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Profiling of *N*-linked glycans from 100 cells by capillary electrophoresis with large-volume dual preconcentration by isotachophoresis and stacking

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ABSTRACT

Glycan structure is changed in response with pathogenesis like cancer. Profiling of glycans from limited number of pathogenetic cells in an early-stage tissue is essential for discovering effective drugs. For analyzing tiny biological samples, we developed sensitive, high-resolution, and salt-tolerant method for analyzing trace level of *N*-linked glycans by coupling capillary electrophoresis (CE), laser-induced fluorescence (LIF) detection, and a new online sample preconcentration (OSP) method named "large-volume dual preconcentration by isotachophoresis and stacking (LDIS)", which is composed of two OSP methods, large-volume sample stacking (LVSS) and transient isotachophoresis (tITP). A typical LDIS-CE-LIF protocol was simple: a short-plug of leading electrolyte (LE) and large-volume sample solution were introduced to a capillary, followed by application of constant voltage. In the analysis of glucose ladder labeled with 8-aminopyrene-1,3,6-trisulfonic acid with 10 mM sodium chloride as LE, up to 2300-fold sensitivity increase was achieved with higher resolution than those in normal CE. By applying pressure assist during preconcentration, both viscous gel electrolyte and salty matrix of up to 10 mM NaCl were acceptable. Finally, *N*-glycans from approximately 100 cells (HeLa, MCF7, and HepG2) were analyzed as the model of localized tumor cells. From 30 to 40 glycans were successfully detected with almost same profile of large-scale sample. *N*-glycan structure could be predicted by searching glucose-unit value via Glycobase database, indicating that HepG2 expressed more sialylated glycans and MCF-7 expressed less glycans respectively, comparing with HeLa cells. It suggests the potential of LDIS-CE-LIF for discovery of disease-specific *N*-linked glycans in microscale environment.

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1. Introduction

Glycosylation is one of the most important post-modification of proteins involved in important cellular functions such as recognition, communication, proliferation, immune response, and differentiation [1–6]. Along with the diseases progression such as cancer and diabetes, structure of *N*-linked glycan is often changed via fucosylation, sialylation, and so on [4–6]. In case of early stage cancer, for example, there are often only limited numbers of primary tumor cells in the tissue and they are localized in 100-μm scale microenvironment [7,8]. For developing effective diagnosis method and drugs, thus, sensitive glycome method for the small population of cells is strongly desired.

Capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detector has been a powerful tool for oligosaccharide analysis due to its high sensitivity and resolution [9–14]. *N*-glycans are typically released by peptide-*N*-glycosidase (PNGase) F, fluorescently labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS), and detected by CE-LIF with sub-nM detectability [13]. Coupled with mass spectrometry (MS) and enzymatic digestion with exo-glycosidase, detailed identification of molecular structure is also possible [14–16]. This structural information is integrated with "glucose unit" (GU) value [17], which is calculated by comparing the migration time of the *N*-glycan with those of glucose oligomers.

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As GU is identical for each structure of *N*-glycans, CE-LIF can be used as an easy and sensitive glycome method [16–18].

Despite the high sensitivity of CE-LIF, injectable volume is normally limited to few nL, which is three orders smaller compared with the prepared sample volume (typically, μL scale). More than 99% sample solution is not used for the analysis, and consequently, the sensitivity potential cannot be fully utilized. To address this issue, online sample preconcentration (OSP) techniques have been developed [19], such as field-amplified sample stacking (FASS) [20], large-volume sample stacking (LVSS) [21–23], dynamic pH junction [24], sweeping [25], and transient isotachophoresis (tITP) [26–28]. In these OSP methods, large-volume sample solution (up to tens μL) is injected and the analytes are focused into a narrow zone (∼few nL), which have provided up to 10,000-fold sensitivity enhancement in oligosaccharide analysis [29–34]. However, these conventional OSP methods lack either resolution or salt-tolerance which are required for practical *N*-glycan profiling. LVSS is one of the most high-resolution OSP methods which keeps more than 90% of resolution compared with normal CE separation. The other advantage of LVSS is its versatile utility for almost all ionic species. However, LVSS is applicable only for lower conductivity sample than 0.02 mS/cm that provides conductivity ratio of BGE/sample higher than 10 [21–23]. It has limited application to *N*-glycan samples typically with greater conductivity than 0.2 mS/cm (equivalent to 1.5 mM NaCl) even if purified with gel filtration or solid phase extraction. LVSS also often provides fronting peaks and disturb peak identification of complicated glycan mixture [29,30]. Therefore, it is required to develop a new OSP method with high salt-tolerance against at least few mM NaCl, and with high sensitivity and resolution.

Here, we got an idea to sequentially combine LVSS with tITP, which provides great salt-tolerance even against sea water which has 0.6 M salt concentration (55 mS/cm) [35]. Generally, tITP seriously lose resolution when large-volume sample is injected. First LVSS preconcentrate salty sample into quite broadened peaks, but it is enough small volume for second tITP focusing without losing resolution. We named this new OSP method as large-volume dual preconcentration by isotachophoresis and stacking (LDIS). It should be noted that other papers have already reported similar OSP methods using multicycle stacking [36,37] and combination of LVSS with dynamic pH junction or sweeping [38–41]. Applicable samples are limited by the employed OSP methods in exchange for higher sensitivity and no salt tolerance was reported. Breadmore reported EOF-balanced isotachophoresis with electrokinetic injection and achieved around 1000-fold sensitivity increase for high-conductivity sample containing 100 mM Cl– [42], although resolution was lowered. Oppositely, LDIS provides high salt-tolerance, high resolution, and versatile applicability for almost all ionic species because of the wide coverage of LVSS and tITP.

In this study, we firstly considered the detailed mechanism of LDIS. Secondly, LDIS was coupled with capillary zone electrophoresis (CZE) separation and LIF detection, where analysis of oligosaccharides was demonstrated to estimate analytical performance such as sensitivity enhancement factor (SEF), resolution, and repeatability. Capillary gel electrophoresis (CGE) was then applied and tolerance of LDIS against viscous background electrolyte (BGE) and salty sample matrix (SM) was evaluated. Demonstration of LDIS-CGE analysis is beneficial to prove the higher analytical flexibility of LDIS not only to oligosaccharide analysis but also many other analyses using viscous gel buffer (e.g., nucleic acid and proteins) and its potential applicability for separation of structural isomers. To demonstrate the glycome analysis of limited amount of sample, finally, around 100 cells of three cancer cell lines were treated in a newly developed protocol as model localized tumor cells. As proteins are involved in tissue-specific glycosylation [16,43], different *N*-glycan profiles were expected. The obtained *N*-glycans were finally analyzed via our developed LDIS-CE-LIF and its potential was discussed.

2. Experimental section

2.1. Materials and chemicals

Fused silica capillaries of 50/375 and 20/375 μm ID/OD were purchased from Polymicro Technologies (Phoenix, AZ, USA); methanol (MeOH), sodium hydroxide (NaOH), tetrahydrofuran (THF), antibiotics (penicillin-streptomycin) solution, 2-[4-(2-Hydroxyethyl)-1-piperazine]ethanesulfonic acid (HEPES), and phosphate-buffered saline (PBS) (-) from Wako (Osaka, Japan); acetonitrile (ACN), 8-aminopyrene-1,3,6-trisulfonic acid (APTS), PVA (M_w = 88,000, 99% hydrolyzed), and bovine ribonuclease (RNase) B and Dulbecco's modified eagle media (DMEM) from Sigma-Aldrich (St. Louis, MO, USA); sodium cyanoborohydride ($NaCNBH_3$) from Tokyo Chemical Industry (Tokyo, Japan); maltodextrin oligosaccharide (glucose ladder) mixture from Elicityl (Crolles, France); sodium chloride (NaCl) from Nacalai Tesque (Kyoto Japan); hydroxypropyl methylcellulose (HPMC) from Alpha Aesar (Heysham, United Kingdom); Agencourt CleanSEQ from Beckman Coulter (Fullerton, CA, USA); PVA-coated (N-CHO) capillary from AB Sciex, (Fullerton, CA, USA); HeLa cell line (JCRB9004) from the Japanese Collection of Research Bioresources Cell Bank; MCF-7 (RBRC-RCB1904), and HepG2 (RBRC-RCB1648) from RIKEN CELL BANK (Tsukuba, Japan); 25 cm^2 cell culture treated flasks (#136196) and trypsin-ethylenediaminetetraacetic acid (EDTA) solution from ThermoFisher Scientific (Waltham, MA, USA); fetal bovine serum (FBS, HyClone SH3007103) from GE Healthcare (Buckinghamshire, UK).

All solutions were prepared with deionized water purified with a Direct-Q System (Nihon Millipore, Tokyo, Japan).

2.2. Sample preparation

Oligosaccharides were released from glycoproteins with PNGase F enzyme using the methods reported previously [29,30]. For fluorescent labeling, oligosaccharides were mixed with 5 μL of 0.1 M APTS in 15% acetic acid and 10 μL of 0.5 M $NaCNBH_3$ in THF. The mixture was kept at 55 °C for 2 h, followed by dilution with water to 100 μL. The obtained sample was further diluted with deionized water or 0.5–100 mM NaCl solution, and injected to a capillary in the following CE experiment.

Cells were cultivated on a plastic flask with DMEM with 10% FBS and 1% antibiotics solution in a humidified atmosphere at 37 °C and 5% CO_2. Cells are harvested by trypsin-EDTA treatment, twice washed with PBS, and suspended to appropriate volume of PBS for cellular concentration around 100 cells/μL. Before dilution, cellular concentration was measured with a C-chip (NanoEntek, Seoul, South Korea). Actual cell number in 1-μL suspension was manually counted via a microscope (Olympus CKX41, Tokyo, Japan). Tens of 1-μL suspension were collected in PCR tubes and remaining suspension was centrifuged to obtain 1.0×10^6 cells pellet, which were stocked under −80 °C until the following preparation. Preparation of *N*-glycan sample was carried out according to previous reports with some optimization (detailed protocol was shown in SI) [17]. It should be noted that no dilution was carried out prior to LDIS preconcentration.

2.3. Capillary electrophoresis

All the experiments were carried out with a PA-800 plus system with 32 Karat Software version 10.1 (AB SCIEX). For the detection, a laser-induced fluorescence (LIF) detector was employed, where

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