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Multi-charged labeling of oligosaccharides and *N*-linked glycans by hexahistidine-based tags for capillary electrophoresis-mass spectrometry analysis☆

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ABSTRACT

The labeling by amino acids and peptides was investigated for sensitive and fast analyses of oligosaccharides and *N*-linked glycans by capillary electrophoresis-mass spectrometry (CE-MS). Peptide tags with a various number of histidine residues were tested for maltooligosaccharide labeling in order to investigate the effect of the size of labels and a number of charges on CE-MS analysis. Nevertheless, the reductive amination labeling of *N*-linked glycans by a hexahistidine tag resulted in a multiple products formation, therefore a peptide tag was modified by hydrazine functionality in order to perform labeling by hydrazone formation chemistry. This labeling approach significantly improved sensitivity with LOD of labeled maltopentaose determined to be 40 nmol/L and also significantly reduced separation time of neutral maltooligosaccharides and *N*-linked glycans released from bovine ribonuclease B. Furthermore, the labeling by this multi-cationic peptide hydrazine tag also allowed performing analysis of acidic glycans by CE-MS in a positive ion mode as demonstrated by separation of sialylated *N*-linked glycans released from bovine fetuin.

1. Introduction

Glycosylation is one of the most common post-translational modifications of proteins and plays crucial role in many biological processes including cell recognition, cell communication, protein stability and protease resistance [1,2]. Since glycans are involved in many patho/physiological conditions, such as cell differentiation, migration, tumor invasion and metastasis, the altered glycosylation might serve as potential biomarker of diseases including cancer [3–5]. Furthermore, glycosylation also affects biological activity, stability, function, clearance from the circulation and antigenicity of recombinant proteins [6].

The extreme complexity and diversity of glycan structures require to use comprehensive analytical methods including high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and mass spectrometry (MS) for detail glycoproteins characterization [7]. The glycoprotein analysis can be performed on several levels such as analysis of intact glycoproteins, glycopeptides and/or glycans. The glycan analysis requires their release from glycoprotein molecule by chemical or enzymatic procedures. Due to the interconversion of α- and β-anomeric forms of the reducing sugar, the lack of chromo- or fluorophores and their low ionization efficiency in MS, a derivatization step is usually involved in the glycan analysis by either LC or CE. The labeling also introduces a charge into the neutral glycan molecules in order to increase the electrophoretic mobility.

Reductive amination is the most commonly used method for labeling of oligosaccharides due to the large number of commercially available labeling tags with an amine functionality such as trisodium 8-aminopyrene-1,3,6-trisulfonate (APTS), 2-aminobenzoic acid (2-AA), 2-aminobezamide (2-AB) and others. The 2-AB tag has been widely applied for chromatographic analysis and an extensive database for a structural assignment has been developed for 2-AB glycans separated by HPLC [8]. However, a major drawback of 2-AB is poor ionization efficiency in a positive ion mode MS. Significantly improved ionization efficiency has been obtained by labeling using procainamide (4-amino-*N*-[2-

Abbreviations: AETMA, (2 aminoethyl)trimethylammonium chloride; DP3, isomaltotriose; DP4, maltotetraose; DP5, maltopentaose; DP6, maltohexaose; DP7, maltoheptaose; PNGase F, peptide-*N*-glycosidase F; RNase B, ribonuclease B.

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(diethylamino)ethyl]benzamide), which has a tertiary amino group in the structure to enhance electrospray ionization (ESI) efficiency [9,10]. Several other cationic labels with different functional groups have been investigated in order to increase the MS sensitivity [11,12]. A (2-aminoethyl)trimethylammonium chloride (AETMA) tag was also used to introduce a quaternary amine functionality into the saccharide molecule followed by ESI/MS, CE/C4D or LC–MS analysis [13–15].

A cationic label Girard's T reagent ((hydrazinocarbonylmethyl)trimethylammonium chloride) was used to improve detection of derivatized oligosaccharides by matrix-assisted laser desorption/ionization (MALDI) and/or electrospray ionization (ESI) MS [16–18]. This labeling reaction is based on a hydrazone formation and introduces a permanent positive charge into the oligosaccharide molecule. Furthermore, Zhao et al. developed several hydrazino-1,3,5-triazine based labels for highly sensitive LC–MS and CE-MS glycan analysis [19,20].

The slow reaction characteristic of reductive amination can be overcome by a rapid reaction of glycosylamines (the precursor of reducing end terminated glycans) with a tag containing a *N*-hydroxysuccinimide group such as InstantAB (a 2-AB analog) [21], InstantPC (a procaine analog) [22], or RapiFluor-MS [23]. RapiFluor-MS contains the tertiary amine group to enhance MS detection and a quinoline part for sensitive fluorescence detection. Triphenylphosphonium derivatives represent another glycosylamine reacting labels [24]. These tags introduce a permanent positive charge to improve ESI–MS sensitivity.

The majority of CE based analysis of *N*-linked glycans has been performed after labeling by APTS followed by CE with LIF detection [25]. On the other hand, there are only few reports dealing with the CE-MS analysis of APTS labeled sugars [26–29] and MS detection in the negative ion mode is considered to be inherently less sensitive [7].

The cationic labels reported for oligosaccharide labeling, usually provide only one positive charge [9,10,13–18,22,23]; however, the analysis of acidic glycans containing several sialic acids by CE-MS performed in a positive ion mode requires attachment of a multicationic tag in order to achieve cationic electrophoretic migration. There are only a few reports focused on oligosaccharide derivatization by multi-cationic labels followed by CE-MS analysis [20].

In this work, we have first tested a labeling method suitable for analysis of acidic *N*-linked glycans by CE-MS in a positive ion mode based on basic amino acids and peptides and reductive amination. Due to the side reaction occurring during *N*-linked glycans labeling by aliphatic amino group containing tags, the hexahistide-based tag was finally modified by hydrazine functionality and hydrazone formation chemistry was used in the final experiments. The applicability of this labeling method was verified by CE-MS analysis of neutral as well as acidic *N*-linked glycans.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were of the highest purity available and used without further purification. Isomaltotriose (DP3), maltotetraose (DP4), maltopentaose (DP5), maltohexaose (DP6), maltoheptaose (DP7), dithiothreitol, peptide-N-glycosidase F (PNGase F), ribonuclease B from bovine pancreas (RNase B), fetuin from fetal bovine serum, histidine, arginine, lysine, and *N*α-Boc-D-lysine were purchased from Sigma-Aldrich (Prague, Czech Republic). Sodium bicarbonate, sodium hydroxide, acetonitrile, methanol, acetic acid, formic acid, sodium tetraborate decahydrate, and hydrochloric acid were purchased from Lach-Ner (Neratovice, Czech Republic). Peptides with an amino acid sequence of HHHHHH, GGGGGHHH were synthetized by Clonestar Peptide Services (Brno, Czech Republic). A peptide with an amino acid sequence of GGHHHHHH was synthetized by Peptide 2.0 (Chantilly, VA, USA). A peptide hydrazine with an amino acid sequence of NH_2NH-GHHHHHHG was synthetized by JPT Peptide Technologies (Berlin, Germany). Bare fused silica capillaries (50 μm and 75 μm ID, 375 μm OD) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Amicon Ultra centrifugal filters (3 kDa and 10 kDa MWCO) were purchased from Merck (Prague, Czech Republic). The HyperSep Hypercarb SPE columns (100 mg) were purchased from Thermo Fisher Scientific (Pardubice, Czech Republic).

2.2. Preparation of glycans from glycoproteins

Standard glycoproteins (2 mg) were dissolved in 400 μL of deionized water. Glycoproteins were denatured before deglycosylation by the addition of a dithiothreitol solution (100 μL, 50 mmol/L in water) and the mixture was incubated at 95 °C for 2 min. Then five units of PNGase F and a 20 mmol/L $NaHCO_3$ solution (500 μL) were added and the reaction mixture was incubated at 37 °C for 24 h. The deglycosylated proteins and enzyme were removed using 3 kDa (RNase B analysis) or 10 kDa (bovine fetuin analysis) MWCO centrifugal filters according to the manufacturer instructions. The collected glycans were subsequently desalted using HyperSep Hypercarb SPE columns. The columns were rinsed by acetonitrile (1 mL) and water (3 mL). The aqueous glycan solutions (1 mL) were loaded and the SPE columns were rinsed by water (3 mL). The glycans were eluted by a solution containing 50% acetonitrile and 1% formic acid (1.5 mL). Acetonitrile was evaporated in the vacuum concentrator (Eppendorf, Ricany u Prahy, Czech Republic) and the aqueous glycan containing solutions were freeze-dried (Martin Christ, Osterode am Harz, Germany). The lyophilized glycans were reconstituted in water at a final concentration of 10 mg/mL of the original glycoprotein.

2.3. Oligosaccharide labeling by reductive amination under acidic conditions

Solutions of maltooligosaccharides (DP3-DP7, 10 μL, 1 mmol/L in water) or released glycans (10 μL, 10 mg/mL of an original glycoprotein solution) were mixed with a label solution (GGHHHHHH, HHHHHH, GGGGGHHH peptide tags or histidine, arginine, lysine, *N*α-Boc-D-lysine amino acids, 10 μL, 0.1 mol/L in water) and acetic acid (10 μL). The mixtures were incubated at 55 °C for 30 min and then a $NaBH_3CN$ solution (10 μL, 1 mol/L in methanol) was added. The reaction mixtures were incubated at 55 °C for 20 h. Finally, the samples were dried in the vacuum concentrator and reconstituted in water (100 μL) before desalting using HyperSep Hypercarb SPE columns and freeze-dried. The lyophilized samples were reconstituted in water before CE-MS analysis.

2.4. Oligosaccharide labeling by reductive amination under alkaline conditions

Solutions of maltooligosaccharides (DP3-DP7, 10 μL, 1 mmol/L in water) or released glycans (10 μL, 10 mg/mL of an original glycoprotein solution) were mixed with a label solution (GGHHHHHH peptide tag, 10 μL, 0.1 mol/L in water). Next a sodium borate buffer (30 μL, 400 mmol/L, pH 8.5) and a $NaBH_3CN$ solution (10 μL, 1 mol/L in a sodium borate buffer) were added. After incubation at 55 °C for 20 h, the mixtures were diluted with a 0.5% formic acid solution (100 μL), desalted by HyperSep Hypercarb SPE columns and freeze-dried. The lyophilized samples were reconstituted in water before CE-MS analysis.

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