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Effects of the redox state of porous graphitic carbon on the retention of oligosaccharides

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ABSTRACT

Retention of hydrophilic compounds on porous graphitic carbon (PGC) is afforded by polar interactions with induced dipoles within this polarizable stationary phase. These interactions depend on the redox state of PGC, which can be influenced by application of an electrical field or by chemical means. We explored the impact of oxidizing and reducing agents on the retention of fluorescence labeled neutral oligosaccharides. Malto-oligosaccharides were employed as simple model system. Subsequently, the effects on the retention of glycans typical for immunoglobulin G (IgG) antibodies were investigated. Chemical oxidation of the PGC surface increased the retention of all analytes tested. Selectivities were significantly altered by the redox treatment, emphasizing the need for controlling the redox state of PGC to achieve reproducible conditions. Furthermore a column pre-conditioning protocol is presented, which allowed for reproducible chromatography of neutral IgG glycans.

1. Introduction

Porous graphitic carbon (PGC) known as very hydrophobic material is a versatile stationary phase for liquid chromatography accomplishing separations of aromatic compounds [1], nucleotides [2], oligosaccharides [3–6] and even inorganic ions [7]. Polar analyte moieties induce dipoles in the highly polarizable graphite-like surface of PGC, resulting in efficient retention even of highly hydrophilic analytes, in contrast to conventional reversed-phases [8]. This effect was termed polar retention effect on graphite.

These polar interactions can be influenced by electric fields, which is exploited in electrochemically modulated liquid chromatography (EMLC) [9]. This technique utilizes modified HPLC columns packed with a conductive stationary phase, which contain a reference and an auxiliary electrode, while the conductive material, e.g. PGC, acts as working electrode. A constant potential in the range of −0.7 to +0.5 V is applied between the stationary phase and the reference electrode, which is controlled by a potentiostat. The applied potential impacts selectivities of charged, but also of neutral analytes and may even alter the elution order, as was shown for corticosteroids [10]. Unintentional alteration of retention on PGC coupled to mass spectrometry was also reported, caused by leakage current from electrospray ionization (ESI) source [11,12].

Similar effects were generated by chemical means [13]. Oxidizing and reducing agents were shown to affect the retention of aromatic sulfonates, while the retention of benzene was only marginally impacted. Also the retention of fluorinated nucleosides [2] was shown to be affected by the PGC redox state.

The redox effect on PGC is often considered to be a polarization effect, meaning a net transfer of electrons towards or from the stationary phase. This charge may be distributed over the conductive surface. The thus generated surface potential alters the adsorption equilibrium of charged analytes according to Coulomb's law. Neutral molecules may interact via permanent or induced dipoles and are expected to be less impacted.

On the other hand, the presence of oxidizable groups was proposed deducing from redox experiments with PGC [13]. Hence functional groups produced by chemical oxidation or reduction of PGC may account for the alteration of selectivities of the stationary phase depending on the redox state.

This publication aims to investigate the influence of the redox state of PGC on the retention of oligosaccharides. Malto-oligosaccharides are studied as simple model system consisting of homogeneous, linear chains containing solely glucose as building block. Furthermore, protein glycans representing typical IgG glycans are studied. All these analytes are labeled with 2-aminobenzamide (2AB) by reductive amination for sensitive detection by fluorescence spectrometry, which is a common strategy for the analysis of protein glycans [14–17]. PGC is reduced and oxidized by chemical means, respectively. The interaction of PGC

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with the oligosaccharides is specified by van't-Hoff plots yielding information about both, enthalpic and entropic contributions to the free energy of adsorption. Furthermore a column pre-conditioning procedure consisting of reduction and subsequent oxidation of the PGC is proposed, which allowed for reproducible analysis of neutral immunoglobulin G (IgG) N-glycans.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were analytical grade or better. Solvents used for chromatography were at least HPLC grade. Malto-oligosaccharides, 2-aminobenzamide (2AB), acetic acid, formic acid, Na[BH_3CN] and $NaBH_4$ were ordered from Sigma–Aldrich (Vienna, Austria). The G0 glycan standard was from Dextra Laboratories (Reading, UK). G0F and G0FB were purchased from PROzyme (Hayward, CA, USA). 2AB-labeled G2F and Man5 were isolated by HILIC chromatography from a 2AB-labeled mAb-glycan sample. The mAb was obtained from in-house development at Sandoz (Kundl, Austria). Acetonitrile (MeCN) and hydrogen peroxide were from Merck (Darmstadt, Germany). Ammonia solution was from AppliChem (Darmstadt, Germany). Water was prepared by a Milli-Q® system (Millipore, Billerica, MA, USA). PD MiniTrap™ G10 gel filtration columns were ordered from GE Healthcare (Vienna, Austria).

2.2. Sample preparation

Malto-oligosaccharides, G0, G0F and G0FB were derivatized with a fluorescent dye to enable sensitive detection. The labeling solution consisted of 50 mg/mL 2AB and 63 mg/mL Na[BH_3(CN)] in dimethyl sulfoxide (DMSO)/acetic acid at a ratio of 7:3. 15 μL of this solution was added to 9 μL of a standard solution of malto-oligosaccharide (1 mg/mL). The mixture was incubated at 37 °C over night. Excess label was depleted by application to custom made PD MiniTrap™ G10 gel filtration columns.

2.3. Instrumentation

Fluorescence chromatograms were recorded on an Agilent 1200 SL system, consisting of a binary pump, a vacuum degasser, an autosampler, a column thermostat and a fluorescence detector. The temperature of the column was measured using an external thermometer with an accuracy of 0.1 °C.

2.4. Chromatographic conditions

The retention study experiments were conducted on a 100 mm × 3 mm (i.d.) PGC column (Hypercarb®, Thermo Electron) packed with 3 μm particles. Mobile phase A was 50 mM ammonium formate at pH 3.8. Mobile phase B additionally contained 50% MeCN. The labeled oligosaccharides were detected by fluorescence spectrometry with excitation at 250 nm and an emission wave length of 428 nm.

For the van't-Hoff plot measurements the PGC column was reduced by switching to a mobile phase containing 50 mM ammonium formate at pH 9.3 and raising the temperature of the column thermostat to 90 °C. Formic acid may take part in a number of redox reaction including oxidation yielding carbon dioxide. In the course of this reaction two electrons are transferred towards an oxidizing agent, which itself is converted to its reduced state. In this case the electrons are transferred towards PGC resulting in reduction of the stationary phase. Due to the generation of two protons this reaction is favored at basic pH. The presence of MeCN was found to be essential to set on the chemical reduction process. Oxidation of the PGC material was accomplished by the injection of 100 μL of a mixture of 3% H_2O_2 and 10% acetic acid in water which leads to the formation of peroxyacetic acid. After both, oxidation and reduction of the PGC surface and PGC column, respectively it was flushed alternately with 10 and 45% MeCN, respectively.

2.5. van't-Hoff plots

For characterization of interactions between the analytes and the stationary phase van't-Hoff plots were produced yielding adsorption enthalpies and entropies. Retention times were measured in duplicate at three temperatures and the logarithm of the retention times was plotted against the inverse temperature (van't-Hoff plot). The slopes of these linear plots correspond to $-\Delta H/R$ and the ordinate intercepts correspond to $\Delta S/R - \ln(\beta)$. Hence, if the phase ratio β is known, van't-Hoff plots yield information about enthalpic and entropic contributions to the free energy of adsorption of the analytes on PGC. The phase ratio of Hypercarb® was taken from literature [18].

2.6. Establishing and testing a stable redox state on PGC

On the basis of an oxidation procedure published recently [2] a method was developed, which generates a reproducible redox state of the PGC column. The mobile phases A and B, as well as the dimensions of the chromatography column were identical to Section 2.4, respectively. In a first step the PGC material was reduced by injection of 50 μL 10% $NaBH_4$ solution and the column was rinsed with approximately seven column volumes 85% B. Subsequently the mobile phase A was changed to 0.071% H_2O_2 in 50mM ammonium formate at pH 3.8, which was maintained for 30 min. Afterwards the starting conditions were re-established. Before starting the first analytical run a blank injection (50 μL H_2O) was performed and the column was equilibrated with the starting conditions.

For testing the stability of the PGC redox state a 2AB-labeled mAb-glycan sample was repeatedly analyzed applying a comparably short gradient. The fraction of eluent B started at 50% and was raised to 95% in 20 min, which was kept for 10 min. Subsequently the column was re-equilibrated with the starting conditions for 19 min. The flow rate was 0.5 mL/min. The column oven temperature was set to 70 °C.

3. Results and discussion

2AB-labeled oligosaccharides possess a secondary, aromatic amino group acting as link between the label and the carbohydrate reducing end. A calculated pK_a-value of 2.62 ± 0.50 for an analogous compound (2AB-labeled galactose) [19], implies that the aromatic amines of the studied analytes are practically non-protonated at pH 3.8 of the running buffer. The content of MeCN in the running buffer, which was at least 25% throughout this study, may further decrease the degree of protonation. Hence, the 2AB-labeled oligosaccharides are considered as neutral but polar molecules under the applied conditions.

3.1. Influence of the redox state of PGC on the retention of malto-oligosaccharides

Malto-oligosaccharides consist of linear chains of α(1,4)-linked glucose units. For these studies oligosaccharides with two to seven glucose units were employed due to their commercial availability. The malto-oligosaccharide standards are referred to as Mal*n*, whereas *n* denotes the number of glucose units.

As indicated by the adsorption enthalpy and entropy values in Table 1, the redox state of PGC significantly impacts the retention behavior of neutral oligosaccharides. Oxidation of PGC increased