masses but also led to a significant broadening of the antibody peaks in the mass spectrum.

3.4 Applications for testing of batch-to-batch consistency and for stability testing

Batch-to-batch consistency is an important quality criterion in the pharmaceutical production. In the biotechnological production of glycoproteins the carbohydrate heterogeneity has to be tested by appropriate analytical methods. For this purpose it is not necessary to assess the exact amount and the structures of all oligosaccharides bound to the protein. Moreover, any separation

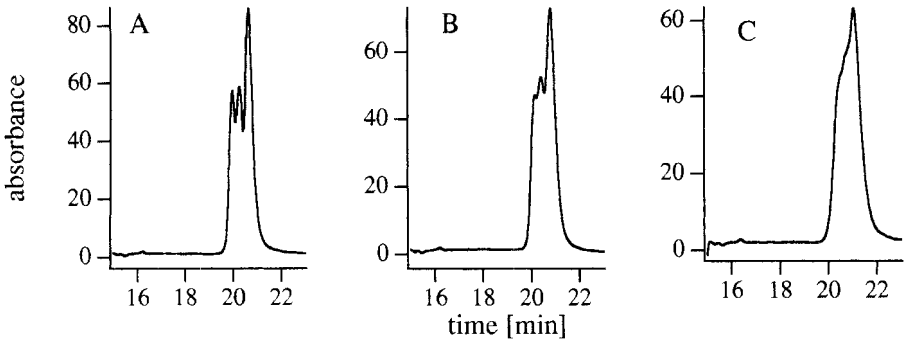


Figure 6 Separation profiles of Ab (batch 1715) after storage in glass vials: (A) 3 months at 2–8 °C, (B) 3 months at room temperature, and (C) 3 months at 37 °C. Experimental conditions: capillary, 48 cm × 75 μm ID, 200 mM sodium tetraborate, voltage 10 kV. Other details as in Section 2.2. The broad peak starting at approximately 24 min represents glycine which was added to the formulation.

method sensitive to heterogeneity provides the information needed. Figure 5 shows the separation profiles of three different batches of the monoclonal antibody. While two profiles (A and B) are quite similar, the third (C) is different. Although all three peaks were detected in the latter profile, the relative peak areas deviated significantly. This technique was also useful for stability testing of the final product in formulations. Figure 6 depicts the antibody separation profiles after three months of storage at different temperatures. Storage at 2–8 °C shows no measurable degradation. Slight degradation was observed at room temperature, and pronounced degradation at 37 °C. Only one of the initial three peaks could be detected clearly. This tendency was even more pronounced after storage at 37 °C for six months. No other degradation products could be detected in these samples. Investigations of the three- and six-month-sample by MALDI-TOF-MS showed no significant decrease of the molecular mass for the first sample but a decreased mass of 145560 ± 178 Da ($n = 4$) for the latter one. Based on the data presented here it can be assumed that this decrease is attributed mainly to degradation of the carbohydrate or sialic acid moiety of the antibody.

4 Concluding remarks

The present study proves the suitability of CE for profiling oligosaccharide-mediated microheterogeneity of glycoproteins. In practice, this technique is extremely simple to apply and provides rapid information. No sample pretreatment or purification is necessary. CE in combination with MALDI-TOF-MS represents a powerful tool for the investigation of glycoprotein structures. Because complex formation of carbohydrates with borate depends on numerous factors, as discussed above, predictions on the separation profile seem to be impossible, even if the structures of the interacting oligosaccharides are well known. In addition, CE of glycoproteins as borate complexes resolves mainly on the basis of structural differences of sugars on the surface of glycoproteins [15]. Thus, sialic acids bound to outer monosaccharides could weaken or even prevent complexation by their negative charges. The glycan moiety represents only about 1% of the total glycoprotein mass. Thus, several complexation reactions need to occur in order to measure an effect of complex formation on the electrophoretic mobility under these conditions. In addition, because it can be assumed that the distribution of sialic acid is not the same in all glycoprotein molecules, this should also influence the migration and complexation behavior.

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