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Parallel analysis and orthogonal identification of N-glycans with different capillary electrophoresis mechanisms

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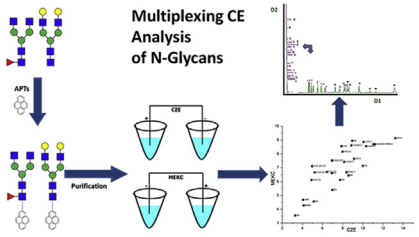
HIGHLIGHTS

- Cross-validation of analytes in complex samples was done with different CE separation mechanisms.
- A simple strategy is used to confirm peak identification and extend capacity of CE separation.
- The method uses small amount of sample, simple instrument and single fluorescent labeling.
- Selection of mechanisms is based on orthogonalities of GU values of glycan standards.
- Micellar electrokinetic chromatography was suitable for analysis of small or highly sialylated glycans.

GRAPHICAL ABSTRACT



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ABSTRACT

The deep involvement of glycans or carbohydrate moieties in biological processes makes glycan patterns an important direction for the clinical and medicine researches. A multiplexing CE mapping method for glycan analysis was developed in this study. By applying different CE separation mechanisms, the potential of combined parallel applications of capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and capillary gel electrophoresis (CGE) for rapid and accurate identification of glycan was investigated. The combination of CZE and MEKC demonstrated enhancing chromatography separation capacity without the compromises of sample pre-treatment and glycan concentration. The separation mechanisms for multiplexing platform were selected based on the orthogonalities of the separation of glycan standards. MEKC method exhibited promising ability for the analysis of small GU value glycans and thus complementing the unavailability of CZE. The method established required only small amount of samples, simple instrument and single fluorescent labelling for sensitive detection. This integrated method can be used to search important glycan patterns appearing in biopharmaceutical products and other glycoproteins with clinical importance.

1. Introduction

Around 70% of the recombinant proteins of interest for human therapy are glycoproteins and it is estimated that ~50% of all

Abbreviations: APTS, 8-Aminopyrene-1,3,6-trisulfonic-acid; GU, glucose unit.

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proteins in mammalian cells are glycosylated at any given time [1]. Glycans or carbohydrate moieties on cell surfaces are involved in many biological processes, such as host-pathogen interactions, cell communication, proliferation and differentiation as well as the initiation of immune responses. Glycosylation of the recombinant protein product influences critical properties such as bio-safety and bio-activity. A well glycosylated protein is believed to have a longer circulatory in vivo half-life and contributes to the recombinant proteins' solubility and stability [2]. The research about glycan had showed huge commercial value as therapeutic monoclonal antibody market has poised for strong double-digit growth [3].

The analysis of glycans is also a new direction for medical research as glycans play important role in biological function, such as development, growth, functioning, or survival of the organism that synthesizes them. Carbohydrates or glycans on the surfaces of breast, prostate and ovarian cancer cells have been investigated [4]. Serums samples from pancreatic cancer patients exhibited an altered sialylation and fucosylation patterns on glycan structures [5]. A method for novel tumor markers was explored by comparing serum antibody levels of Hodgkin's lymphoma patients against 37 glycan structures [6]. Glycan analysis was applied to the diagnosis of infectious diseases too. Serum samples from *Salmonella* [7], *Burkholderia pseudomallei* and *B. mallei* [8] were identified using anti-carbohydrate antibodies. The specificity for host cells of Influenza A virus strains were compared by the abilities of viral surface glycoprotein hemagglutinin binding to receptors containing glycans bearing terminal sialic acids [9,10], which indicating that glycan information can be used to map the evolution of new pathogenic influenza strains. One antibody, 2G12, binding exclusively to carbohydrates of gp120, was used to neutralize the sugar residues responsible for the entry of HIV virus into host cells [11,12]. Moreover, carbohydrates those are on the surface of pathogens represent valuable targets for direct detection of pathogens [13–15], such as the O-antigen moiety of endotoxins or lipopolysaccharides on gram-negative bacteria, which exist widely in air, water and soil.

Glycosylation is a non-template driven process, compared to protein sequences, and thus the structure cannot be directly predicted. In addition, each glycosylation site contains a variety of glycan structures, leading to a highly complex mixture. Different techniques are in use in different laboratories for glycan analysis, such as high performance anion exchange chromatography (HPAEC) [16], matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [17], CE [18–20] and normal phase liquid chromatography (NPLC) [21,22]. These techniques are not directly comparable and hence are not suitable for validation purposes. As most glycans are released at nano-gram quantities and difficult to be amplified like DNA by PCR, research advances about specific glycan structures to functions have been slow because of a lack of suitable methodologies for quantification.

To analyze complicated samples, researchers had previously explored two dimensional separations, such as 2-D gel electrophoresis, 2-D HPLC, and 2-D LC/CE. Most research studies on multi-dimensional separation are based on the collection of eluents from the first separation step and the subsequent separation of the collected eluents in the second column or capillary [23–25]. The whole procedure is time-consuming and the coupling of the two techniques can only be achieved by compromising the separation efficiencies of individual techniques. The coupling of chromatography and mass spectrometer can provide another method for 2-D separation. However, there are also some disadvantages for mass spectrometers, like the high cost, potential loss of sensitivity due to the difficulties of ionization of some compounds and the incompatibility for inorganic solutions.

Alternatively, multiplexing mapping methods had been explored for the analysis of glycan samples. Neutral and mono-, di-, tri- and tetrasialyl oligosaccharides were separated by an octadecylsilica (ODS-silica) column and each elution was separated further on an amide-silica column [26]. Two or three dimensional glycan map could be generated for identifying glycans.

This work focuses on the development of multiplexing CE-laser induced fluorescence (LIF) methods for the identification of N-glycans, similar to the parallel analysis of glycans by the combination of HPLC and CE separations [27,28]. Different from most of traditional multi-dimension separation methods, the multiplexing technique provides a relatively simple solution for analyzing complicated samples. Without the need of fraction collection, samples were separated by two or more different mechanisms at the same time and it is possible to develop an analytical platform so that results from different separation mechanisms can be corroborated to give convincing glycan profiling results. In this study, the best combination of different separation mechanisms was selected and optimized. Only one fluorescent tagging agent 8-aminopyrene-1,3,6-trisulfonic-acid (APTS) was applied. This multiplexing method is very helpful for complicated glycan profiling as it is high resolution, rapid and also an efficient cross-validation platform. This method required only simple instrumentation and the low sample consumption makes it an ideal technique for other clinical samples. The “dual-core” method can enhance greatly the capacity of CE or other chromatography techniques to handle complicated samples.

2. Materials and methods

2.1. Chemicals and reagents

Dextran (150,000 Da), 8-aminopyrene-1,3,6-trisulfonic-acid, sodium dodecyl sulfate (SDS), sodium cyanoborohydride, sodium hydroxide, Hydroxypropyl cellulose (HPC, average 100,000 MW), Human IgG (I4506) were purchased from Sigma (St. Louis, USA). Human serum IgG was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Sodium tetraborate anhydrous, citric acid, Hydroxyethyl cellulose (HEC, medium viscosity) were products of Fluka (Buchs, Switzerland). 2-(N-morpholino) ethane sulfonic acid (MES) was purchased from TCI Kasei Tokyo (Tokyo, Japan). Tris(hydroxymethylamino-methane) (TRIS) was purchased from Fisher Biotech (New Jersey, USA). Polyvinyl alcohol (PVA) 31,000–50,000 MW was obtained from Aldrich (Milwaukee, WI, USA). Ammonium acetate (NH_4Ac) was the product of Merck (Darmstadt, Germany). 26 N-glycan standards were purchased from Dextra Laboratories (West Berkshire, UK). Galactosidase S (P0745S), Neuraminidase A (P0722S), Fucosidase (P0748S) and PNGase F kit were from New England Biolabs (Hitchin, UK). Deionized water used throughout the experiments was supplied by a Direct-Q Millipore water purification system (Molsheim, France).

2.2. CE separation buffers

Borate buffers with SDS used in MEKC method contained variable SDS concentrations in 50 mM tetraborate buffer. 10 mM SDS in 50 mM tetraborate buffer was prepared by adding 0.5 mL of 0.4 M SDS into 10 mL of 0.1 M sodium tetraborate and diluted with pure water up to 20 mL. Subsequently, pH measurement was conducted and adjustment was made to reach pH 9.33. The other two borate buffers containing SDS (25 mM and 50 mM) were prepared by the same procedures with adjusted calculation in the total volume of borate and SDS used to obtain desired concentrations. Acetate buffers with SDS for MEKC were prepared by adding different concentrations of SDS (10 mM, 25 mM and 50 mM) in 25 mM acetate buffer, using similar method. The pH value was adjusted to 4.50 by 1 M acetic acid.