Capillary Electrophoresis and UV-Pulsed Lasers or UV-Continuous Light Emitting Diodes Technologies to Induce Fluorescence for the Characterization of Oligosaccharides Labelled With 2-Amino-1-Naphthalenesulfonic Acid

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Introduction

Glycan characterization of therapeutic proteins is important due to the role of oligosaccharides in protein stability, half-life, biological activity and efficacy [1]. Glycans are typically characterized by releasing them from the therapeutic protein of interest and analyzing them after they have been fluorescently derivatised [2]. The most widely, currently used fluorescent label for glycans analysis by capillary electrophoresis and laser induced fluorescence detection (CE-LIF) studies and mass spectrometry. The main problem with CE-LIF is obtaining a light source that will achieve sufficient excitation to detect labelled oligosaccharides. Using 2-amino-1-naphthalenesulfonic acid (2-ANSA) labelled oligosaccharides, we performed a comparison on CE between pulsed UV laser-induced fluorescence (266 nm) and a UV Light emitting diode (LED) induced fluorescence (280 nm). We show that the continuous LED (2 mW) as an excitation source is as sensitive as with a pulsed laser (5 mW). The CE separation conditions used consisted of a buffer containing 40 mM ε-aminocaproic acid, pH 4.5 and 0.02% hydroxypropylmethylcellulose (4000 cP) run using reverse polarity (-20 kV). This study shows that 2-ANSA is well adapted for allowing labelled oligosaccharides to be run on CE-LEDIF and also to be used for MALDI-MS studies. Applications were realized on oligosaccharides that had been released from human IgG.

Keywords: Biomolecule; Oligosaccharide; Capillary electrophoresis; UV light emitting diodes; Fluorescence; Mass spectrometry

Abstract

Aminonaphthalene sulfonic acids are relatively unknown dyes that can be used to derivatize oligosaccharides for capillary electrophoresis with laser induced fluorescence detection (CE-LIF) studies and mass spectrometry. We show that the continuous LED (2 mW) as an excitation source is as sensitive as with a pulsed laser (5 mW). The CE separation conditions used consisted of a buffer containing 40 mM ε-aminocaproic acid, pH 4.5 and 0.02% hydroxypropylmethylcellulose (4000 cP) run using reverse polarity (-20 kV). This study shows that 2-ANSA is well adapted for allowing labelled oligosaccharides to be run on CE-LEDIF and also to be used for MALDI-MS studies. Applications were realized on oligosaccharides that had been released from human IgG.
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Materials and Methods

CE/UV-LIF or CE/UV-LEDIF

A CE7100 (Agilent Technologies, Walbronn, Germany) was used as the CE separation system equipped with a Zetalif™ detector from Picometrics (Toulouse, France) using either a 266 nm pulsed laser (4 mW, 10 kHz, Teem Photonics, Meylan, France) or continuous 280 nm LED (3 mW, Picometrics). The Picometrics detector uses a 2 mm diameter silica ball lens which focuses the light source beam into the inner diameter of the capillary and collects the fluorescence with a high numerical aperture. Analyses were performed using a fused silica capillary (Polymicro Technologies Phoenix, Arizona): 50 µm ID, 65 cm total length, 50 cm effective length. The buffer is 40 mM ε-aminocaproic acid pH 4.5 adjusted with pure glacial acetic acid and 0.02% hydroxypropylmethylcellulose (4000 cP, Sigma, Saint Quentin Fallavier, France). Using this pH and viscosity means the electroosmotic flow is very low. The separation was run at -20 kV, the sample is injected at 0.5 psi, 10 s and the temperature is 20°C. N-glycan Standards, A2F, A1F, FA2G2, FA2G1, FA2, and IgG N-glycan library and fetuin N-glycan library were provided by Ludger Ltd.

2-ANSA labelling

Labelling was performed with a Ludger Tag 2-ANSA labelling kit (Ludger Ltd, Abingdon, UK). Briefly, oligosaccharide standards (5-25 µg amounts) were labelled with 2-ANSA in a solution of dimethyl sulfoxide (DMSO) and acetic acid in the presence of 2-picoline borane (2PB) for 3 hours at 65°C. Labellled oligosaccharides were cleaned up using LudgerClean T1 Cartridges (LC-T1-A6, Ludger Ltd).

MS experiments on 2-ANSA labelled oligosaccharides

A MALDI TOF experiment, to confirm the mass of the different 2-ANSA labelled oligosaccharides, was performed using a Shimadzu Biotech Axima Resonance, by spotting 0.5 µl sample on top of 0.5 µl 2,5-dihydroxybenzoic acid (DHB) matrix (10 mg/ml DHB in 50% acetonitrile, 0.01% trifluoroacetic acid) and drying under vacuum. MALDI-MS was performed in negative ion mode with the instrument set at the optimised 850 mass window with a range m/z 850-2000, pulsed excitation off, reflectron mode, Gate off, Laser repeat rate 5Hz. The table 1 presents the different m/z values of the different labelled saccharides.

Results and Discussion

Separation of the derivatized oligosaccharides using capillary electrophoresis

Using the CE parameters described in CE/UV-LIF or CE/UV-LEDIF, a typical CE-LIF profile of a mixture of the 2-ANSA labelled oligosaccharide standards is shown in Figure 1. In this profile, the major neutral oligosaccharide structures, FA2, FA2G1 and FA2G2, and sialylated oligosaccharide structures, A1F and A2F, mono-sialylated and di-sialylated respectively, are well resolved. The CE-LIF method can also be used for separation of 2-ANSA labelled oligosaccharides released from biological samples. A CE-LIF profile of 2-ANSA labelled oligosaccharides released from human IgG is shown if Figure 2A. The oligosaccharides released from human IgG are a mixture of neutral glycans and sialylated glycans (mono-sialylated and di-sialylated) that are separated, predominately, due to the negative charge imparted by the 2-ANSA moiety. Although the di- and mono-sialylated oligosaccharides are the first to migrate, the migration order of the neutral oligosaccharides is dependent on the number of glycan residues in the labelled oligosaccharide. Neutral oligosaccharides with a greater number of residues are the singly charged species with the longest migration time.

Comparison between CE/UV-LIF and CE/UV-LEDIF

Currently no other studies have been performed that focus on the separation of oligosaccharides labelled with 2-ANSA using CE/UV-LIF as the detection method. Certainly, this is because pulsed UV lasers are not used in CE-LIF studies of labelled oligosaccharides. The use of UV-LED has also not been reported for this method. Their use in CE studies is very low.
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bovine serum albumin (BSA) and myoglobin, and a tryptic digest of BSA labeled with ortho-phthalaldehyde (OPA), the emission wavelength of the LED was 365 nm (bandwidth 8 nm) which is well adapted for the excitation of the OPA compounds. The LOD for Glu and Asp were reported to be 47 nM [10]. In this final study, a very near wavelength UV-LED (380 nm, bandwidth 14 nm, 1.2-2 mW) was used for riboflavin and phenylglyoxal Trp derivative separation, 0.4 µM and 3 µM respective LODs were obtained [11]. In our study we estimate the LOD to be 200 nM.

Our goal was to compare the 2-ANSA labelled oligosaccharides from human IgG using both induced fluorescence by a 266 nm pulsed laser or a continuous 280 nm LED. Figures 2A and 2B because they are still as expensive as a UV pulsed 266 nm laser.

In a recent CE study, isoquinoline alkaloids were analyzed using UV-LEDIF detection with an excitation source exciting at 280 nm (bandwidth of 15 nm) with a power of 0.5 mW and controlled temperature [8]. The limits of detection (LOD) were in the range of 150 nM to 1.5 µM depending on the molecule studied. In a second study [9] researchers analyzed adenosine triphosphate and diphosphate and guanosine diphosphate using a 255 nm (bandwidth 25 nm) UV-LED with a low power of 0.014 mW. Even with this low light power, the LOD were in the range 40-407 µM.

In another study a much more powerful UV-LED (12 mW) was used for the detection of amino acids in two different proteins, A and D detection with a 266 nm laser B and D detection with a 280 nm LED.

Figure 2: CE-LIF analysis of 2-ANSA labeled IgG glycans with detection by A) a 266nm laser and B) a 280nm LED.

Figure 3: MALDI mass spectrum of 2-ANSA labeled human IgG glycans. Negative ion mode.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Structure</th>
<th>Theoretical m/z (M-H)-2-ANSA derivatives (monoisotopic mass)</th>
<th>Measured m/z (M-H)-2-ANSA derivatives</th>
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Table 1: Structure of the studied N-linked glycans: Complex type biantennary N-glycans are named using the abbreviation “FAaBGgSs” where “a” is the number of antennae linked to the trimannosyl core A, “s” the number of sialic acid residues (S), “g” the number of galactose residues (G), and “F” indicates core-fucosylation (F). B is a GlcNAc linked on the central mannose from the trimannosyl core.
show the comparison between the labelled oligosaccharides analyzed by the two different light sources, under the separation conditions, pH 4.5 with the semi-viscous buffer and reverse polarity. The comparison of the electropherogram of 2-ANSA labelled oligosaccharides from human IgG (Figure 2) with the electropherogram of the five 2-ANSA labelled oligosaccharide standards (Figure 1) has allowed us to structurally identify the five major peaks in the human IgG.

However, further analysis using CE-MS would be required to allow the full identification of all the different glycans. The electropherograms show that the first species to migrate are the di-sialylated oligosaccharides, followed by the mono-sialylated structures and then the neutral, non-sialylated structures. The migration order is reversed compared to a similar strategy reported in the literature concerning the labelling of glycans with a neutral dye, 9-fluorenylmethyl chloroformate of the glycan part of an antibody during the enzymatic hydrolysis by the PNGase [12]. In this case, where the buffer was 50 mM ammonium acetate (+30 kV), the non-sialylated oligosaccharide structures migrate first and are not separated (as electroosmotic flow markers) followed by mono-, di-, tri-, and then tetra-charged structures (i.e. containing four sialic acid residues). In our experiments, calculating the signal to noise (S/N) of the FA2G1 peak, we obtain 1084 with the 266 nm laser excitation and 898 with the 280 nm LED. The similar LODs show that the sensitivity is comparable for both experiments.

MALDI Mass spectrum of 2-ANSA derivatives

2-ANSA labelled oligosaccharides can be analyzed by MALDI mass spectrometry as shown in Figure 3, where the MALDI mass spectrum is shown of 2-ANSA labelled oligosaccharides released from human IgG (Table 1). To obtain this spectrum approximately 14 pmol (25 ng) of 2-ANSA labelled IgG glycans were spotted onto the plate. Moreover, 2-ANSA derivatization allows an easy identification of both neutral and sialylated oligosaccharides in a single negative ion mode spectrum.

Conclusion

In this study, an electrophoretic method was developed for the separation and characterization of oligosaccharides. The single negative charge of the 2-ANSA label proved to be highly advantageous and was able to affect the separation of both neutral and sialylated oligosaccharides by CE-LIF, CE-LEDIF and MALDI-MS. The fluorescent properties of the 2-ANSA label enable its use for the separation of oligosaccharides by CE, with the use of both a 266 nm laser and a 280 nm LED, with high resolution. LED technology providing a continuous wavelength, results in similar sensitivity in fluorescence to that obtained from conventional LIF technology using a pulsed laser.

Funding Sources

This work was partially funded by the European Union’s Seventh Framework Program (FP7-Health-F5-2011) under Grant agreement 278535 (HighGlycan)

References