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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

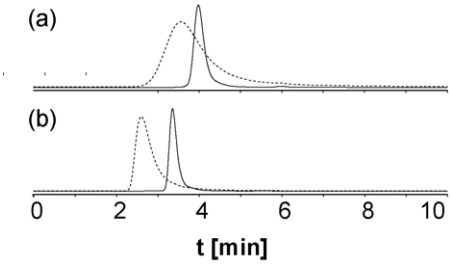

Fig. 1. Chromatograms of Mal3 (solid line) and Mal7 (dashed line) on (a) oxidized PGC and (b) reduced PGC, respectively. The column temperature was 29.6 °C.

retention of all oligosaccharides compared to reduced PGC due to lower adsorption enthalpies, which overcompensate for the lower gains of entropy within the studied temperature range. Since all malto-oligosaccharides are similarly affected selectivities are only slightly altered, as illustrated in Fig. 1 for Mal3 and Mal7.

Generally, the differences in adsorption enthalpies on oxidized and reduced PGC increase with the number of glucose units, which is in accordance with the proposed retention mechanism. The more polar (hydroxyl) groups the analyte possess the higher are the decreases of adsorption enthalpy and entropy detected. This trend is most pronounced for the smaller oligosaccharides reaching approximately constant differences for the higher oligomers Mal6 and Mal7.

According to our previous investigations, the gain of entropy correlates with the area of interaction between the analyte and the PGC surface [20]. Hence lower entropy values, as resulting from oxidation of PGC, may indicate diminished interactions between the adsorbed molecules and the stationary phase. However, since retention is increased and the adsorption enthalpies are decreased (i.e. more favorable) we propose that the lowering of the entropies is caused by increased interactions between the hydroxyl groups of the analytes and the oxidized PGC surface. The stronger binding to the surface may cause a decrease of the degrees of freedom of the analytes thus explaining the lower entropy values measured. Consequently, the differences between adsorption enthalpies on reduced and oxidized PGC may correspond to the contribution of the polar retention effect on graphite to the overall retention.

The data obtained by this experiment series are qualitatively in accordance with the data from our previous study [20], since enthalpy and entropy values are all positive and increase with the number of glucose units. But quantitative comparison reveals discrepancies, as data with both, reduced and oxidized PGC are significantly lower than data from untreated PGC. For the previous study a PGC column was used, which could not be reduced by the procedure described. This finding indicates an irreversible alteration of the stationary phase. The phenomenon of column "aging" was reported for PGC columns leading to a decreased retention of oligosaccharides and nucleotides [2,12]. By contrast, our data demonstrated increased retention on the "aged" column. Therefore it remains unclear whether these reports correspond to the same type of initiated surface modification.

Table 1
Adsorption enthalpies and entropies of 2AB-labeled malto-oligosaccharides on oxidized and reduced PGC, respectively. The fraction of MeCN in the mobile phase was 35% MeCN.

	PGC	Mal3	Mal4	Mal5	Mal6	Mal7
ΔH [kJ/mol]	Oxidized	12.6	15.6	16.9	17.5	18.8
	Reduced	15.3	19.0	21.5	22.6	24.0
ΔS [J/(mol K)]	Oxidized	53.2	62.4	65.9	67.1	71.7
	Reduced	60.0	71.2	77.8	80.3	84.6

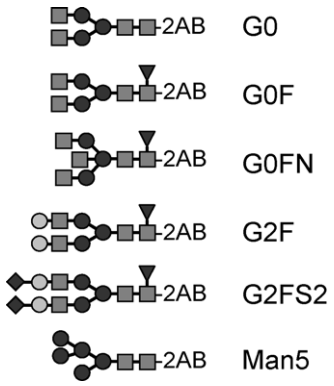

Fig. 2. Illustration of N-glycan structures. (■) N-acetylglucosamine, (▼) fucose, (●) mannose, (○) galactose, and (◆) N-acetylneuraminic acid.

3.2. Influence of the column temperature on the retention of N-glycans

Due to its flat surface, PGC offers interesting selectivities for analytes differing in their three-dimensional structure. Thus, it is often employed for the separation of branched oligosaccharides, e.g. of protein glycans [3]. These molecules differ from the malto-oligosaccharides not only by the presence of branchings, but also because they are composed of several types of monosaccharides (mannose, N-acetylglucosamine—GlcNAc, fucose, galactose, etc.).

For our studies we utilized five protein N-glycans typically for IgG antibodies [21] (G0, G0F, G0FN, G2F, G2FS2, Man5; see Fig. 2). The G0 glycan is the core structure for biantennary glycans, which may be expanded in living cells by glycosyltransferases. G0F possesses an additional core-fucose, which is known to increase the retention on PGC [4], whereas, the bisecting N-acetylglucosamine of G0FN was reported to decrease the retention [22]. Thus, G0 and G0FN exhibit similar retention on PGC even though they differ in their composition by two monosaccharide units. G2F exhibits a galactose attached to both branches of core structure and a core-fucose. Man5 is a high-mannose type glycan, which is typically present in antibodies in the low percent range.

In the studied temperature range G0 and G0FN exhibited similar retention times. Indeed, at a temperature of approximately 64 °C these glycans co-eluted from the PGC column, but due to the difference in adsorption enthalpy G0FN eluted first at higher temperature and vice versa. Man5 was strongly retained on PGC eluting close to G2F. However, the adsorption enthalpy of the high-mannose glycan was significantly lower, implying a correspondingly lower increase of the retention factor with temperature. As these examples clearly demonstrate the column temperature is an important parameter for the selectivities of PGC for N-glycans.

3.3. Influence of the redox state of PGC on the retention of N-glycans

Consistent with the findings employing malto-oligosaccharides, oxidation of PGC resulted in increased retention of all glycans,

Table 2
Adsorption enthalpies and entropies of 2AB-labeled N-glycans on reduced and oxidized PGC, respectively. MeCN was used as organic modifier.

	PGC	G0	G0F	G0FN	G2F	Man5
ΔH [kJ/mol]	Oxidized	40.9	49.1	30.3	54.7	34.0
	Reduced	45.2	53.5	34.4	63.6	44.4
ΔS [J/(mol K)]	Oxidized	132	160	100	178	120
	Reduced	141	170	109	201	146

Table 3
Average retention times and average drifts per run based on six consecutive runs on two pre-conditioned PGC columns, respectively.

Glycan	Column 1		Column 2	
	Average retention time [min]	Average drift per run [min]	Average retention time [min]	Average drift per run [min]
G0F	9.92	0.00	10.19	0.01
G1F	10.40	0.00	10.64	0.01
G2F	10.74	0.00	10.95	0.01
Man5	11.10	0.00	11.43	0.03
G2FS2	22.10	0.26	15.50	0.12

due to a decrease in adsorption enthalpies overcompensating the reduction of entropy. The enthalpies of G0, G0F and G0FN exhibited comparable increases, whereas the increase of the enthalpy of G2F was approximately twice as much. Man5, a high-mannose type glycan, revealed the largest increase in adsorption enthalpy by oxidation of PGC. The changes in adsorption entropies exhibited a very similar pattern as the adsorption enthalpies.

In analogy to the reasoning for the retention behavior of malto-oligosaccharides (see Section 3.1), the decrease of the adsorption enthalpies by oxidation of PGC is assigned to enhanced polar interactions of the glycans with the oxidized PGC surface. These increased interactions reduce the degree of freedom of the adsorbed molecules, resulting in lower adsorption entropy values (Table 2).

These results have implications for the application of PGC to the analysis of glycans. Changes of the PGC redox state significantly impact selectivities due to alterations of the adsorption enthalpies and entropies. Additional experiments showed, that reduction of PGC is more quickly achieved by injecting 50 μL of a 10% $NaBH_4$ solution onto the column. The effects for neutral glycans were identical to the procedure employing ammonium formate. As demonstrated in Fig. 3, the status of a "reduced" column enabled a proper separation of the two neutral glycans G0 and G0FN which were not resolved on the "oxidized" column. G2FS2 on the other hand, co-eluted with G0FN on the reduced column but was not detected after oxidation of the stationary phase. Similar results were reported for the effect of an ESI voltage on the retention of reduced glycans [12]. The applied voltage increased the retention of G2FS2 so that it could not be eluted. Notably, also at negative ESI voltage the retention on PGC was increased, which may be explained by redox reactions occurring on the surface of conductive materials within electric fields [23]. According to the proposed model the potential difference along the column within the mobile phase results in oxidation of the PGC stationary phase at one end of the column and reduction at the other end. Thus charged glycans may not be eluted from the PGC column irrespectively of the polarity of the applied voltage.

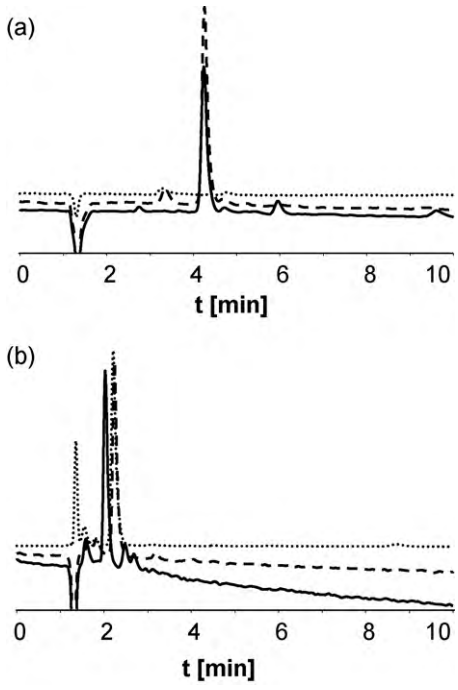

Fig. 3. Overlay of chromatograms of G0 (solid line), G0FN (dashed line) and G2FS2 (dotted line) at 64 °C on (a) oxidized PGC and (b) reduced PGC.

The mentioned examples highlight the importance of adequate control of the PGC redox state for the separation of both, neutral and acidic glycans. Recently an oxidation procedure was proposed for the reproducible chromatographic analysis of nucleosides and nucleotides on PGC [2]. The column was oxidized by applying hydrogen peroxide in the running buffer prior to the analysis. However this procedure did not provide reproducible chromatograms in our case. Retention times markedly decreased for consecutive runs, which is typical for oxidized PGC columns according to our experience. Thus, an alternative procedure was developed. The column was reduced by the injection of sodium borohydride solution prior to the oxidation by hydrogen peroxide. Borohydride is a strong reducing agent and thus results in complete reduction of PGC independently from its initial state. On reduced PGC columns retention times increased from run to run. Hence a mobile phase containing hydrogen peroxide was delivered to the column. If the column was oxidized beyond the equilibrium state retention times decreased from run to run. Thus the concentration of the oxidizer as well as the incubation time requires accurate adjustment to achieve repeatable retention times for neutral glycans. The final method is described in Section 2.5. The general applicability of the column conditioning method was verified by performing this method on a second column.

For both columns average retention times and average drifts of the retention times of individual glycans are shown in Table 3. The differences in the average retention times are below 3% for all neutral glycans, which demonstrates that a reproducible redox state was achieved by the redox treatment. The low drift values demonstrate minimal aberrations of the retention times of neutral glycans from run to run. However retention data of G2FS2 indicate much worse reproducibility for charged glycans. The average retention times differ by approximately 6.6 min. Additionally a significant drift towards higher retention times was observed on both columns. Hence the pre-conditioning of the PGC material by reduction and subsequent oxidation, which works well for neutral glycans, may not be appropriate for charged glycans.

4. Conclusion

The redox state of PGC was demonstrated to impact the retention of neutral 2AB-labeled malto-oligosaccharides on this stationary phase. Oxidation results in lower adsorption enthalpy and entropy values, which is assigned to increased interactions between the hydroxyl groups of the carbohydrates and the

oxidized PGC surface. The stronger binding of the polar analyte to the PGC surface may reduce the degrees of freedom of the adsorbed molecules resulting in lower adsorption entropies.

Retention studies of protein N-glycans typical for h-IgG confirmed the same retention behavior for this group of oligosaccharides. The core-fucose, which is attached to the C6-atom of the reducing end GlcNAc, extensively interacts with PGC, which can be rationally explained by the relatively high hydrophobicity of this deoxyhexose. The reducing end GlcNAc is derivatized with 2AB it exhibits an open-chain conformation. Hence, the fucose is relatively free to move allowing for intensive interactions with the rigid stationary phase. The bisecting GlcNAc, on the other hand, obviously prevents interactions of the residual glycan, as demonstrated by markedly reduced adsorption enthalpies and entropies, thus decreasing retention of glycans carrying this moiety in the studied temperature range.

For neutral glycans selectivities were shown to be affected by the temperature and partially also by the redox state, but to a minor extent. Even though these comparably small differences in selectivities may increase or diminish resolution of critical pairs of analytes, as was shown for G0 and G0FN. In contrast, an acidic glycan exhibiting a similar retention factor as the neutral glycans on reduced PGC could not be eluted from oxidized PGC. Since similar results were reported provoked by an electrical potential the emergence of a surface charge is proposed to induce increased retention of oligosaccharides on oxidized PGC. Hence, atmospheric oxygen, which readily dissolves in organic solvents, should be considered as potential source of interference for the retention properties of PGC.

To achieve reproducible conditions on PGC a column preconditioning procedure was developed including reduction and subsequent oxidation of the stationary phase. This procedure resulted in similar and repeatable retention times of neutral glycans on to different columns. On the other hand the charged glycan species G2FS2 still exhibited significantly different and drifting retention times.

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