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Short Communication

Affinity capillary electrophoresis for the assessment of binding affinity of carbohydrate-based cholera toxin inhibitors

Developing tools for the study of protein carbohydrate interactions is an important goal in glycobiology. Cholera toxin inhibition is an interesting target in this context, as its inhibition may help to fight against cholera. For the study of novel ligands an affinity capillary electrophoresis (ACE) method was optimized and applied. The method uses unlabeled cholera toxin B-subunit (CTB) and unlabeled carbohydrate ligands based on ganglioside GM1-oligosaccharides (GM1os). In an optimized method at pH 4, adsorption of the protein to the capillary walls was prevented by a polybrene-dextran sulfate-polybrene coating. Different concentrations of the ligands were added to the BGE. CTB binding was observed by a mobility shift that could be used for dissociation constant (K_d) determination. The K_d values of two GM1 derivatives differed by close to an order of magnitude (600 ± 20 nM and 90 ± 50 nM) which was in good agreement with the differences in their reported nanomolar IC_{50} values of an ELISA-type assay. Moreover, the selectivity of GM1os towards CTB was demonstrated using Influenza hemagglutinin (H5) as a binding competitor. The developed method can be an important platform for preclinical development of drugs targeting pathogen-induced secretory diarrhea.

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Affinity capillary electrophoresis / Cholera toxin inhibitors / Protein-carbohydrate interaction / Secretory diarrhea
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Protein–carbohydrate interactions meditate many biological processes including infections, immune responses, tumorigenesis, cell communication, cell trafficking, and fertilization [1]. For this reason it is becoming increasingly relevant to develop analytical tools that can accurately and rapidly determine these interactions. Such tools are valuable for carbohydrate recognition in glycobiology [2], for biomarker discovery, and for drug development [3]. A protein that needs a cost effective intervention is the cholera toxin [4], which causes cholera [5]. Cholera affects 1.4–4.3 million people worldwide, of which 28 000–143 000 die each year [6]. Numerous inhibitors for cholera toxin have been developed [7]. Of these, multivalent inhibitors based on the ganglioside GM1-oligosaccharide (GM1os) have shown the highest inhibitory potency [8, 9], however more development of cheaper and still effective compounds is expected, for which new assay methods are required. Recently we demonstrated that an assay based on the swelling of intestinal organoids provides a biorelevant alternative to the in vivo rabbit ileal loop assay [10], which is notoriously time-consuming, difficult, and stressful for the animals [11]. A drawback is the limited availability of the organoids. We here report an advance for in vitro inhibitor evaluation based on ACE with solution based dissociation constant (K_d) determinations instead of the half maximal IC_{50}. It has the potential for the simultaneous determination of the affinity of mixture components and can be used with small quantities. Furthermore, the assay can be run with unlabeled components, in contrast, for example, to the ELISA [9] involving immobilized GM1 and CTB_5 (cholera toxin B-subunit) linked to horseradish peroxidase for signal generation.

ACE is a powerful analytical tool for the study of ligand-protein interactions [12, 13]. This technique is based on the fact that the effective electrophoretic mobility (μ_{eff}) of the protein–ligand complexes differs from the μ_{eff} of the intact

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Abbreviations: μ_{eff}, electrophoretic mobility; **CTB**, cholera toxin B-subunit; **GM1os**, ganglioside GM1-oligosaccharide; **H5**, influenza hemagglutinin; K_d, dissociation constant

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proteins or ligands. ACE has been used for the analysis of carbohydrate–protein interactions in essentially two different scenarios. One is the study of relatively high molecular weight (2–30 kDa) glycans like heparin with anticoagulant properties binding to serum proteins [14]. Ligands are added to the BGE and protein mobility shifts are measured. In the other scenario carbohydrates are injected and the protein is added to the BGE. As carbohydrates are neutral at physiological pHs, a derivatization step is required to charge and give them better detection properties. However, derivatization can affect the binding behavior [13, 15, 16]. In our studies we evaluated the shifts of CTB as a function of underivatized relatively low Mw glycan ligands (Supporting Information Fig. S1).

In this work, CE analyses were carried out using a Beckman PA 800 instrument with ultraviolet detection. Detailed experimental procedure is presented in Supporting Information. Firstly, CTB analysis was optimized for relatively high μ_{eff} values in order to enhance mobility shifts upon ligand binding, thus enabling K_d determination. To this end, different BGEs consisting of ammonium acetate, sodium dihydrogen phosphate, or ammonium hydroxide at concentrations ranging from 25 to 150 mM and at a pH range of 2–12 were studied. One of the possible problems of CE with proteins is that the separation can be hampered by adsorption of the proteins onto the capillary wall. To avoid that, capillaries were coated with a coating depending upon the BGE pH. As the p*I* of CTB is 7.8 [17], a positive polybrene-dextran sulfate-polybrene coating was used for the pH range 2–7, while a negative polybrene-dextran coating was used for pHs 7–12; both coatings previously developed by our group [18, 19]. Reverse polarity was used for the positive coating and normal polarity for the negative coating. In order to calculate the μ_{eff}, a neutral EOF marker (formamide at 0.05%) was included in the sample.

The μ_{eff} of CTB about pH 7 was found to be zero. The protein had no charge at neutral pH conditions (p*I* 7.8) and it migrated with the EOF. In addition, repeatability problems were found with high buffer concentrations (100–150 mM), as the currents became unstable. Relatively high μ_{eff} values were observed at pH 2 ammonium acetate 25 mM (-1.56×10^{-8} $m^2V^{-1}s^{-1}$), pH 4 sodium phosphate 50 mM (-1.16×10^{-8} $m^2V^{-1}s^{-1}$), pH 10 sodium phosphate 25 mM (8.33×10^{-9} $m^2V^{-1}s^{-1}$), and pH 12 sodium phosphate 25 mM (8.44×10^{-9} $m^2V^{-1}s^{-1}$).

For affinity studies, two different cholera toxin inhibitors based on GM1os, a monovalent carbohydrate (**1**), and a bivalent carbohydrate (**2**) (Supporting Information Fig. S1) were added to the optimized BGEs and the protein CTB was injected. High concentrations of ligands were selected to ensure ligand–protein interactions. As the concentration of the protein is not used to obtain K_d values, we used high concentration of CTB, to ensure sufficient signal to measure the μ_{eff} reproducibly. At pH 4 (Fig. 1A), the addition of the carbohydrates lead to a significantly reduced μ_{eff} and symmetric non-broadened peaks (Fig. 1B and C), indicative of fast kinetics. The reduction of the migration times can be explained by the partial negative charge of the carbohydrates at pH 4 in combination with the positively charged capillary. At all the other pH conditions no considerable μ_{eff} shifts were observed. As a result, pH 4 was selected for further studies.

The repeatability of the developed method was assessed by measuring the μ_{eff} (n = 6) in the absence and presence of carbohydrates (48.2 μM carbohydrate **1**, or 27.4 μM carbohydrate **2**), obtaining RSDs of 2, 2, and 4%, respectively. The reproducibility was also assessed determining the μ_{eff} on six different days, including the installation of new capillaries and the preparation of fresh buffers. RSD values were 2, 6, and 6%, respectively. Raw data are presented in Supporting Information Table S1.

For K_d determination BGEs containing increasing concentrations of carbohydrate **1** or carbohydrate **2** were prepared and used for the analysis of CTB (62.5 μM). The carbohydrate concentrations in the BGEs were varied between the low nanomolar range to the micromolar range to cover a broad

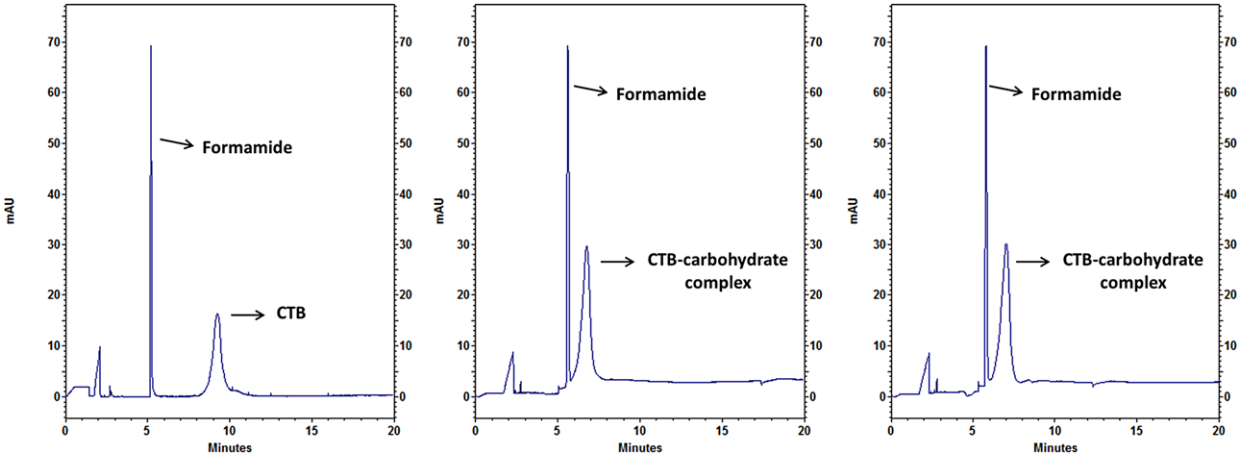

Figure 1. CE analysis of CTB (62.5 μM) using a polybrene-dextran sulfate-polybrene-coated capillary and a BGE of 50 mM sodium dihydrogen phosphate (pH 4) containing no carbohydrate (A), 48.2 μM of carbohydrate 1 (B), or 27.4 μM of carbohydrate 2 (C). The first peak in the electropherograms is an injection related peak.

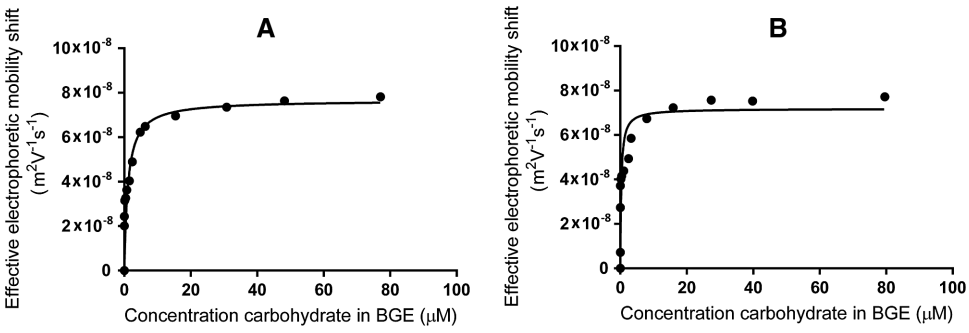

Figure 2. ACE binding curves for CTB-carbohydrate complexes obtained by plotting μ_{eff} shifts against the concentration of carbohydrates in the BGE. Carbohydrate 1 (A), carbohydrate 2 (B).

spectrum of possible K_d. For each carbohydrate and each studied concentration, the difference of the measured μ_{eff} of CTB with the μ_{eff} obtained without carbohydrate was determined (Supporting Information Table S2). The obtained values were plotted versus the carbohydrate concentration, and fitted using nonlinear regression (Fig. 2). Using the CE instrument as a viscosimeter, no significant differences in viscosity were observed for these concentrations of carbohydrates in the BGE. Consequently, no viscosity correction to calculate the μ_{eff} was needed. This way, a K_d value of 600 ± 20 nM was obtained for carbohydrate **1** and a K_d value of 90 ± 50 nM for carbohydrate **2**. While we cannot directly compare K_d values with IC_{50} values of competition experiments or swelling inhibition, the differences between the two compounds of close to an order of magnitude are in reasonable agreement with those of our previously described IC_{50} values using the common ELISA assay and the newly developed assay based on organoid swelling inhibition [10]. As such the method is capable of differentiating compounds of relatively similar binding or inhibitory potency, even though the assay was run at pH 4 rather than the usual neutral condition in, for example, the ELISA assay.

The selectivity of GM1os towards CTB was demonstrated using influenza hemagglutinin (H5) as a binding competitor. This glycoprotein has an affinity for sialic acids [20], which are present in both carbohydrate **1** and **2**. However, since CTB binds to more saccharides of GM1 than just the sialic acid it is expected to bind far stronger to **1** and **2** than H5. The mixture of H5 and CTB was analyzed by CE, and affinity studies were carried out by adding the carbohydrates **1** and **2** to the BGE. As shown in Fig. 3, H5 and CTB have different μ_{eff} values (Fig. 3A). However, when carbohydrate **I** was added to the BGE, the CTB peak shifted towards EOF, obtaining the same μ_{eff} as H5, which did not shift (Fig. 3B). Similar results were obtained using carbohydrate **2**. Moreover, experiments were carried out injecting only H5, but no mobility shifts were observed. This was expected, as H5 can interact with sialic acids in the low millimolar range [21], and our measurements were carried out at nM–μM concentrations. With this approach, the selectivity of GM1os towards CTB was demonstrated.

In summary, the ACE method was effective at detecting relatively subtle ligand changes, such as those between

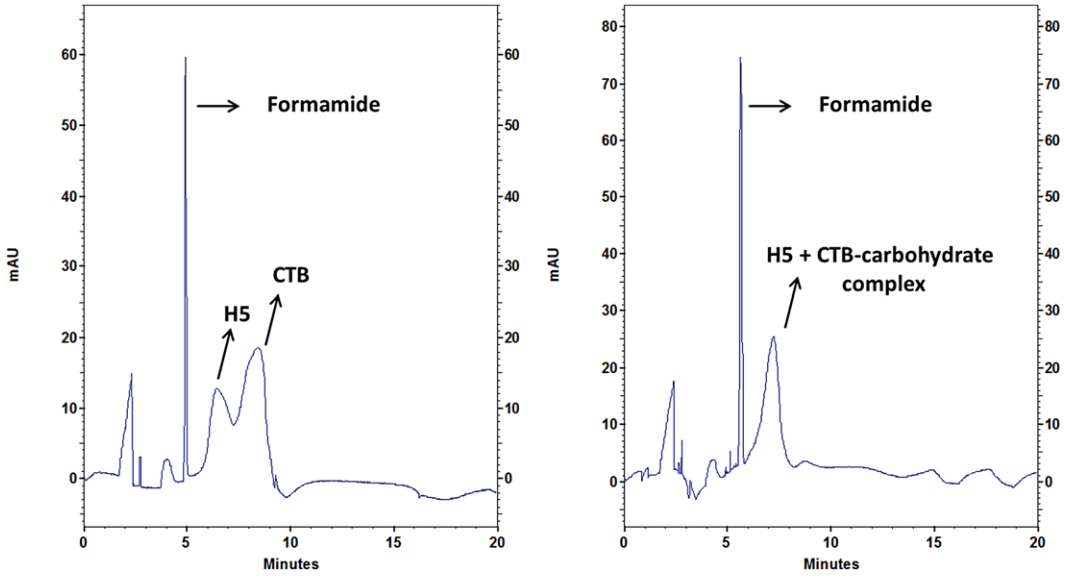

Figure 3. CE analysis of H5 (10 μM) and CTB (62.5 μM) using a polybrene-dextran sulfate-polybrene-coated capillary and a BGE of 50 mM sodium dihydrogen phosphate (pH 4) containing no carbohydrate (A), 10 μM of carbohydrate 1 (B). The first peak in the electropherograms is an injection related peak, and other minor peaks like the one at 4 min, are impurities.

monovalent ligand **1** and the slightly stronger binder and non-spanning [22] ligand **2**, that may be involved in bridging between toxins as previously seen for heterobivalent ligands [23]. It is therefore likely that the method can discriminate amongst newly designed derivatives in a straightforward fashion. The method was also able to demonstrate the selectivity of GM1os towards CTB. Overall, the present study demonstrates that ACE is a powerful tool for the study of protein–carbohydrate interactions that exhibit fast equilibrium kinetics, giving the possibility to measure K_d values even in the nanomolar range. In the past (see above) ACE has been used for the study of interaction between a protein and glycans as heparin but in the present paper the potential for low-molecular weight carbohydrates has been demonstrated. The developed platform can be important for preclinical development of drugs targeting pathogen-induced secretory diarrhea as shown here for compounds targeting cholera toxin and can be extended to other studies involving protein–carbohydrate interactions. Moreover, it can be combined with MS to obtain conformational information on the separated compounds [12].

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