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DISSERTATION

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„Investigation of High-Performance Liquid Chromatography Methods for the Analysis of Protein N-glycans“

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1 Introduction

In contrast to small-molecule drugs therapeutic proteins generally exhibit intrinsic variability, due to post-translational modifications, including N-glycosylation, O-glycosylation, disulfide bond formation, amidation, de-amidation, etc.. Particularly glycans cause a high degree of heterogeneity, because their synthesis is not template driven, but the result of the catalytic activity of a number of enzymes. Furthermore it depends on the concentrations of the activated monosaccharides necessary for the enzymatic reactions. Thus a biopharmaceutical drug product can not be considered a single entity, but consists of a high number of variants, which may significantly differ in their pharmaceutical properties, e.g. target affinity, bioactivity or serum half-life. The high degree of heterogeneity constitutes a major challenge for the biopharmaceutical industry. Particularly manufacturers of biosimilar drugs have an outstanding interest to demonstrate physico-chemical equivalence, because this allows for referring to clinical data of original drugs, which have been approved by the *European Medicines Agency* (EMEA). Hence for the development of biosimilars a comprehensive characterization of the variants of the originator and of the product under development is mandatory. Furthermore fast profiling methods are needed to verify batch-to-batch consistency for in-process control and quality control purposes.

Chromatographic techniques are indispensable tools for analysis in the biopharmaceutical industry enabling separation of variants from the micro to (semi-)preparative scale. The diversity of chromatography modes allows for discrimination according to a number of parameters, e.g. size, charge and hydrophobicity. Recent developments in particle technology (sub-2µm particles [1], porous-shell / fused-core particles [2]) and instrument technology reduce run times and thus increase the throughput. Furthermore *hydrophilic-interaction chromatography* (HILIC) has become a valuable instrument for the analysis of hydrophilic compounds adding alternative selectivities to established chromatography modes.

This introduction provides an overview of the glycosylation of biopharmaceuticals and discusses the impact of the glycans on the pharmaceutical properties. Relevant parameters describing the glycan pattern are presented. Finally methods for glycan analysis on the levels of intact proteins, (glyco-)peptides, and free glycans are presented.

1.1 Biosynthesis of N-glycans

In eukaryotes the biosynthesis of N-glycans starts at the cytosolic side of the *endoplasmic reticulum* (ER) [3]. A precursor glycan consisting of *N-acetylglucosamin*e (GlcNAc) and *mannose* (Man) monosaccharides is built attached to dolichol via a pyrophosphate. Then the precursor flips into the ER lumen, where it is further extended by mannose and glucose units and consequently transferred to an evolving protein chain containing the Asn-X-Ser/Thr consensus sequence, whereas X is any amino acid without proline. Hence the N-glycosylation of proteins is a co-translational event [4], even though it is usually counted among post-translational modifications. Within the ER lumen the terminal glucose units are hydrolyzed by exoglycosidases. Only glycoproteins, which do not contain these glucoses are further transported into the Golgi.

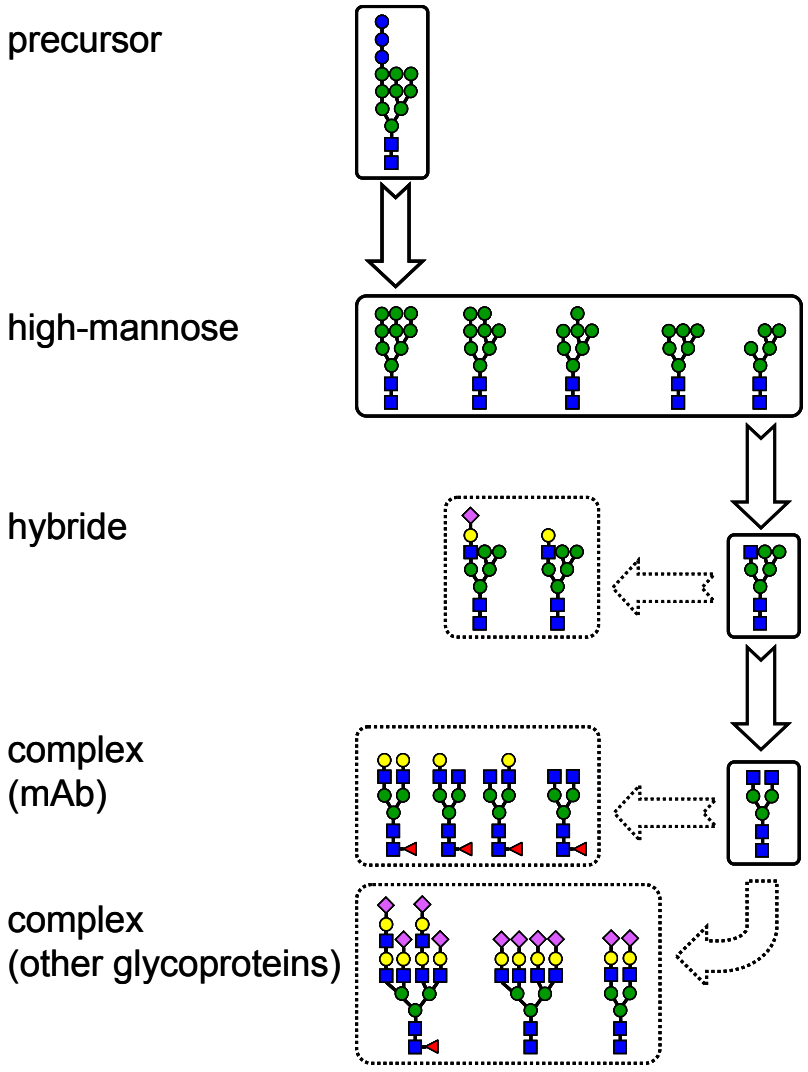

Figure 1. Biosynthesis of protein N-glycans in eukaryotic cells.

The Golgi typically contains a high number of glycan processing enzymes, which either catalyze the transfer of a monosaccharide unit towards a glycan (glycosyltransferases) or the hydrolysis of a monosaccharide (exoglycosidases). Glycosyltransferases generally offer high selectivities for the monosaccharide donor and the anomeric configuration and linkage of the glycosidic bond established. Thus the glycosidic linkage can be deduced from the monosaccharide sequence. Sialic acids attached to terminal galactoses represent an exception, because two different exoglycosidases may be present in mammalian cells specific for α-2,3- and α-2,6-linkage, respectively.

Nevertheless the resulting glycan pattern depends on numerous parameters, e.g. the expression level of the glycosyltransferases, the availability of monosaccharide-donors and the properties of the growing polypeptide chain. Hence it is susceptible to changes in cell culture conditions and must therefore be monitored to demonstrate batch-to-batch consistency of the production.

1.2 N-glycans in mammalian cells

1.2.1 The structure of mammalian N-glycans

The complexity of the anabolic pathway of protein N-glycosylation gives rise to a great variability of possible glycan structures. Nonetheless all N-glycans share a core structure of two GlcNAc and three man units, illustrated in Figure 2. Three major groups are discerned: high-mannose, complex and hybrid-type glycans.

High-mannose glycans resemble the structure of the precursor transferred from dolichol phosphate to the polypeptide chain within the ER. They differ only in the number of mannose units released by exoglycosidases. Typically they are termed *ManX,* whereas X counts the number of mannose units. For Man6, Man7, and Man8 several isomers may exist, which may be differentiated by explicitly giving the sites of the glycosidic bonds resulting in complex notations. Hence the structures are often illustrated and assigned to arbitrary names, e.g. Man7a.

Hybrid-type glycans are also incompletely processed glycans, which exhibit a complex-type (1,3)-branch and a high-mannose type (1,6)-branch. Similarly to high-mannose glycans no consistent nomenclature was developed for hybrid-type glycans. Thus referring to structures is typically accomplished by illustrating and numbering the structures of interest.

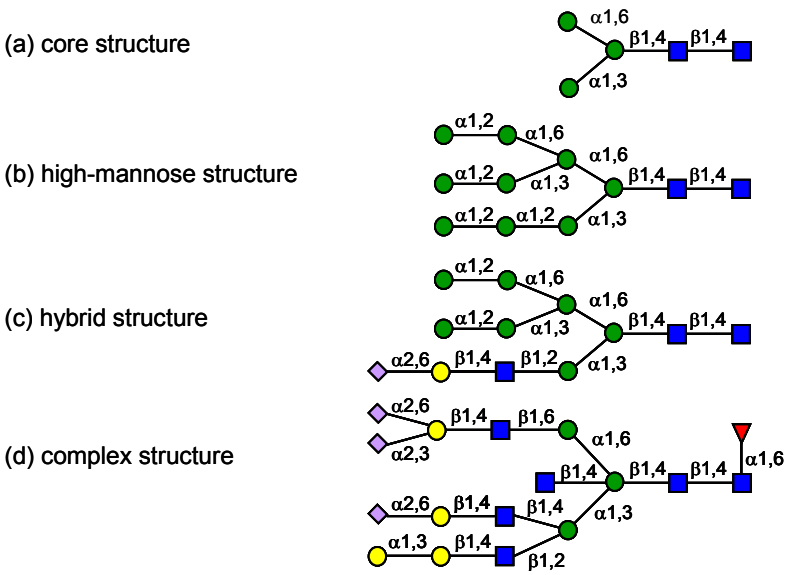

Figure 2 Illustration of (a) the glycan core structure, as well as (b) high-mannose, (c) hybrid, and (d) complex type glycans.

Complex glycans represent the most variable group of N-glycans. This group is characterized by the attachment of at least one GlcNAc to both mannose units at the non-reducing end of the core structure forming the antennae. Galactose units are generally added to the terminal GlcNAc units. Additional GlcNAc units can be added to the branching mannose units, which are further processed in the same manner as the other branches. In contrast the so-called "bisecting GlcNAc" which is linked via a β-1,4-glycosidic bond to the central mannose unit is not further processed.

Further modifications of the antennae include the addition of α-linked galactose, *N-acetyllactosamine* (LacNAc) disaccharides or sialic acids of which two different forms are commonly found in mammalian cells, *N-acetylneuraminic acid* (NANA) and *N-glycolylneuraminic acid* (NGNA). Human cells are not able to produce NGNA. Even though these monosaccharides can be found in human glycoprotein, which is explained by dietary uptake. As stated in section 1.1 sialic acids are either linked via α-2,3 or via α-2,6 glycosidic bonds. α-galactose is a non-human glycan modification and thus may cause an adverse immune response [5].

1.2.2 Glycans of important expression systems

Glycosylation is a very common post-translational modification of proteins expressed in eukaryotic cells. Furthermore archaea and certain are capable of protein glycosylation, even though their glycans significantly differ from glycans found in mammalian cells [6-8]. Thus therapeutic glycoproteins are typically produced in eukaryotic expression systems [9].

Yeast cells

Yeast cells provide high product titers and are often used for the expression of proteins, which are not correctly folded when expressed in bacteria. As eukaryotic cells they are also glycosylation-competent and recognize the same consensus sequence as mammalian cells, but the structure of the glycans significantly differ from human glycans.

In contrast to higher eukaryotes the Man8 structure imported into the Golgi is not trimmed, but extended by additional mannose units. The resulting glycans can contain up to 200 mannose units [8]. Furthermore these hyper-mannose glycans may exhibit terminal Man-α1,3-linkages, which are immunogenic. Hence, therapeutic glycoproteins are usually not expressed in yeast cells.

Mouse cells

In mouse cells the N-glycans are quite similar to human glycans, but certain differences exist. A study comparing the glycosylation of *immunoglobulin G* (IgG) between several species found only NGNA and no NANA. Furthermore no glycans containing bisecting GlcNAc were detected, in contrast to human IgG [10]. An antibody expressed in mouse cells additionally exhibits Gal-a1,3-Gal [11], which is immunogenic in humans [12].

Chinese hamster ovary cells

Chinese hamster ovary (CHO) cells are predominantly employed for expression of therapeutic glycoproteins. Even though they contain the gene encoding Gal-α1,3-transferase, this gene is not active in CHO cells. Furthermore the ration of NANA/NGNA is comparably high, although it depends on the culture conditions [13].

1.2.3 Glycans of therapeutic glycoproteins

Tissue plasminogen activator

The *tissue plasminogen activator* (tPA) contains 527 amino acids was one of the first glycoproteins produced for pharmaceutical application. It contains three glycosylation sites [14], which differ not only in their glycan pattern, but also in the occupancy with glycans. High-mannose glycans, which are present in high amounts in only one of these sites reduced the serum half-life of tPA by binding to the mannose receptor on liver endothelial cells [15]. In a genetically modified variant the translocated glycosylation site was occupied by sialylated complex type glycans. This variant offered an increased serum half-life allowing for the reduction of the dose [16].

Erythropoietin

Erythropoietin (EPO) is composed of 165 amino acids and contains three N-glycans and one O-glycan. The structure is depicted in Figure 3. The N-glycans are of the complex type, core-fucosylated, typically with three to four antennae and a variable number of LacNAc units [17]. EPO glycans are heavily sialylated and the degree of sialylation was demonstrated to impact the affinity to the acceptor and the clearance rate. The higher the number of sialic acids the lower is the affinity to the receptor [18]. On the other hand EPO is eliminated from the plasma by an asialoglycoprotein receptor in the liver [8, 18, 19]. Hence the efficacy of EPO increases with the degree of sialylation.

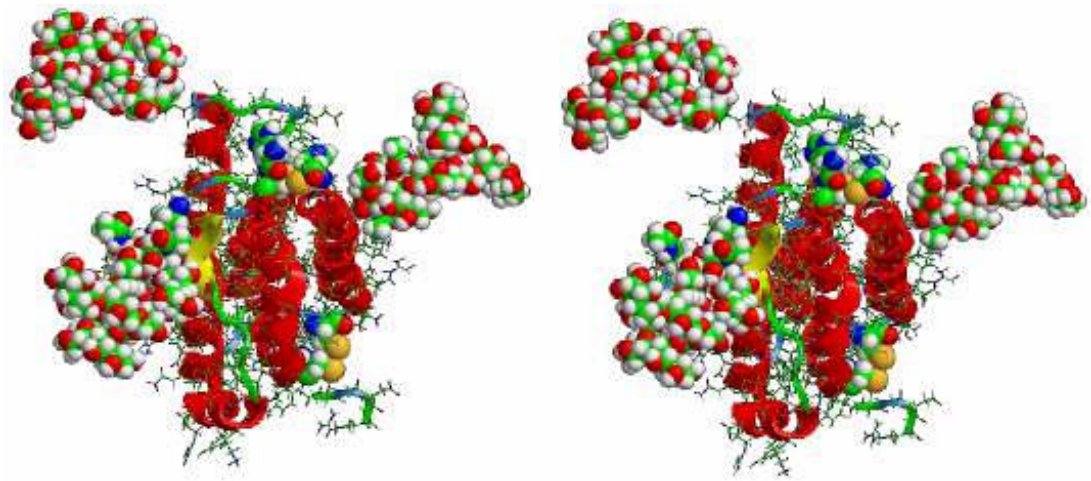

Figure 3. Structure of glycosylated rhEPO. The two pictures generate a 3D-image when viewed applying the crossed-eye technique.

Monoclonal antibodies – Fc parts

Monoclonal antibodies (mAbs)are a promising group of biopharmaceuticals and have already proven their usefulness in the therapy of a number of serious diseases, e.g. rheumatoid arthritis and cancer. Even compared to other therapeutic proteins mAbs are quite complex molecules consisting of four peptide chains, which are connected by disulfide bonds. Most therapeutic antibodies are of the IgG1 type [20], consisting of two heavy chains and two light chains, which are linked by disulfide bridges. The Fc part of the heavy chain contains a conserved N-glycosylation site, which is mainly occupied by complex type, fucosylated, biantennary glycans with variable galactosylation. The glycosylated Fc part is illustrated in Figure 4.

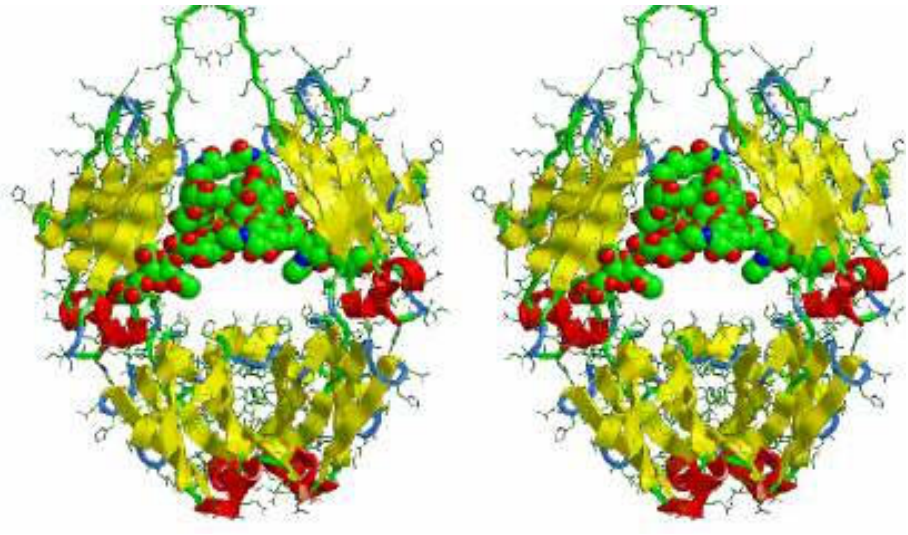

Figure 4. Illustration of a glycosylated mAb Fc part. The two pictures generate a 3D-image when viewed applying the crossed-eye technique.

Human IgG glycans also exhibit an additional GlcNAc moiety at the branching Man – a so-called bisecting GlcNAc, which is typically absent in recombinant antibody products. Even though the Fc glycans are embedded between the two protein chains, they significantly impact the interaction with effector cells and thus the bioactivity [20] and the clearance rate [21] of the antibody. The absence of the core Fuc increases the activity of the antibody in *antibody-dependent cellular cytotoxicity* (ADCC) assays [22-24]. Also the galactosylation level was shown to impact the *complement-mediated cell lysis* (CMCL) assay [16]. Sialylation was reported to impact the inflammation activity of the antibody [25], but in contrast to other glycoproteins serum half-lives are not significantly affected [12]. The glycans are shielded by the peptide chains diminishing interactions with receptors responsible for plasma clearance, which may also explain the increased plasma half-lives of high-mannose glycan containing antibodies [12].

Monoclonal antibodies – Fab parts

Antibodies may also contain glycans in the Fab region in addition to the conserved N-glycosylation site in the Fc part. In human plasma about 30 % of the IgG exhibit Fab glycans [12]. A therapeutic antibodies exhibiting glycans in the Fab region was also approved (Cetuximab). In contrast to Fc glycans these glycans are extensively galactosylated, exhibit a high degree of sialylation and are of the biantennary or triantennary complex type [11]. Notably the presence of Gal-α-1,3-Gal moiety was reported, which may induce an immune response in humans representing a considerable concern for the safety of the product

1.2.4 Relevant parameters of glycosylation

As illustrated by the samples in the former section glycosylation significantly impacts the efficacy and the safety of therapeutic glycoproteins [13, 20, 26, 27]. Generally a high degree of sialylation offers significantly increased serum half-lives due to decreased affinity to asialoglycoprotein receptor, but receptor affinities necessary for the function of the product may also be reduced. High-mannose glycans represent incompletely processed glycans and are thus rapidly removed from circulation, which affects efficacy.

The Fc glycans of antibodies are an exception due to their unique position enclosed by the polypeptide chains resulting in decreased interactions with glycan receptors. Nevertheless mAb-Fc glycans significantly impact the affinity of effector cells towards the antibody. This issue was also accounted for by the EMEA in the *Guideline on development, production, characterisation and specifications for monoclonal antibodies and related products* [28], claiming the characterization of the glycans in respect to mannosylation, galactosylation, fucosylation and sialylation. These parameters are considered to influence the efficacy of the antibody. Furthermore non-human glycan structures are a safety issue, because they may induce a immune response.

NGNA is a common sialic acid in mammals, but can not be produced by human cells due to a mutational inactivation of a necessary enzyme [8]. Even though this monosaccharide is also incorporated in human glycans likely via dietary uptake. Low levels (1 % of total sialic acids) of NGNA do not induce an immune response, while higher amounts may be critical [8, 13, 19]. In contrast glycans exhibiting the Gal-α1,3-Gal moiety have been clearly demonstrated to induce severe immune responses even at low levels due to high concentrations of antibodies directed against this epitope in human serum [5]. Thus the levels of the immunogenic structures are further important parameters for glycan analysis during development and for quality control.

1.3 Analysis techniques for protein N-glycans analysis

The characterization of glycans of biopharmaceuticals requires highly selective analytical methods, which can discern closely related glycans. Furthermore relative quantitation must be performed to assess the critical parameters described in the former section. This section summarizes several *mass spectrometry* (MS) and *high-performance liquid chromatography* (HPLC) techniques providing different levels of detail on the glycan pattern. Detailed information can be found in several review articles [9, 29-34]. Furthermore *ion mobility spectrometry* represents an emerging technique for

glycan analysis, as several publication in this field demonstrate [35-37].

1.3.1 Mass spectrometry based techniques

Modern MS instruments nowadays allow for direct analysis of intact proteins providing a quick evaluation of their glycan pattern [37-41]. The relative intensities of the main glycans G0F, G1F and G2F, as well as Man5 can be taken directly from the de-convoluted mass spectrum of the intact mAb. Limited proteolysis of the mAb with papain cleaves the heavy chains in the hinge region generating one Fc and two Fab fragments. The mass of the detected Fc molecule is approximately one third of the intact mAb resulting in increased resolution of isoforms.

A similar level of detail is attained by MS analysis of the heavy chain [37, 38, 41], which is obtained by reduction of the disulfide bridges of the antibody. But in contrast to MS of the Fc the heavy chain contains a single glycan. The reduced heterogeneity allows for differentiation between glycans with similar mass (e.g. G0F-GlcNAc and Man5 corresponding to a difference of about 25 Da). Nonetheless information of correlations between the glycan compositions of the two heavy chains is lost by this method.

The combination of papain digest and reduction yields heavy chain fragments of approximately 26 kDa containing one glycan [38, 42]. The resolution of *modern time-of-flight* (TOF) mass analyzers enables for unambiguous discrimination of glycans with similar mass, e.g. G0F and G1 with a mass difference of approximately 16 Da. Thus this method yields a detailed glycan pattern, while demanding comparably little sample preparation. Even though this method may not be appropriate for detection of minor glycan variants, as well as for quantitative analysis particularly of acidic glycans.

The examples given demonstrate that MS is an important technique for the analysis of protein glycosylation and offers a relatively high degree of detail depending on the mass of the glycoprotein. But due to differences in ionization efficiencies and suppression effects the signal intensities do not necessarily reflect the ratios of the glyco-isoforms.

1.3.2 Capillary electrophoresis based techniques

Capillary electrophoresis (CE) provides orthogonal selectivities and thus complementary information to chromatographic techniques. Its capability to separate intact glycoprotein isoforms according to the number of sialic acids is particularly useful for high-throughput analysis e.g. for quality control. Coupling to MS allows for detection of additional glyco-isoforms. CE-MS

techniques were reviewed recently [43]. This review also covers separations of glycopeptides and free, fluorescence labeled glycans (*glycan mapping)*.

Since glycan mapping by CE necessitates charged analytes 2AA or higher aromatic compounds containing several sulfonic acid moieties, e.g. *8-aminopyrene-1,3,6-trisulfonic acid* (APTS), are used as fluorescence label. The increased charge of the latter label allows for short analysis times and increases the resolution of neutral glycans [44]. Furthermore the excitation of APTS is compatible with commercial lasers (argon ion laser, emission wave length 488 nm) for very sensitive detection by *laser-induced fluorescence* (LIF). Detection by mass spectrometry was also reported [43].

1.3.3 High-performance liquid chromatography based techniques

Peptide mapping

Peptide mapping of proteins has become a routine method in the biopharmaceutical industry for the proof of identity as well as for the identification of variants, e.g. de-amidations. Information on the glycan pattern can also be obtained by this means [45]. Glyco-isoforms of one peptides are usually not or only marginally separated in the *reversed-phase chromatography* (RPC) typically applied [38]. Even though the attachment of glycans may reduce the ionization efficiency of the electrospray ionization a detailed glycan pattern can be obtained. However signal intensities may not reflect the real proportions of the glycans, because sialic acids influence the charge state of the peptide. For glycoproteins containing more than one glycosylation site this method provides site-specific glycosylation patterns, which are important e.g. in case of additional glycosylation sites in the Fab fragment of a therapeutic mAb.

Glycan mapping

The term glycan mapping refers to the analysis of released glycans by a high-performance separation technique. Two dimensional separations may be utilized for identification of glycans without the use of an MS [46, 47]. Glycan samples can further be fractionated by anion-exchange chromatography prior to 2D-LC according to the number of sialic acids [48], which then corresponds to a three-dimensional mapping technique. The selectivities and performances of RPC, HILIC and PGC chromatography, which are typically employed in glycan mapping methods, were compared in Manuscript IV. Typically the glycans are labeled with a fluorescence dye for sensitive detection and straight-forward relative quantitation.

Glycan mapping provides the most detailed information on the glycan pattern. However the sample preparation is quite time consuming and necessitates extensive sample manipulation. Differences in recoveries and reaction rates may result in systematic aberrations of the glycan pattern. Thus it is not surprising that inter-laboratory studies revealed significant differences in glycan patterns determined by different laboratories applying glycan mapping techniques [49, 50].

For glycan mapping the N-glycans are released by treatment with an appropriate glycosidase, typically PNGase-F, which can be applied to all mammalian N-glycans. In contrast N-glycans of plant and insect cells may exhibit Fuc-α1,3 attached to the reducing end GlcNAc, which necessitates the application of another glycosidase (PNGase-A) [9]. The enzymatic release reaction was associated with the detection of artifacts in subsequent analyses [51]. Much shorter reaction times in the low minutes range can be obtained by an PNGase-F containing *immobilized enzyme reactor* (IMER) [52]. As an alternative the glycoprotein can be digested by pronase, which un-specifically hydrolyzes peptide bonds of proteins yielding glycans attached to the asparagine or a very short peptide. The application of an IMER provided a convenient technique for glycan mapping [53]. Note that the information on the glycosylation site is lost by this procedure.

Glycans released by PNGase-F exhibit an intact aldehyde group at the reducing end. The kinetics of the inter-conversion of the corresponding anomers is slow at neutral or acidic pH leading to broadened or split peaks in chromatography [54-56]. This can either be avoided by direct reduction yielding an oligosaccharide alditol or by derivatization of the aldehyde group with an aromatic amine, which allows for sensitive and straight-forward relative quantitation by fluorescence spectrometric detection. To increase the throughput of this laborious sample preparation a 96-well plate platform was recently developed [57].

For chromatography typically *2-aminobenzoic acid* (2AA) or their derivatives (e.g. *2-aminobenzamide* = 2AB) are employed, but several alternatives are available [58-61]. Derivatization with these hydrophobic compounds also enables for analysis of the glycans by RPC, which are not sufficiently retained on these stationary phases in their native form.

Hydrophilic interaction chromatography

In chromatography several types of stationary phases are available for the analysis of glycans. Typically HILIC is applied for these polar analytes. In HILIC glycans are retained according to the number and accessibility of polar groups, primarily hydroxyl groups. Thus HILIC retention times correlate with the size of the glycans, with additional selectivities for structural restrictions enabling

also the separation of isomers. Furthermore several HILIC stationary phases provide charges (bare silica, ZIC-HILIC [62], amine phases [63]), which impact the selectivities for sialylated and sulfated glycans. Also ion-exchange columns operated under HILIC conditions offer a HILIC-like retention and additionally ionic interactions [64].

Very recently HILIC sub-2µm particles dedicated to the analysis of glycans were commercialized accomplishing ultra-high performances [1]. Fused-core particles, which represent an alternative for achieving very high performance [2], are solely available in non-derivatized form (bare silica), which provides lower retention than e.g. amide bonded phases [65]. Also the development of a *porous layer open tubular* (PLOT) HILIC column was recently reported [66].

In the biopharmaceutical industry HILIC is the standard separation method for glycan mapping. Manuscript III in this thesis reports on the development and validation of a glycan mapping method employing HILIC chromatography for the separation of the glycans. Closely related or even isomeric glycans can be discriminated by HILIC and the mobile phases applied are well suited for on-line coupling to MS allowing for reliable determination of the monosaccharide compositions of separated glycans.

Reversed phase chromatography

As stated above fluorescence labeled glycans can also be analyzed by RPC [67-70]. Generally retention of glycans is very low, which limits the fraction of the organic solvent in the mobile phase to below ten percent. Furthermore only shallow gradients can be applied, which impairs the peak capacity of the method. Nonetheless sub-2µm materials are available, which provide sufficient resolution to effectively monitor glycans by RPC [71]. Furthermore for a couple of bonded phases fused-core particles are available for operation in RPC mode.

Porous graphitic carbon chromatography

Porous graphitic carbon (PGC) was originally developed as an alternative stationary phase to silica bonded phases in RPC. Its properties were recently reviewed in detail [72]. In contrast to conventional silica-bonded alkyl-phases PGC consists to nearly 100 % of carbon and thus does not contain silanols. The surface is similar to crystalline graphite and consists of hexagonally arranged carbon atoms, which are connected by 1.5-order bonds. Non-polar analyte molecules adsorb on the surface due to van-der-Waals interactions, while PGC offers additional interactions with polar moieties via induced dipoles, which was termed *polar retention effect on graphite* (PREG). Hence both hydrophobic and hydrophilic groups may increase the retention on PGC [73]. The interaction

of oligosaccharides strongly depends on this phenomenon. But certainly steric properties of the analyte play a major role for the interaction with the rigid surface of PGC. Manuscripts I and II investigate the impacts of the column temperature, the composition of the mobile phase and the redox state of the graphitic stationary phase on the retention of malto-oligosaccharides, as well as N-glycans typical for immunoglobulin G. Deducing from the observed effects a retention model for oligosaccharides on PGC is developed, which includes the influence of the organic modifier as important parameter on retention.

In glycan analysis PGC is typically utilized for the analysis of native or reduced oligosaccharides [74]. The retention order is usually not predictable, but acidic moieties (e.g. sialic acids, sulfate moieties) increase retention [75]. The glycans are usually detected by online MS via *electrospray ionization* (ESI) interface, which may suppress the elution of charged glycans due to polarization of the conductive PGC surface by leakage currents [76, 77]. The separation of labeled glycans on PGC was also reported [78] even though they were detected by MS.

1.4 References

[1] J.Ahn, J.Bones, Y.Q.Yu, P.M.Rudd, M.Gilar, J.Chromatogr. B 878 (2010) 403.

[2] J.M.Cunliffe, T.D.Maloney, J. Sep. Sci. 30 (2007) 3104.

[3] M.E.Taylor, Drickhammer K, Introd. Glycobiology. 2006.

[4] A.J.Petrescu, M.R.Wormald, R.A.Dwek, Cur. Opin. Struct. Biol. 16 (2006) 600.

[5] B.A.Macher, U.Galili, Biochim. Biophys. Acta, Gen. Subj. 1780 (2008) 75.

[6] R.H.Langdon, J.Cuccui, B.W.Wren, Future Microbiol. 4 (2009) 401.

[7] C.Schaffer, M.Graninger, P.Messner, Proteomics 1 (2001) 248.

[8] P.P.Jacobs, N.Callewaert, Cur. Mol. Med. 9 (2009) 774.

[9] E.Higgins, Glycoconjugate J. 27 (2010) 211.

[10] T.S.Raju, J.B.Briggs, S.M.Borge, A.J.S.Jones, Glycobiology 10 (2000) 477.

[11] J.Qian, T.Liu, L.Yang, A.Daus, R.Crowley, Q.Zhou, Anal. Biochem. 364 (2007) 8.

[12] R.Jefferis, Nat. Rev. Drug Discovery 8 (2009) 226.

[13] P.Hossler, S.F.Khattak, Z.J.Li, Glycobiology 19 (2009) 936.

[14] O.V.Borisov, M.Field, V.T.Ling, R.J.Harris, Anal. Chem. 81 (2009) 9744.

[15] P.M.Rudd, R.J.Woods, M.R.Wormald, G.Opdenakker, A.K.Downing, I.D.Campbell, R.A.Dwek, Biochim. Biophys. Acta, Protein Struct. Mol.r Enzymol. 1248 (1995) 1.

[16] R.G.Werner, K.Kopp, M.Schlueter, Acta Paediatr. 96 (2007) 17.

[17] E.Llop, R.Gutierrez-Gallego, J.Segura, J.Mallorqui, J.A.Pascual, Anal. Biochem. 383 (2008) 243.

[18] C.T.Yuen, P.L.Storring, R.J.Tiplady, M.Izquierdo, R.Wait, C.K.Gee, P.Gerson, P.Lloyd, J.A.Cremata, Br. J. Haematol. 121 (2003) 511.

[19] G.Walsh, R.Jefferis, Nat. Biotechnol. 24 (2006) 1241.

[20] P.Chames, M.Van Regenmortel, E.Weiss, D.Baty, Br. J. Pharmacol. 157 (2009) 220.

[21] X.Y.Chen, Y.D.Liu, G.C.Flynn, Glycobiology 19 (2009) 240.

[22] M.Peipp, J.J.L.van Bueren, T.Schneider-Merck, W.W.K.Bleeker, M.Dechant, T.Beyer, R.Repp, P.H.C.van Berkel, T.Vink, J.G.J.van de Winkel, P.W.H.I.Parren, T.Valerius, Blood 112 (2008) 2390.

[23] Y.Kanda, T.Yamada, K.Mori, A.Okazaki, M.Inoue, K.Kitajima-Miyama, R.Kuni-Kamochi, R.Nakano, K.Yano, S.Kakita, K.Shitara, M.Satoh, Glycobiology 17 (2007) 104.

[24] A.Okazaki, E.Shoji-Hosaka, K.Nakamura, M.Wakitani, K.Uchida, S.Kakita, K.Tsumoto, I.Kumagai, K.Shitara, J. Mol. Biol. 336 (2004) 1239.

[25] Y.Kaneko, F.Nimmerjahn, E.V.Ravetch, Science 313 (2006) 670.

[26] H.Li, M.d'Anjou, Cur. Opin. Biotechnol. 20 (2009) 678.

[27] T.S.Raju, Cur. Opin. Immunol. 20 (2008) 471.

[28] Production and Quality Control of Monoclonal Antibodies and Related Substances (2009) European Medicines Agency, London, United Kingdom. Accessed 22 April 2010.

[29] H.Geyer, R.Geyer, Biochim. Biophys. Acta, Proteins Proteomics 1764 (2006) 1853.

[30] M.Wuhrer, A.M.Deelder, C.H.Hokke, J. Chromatogr. B 825 (2005) 124.

[31] S.A.Brooks, Mol. Biotechnol. 43 (2009) 76.

[32] M.V.Novotny, Y.Mechref, J. Sep. Sci. 28 (2005) 1956.

[33] C.Huhn, M.H.J.Selman, L.R.Ruhaak, A.M.Deelder, M.Wuhrer, Proteomics 9 (2009) 882.

[34] J.Zaia, Chem. Biol. 15 (2008) 881.

[35] M.D.Plasencia, D.Isailovic, S.I.Merenbloom, Y.Mechref, D.E.Clemmer, J. Am. Soc. Mass Spectrom. 19 (2008) 1706.

[36] M.L.Zhu, B.Bendiak, B.Clowers, H.H.Hill, Anal. Bioanal. Chem. (2009) 1853.

[37] P.Olivova, W.B.Chen, A.B.Chakraborty, J.C.Gebler, Rapid Commun. Mass Spectrom. (2008) 29.

[38] S.Sinha, G.Pipes, E.M.Topp, P.V.Bondarenko, M.J.Treuheit, H.S.Gadgil, J. Am. Soc. Mass Spectrom. 19 (2008) 1643.

[39] E.Wagner-Rousset, A.Bednarczyk, M.C.Bussat, O.Colas, N.Corva∩a, C.Schaeffer, A.Van Dorsselaer, A.Beck, J. Chromatogr. B 872 (2008) 23.

[40] W.Morelle, K.Canis, F.Chirat, V.Faid, J.C.Michalski, Proteomics 6 (2006) 3993.

[41] C.W.N.Damen, W.B.Chen, A.B.Chakraborty, M.van Oosterhout, J.R.Mazzeo, J.C.Gebler, J.H.M.Schellens, H.Rosing, J.H.Beijnen, J. Am. Soc. Mass Spectrom. 20 (2009) 2021.

[42] A.Lim, A.Reed-Bogan, B.J.Harmon, Anal. Biochem. 375 (2008) 163.

[43] S.Amon, A.D.Zamfir, A.Rizzi, Electrophoresis 29 (2008) 2485.

[44] S.Kamoda, K.Kakehi, Electrophoresis 29 (2008) 3595.

[45] S.Itoh, N.Kawasaki, M.Ohta, T.Hayakawa, J. Chromatogr. A 978 (2002) 141.

[46] N.Tomiya, J.Awaya, M.Kurono, S.Endo, Y.Arata, N.Takahashi, Anal. Biochem. 171 (1988) 73.

[47] K.Deguchi, T.Keira, K.Yamada, H.Ito, Y.Takegawa, H.Nakagawa, S.I.Nishimura, J. Chromatogr. A 1189 (2008) 169.

[48] H.Nakagawa, Y.Kawamura, K.Kato, I.Shimada, Y.Arata, N.Takahashi, Anal. Biochem. 226 (1995) 130.

[49] Y.Wada, P.Azadi, C.E.Costello, A.Dell, R.A.Dwek, H.Geyer, R.Geyer, K.Kakehi, N.G.Karlsson, K.Kato, N.Kawasaki, K.H.Khoo, S.Kim, A.Kondo, E.Lattova, Y.Mechref, E.Miyoshi, K.Nakamura, H.Narimatsu, M.V.Novotny, N.H.Packer, H.Perreault, J.Peter-Katalinic, G.Pohlentz, V.N.Reinhold, P.M.Rudd, A.Suzuki, N.Taniguchi, Glycobiology 17 (2007) 411.

[50] S.Thobhani, C.T.Yuen, M.J.A.Bailey, C.Jones, Glycobiology 19 (2009) 201.

[51] Y.Liu, O.Salas-Solano, L.A.Gennaro, Anal. Chem. 81 (2009) 6823.

[52] J.Krenkova, N.A.Lacher, F.Svec, J. Chromatogr. A 1216 (2009) 3252.

[53] C.Temporini, E.Perani, E.Calleri, L.Dolcini, D.Lubda, G.Caccialanza, G.Massolini, Anal. Chem. 79 (2007) 355.

[54] J.Q.Fan, A.Kondo, I.Kato, Y.C.Lee, Anal. Biochem. 219 (1994) 224.

[55] M.Wuhrer, C.A.M.Koeleman, A.M.Deelder, C.N.Hokke, Anal. Chem. 76 (2004) 833.

[56] J.Zhao, W.L.Qiu, D.M.Simeone, D.M.Lubman, J. Proteome Res. 6 (2007) 1126.

[57] L.Royle, M.P.Campbell, C.M.Radcliffe, D.M.White, D.J.Harvey, J.L.Abrahams, Y.G.Kim, G.W.Henry, N.A.Shadick, M.E.Weinblatt, D.M.Lee, P.M.Rudd, R.A.Dwek, Anal. Biochem. 376 (2008) 1.

[58] K.R.Anumula, Anal. Biochem. 350 (2006) 1.

[59] M.Pabst, D.Kolarich, G.Poltl, T.Dalik, G.Lubec, A.Hofinger, F.Altmann, Anal. Biochem. 384 (2009) 263.

[60] C.T.Yuen, C.K.Gee, C.Jones, Biomed. Chromatogr. 16 (2002) 247.

[61] K.Kakehi, T.Funakubo, S.Suzuki, Y.Oda, Y.Kitada, J. Chromatogr. A 863 (1999) 205.

[62] Y.Takegawa, K.Deguchi, T.Keira, H.Ito, H.Nakagawa, S.Nishimura, J. Chromatogr. A 1113 (2006) 177.

[63] G.G.Pan, L.D.Melton, J. Chromatogr. A 1077 (2005) 136.

[64] D.C.A.Neville, R.A.Dwek, T.D.Butters, J. Proteome Res. 8 (2009) 681.

[65] T.Ikegami, K.Tomomatsu, H.Takubo, K.Horie, N.Tanaka, J. Chromatogr. A 1184 (2008) 474.

[66] Q.Luo, T.Rejtar, S.L.Wu, B.L.Karger, J. Chromatogr. A 1216 (2009) 1223.

[67] N.Tomiya, M.Kurono, H.Ishihara, S.Tejima, S.Endo, Y.Arata, N.Takahashi, Anal. Biochem.163 (1987) 489.

[68] X.Y.Chen, G.C.Flynn, Anal. Biochem. 370 (2007) 147.

[69] T.Murakami, S.Natsuka, S.Nakakita, S.Hase, Glycoconjugate J. 24 (2007) 195.

[70] T.Suzuki, I.Matsuo, K.Totani, S.Funayama, J.Seino, N.Taniguchi, Y.Ito, S.Hase, Anal. Biochem. 381 (2008) 224.

[71] B.D.Prater, H.M.Connelly, Q.Qin, S.L.Cockrill, Anal. Biochem. 385 (2009) 69.

[72] L.Pereira, J. Liq. Chromatogr. Relat. Technol. 31 (2008) 1687.

[73] M.C.Hennion, V.Coquart, S.Guenu, C.Sella, J. Chromatogr. A 712 (1995) 287.

[74] L.R.Ruhaak, A.M.Deelder, M.Wuhrer, Anal. Bioanal. Chem. 394 (2009) 163.

[75] K.A.Thomsson, M.Ba¦êckstro¦êm, J.M.Holme¦ün Larsson, G.C.Hansson, H.Karlsson, Anal. Chem. 82 (2010) 1470.

[76] A.Tornkvist, S.Nilsson, A.Amirkhani, L.M.Nyholm, L.Nyholm, J. Mass Spectrom. 39 (2004) 216.

[77] M.Pabst, F.Altmann, Anal. Chem. 80 (2008) 7534.

[78] J.Yuan, N.Hashii, N.Kawasaki, S.Itoh, T.Kawanishi, T.Hayakawa, J. Chromatogr. A 1067 (2005) 145.

2 Objective

The aim of the thesis was to investigate *Porous Graphitic Carbon* (PGC) as a stationary phase in HPLC for the analysis of protein N-glycans and to compare it to more established methods, namely *hydrophilic-interaction chromatography* (HILIC) and reversed-phase chromatography (RPC).

In a first step the retention properties of PGC for oligosaccharides were systematically investigated using malto-oligosaccharide and also N-glycan standards. The results of these experiments were reported in the first two manuscripts.

The third publication describes a glycan analysis procedure developed to meet the regulatory requirements in the biopharmaceutical industry. The focus was laid on the optimization of the sample preparation to maintain the glycan pattern of the original sample.

The chromatography systems typically employed in glycan analysis are compared in the fourth publication. In HILIC and RPC columns packed with sub-2µm particles were applied to achieve the optimum performance.

3 List of publications

3.1 Mauscript I

Melmer M., Stangler T., Premstaller A., Lindner W. *Solvent effects on the retention of oligosaccharides in porous graphitic carbon liquid chromatography*
transmitted to the *Journal of Chromatography A*

3.2 Mauscript II

Melmer M., Stangler T., Premstaller A., Lindner W. *Effects of the redox state of porous graphitic carbon on the retention of oligosaccharides*
transmitted to the *Journal of Chromatography A*

3.3 Mauscript III

Melmer M., Stangler T, Schiefermeier M, Brunner W., Toll H., Rupprechter A., Lindner W., Premstaller A. *HILIC analysis of fluorescence labeled N-glycans from recombinant biopharmaceuticals*
Analytical and Bioanalytical Chemistry (2010) accepted manuscript

3.4 Mauscript IV

Melmer M., Stangler T., Premstaller A., Lindner W. *Comparison of Hydrophilic-Interaction, Reversed-Phase and Porous Graphitic Carbon Chromatography for Glycan Analysis*
transmitted to the *Journal of Chromatography A*

Appendix I

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Manuscript I

Solvent effects on the retention of oligosaccharides in porous graphitic carbon liquid chromatography

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Abstract

Porous graphitic carbon (PGC) is known as well suited adsorbent for liquid chromatography of carbohydrates. In this work we report on systematic investigations of solvent effects on the retention mechanism of fluorescence labeled malto-oligosaccharides on PGC. The adsorption mechanism was found to depend on the type of organic modifier used in the mobile phase. Positive adsorption enthalpies and entropies, which have already been reported in the literature, were solely produced using acetonitrile. Both alternative solvents (tetrahydrofuran, 2-propanol) yielded in contrast negative enthalpies. As plausible retention mechanism for oligosaccharides on PGC applying acetonitrile as mobile phase component we propose the formation of a dense and highly ordered solvation layer of the PGC surface with the linear acetonitrile molecules. Adsorption of analyte molecules requires a displacement of numerous acetonitrile molecules, which explains the positive enthalpy and entropy values measured. The interplay of enthalpic and entropic contributions to the overall adsorption phenomena results in strongly temperature dependent chromatographic selectivity values.

Keywords

Porous graphitic carbon, Temperature, Organic modifier, Malto-oligosaccharides, Van't-Hoff Plots

Introduction

Porous Graphitic Carbon (PGC) was introduced in the 1980's as a novel stationary phase for liquid chromatography. Aside its advantageous physical and chemical properties it also exhibits increased selectivities for methylene groups of aromatic hydrocarbons compared to conventional reversed phases [1]. Further investigations with substituted aromatic hydrocarbons showed additional interactions of induced dipoles on the PGC surface with polar moieties causing an increase of retention [2]. This effect was termed "polar retention effect on graphite" (PREG). Exploiting this phenomenon, separations of very polar analytes, even of inorganic ions can be accomplished on this totally apolar material [3]. Hence, PGC proved especially useful for analysis of very polar molecules, which are not, or only poorly retained in reversed-phase chromatography, e.g. oligosaccharides [4]. These groups otherwise represent challenges for separation sciences.

Native oligosaccharides are not retained in reversed-phase chromatography. Even when derivatized with a fluorescent dye retention is quite low limiting the fraction of organic solvents in the mobile phase below ten percent. Such a low organic content compromises sensitivity of detection by mass spectrometry, which is often necessary to identify analytes. Alternative chromatography methods for oligosaccharide analysis, e.g. high-pH anion exchange chromatography (HPAEC) or hydrophilic interaction liquid chromatography (HILIC), usually exhibit low selectivities for isomers, while PGC has demonstrated to offer remarkable selectivities for isomeric oligosaccharides, e.g. for complex protein glycans [5-9].

Despite its frequent use in this field the retention mechanism of carbohydrates on PGC is not fully understood. General aspects of the retention properties of PGC were reviewed recently [10,11] discussing hydrophobic interactions as well as the polar retention effect. Another review focusing on oligosaccharide analysis on PGC briefly summarizes findings concerning the retention principle for oligosaccharides [4]. But due to the limited data available the parameters determining the retention properties of free and fluorescence labeled oligosaccharides are still unclear. Very recently a paper was published investigating the retention properties of PGC for certain linear oligosaccharides derived from cell wall polysaccharides [12], demonstrating the influence of monosaccharide composition, charges and acetylation of hydroxide groups on the retention.

An interesting observation in PGC chromatography is the increase of retention of oligosaccharides with higher temperature [13], this is in contrast to results derived for other analytes, as e.g. for aromatic hydrocarbons [14]. A study on glucosaminoglycan disaccharides reported positive and negative adsorption enthalpies depending on the pH [15].

Herein we report on investigations of solvent effects on the retention of fluorescently labeled malto-oligosaccharides (maltotriose –Mal3, maltotetraose –Mal4, maltopentaose –Mal5, maltohexaose –Mal6, maltoheptaose –-Mal7) on PGC. These compounds were selected as simple model system consisting of homogeneous, linear chains containing analytes composed of only glucose units as building blocks.

The analytes are labeled with 2-aminobenzamide (2AB) by reductive amination for sensitive detection by fluorescence, which is a common strategy for the analysis of protein glycans [16]. The interaction of PGC with the oligosaccharides in different organic modifiers (acetonitrile - MeCN, propionitrile - EtCN, 2-propanol – 2-POH, tetrahydrofuran - THF) is specified by van't-Hoff plots. Hence, the logarithm of the retention factor k is plotted against the inverse temperature ($1/T$). The slopes of these 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 reported in literature [17].

Experimental

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, tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), $NaBH_4$, $Na[BH_3CN]$ and formic acid were ordered from Sigma-Aldrich (Vienna, Austria). Acetonitrile (MeCN), propionitrile (EtCN) and 2-propanol (2-POH) were from Merck (Darmstadt, Germany). Ammonia solution was from AppliChem (Darmstadt, Germany). Water was prepared by a Milli-Q® system (Millipore MA, USA). PD MiniTrap™ G10 columns were ordered from GE Healthcare (Vienna, Austria).

Sample preparation

Oligosaccharides were derivatized with 2-aminobenzamide (2AB) to enable sensitive fluorescence detection. The labeling solution consisted of 50 mg/mL 2AB and 63 mg/mL $Na[BH_3(CN)]$ in DMSO / acetic acid at a ratio of 7:3. 15 µL of this solution were 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 PD MiniTrap™ G10 gel filtration columns.

For the control experiment employing non-labeled oligosaccharide alditols the corresponding standards were reduced by a 1 % $NaBH_4$ solution at room temperature for four hours. Excess

reagent was neutralized with acetic acid. Reduced malto-oligosaccharide alditols are referred to as Mal3 (maltotriose), Mal4 (maltotetraose), Mal5 (maltopentaose), Mal6 (maltohexaose) and Mal7 (maltoheptaose).

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. Non-fluorescent labeled oligosaccharide alditols were chromatographed on an Agilent 1100 system with an analogous setup, but with a corona charged aerosol detector (CAD).

Chromatographic conditions

The retention study experiments were conducted on PGC columns (Hypercarb®, Thermo Electron Corporation). For experiments with EtCN a 100 mm x 3 mm (i.d.) column with 3 µm particles was used. All other experiments were performed on a 150 mm x 4.6 mm (i.d.) column with 5 µm particles.

Mobile phase A was 50 mM ammonium formate at pH 3.8. Mobile phase B additionally contained the respective organic modifier. The fraction of organic solvent was adjusted to achieve adequate retention times for all analytes in the studied temperature range. For 2AB-labeled malto-oligosaccharides a fraction of 35 % MeCN, 17.5 % 2-POH 10 % THF and 9.5 % EtCN were applied, respectively. Since EtCN is only slightly soluble in water, 0.1 % HCOOH was added to the mobile phases instead of a buffer to avoid separation of aqueous and organic phase in this case.

The temperature of the column was measured using an external thermometer with an accuracy of 0.1 °C.

2AB-labeled oligosaccharides were detected by fluorescence spectrometry with excitation at 250 nm and an emission wave length of 428 nm.

Results and Discussion

Acetonitrile as organic solvent

Typically, MeCN is used as organic modifier in the running buffer for PGC chromatography of free oligosaccharides [4, 5, 18-20]. Other solvents are only rarely applied [8, 21]. Methanol being polar protic, exhibits a low elution strength and is therefore not suitable for higher or charged oligosaccharides. Higher alcohols act as stronger eluents, so 2-POH is occasionally utilized, sometimes mixed with MeCN to reduce the viscosity [8, 22]. THF, being polar non-protic, is described as a very strong eluent on PGC and is therefore employed as an alternative solvent in this

study.

Applying MeCN as organic modifier the slopes of the van't-Hoff plots of the labeled oligosaccharides are negative, corresponding to positive adsorption enthalpies, as illustrated in Figure 1. The linearity of the plots was excellent in the investigated temperature range. Usually adsorption enthalpies in HPLC are negative, because retention is driven by a gain of energy. But using MeCN as component in a hydro-organic mobile phase positive adsorption enthalpies have been reported [13], which is affirmed by our results (Table 1). Additional glucose units cause a steady increase of the adsorption enthalpy for this series of analytes. The highest enthalpy value measured (Mal7) was 156 % higher than the value corresponding to Mal3.

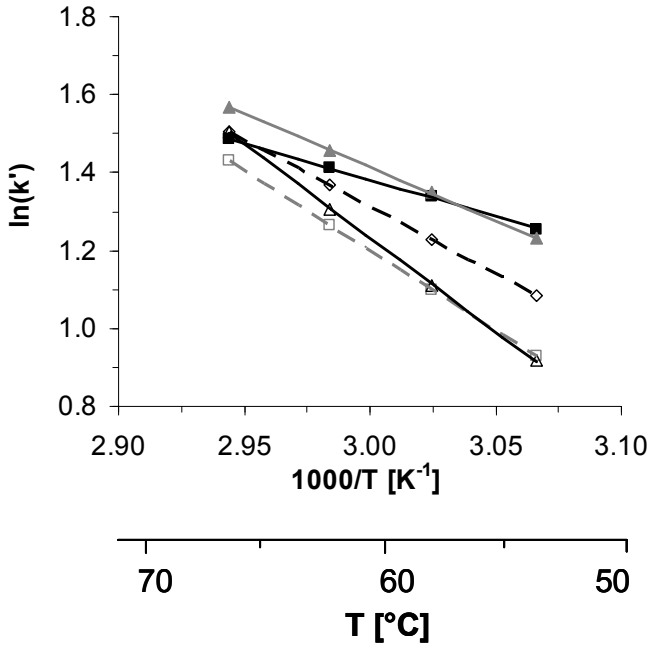

Figure 1. Van't-Hoff plots of 2AB-labeled malto-oligosaccharides (maltotriose ■, maltotetraose ▲, maltopentaose ◇, maltohexaose □ and maltoheptaose △) in 35 % acetonitrile. The second abscissa shows the corresponding temperature.

Since the adsorption of the labeled oligosaccharides is endothermic the retention is driven by an increase of the entropy of the system. Similar to the enthalpy values, the gain of entropy increases with the number of glucose units of the analyte, as shown in Table 1. Thus Mal7 exhibits a 105 % higher adsorption entropy than Mal3.

An additional glucose unit results in both, an increase of the adsorption enthalpy, represented by a steeper graph in the van't-Hoff plot, and an increased adsorption entropy, represented by a larger

intercept of the ordinate. Hence, selectivities and even elution orders of oligosaccharides may highly depend on the column temperature, as illustrated by the van't-Hoff plots in Figure 1.

Concluding from the van't-Hoff data, we propose that the strong interaction of the nitrogen lone pair electrons with the PGC surface causes a layer of MeCN molecules oriented with the nitrogen atom towards the PGC surface, which may also be expressed by a quasi face-to edge Π-Π interaction model. The approximately linear shape of the MeCN molecules affords a high degree of order within this layer. Adsorption of big analyte molecules, e.g. an oligosaccharide, premises the displacement of a high number of MeCN molecules requiring an accordingly high amount of energy, which significantly exceeds the energy gain by the interaction of the labeled carbohydrate with PGC. Thus, the increase of entropy generated by desorbed MeCN molecules causes negative changes of free enthalpy, and thus retention. This hypothesis explains the positive adsorption enthalpies and entropy values for oligosaccharides on the MeCN-PGC system, whereas aromatic small molecules, as used in several studies, do not comply with this model.

Table 1. Adsorption enthalpies and entropies for 2AB-labeled malto-oligosaccharides with different organic solvents in the running buffer: acetonitrile – MeCN, 2-propanol – 2-POH, tetrahydrofuran – THF, propionitrile – EtCN.

	Solvent	Mal3	Mal4	Mal5	-Mal6	Mal7
ΔH [kJ/mol]	35 % MeCN	15.7	22.8	28.6	34.2	40.1
	17.5 % 2-POH	-21.0	-20.1	-19.6	-20.0	-19.9
	10 % THF	-35.0	-36.4	-37.3	-38.7	-41.3
	20 % MeCN / 5 % THF	-4.10	-2.58	-1.97	-2.00	-2.57
	9.5 % EtCN	1.78	2.58	3.13	3.42	3.40
ΔS [J/mol*K]	35 % MeCN	64.1	85.9	102	118	136
	17.5 % 2-POH	-47.5	-45.1	-44.5	-46.1	-45.7
	10 % THF	-88.7	-93.5	-97.6	-102.5	-110
	20 % MeCN / 5 % THF	4.22	8.70	9.80	9.12	7.88
	9.5 % EtCN	20.3	22.3	22.6	22.3	22.0

Comparison to alternative organic solvents

A comparison of MeCN with THF, as well as with the protic solvent 2-POH revealed that these two alternative solvents exhibit similar properties but with clear differences to MeCN (Table 1). Adsorption enthalpies and entropies are negative and the differences in the thermodynamic data of

individual analytes are comparably small for these solvents. In 2-POH both, enthalpy and entropy values vary by about 7 % between the analytes, without noticeable trend. Adsorption is driven by a gain of energy, which is almost independent from the oligosaccharide moiety. We therefore assume that the interaction of the label with the PGC surface dominates the retention of the analytes, while the carbohydrate moiety does not significantly contribute to the overall observed retention characteristics.

In THF tendencies are observed within the analyte series. The (negative) values for the adsorption enthalpies and entropies decrease with every elongation of the oligosaccharide chain, as shown in Table 1. The adsorption enthalpy of the smallest (Mal3) oligosaccharide is 15 % higher than the enthalpy of the biggest oligosaccharide (Mal7), while the adsorption entropy is 19 % higher. Although THF is an even stronger eluent than 2-POH, the oligosaccharide moieties interact with the stationary phase and thereby contribute to the retention by increasing the gain of enthalpy of adsorption, but only to a lower extent compared to MeCN.

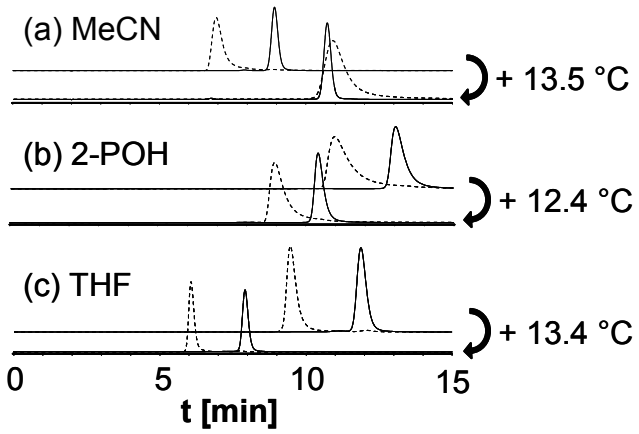

Figure 2. Overlaid chromatograms of 2AB-labeled maltotriose (solid line) and maltoheptaose (dashed line) at the highest and the lowest temperature of the van't-Hoff plots, respectively. The organic modifiers in the mobile phase were (a) acetonitrile, (b) 2-propanol and (c) tetrahydrofuran.

The impact of the column temperature on the selectivity for Mal3 and Mal7 in MeCN, POH and THF is illustrated in Figure 2. Because of the small differences in adsorption enthalpies observed using 2-POH and THF, respectively, the selectivity is only marginally impacted by the column temperature. By contrast, in MeCN the adsorption enthalpy of Mal7 is more than 150 % higher than the enthalpy of Mal3, resulting in changes of the elution orders within the studied temperature range.

Control experiments

Additional experiment series were carried out to challenge the developed retention model. Firstly, a mixture of MeCN and THF (4:1) was used as organic solvent and the adsorption enthalpies and entropies of the malto-oligosaccharides were determined. According to our retention model the small fraction of THF disturbs the ordered structure of MeCN in proximity to the surface due to its high affinity towards PGC. Thus, significant decreases in enthalpy and entropy values are expected compared to pure MeCN. Indeed, the measured enthalpy values even were negative, while adsorption entropies were significantly reduced, but still in the positive range (Table 1).

In another experiment EtCN instead of MeCN was applied as the organic modifier in the aqueous mobile phase. As a homologue of MeCN it should possess similar chemical properties, but the shape of the molecule is not linear anymore. Therefore adsorbed EtCN molecules may not form such densely packed structured layers on the PGC surface as MeCN. Analogous to the MeCN/THF mixture, adsorption enthalpies and entropies were significantly reduced for the EtCN system compared to the MeCN system (Table 1). Moreover enthalpies do not further increase for oligosaccharides with more than five glucose units, while entropies are approximately constant for all tested analytes. This may be due to decreased interactions between the carbohydrate moiety and the PGC surface.

As clearly demonstrated by these data, the adsorption mechanism of oligosaccharides on PGC strongly depends on the nature of the organic solvent and thus of the "solvated" PGC surface. Neither THF nor 2-POH produced positive adsorption enthalpies. Therefore, we assume that positive enthalpy and entropy values are specific for MeCN. The properties of EtCN lie between MeCN and THF or 2-POH, which also complies with the proposed retention model.

Table 2. Adsorption enthalpy and entropy values of reduced malto-oligosaccharides (alditols) on PGC applying 15 % acetonitrile in the running buffer.

	Mal3	Mal4	Mal5	Mal6	Mal7
ΔH [kJ/mol]	6.96	16.2	23.6	29.4	34.0
ΔS [J/mol*K]	17.5	49.4	75.8	97.0	113

Experiments with (non-labeled) oligosaccharide alditols affirmed that the effects were not caused by the fluorescence label, as these compounds also exhibit positive values and a steady increase by

the elongation of the glucose chain using MeCN (Table 2). Anyhow, the aromatic moiety of the labeled oligosaccharides was found to interact strongly with the PGC surface, as the organic fraction has to be more than doubled to achieve elution of the labeled oligosaccharides. Relative differences between the oligosaccharide alditols were found to exceed those of the labeled derivatives indicating strong interactions between the label and the stationary phase causing a retention increment.

Conclusion

For the analysis of oligosaccharides by PGC-HPLC acetonitrile (MeCN) is typically applied as component of a hydro-organic mobile phase. Compared to 2-propanol (2-POH) and tetrahydrofuran (THF), which are described in literature as alternative solvents with sufficient elution strength, MeCN offers unique properties as organic solvent. As concluded from the presented data, the virtually linearly shaped MeCN molecules form a highly ordered layer on the PGC surface representing thus its "solvation" status. According to this model analyte molecules adsorbing on the surface displace a number of these solvent molecules, which necessitates a high amount of energy, proportional to the interaction area. This explains the positive enthalpy values measured as well as the increments of adsorption enthalpy with every glucose unit. The liberated MeCN molecules increase the entropy of the system, resulting in negative free energy values and therefore strong retention of these highly polar compounds.

Addition of a stronger eluent disturbs the structured arrangement of MeCN molecules, reducing the contribution of the entropy to the adsorption. A similar effect was observed when replacing MeCN with EtCN. Even though EtCN possesses similar chemical properties as MeCN, its carbon chain is not linear, which may somewhat hinder the formation of very ordered layers. Entropy and enthalpy values are lower than for MeCN, but entropy significantly contributes to adsorption.

The aromatic fluorescence label significantly increases the overall observed retention, as demonstrated by the comparison of 2AB-labeled malto-oligosaccharides with the corresponding reduced oligosaccharides. For the elution of the alditols a fraction of 15 % MeCN was found sufficient, whereas labeled oligosaccharides were eluted in 35 % MeCN. Nonetheless, qualitatively the data were quite similar, characterizing also the retention of the alditols as entropy driven with enthalpy and entropy increments for every glucose unit.

The column temperature was identified as important factor impacting the selectivity of PGC at least with MeCN in the running buffer. With THF only minor variations of selectivities for malto-

oligosaccharides with the temperature were observed (below 4 %), whereas no significant variations were detected employing 2-POH as solvent.

The robustness of the PGC chromatographic system using MeCN as organic modifier is quite sensitive to the column temperature, so special care is needed to keep it constant. However, also other effects seem to influence the retentivity and selectivity of PGC chromatographic systems and of the PGC surface, respectively, but which are subject of additional investigation.

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Reference List

[1] J.H.Knox, P.Ross, Adv. Chromatogr. 37 (1997) 73.

[2] C.Lepont, A.D.Gunatillaka, C.F.Poole, Analyst 126 (2001) 1318.

[3] T.Takeuchi, T.Kojima, T.Miwa, J. High Resolut. Chromatogr. 23 (2000) 590.

[4] L.R.Ruhaak, A.M.Deelder, M.Wuhrer, Anal. Bioanal. Chem. 394 (2009) 163.

[5] J.Stadlmann, M.Pabst, D.Kolarich, R.Kunert, F.Altmann, Proteomics 8 (2008) 2858.

[6] L.Budai, F.Pollreisz, O.Ozohanics, K.Ludanyi, L.Drahos, K.Vekey, Eur. J. Mass Spectrom. 14 (2008) 419.

[7] M.Pabst, J.S.Bondili, J.Stadlmann, L.Mach, F.Altmann, Anal. Chem. 79 (2007) 5051.

[8] C.E.Costello, J.M.Contado-Miller, J.F.Cipollo, J. Am. Soc. Mass Spectrom. 18 (2007) 1799.

[9] N.Kawasaki, M.Ohta, S.Itoh, M.Hyuga, S.Hyuga, T.Hayakawa, Biologicals 30 (2002) 113.

[10] L.Pereira, J. Liq. Chromatogr. 31 (2008) 1687.

[11] C.West, C.Elfakir, M.Lafosse, J. Chromatogr. A 1217 (2010) 3201.

[12] Y.Westphal, H.A.Schols, A.G.J.Voragen, H.Gruppen, J. Chromatogr. A 1217 (2010) 689.

[13] M.Pabst, F.Altmann, Anal. Chem. 80 (2008) 7534.

[14] Y.N.Zhang, V.L.McGuffin, J. Liq. Chromatogr. 30 (2007) 1551.

[15] P.Koivisto, M.Stefansson, Chromatographia 57 (2003) 37.

[16] H.Geyer, R.Geyer, Biochim. Biophys. Acta: Proteins Proteom. 1764 (2006) 1853.

[17] C.Andre, Y.C.Guillaume, J. Chromatogr. A 1029 (2004) 21.

[18] C.S.Chu, M.R.Ninonuevo, B.H.Clowers, P.D.Perkins, H.J.An, H.F.Yin, K.Killeen, S.Miyamoto, R.Grimm, C.B.Lebrilla, Proteomics 9 (2009) 1939.

[19] M.S.Bereman, T.I.Williams, D.C.Muddiman, Anal. Chem. 81 (2009) 1130.

[20] Y.G.Kim, K.S.Jang, H.S.Joo, H.K.Kim, C.S.Lee, B.G.Kim, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 850 (2007) 109.

[21] C.W.Reid, J.Stupak, C.M.Szymanski, J.J.Li, Anal. Chem. 81 (2009) 8472.

[22] S.Robinson, E.Bergstrom, M.Seymour, J.Thomas-Oates, Anal. Chem. 79 (2007) 2437.

Appendix II

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Manuscript II

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.

Keywords

Malto-oligosaccharides, N-glycans, Porous graphitic carbon, Redox state, Temperature, Van't-Hoff plots

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 effected 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 diploes 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 with the oligosaccharides is specified by van't-Hoff plots yielding information about both, enthalpic and entropic contributions to the free energy of adsorption. The phase ratio of Hypercarb® was taken from literature [18]. 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.

Experimental

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).

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 were added to 9 µL of a standard solution of malto-oligosaccharide (1 mg/mL). The mixture was incubated at 37 °C over night. Access label was depleted by application to custom made PD MiniTrap™ G10 gel filtration columns.

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.

Chromatographic conditions

The retention study experiments were conducted on a 100 mm x 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. 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.

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 50 mM 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.

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 pKa-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.

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 Figure 1, the redox state of PGC significantly impacts the retention behavior of neutral oligosaccharides. Oxidation of PGC increased 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 Figure 1 for Mal3 and Mal7.

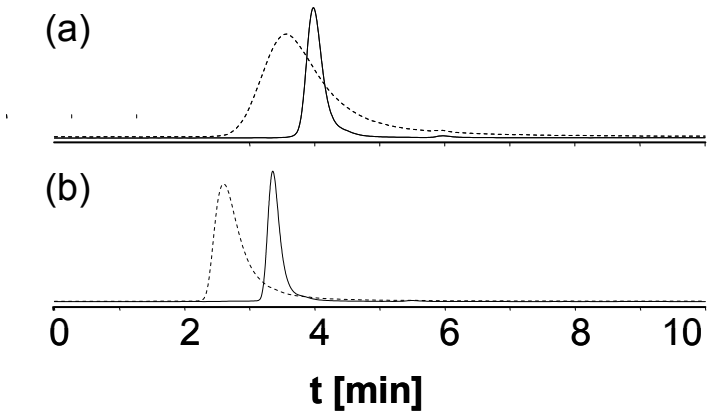

Figure 1. Chromatograms of Mal3 (solid line) and Mal7 (dashed line) on (a) oxidized PGC and (b) reduced PGC, respectively. The column temperature was 29.6 °C.

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.

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	oxidized	12.6	15.6	16.9	17.5	18.8
[kJ/mol]	reduced	15.3	19.0	21.5	22.6	24.0
ΔS	oxidized	53.2	62.4	65.9	67.1	71.7
[J/mol*K]	reduced	60.0	71.2	77.8	80.3	84.6

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.

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 Figure 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.

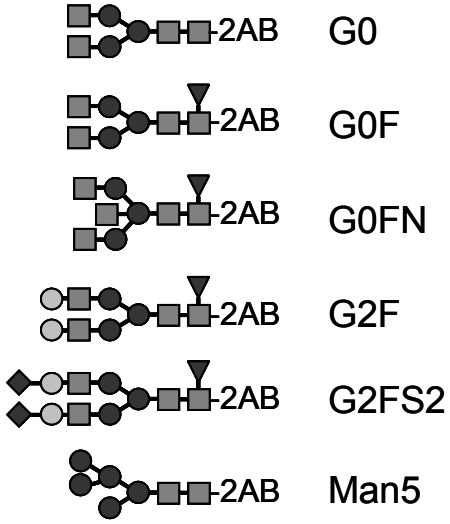

Figure 2. Illustration of N-glycan structures. ■ N-acetylglucosamine, ▼ fucose, ● mannose, ○ galactose, ◆ N-acetylneuraminic acid.

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.

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, 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. 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	oxidized	40.9	49.1	30.3	54.7	34.0
[kJ/mol]	reduced	45.2	53.5	34.4	63.6	44.4
ΔS	oxidized	132	160	100	178	120
[J/mol*K]	reduced	141	170	109	201	146

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 was identical to the procedure employing ammonium formate. As demonstrated in Figure 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.

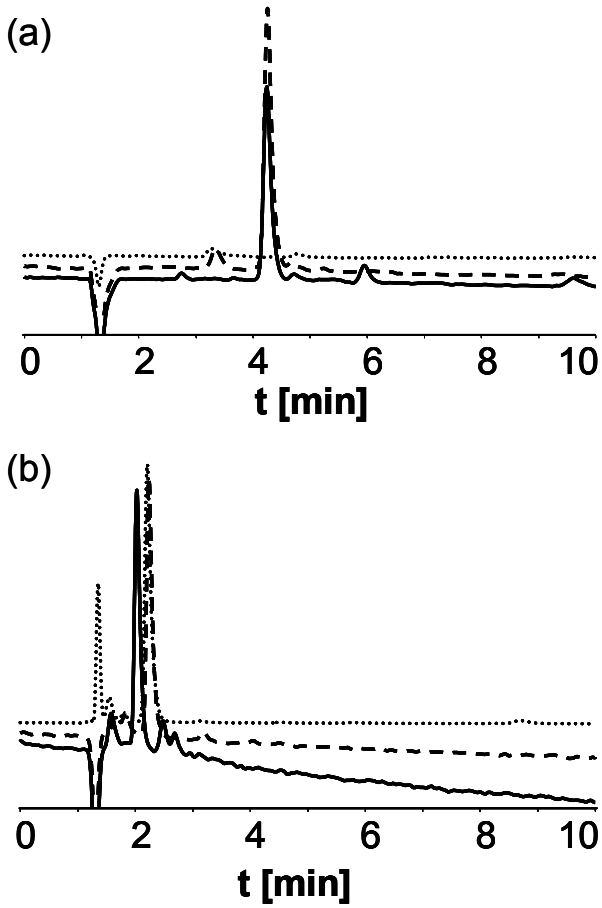

Figure 3. Overlay of chromatograms of G0 (solid line), G0FN (dashed line) and G2FS2 (dotted line) at 64 °C on (a) oxidized PGC and (b) reduced PGC.

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. 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. Subsequently a mobile phase containing hydrogen peroxide was delivered to the column. The concentration of the oxidizer was adjusted 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.

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

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.

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 effected 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 pre-conditioning 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.

Reference List

[1] M.C.Hennion, V.Coquart, S.Guenu, C.Sella, J. Chromatogr., A 712 (1995) 287.

[2] R.S.Jansen, H.Rosing, J.H.M.Schellens, J.H.Beijnen, J. Chromatogr., A 1216 (2009) 3168.

[3] L.R.Ruhaak, A.M.Deelder, M.Wuhrer, Anal. Bioanal. Chem. 394 (2009) 163.

[4] J.Stadlmann, M.Pabst, D.Kolarich, R.Kunert, F.Altmann, Proteomics 8 (2008) 2858.

[5] A.Antonopoulos, P.Favetta, W.Helbert, M.Lafosse, J. Chromatogr., A 1147 (2007) 37.

[6] S.Robinson, E.Bergstrom, M.Seymour, J.Thomas-Oates, Anal. Chem. 79 (2007) 2437.

[7] T.Takeuchi, T.Kojima, T.Miwa, J. High Resol. Chromatogr. 23 (2000) 590.

[8] L.Pereira, J. Liq. Chromatogr. Relat. Technol. 31 (2008) 1687.

[9] J.A.Harnisch, M.D.Porter, Analyst 126 (2001) 1841.

[10] E.Y.Ting, M.D.Porter, Anal. Chem. 69 (1997) 675.

[11] A.Tornkvist, S.Nilsson, A.Amirkhani, L.M.Nyholm, L.Nyholm, J. Mass Spectrom. 39 (2004) 216.

[12] M.Pabst, F.Altmann, Anal. Chem. 80 (2008) 7534.

[13] A.Tornkvist, K.E.Markides, L.Nyholm, Analyst 128 (2003) 844.

[14] H.Geyer, R.Geyer, Biochim. Biophys. Acta, Proteins Proteomics 1764 (2006) 1853.

[15] C.Huhn, M.H.J.Selman, L.R.Ruhaak, A.M.Deelder, M.Wuhrer, Proteomics 9 (2009) 882.

[16] D.C.A.Neville, V.Coquard, D.A.Priestman, D.J.M.te Vruchte, D.J.Sillence, R.A.Dwek, F.M.Platt, T.D.Butters, Anal. Biochem. 331 (2004) 275.

[17] J.Ahn, J.Bones, Y.Q.Yu, P.M.Rudd, M.Gilar, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 878 (2010) 403.

[18] I.Clarot, D.Cledat, L.Boulkanz, E.Assidjo, T.Chianea, P.J.P.Cardot, J. Chromatogr. Sci. 38

(2000) 38.

[19] Scifinder, Version 7.2. Chemical Abstracts Service, Columbus, Ohio, USA. RN 888240-17-3, accessed Feb 10, 2010; calculated using ACD/Labs software version 9.02 for Solaris,© 1994-2010 ACD/Labs, Toronto, Ontario, Canada.

[20] M.Melmer, T.Stangler, A.Premstaller, W.Lindner, J.Chromatogr. A (2010) in press.

[21] R.Jefferis, Nat. Rev. Drug Discovery 8 (2009) 226.

[22] C.S.Chu, M.R.Ninonuevo, B.H.Clowers, P.D.Perkins, H.J.An, H.F.Yin, K.Killeen, S.Miyamoto, R.Grimm, C.B.Lebrilla, Proteomics 9 (2009) 1939.

[23] W.Z.Lu, R.M.Cassidy, Anal. Chem. 66 (1994) 200.

Appendix III

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Manuscript III

HILIC analysis of fluorescence labeled N-glycans from recombinant biopharmaceuticals

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In contrast to conventional drugs, biopharmaceuticals are highly complex molecules exhibiting a remarkable heterogeneity. Protein glycosylation represents an inherent source of this heterogeneity and also impacts safety, efficacy and serum half-life of therapeutic glycoproteins. Therefore the analysis of the glycan pattern is an important issue for characterization and quality control in the biopharmaceutical industry. In this publication we describe a complete workflow for the analysis of protein N-glycans. The sample preparation procedure, consisting of the release of the N-glycans by PNGase-F, followed by fluorescence labeling with 2-aminobenzamide and depletion of the access label, was optimized to avoid alteration of the glycan sample. Subsequently, labeled glycans are analyzed by hydrophilic-interaction liquid chromatography (HILIC) with fluorescence detection. To demonstrate the accuracy of the method an antibody sample was additionally analyzed by an orthogonal method. The antibody was digested with lysyl endopeptidase and the (glyco-) peptides were analyzed by RP-HPLC-MS. The consistency of the results of these two methods demonstrates the reliability of the herein introduced glycan analysis method.

2-aminobenzamide, biopharmaceutical, fluorescence labeling, HILIC, N-glycans, sample preparation

Introduction

Compared to other biomolecules, like proteins or nucleic acids, significantly less effort has been made in the development of analysis methods for protein glycosylation. But recently, the influence of glycans on the properties of glycoproteins has become obvious, as was shown for the bioactivity of therapeutic proteins [1-3]. Recently, the European Medicines Agency (EMEA) published a guideline for development, production, characterization and specifications of therapeutic monoclonal antibodies [4] claiming the characterization and control of glycans with respect to mannosylation, galactosylation, fucosylation and sialylation. Thus, there is an urgent need for a robust analysis method, which can be validated for the use in the biopharmaceutical industry. Considering the challenges in glycan analysis it is not surprising that recent studies on the characterization of protein glycosylation demonstrated significant aberrations not only between different methods, but also between laboratories applying the same method e.g. MALDI-MS or HPLC-FL [5,6].

For fluorescence detection glycans are released from the glycoprotein prior to labeling with a fluorescent dye by reductive amination [7]. Furthermore, the reagent is usually depleted by gel filtration or solid phase extraction. Hence, the sample preparation for HPLC-FL is quite sophisticated and implicates a number of pitfalls, which may compromise the integrity of the glycan sample. Since the quantitation by HPLC-FL is quite straight forward the diverging results reported in afore mentioned studies are likely to originate from sample preparation. Quantitation by HPLC-FL was also used as a reference method for glycan quantitation in biopharmaceutical applications [8].

Hydrophilic interaction liquid chromatography (HILIC) is widely utilized for the analysis of very polar compounds, particularly for protein glycans [9,10]. It offers high selectivities for the typical antibody glycans and their un-fucosylated analogs, e.g. G0F/G0. On the other hand selectivities for glycans exhibiting similar polarities are very low complicating the separation of minor glycan variants, which may be important for bioactivity.

In this paper we present an analysis method for protein glycans meeting the scientific and regulatory requirements in the biotech industry. The method can be applied to glycans released from monoclonal antibodies as well as to even more heterogeneous glycan pools released from other glycoproteins containing larger glycans. In particular the sample preparation was optimized to avoid alterations of the glycan sample. It consists of a releasing step of the N-glycans by PNGase F,

labeling with 2-aminobenzamide (2-AB) and depletion of the free label by gel filtration. Every step of the sample preparation procedure is demonstrated to be non-selective, thus providing a representative glycan sample.

The resolution of the subsequent HILIC method was optimized to enable the separation of pharmacological relevant glycan variants, while fluorescence spectrometry enables for their sensitive quantitation at a level of 0.1 %. Furthermore this analysis method was validated for the characterization of glycans released from monoclonal antibodies. The reproducibility of the method is demonstrated by the analysis of a mAb glycan sample in six different laboratories. Additionally, hyphenation to a highly accurate and sensitive mass spectrometer is easily achieved, allowing for determination of the masses and thus the monosaccharide compositions of these minor constituents. Finally, the comparison of the results to those of an orthogonal method, namely RP-HPLC-MS analysis of glycopeptides, demonstrated the accuracy of the established method and its suitability for routinely relative quantitation of protein N-glycans.

Materials and methods

Materials

N-Glycosidase F (PNGase F) was ordered from Roche (Roche Diagnostics, Vienna, Austria). The glycoprotein samples (mAb, rhEPO) were obtained by in-house development. For ultracentrifugation Microcon® centrifugal filter devices with YM-30 membrane from Millipore were used. PD MiniTrap™ G10 size exclusion columns were ordered from GE Healthcare (Vienna, Austria).

Release of glycans by PNGase-F

For the reaction 2.25 mg of desalted mAb (corresponding to approximately 15 nmol) were incubated with 360 U/mL PNGase-F in 15 mM Tris/HCl pH 7.0 at 37 °C for 17 h. Afterwards the released glycans were separated from the proteins by ultrafiltration using Microcon® centrifugal filter devices with YM-30 filter membranes. For the derivatization reaction the solvent was removed on a centrifugal evaporator at room temperature.

Fluorescence labeling of N-glycans by reductive amination with 2-aminobenzamide (2-AB)

2-AB and $Na[BH_3(CN)]$ were dissolved in dimethylsulfoxide / acetic acid (7:3) to give a concentration of 50 mg/mL and 63 mg/mL, respectively. 15 µL of this labeling solution together with 10 µL of deionized water were added to about 30 nmol mAb N-glycans (2 mol N-glycans per

1 mol mAb). The reaction temperature was set to 37 °C for 17 h (over night) to avoid loss of sialic acids [1,2] (see also Results and discussion).

Excess reagent was depleted by gel filtration on PD MiniTrap™ G10 size exclusion columns. The columns were conditioned by flow-through of 10 mL deionized water. The sample was applied in a maximum volume of 100 µL. Then the column was rinsed with water, the first 700 µL were discarded and the next 600 µL containing the analytes were collected.

HILIC-HPLC analysis of 2-AB-labeled glycans

HILIC chromatography was performed employing a TSKgel® Amide-80 HPLC-column, 150 mm x 2.0 mm (i.d.) from Tosoh Bioscience with 3 µm packing material. Mobile phase A is 60 mM ammonium formate in 75 % acetonitrile, mobile phase B is 115 mM ammonium formate in 54 % acetonitrile. The flow rate was 200 µL/min and the temperature of the column compartment was set to 45 °C. For the analysis of mAb N-glycans the following gradient was applied: 0 min – 21 % B, 80 min – 53 % B, 81 min – 100 % B, 92 min – 100 % B, 93 min – 21 % B, which was kept constant for 20 min for column equilibration. The analytes were detected by fluorescence spectrometry with an excitation wave length of 250 nm and an emission wave length of 428 nm. Only 2 µL of the eluate from the G10 cartridge were injected for routine analysis. Such a small volume can be injected directly without dilution with acetonitrile, which is only mandatory for bigger amounts to maintain the peak shape in the HILIC chromatogram. For MS analyses a higher amount is needed. Thus, the sample is diluted 1:4 with acetonitrile and 30 µL are injected. Analysis of unknown glycoprotein N-glycans, which may contain a higher number of monosaccharide subunits, is performed using an elongated gradient: 0 min – 21 % B, 150 min – 81 % B, 151 min – 100 % B, 162 min – 100 % B, 163 min – 21 % B, 183 min – 21 % B.

This method was run with comparable results on different high-performance chromatography systems. The fluorescence chromatograms, without MS detection, in this paper were recorded using an Agilent 1100 chromatography system with a vacuum degasser, a binary pump, an autosampler, a thermostated column compartment and a fluorescence detector.

HPLC-FL-MS analyses were performed on a Dionex Ultimate® 3000 HPLC-system, consisting of a degasser, a quaternary pump, a thermostated autosampler, a column thermostat and an external fluorescence detector. The chromatography system was directly coupled to a LIT-Orbitrap® (LTQ-Orbitrap®, Thermo Scientific) mass spectrometer without solvent splitting by a standard ESI source equipped with a stainless steel emitter capillary. The mass spectrometer was operated in positive mode with the ESI-voltage at 4.5 kV. The capillary voltage was 48 V and the temperature of the

heated capillary was 275 °C. Sheath gas flow, auxiliary gas flow and sweep gas flow were at 21.0, 7.5 and 9.5 arb (arbitrary units), respectively. The nominal resolution was set to 100.000.

RP-HPLC-MS analysis of glycopeptides

Glycopeptides were analyzed after lysyl endopeptidase digest by RP-HPLC-ESI-MS on an ODS-column (Vydac® C18 5 µm particle diameter, 150 mm x 2.1 mm). The HPLC-MS system used was the same as for HILIC-MS (Dionex Ultimate® 3000) without the fluorescence detector. The column was thermostated at 60°C. Mobile phase A was water with 0.1 % TFA, mobile phase B was acetonitrile with 0.1 % TFA. The flow rate was 200 µL/min. Elution was achieved by the following gradient: 0 min – 0 % B, 5 min – 0 % B, 10 min – 2 % B, 15 min – 5 % B, 60 min – 20 % B, 95 min – 22 % B, 130 min – 33 % B, 150 min – 35 % B, 155 min – 70 % B, 160 min – 100 % B, 165 min – 100 % B, 166 min – 0 % B, 176 min – 0 % B.

For quantitation by an IT-MS precautions must be taken to maintain conditions during the whole measurement. In particular, the ion inject time, which corresponds to the time frame for accumulating ions within the ion trap, must be kept constant, because longer ion inject times cause higher signal intensities. Thus, only scans with the same ion inject time can be combined to gain quantitatively meaningful results. For glycopeptide quantitation the ion inject time was kept at a constant value of 1.000 ms over the elution range of the glycopeptides, allowing for quantitative analysis of the glycan pattern.

Results and discussion

Release of glycans from mAb with PNGase F

The N-glycans were released from the protein by treatment with N-glycosidase F (PNGase-F) at 37°C over night (17 h). The completeness of the reaction was verified by the analysis of the reduced antibody by CE according to the manufacturer [11]. In the untreated sample there was approximately 0.5 % non-glycosylated heavy chain (see Figure 1) present, which is not unusual for mAb's. After the PNGase-F digest no glycosylated heavy chain was detected, demonstrating complete release of the glycans from the protein.

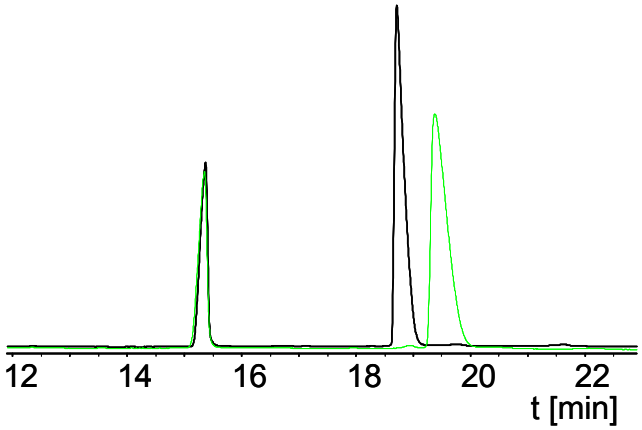

Fig 1 Electropherogram of the reduced antibody before (green line) and after (black) PNGase-F digest. After the digest no glycosylated heavy chain was detected.

The released glycans were separated from the proteins by ultrafiltration using centrifugal filter devices with YM-30 filter membranes (30 kDa nominal molecular weight limit). Glycoproteins with a lower molecular weight may require filters with a lower nominal weight limit. For the subsequent derivatization reaction the solvent was removed on a centrifugal evaporator.

Labeling with 2-aminobenzamide

Labeling of glycans with a fluorescent dye by reductive amination allows for both, sensitive detection and relative quantitation. The reaction was shown to be non-selective, by comparison with radioactive labeling by reduction with NaB^3H_4 [12]. Side reactions are another potential concern that may influence the sample composition. Especially sialic acids are known to hydrolyze at elevated temperatures, which are often applied to achieve short reaction times. Studies showed serious loss of sialic acids if the temperature is raised above 70 °C [12]. This side reaction shifts the results towards lower contents of sialic acids and thus causes systematic errors. To completely avoid these errors the temperature of the labeling reaction was set to 37 °C, as proposed in literature [13]. Applying these conditions eliminates the hydrolysis of sialic acids.

Kinetic studies were conducted to determine the optimum reaction time. Six aliquots of a mAb glycan sample were labeled by the described procedure for one to 24 hours. 2-AB labeled maltopentaose was added to each aliquot as an internal standard prior to the labeling reaction. The resulting data were fitted according to a kinetic first-order model yielding the final concentrations and the reaction rate constants. The half-lives in Table 1 indicate the reaction rate for each glycan. Interestingly non-fucosylated glycans exhibit higher half-lives (lower reaction rates) than fucosylated glycans. Thus the relative intensities of non-fucosylated glycans increase with the

reaction time until a constant level is reached after approximately ten hours, as illustrated in Figure 2. Hence reaction times significantly below this value may produce aberrant glycan patterns. At very long reaction times (24 h) the reaction yields decreased slowly. As a consequence the reaction time is conducted over night (approximately 17 h) to achieve reproducible results.

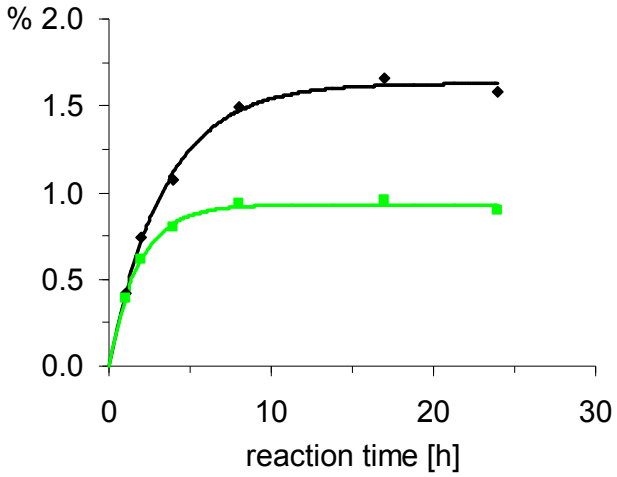

Fig 2 Plots of the corrected area of Man5 (black line) and G2FS1 (green line). Both glycans exhibit similar peak areas after one hour reaction time, but distinct final values.

Table 1 Half-lives of individual glycans in the labeling reaction with 2-AB at 37 °C

Glycan	**$\tau_{1/2}$ [h]**
G0F-GlcNAc	0.98
G0	2.3
G0F	1.2
Man5	2.4
(1,6)G1F	1.2
(1,3)G1F	1.2
G2F	1.2
G2FS1	1.3
G2FS2	1.1

For removal of excess label reagent several methods were evaluated with regard to yield, degree of label depletion and influence on the glycan composition. Initial screenings included several types of

solid phase extraction methods (reversed phase, HILIC) as well as size-exclusion methods (Sephadex® G10 and G25). The clean-up methods were evaluated by application to a glycan sample containing a broad range of analytes, from bi-antennary glycans to tetra-antennary glycans with up to two LacNAc's. Accubond II Cyano SPE columns (Agilent) exhibited no influence on the glycan pattern, but recoveries were below 75 %. Furthermore the label was not completely depleted. Cellulose disc SPE material (Ludger) performed better in our experiments regarding to both, depletion of free label and to recoveries. No influence on the glycan pattern was observed. But as a drawback the procedure was quite time consuming. Reversed phase SPE materials were not capable of depleting the free label and recoveries were low. Thus they were not considered for application in the analysis method. Also on porous graphitic carbon, which is commonly employed for desalting of oligosaccharide samples [14], the free label was insufficiently depleted.

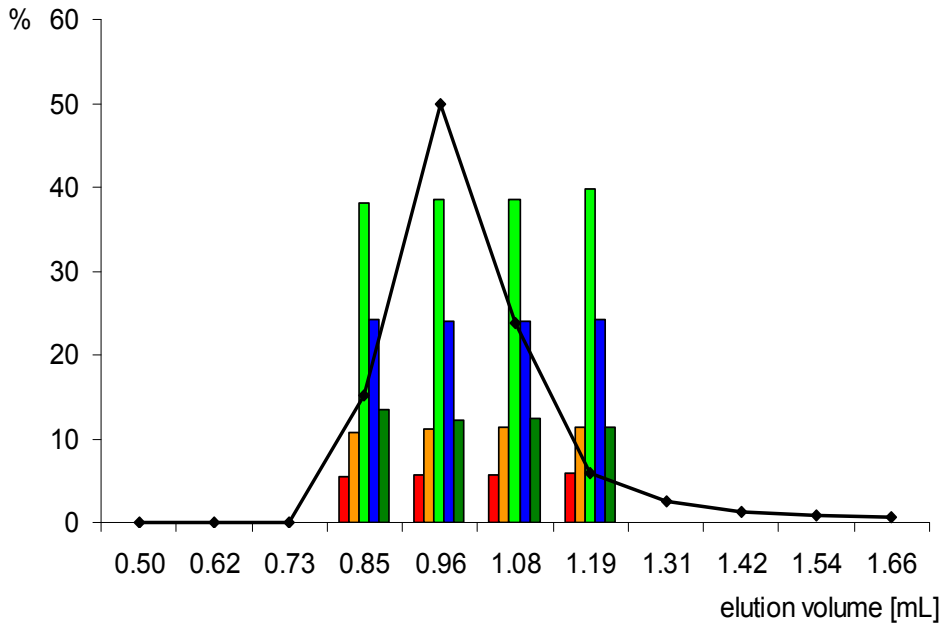

Fig 3 Glycan pattern of different fractions of eluate from size-exclusion columns, showing a constant pattern. The black line indicates the percentage of the total glycans in each fraction.

Apart from SPE procedures size exclusion on Sephadex® G10 and G25 was tested for sample clean-up. In conclusion, gel-filtration columns containing Sephadex® G10 material exhibited the best performance. Figure 3 shows the glycan pattern of several fractions eluting from the G10 column. All glycans elute within approximately 600 µL without detectable differences according to their size. Thus, if the elution range is shifted on singular columns this may compromise the recovery,

but will not alter the relative quantitation. The glycans elute prior to the free label, which is quantitatively removed by this procedure. Consistency of the pre-packed columns was tested by fractionation of a glycan sample on four columns. When collecting the eluate in the range of 700 – 1300 µL, recoveries typically were above 95 % while avoiding excessive sample dilution (data not shown).

HILIC-FL

Hydrophilic interaction liquid chromatography is at present the method of choice for detailed characterization of protein glycans [5,6,10,15]. The elution order follows the polarity and thus generally the size of the oligosaccharides, with additional selectivities for different monosaccharide units and linkages [10,16]. This makes method development quite straightforward and allows for estimation of the identity of unknown glycans by their retention times. As a drawback, pairs of analytes with similar hydrophilicity, e.g. G0/G0F-GlcNAc or G1FS1/G2F, are sometimes difficult to separate [17,18]. In addition selectivities sometimes slightly vary between different column batches. The Amide-80 chromatography column used in this study is a neutral HILIC stationary phase. Thus, it exhibits typical HILIC-selectivities for glycans and furthermore higher peak capacities than other HILIC columns tested. These properties make it suitable for the analysis of complex glycan samples. The outstanding separation power of the Amide-80 column is illustrated in Figure 4 (A). Using columns packed with 3 µm particles enables for the separation of critical analytes, e.g. G0 from G0F-GlcNAc. The former was shown to increase the antibody-dependent cellular cytotoxicity (ADCC) activity significantly [1] and is therefore of major interest for pharmaceutical applications. To the best of the authors knowledge, this is the first HILIC-method in the literature, which was shown to separate these two closely related glycans. The major glycans G0F, (1,3)G1F, (1,6)G1F and G2F are well separated, allowing robust quantitation of the degree of galactosylation, while Man5, Man6 and Man7 remain a challenge, because they elute close to the main glycans G0F, G1F and G2F, respectively. Nonetheless, Man5, which represents the most abundant high-mannose glycan, can be robustly quantified, as demonstrated in the Reproducibility section.

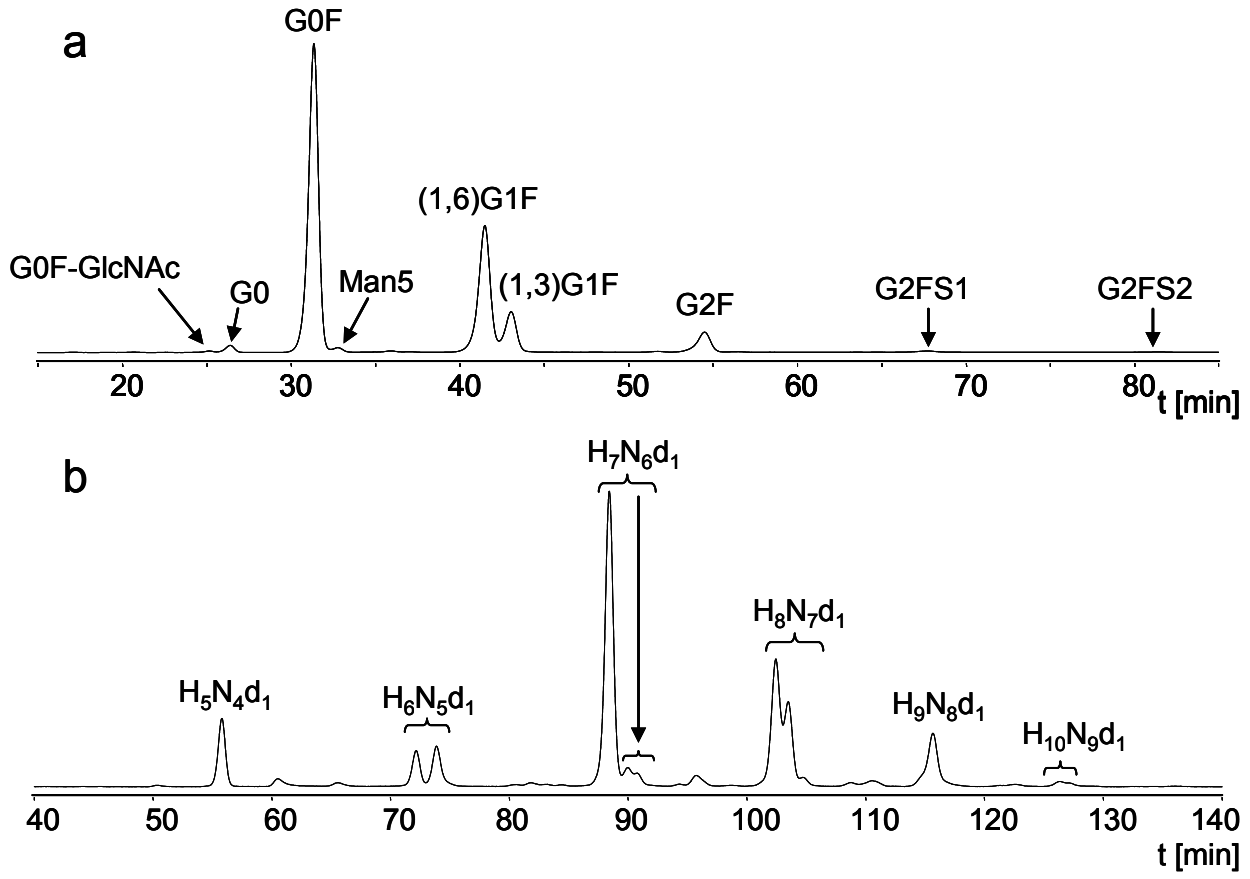

Fig 4 HILIC chromatograms of glycans from (a) a mAB and (b) a rhEPO

The labeled glycans are detected by a fluorescence detector, which enables for relative quantitation even of minor glycans. Aminobenzoic acid derivatives exhibit two maxima for the excitation of fluorescence [19]. Usually for 2-AB-labeled glycans the higher excitation wave length of about 330 nm is preferred [7,12] but according to our experience, excitation at 250 nm exhibits a higher response and therefore more intense peaks and a better signal-to-noise ratio. Generally, excitation at the lower wave length generates higher signals, but also causes a higher noise [19]. Thus the optimum excitation wave length may depend on the fluorescence detector used, as well as signal intensities. On our instrument the optimum sensitivity for fluorescence detection was achieved with the excitation wave length at 250 nm and the emission wave length at 428 nm. Using these parameters quantitation of peaks, which represent only 0.1 % of overall glycans was performed.

Additionally, a gradient was developed for the analysis of desialylated glycoprotein glycans. These glycans may exhibit up to three or four antennaries and, in addition, a variable number of LacNAc repeats (Gal-(α1,4)-GlcNAc-(β1,2)-). Therefore, the gradient is prolonged, while maintaining the same gradient slope, as described in Materials and methods. Thus this chromatography method is

suitable for very heterogeneous glycan samples of previously unknown compositions. Figure 4 (B) shows the chromatogram of glycans from a rhEPO, measured employing this gradient. As already mentioned before, in HILIC-mode glycans are primarily retained according to their size, but this chromatogram further illustrates the ability of the Amide-80 column to separate glycan isomers. This affirms the suitability of this column also for the analysis of more complex asialo glycan samples. However, in-depth analysis of sialylated glycan samples is complicated due to the additional heterogeneity of sialic acid linkages.

High resolution mass spectrometry

For identification of the glycans the chromatography system was coupled to a LIT-Orbitrap® hybrid-type mass spectrometer. The LIT mass spectrometer is capable of MS^n and the Orbitrap® additionally provides high mass accuracy in the low ppm-range and high sensitivity. The measured m/z-values were compared to data from current literature to assign peaks to glycan structures. Also in this case quantitation was based on the fluorescence signal.

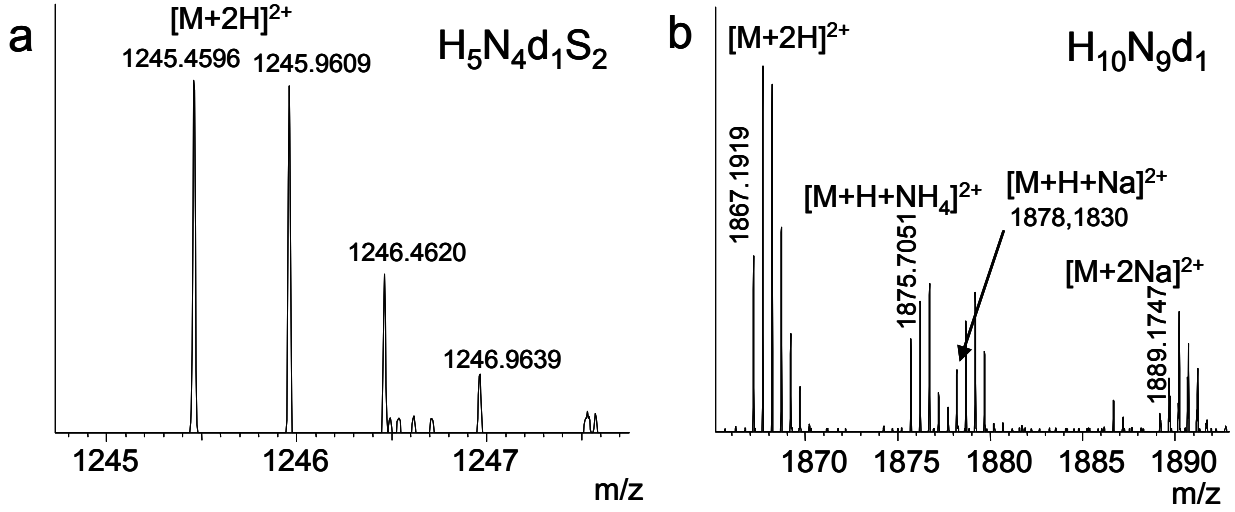

Fig 5 MS spectra from HILIC-MS analyses. (a) G2FS2, corresponding to 0.1 % of total glycans and **(b) a tetra-antennary glycan with three LacNAc repeats**

Figure 5 (A) shows the base peak chromatogram of the 2-AB-labeled N-glycans of a mAb, as well as a MS-spectrum of G2FS2, which corresponds to about 0.1 % of total glycans in this sample. The inset illustrates that chromatographic resolution is not significantly diminished by the MS coupling, as G0F-GlcNAc and G0 are well separated. The superior signal-to-noise ratio allowed for the measurement of the accurate mass of this glycan with an error of only 0.003 m/z, which corresponds to 2.5 ppm. This pronounced mass accuracy for a minor glycan variant illustrates the

high sensitivity, which allows certain detection of glycans down to 0.1 % and also highlights the reliability of identification, avoiding false positives. Furthermore, integration of the fluorescence signal enables for robust quantitation of individual glycans. Direct quantitation by MS is complicated by the fact, that the signals of higher glycans are systematically reduced, because bigger glycans exhibit a higher number of adduct ions. While most glycans generate almost exclusively singly protonated ions, higher glycans also occur as doubly charged species containing several combination of cations ($[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+2H]^{2+}$, …), thus complicating quantitation as exemplified by a chromatogram of a rhEPO glycan in Figure 5 (B).

Reproducibility

The optimized analysis method was validated according to the ICH-guideline [20]. The reproducibility of the method was tested by analyzing aliquots of the same mAb sample in six different laboratories employing the herein published method. Each technician performed six analyses including sample preparation, respectively. The results of this reproducibility study are illustrated in Figure 6, showing the average relative peak areas of the identified glycans reported by the six contributing laboratories, while the error bars represent the standard deviations. This standard deviations indicate the cumulative variations over the whole workflow including enzymatic release, labeling reaction, depletion of excess label, HILIC-FL analysis on different HPLC systems and evaluation of the chromatographic data.

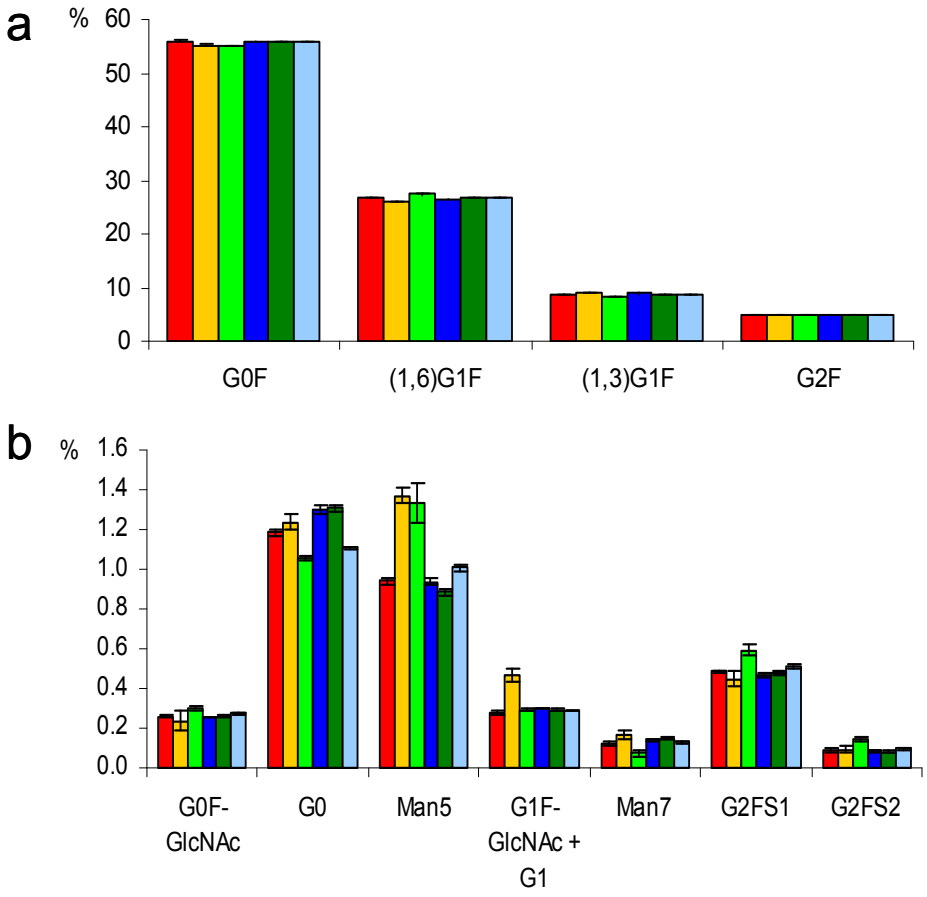

Fig 6 Results from repeatability studies between six different laboratories. The error bars indicate the total standard deviation of the procedure including sample preparation, HILIC analysis and data evaluation

Furthermore, Table 2 shows critical parameters of the glycan sample calculated from the data reported by each laboratory. The galactosylation parameter, which corresponds to the distribution between G0F, G1F and G2F, can be determined very repeatably. The sum of the areas of peaks corresponding to fucosylated glycans (fucosylation), which is of particular importance for the bioactivity (ADCC), is also measured very precisely. The quantitation of the mannosylation is impeded by the close elution of G0F and Man5, which is typically the most abundant high-mannose glycan in mAb glycan samples. Thus the mannosylation values reported are in the range of 1.0 to 1.5 %. Furthermore the Z-number is given as a measure for sialylation.

Table 2 Parameters from repeatability study characterizing the glycan pattern (Galactosylation = G1F + 2 x G2F; Fucosylation = G0F + G1F + G2F + G0F-N + G2FS1 + G2FS2; Mannosylation = Man5 + Man7; Z-number = G2FS1 + 2 x G2FS2)

Lab	Galactosylation	Fucosylation	Mannosylation	Z-number
Lab 1	45	92	1.1	0.67
Lab 2	45	91	1.5	0.63
Lab 3	46	92	1.4	0.87
Lab 4	45	92	1.1	0.64
Lab 5	45	92	1.0	0.65
Lab 6	45	92	1.1	0.70

The variations between the results reported by the six labs are remarkably small, especially for the main glycans. But even for G2FS2, which corresponds to approximately 0.1 % of total glycans, standard deviations in the range of 0.004 to 0.014 % were reported. The standard deviation of the reported average values was 0.021 %. These results demonstrate the high sensitivity of the fluorescence spectrometric detection, which compares well to detection limit of the Orbitrap® MS. Thus all glycans identified by MS could be quantified by their fluorescence signal. Furthermore these data illustrate the robustness of the analysis method, which makes it suitable for implementation in a quality control environment.

RP-HPLC-MS of glycopeptides

Reversed-phase chromatography of glycopeptides is a common tool for site-specific analysis of glycans [21]. We utilized this orthogonal method to assess the accuracy of the developed analysis method for 2-AB-labeled glycans. The results of the HILIC-FL method were compared to RP-HPLC-MS data of the lysyl endopeptidase digest of the same mAb sample. The glycoforms of the glycopeptide are only partially separated by RP-HPLC. Selective detection is achieved by mass spectrometry. Quantitation by MS requires the strict control of a number of operational parameters to obtain meaningful results. All glycoforms of the glycopeptide were eluted in a narrow time window of 2-3 min, and thus at approximately the same fraction of acetonitrile in the mobile phase, which is a prerequisite for similar ionization efficiencies. Since the bigger part of the total mass of the glycopeptides stems from the peptide moiety the m/z-values of all ions are quite similar, minimizing selectivities of the ion optics of the MS. Furthermore, the ion injection time was kept constant at 1.000 ms over the elution range of the glycopeptides. Thus, the intensity of the MS signal depends only on the concentration of the analyte and not on the ion inject time.

For quantitation two different methods of data evaluation were tested. Firstly, the mass spectra of the whole elution range of the glycopeptides were averaged. The signal heights of the most abundant isotopic peak were directly used for quantitation. Secondly, the extracted ion chromatograms of the triply charged glycopeptides were integrated. The latter method utilizes signals from the whole isotopic pattern, whereas the former method measures only the highest isotopic signal. In Figure 7, the data evaluation modes are indicated as relative height (MS) and relative area (MS), respectively.

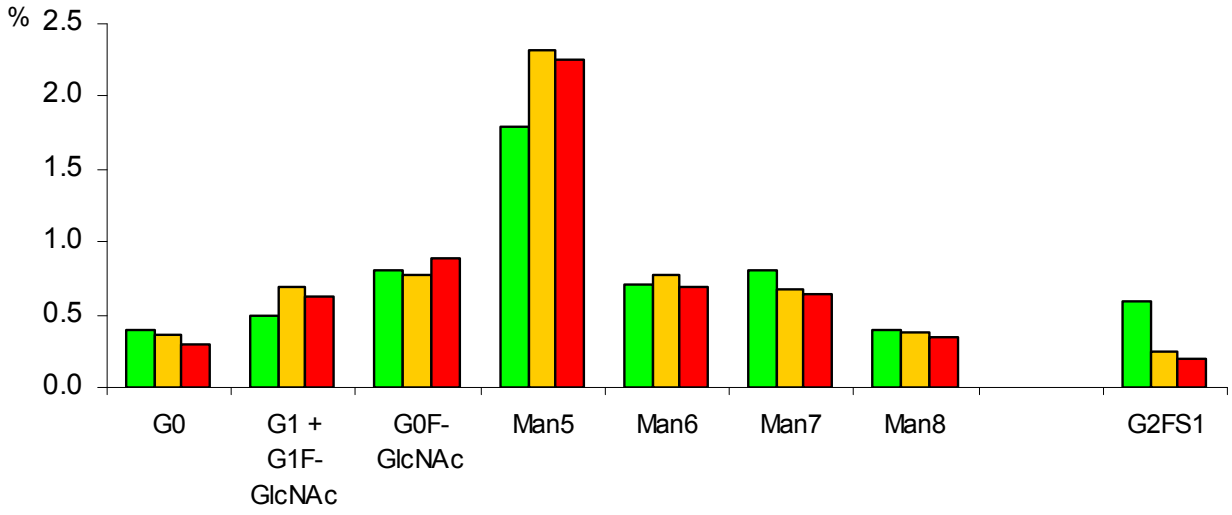

Fig 7 Quantitation of minor glycan variants by RPC-MS analysis of (glyco-)peptides compared to HILIC-FL

The results of both integration methods for all neutral glycans detected correspond well with the data obtained by HILIC-FL. For the abundant glycans G0F, G1F and G2F peak areas determined by HILIC-FL were 52.2, 35.7 and 5.4 % respectively. The RPC-MS method yielded 55.5, 33.6, and 4.8 % area and 56.1, 33.1 and 4.4 % for quantitation by signal height, respectively. For the glycans present in small amounts, the differences were in the range of 2.2 to 24.9 % of the respective peak area. Quantitation by peak height in MS resulted in higher deviations in the range of 3.5 to 39.7 % Serious aberrations were solely observed for sialylated glycans, namely G2FS1, which was assigned 0.6 % by HILIC-FL, but only 0.2 and 0.3 % by RPC-MS with quantitation by area and by height, respectively. Apparently, the ionization efficiencies for glycopeptides with glycans containing a negative charge are significantly lower than for glycopeptides with neutral glycans. Notably in both modes, the HILIC-FL method for 2-AB-labeled glycans and the peptide map

method glycans were detected, which could not be detected by the other method. E.g. G2FS2 could be detected in the HILIC method but not in the peptide map, while G1 and G1F-GlcNAc were not resolved on the HILIC column, but were easily distinguished by MS in the RP-HPLC-MS method for lysyl endopeptidase peptides. The congruence of the data obtained by the highly orthogonal RP-HPLC-MS method confirms the correctness of quantitation of the herein published HILIC-FL method for mAb glycans.

Conclusion

In this paper we presented a method for the quantitative analysis by HILIC-FL of 2-AB labeled mAb N-glycans. Since the eluent is MS compatible hyphenation to MS can be easily achieved via ESI coupling. Much effort has been made to establish a sample preparation procedure, which enables for sensitive in-depth analysis of the glycosylation, but avoids alteration of the glycan pattern, thus providing a representative glycan sample. Every step was optimized in respect to preclude selective loss of analytes. In the first step the glycans are released by PNGase F and subsequently labeled with 2-AB under mild conditions (at 37 °C for 17 h) to avoid hydrolysis of sialic acids. The excess label is depleted on gel filtration columns, packed with Sephadex® G10 material, which offer high recoveries and completely deplete free 2-AB. Analysis of the labeled glycans is performed by HILIC, which is the method of choice for high-performance separation of oligosaccharides due to its separation power and the straightforward method development. Glycans are detected by fluorescence spectrometry, which allows for straight forward quantitation. The reported results illustrate that employing this method peaks corresponding to only 0.1 % of total glycans can be reproducibly quantified. Although dedicated to mAb-glycans, the analysis method is demonstrated to be also applicable to the analysis of more complex asialo glycans, e.g. from desialylated rhEPO, by adjusting the gradient of the HILIC method.

The entire workflow for glycan analysis, consisting of cleavage of glycans from the mAb by PNGase-F, labeling with 2-AB, depletion of free label and subsequent analysis by HILIC-FL was validated as characterization method for mAb glycans and will form the basis of batch release analysis procedures. For testing the robustness of the analysis method a mAb sample was analyzed by six operators in different laboratories. This test showed e.g. that it allows for precise quantitation of G0, which corresponds to only 1.0 +/- 0.1 % of total glycans. The standard deviation of 0.1 % includes variation for the sample preparation method, analysis on different HPLC systems and also between performing technicians. From these data it is obvious, that the level of this glycan, which is

known to remarkably influence the bioactivity (ADCC) of mAbs, can be monitored very reliably with the herein published method.

For identification of glycans mass spectrometric analyses are obligatory. Since the eluents of the HILIC method contain a volatile buffer and a high fraction of organic solvent it is ideally suited for ESI-MS coupling. The LTQ-Orbitrap® hybrid-type mass spectrometer, which was employed in this study, provides accurate mass measurements and therefore reliable determination of glycan composition. The high sensitivity of the instrument allows for the identification of glycans which correspond to only 0.1 % of total glycans. The glycan pattern obtained by this analysis method was compared to results of RPC-MS analyses on the glycopeptide level of the same sample. In contrast to literature [5], the results were in remarkably good agreement, which is a strong indicator for the accuracy of both methods.

Reference List

1. Peipp M, van Bueren JJL, Schneider-Merck T, Bleeker WWK, Dechant M, Beyer T, Repp R, van Berkel PHC, Vink T, van de Winkel JGJ, Parren PWHI, and Valerius T (2008) Blood 112:2390-2399

2. Huhn C, Selman MHJ, Ruhaak LR, Deelder AM, and Wuhrer M (2009) Proteomics 9:882-913

3. Jefferis R (2009) Nat Rev Drug Discovery 8:226-234

4. Production and Quality Control of Monoclonal Antibodies and Related Substances (2009) European Medicines Agency, London, United Kingdom. Accessed 22 April 2010

5. Sinha S, Pipes G, Topp EM, Bondarenko PV, Treuheit MJ, and Gadgil HS (2008) J Am Soc Mass Spectrom 19:1643-1654

6. Wada Y, Azadi P, Costello CE, Dell A, Dwek RA, Geyer H, Geyer R, Kakehi K, Karlsson NG, Kato K, Kawasaki N, Khoo KH, Kim S, Kondo A, Lattova E, Mechref Y, Miyoshi E, Nakamura K, Narimatsu H, Novotny MV, Packer NH, Perreault H, Peter-Katalinic J, Pohlentz G, Reinhold VN, Rudd PM, Suzuki A, and Taniguchi N (2007) Glycobiology 17:411-422

7. Anumula KR (2006) Anal Biochem 350:1-23

8. Viseux N, Hronowski X, Delaney J, Domon B (2001) Anal Chem 73:4755-4762

9. Hemstrom P and Irgum K (2006) J Sep Sci 29:1784-1821

10. Wuhrer M, de Boer AR, and Deelder AM (2009) Mass Spectrom Rev 28:192-206

11. IgG Purity/Heterogeneity Assay Standard Operating Protocol (2006) Beckman-Coulter. Accessed 22 April 2010

12. Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, and Parekh RB (1995) Anal Biochem 230:229-238

13. Watanabe T, Inoue N, Kutsukake T, Matsuki S, and Takeuchi M (2000) Biol Pharm Bull 23:269-273

14. Packer NH, Lawson MA, Jardine DR, and Redmond JW (1998) Glycoconjugate J 15:737-747

15. Wagner-Rousset E, Bednarczyk A, Bussat MC, Colas O, Corvaïa N, Schaeffer C, Van Dorsselaer A, and Beck A (2008) J Chromatogr B 872:23-37

16. Barnes CAS and Lim A (2007) Mass Spectrom Rev 26:370-388

17. Deguchi K, Keira T, Yamada K, Ito H, Takegawa Y, Nakagawa H, and Nishimura SI (2008) J Chromatogr A 1189:169-174

18. Ruhaak LR, Huhn C, Waterreus WJ, de Boer AR, Neususs C, Hokke CH, Deelder AM, and Wuhrer M (2008) Anal Chem 80:6119-6126

19. Yuen CT, Gee CK, and Jones C (2002) Biomed Chromatogr 16:247-254

20. Q2(R1): Validation of Analytical Procedures: Text and Methodology (1994) ICH, Geneva, Switzerland. Accessed 22 April 2010

21. Morelle W, Canis K, Chirat F, Faid V, and Michalski JC (2006) Proteomics 6:3993-4015

Appendix IV

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Manuscript IV

Comparison of Hydrophilic-Interaction, Reversed-Phase and Porous Graphitic Carbon Chromatography for Glycan Analysis

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Abstract

Hydrophilic-interaction liquid chromatography (HILIC), reversed-phase chromatography (RPC) and porous graphitic carbon (PGC) chromatography are typically applied for liquid chromatographic separations of protein N-glycans. Hence the performances of these chromatography modes for the separation of fluorescently labeled standard glycan samples (monoclonal antibody, fetuin, ribonuclease-B) covering high-mannose and a broad range of complex type glycans were investigated. In RPC the retention of sialylated glycans was enhanced by adding an ion-pairing agent to the mobile phase, resulting in improved peak shapes for sialylated glycans compared to methods recently reported in literature. For ion pairing RPC (IP-RPC) and HILIC ultra-high performance stationary phases were utilized to maximize the peak capacity and thus the resolution. But due to the shallow gradient in RPC the peak capacity was lower than on PGC. Retention times in HILIC and IP-RPC could be correlated to the monosaccharide compositions of the glycans by multiple linear regression, whereas no adequate model was obtained for PGC chromatography, indicating the significance of the three-dimensional structure of the analytes for retention in this method. Generally low correlations were observed between the chromatography methods, indicating their orthogonality. The high selectivities, as well as the commercial availability of ultra-high performance stationary phases render HILIC the chromatography method of choice for the analysis of glycans. Even though for complete characterization of complex glycan samples a combination of chromatography methods may be necessary.

Keywords

Fluorescence labeling, Hydrophilic interaction chromatography, Ion-pairing reversed-phase, Multiple linear regression, N-glycans, Porous graphitic carbon

Introduction

Protein glycosylation has implications for a variety of biological functions, e.g. cell-cell signaling, protein stability and solubility and affinity to target molecule. The glycan profile is particularly relevant for therapeutic proteins, because it impacts efficacy and safety of the product [1-3]. Thus, glycans of biopharmaceuticals must be characterized and also monitored during production, requiring both, in-depth characterization methods and fast profiling methods.

Chromatographic analysis of glycans is typically accomplished utilizing hydrophilic-interaction chromatography (HILIC), but may also be performed on conventional reversed-phase or porous graphitic carbon (PGC) stationary phases. The glycans are typically labeled with a fluorescence dye prior to their analysis. In reversed-phase chromatography (RPC) labeling with an aromatic tag is mandatory to generate retention of these highly hydrophilic analytes. In contrast PGC is typically employed also for reduced glycans, which are detected by mass spectrometry (MS). This fact is particularly remarkable, because interferences of the separation of glycans on PGC with the high-voltage of the electrospray ionization source were reported [4, 5]. Nevertheless PGC is also capable of separating fluorescence labeled glycans . This aromatic label may then be utilized to introduce isotope tags for mass spectrometric detection [6].

In HILIC the fluorescent labeled glycans are still retained according to the number and accessibility of polar groups. Several commercially available phases possess charged moieties additionally providing coulomb interactions [7]. Retention is generally correlated to size of the glycans with certain selectivities for isomers. Due to the high fraction of organic solvent in HILIC (usually acetonitrile) the column back pressure is comparably low, allowing for the use of ultra-high-performance liquid chromatography (UHPLC) columns with standard HPLC equipment at least at elevated temperature. Furthermore, remarkable resolution of a highly sialylated glycan sample was achieved on a weak anion-exchange column operated in HILIC mode, illustrating the potential of combinations of retention mechanisms [8].

In RPC the glycans elute in groups according to their structure elements [9]. The elution order may depend on specific column properties, e.g. end-capping or silanol activity, because retention order varies between ODS columns of different brands [10]. Furthermore retentivity of fluorescently labeled glycans on conventional RP columns is quite low thus limiting the fraction of organic solvent in the mobile phase.

Porous graphitic carbon (PGC) is employed for glycan analysis mainly in the academic field. This stationary phase solely consists of graphite type carbon and offers remarkable selectivities for

isomeric glycans and increased retention particularly for charged glycans [11, 12]. The strong adsorption of polar analytes on PGC compared to conventional reversed-phases even allows for the analysis of native and reduced oligosaccharides [13, 14]. In previous experiments we identified the nature of the organic modifier, the column temperature and the redox state of the PGC material as important parameters impacting retention of oligosaccharides [15, 16].

The development of UHPLC equipment significantly increases the peak capacities achievable in liquid chromatography and enables for shorter analysis times. For several years the range of UHPLC columns was restricted to RPC, which have been investigated for high-throughput analysis of fluorescently labeled immunoglobulin G (IgG) glycans [10]. But recently a HILIC type UHPLC stationary phase dedicated to glycan analysis has become available, which was tested for RNase-B, fetuin and IgG glycans [17].

In this contribution we report on the investigation of HILIC, ion pairing RPC (IP-RPC) and PGC chromatography for the analysis of 2-aminobenzamide (2-AB) labeled glycans. The retention times of 2-AB labeled glycans from fetuin, RNase-B and a mAb in HILIC, IP-RPC and PGC chromatography, respectively, were determined applying linear gradients. Based on this broad data set the retention properties of the stationary phases regarding protein N-Glycans were evaluated and compared.

Experimental

Chemicals and Reagents

All chemicals used were at least analytical grade. RNase-B was purchased from Worthington Biochemical (Lakewood, NJ, USA), fetuin was ordered from Sigma (Steinheim, Germany). The mAb was obtained from in-house development at Sandoz (Sandoz, Kundl, Austria). PNGase-F and neuraminidase were ordered from Roche (Roche Diagnostics, Vienna, Austria). Water was purified by a MilliQ® system (Millipore; Billerica, MA, USA). Gradient grade acetonitrile and methanol (p.a.) were purchased from Merck (Darmstadt, Germany).

Sample preparation

The glycans of a mAb were released by incubation with PNGase-F at 37 °C in 15 mM Tris/HCl pH 7.0 over night. Afterwards the glycans were separated from the proteins by ultrafiltration using Microcon® YM-30 centrifugal filter devices. The reducing end was labeled with 50 mg/mL 2-aminobenzamide and 63 mg/mL $Na[BH_3(CN)]$ in dimethylsulfoxide/ acetic acid 7:3 at 37 °C over night. The excess label was depleted by gel filtration on PD MiniTrap™ Sephadex® G-10 column.

Fetuin and RNase-B were denaturated in 6 M guanidine chloride at 80 °C for 2 h prior to the PNGase-F digest.

An aliquot of fetuin glycans was treated with neuraminidase, according to the instructions of the manufacturer, to remove the sialic acids. The resulting sample is termed “asialo fetuin” within this publication.

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 excitation wavelength was 250 nm and the emission was recorded at 428 nm.

Hydrophilic interaction chromatography

The glycan samples from fetuin, asialo fetuin and RNase-B were fractionated on a Waters Acquity UPLC™ BEH Glycan column (2.1 mm i.d., 150 mm length) packed with 1.7 µm. Fractions were collected manually. Solvent A was acetonitrile and solvent B was 150 mM formic acid titrated to pH 4.4 with ammonia solution (25 %). Retention times of the individual glycans were determined by re-injection on the same column. The method started with 30 % B, which was increases by 0.5 %/min. The column temperature was maintained at 60 °C. Retention times determined by re-injection were employed for data analysis.

For analysis by IP-RP and PGC chromatography the fractions were concentrated in a centrifugal evaporator. For re-chromatography the fractions were diluted with water 1:10. Samples exhibiting unsatisfactory peak shape when re-chromatographed were diluted with water 1:50.

Ion pairing reversed-phase chromatography

For the reversed-phase separation a Waters Acquity® UPLC® BEH300 C18 column (2.1 mm i.d., 100 mm length) packed with 1.7 µm particles was utilized. As for all chromatography methods, a linear gradient was developed to achieve elution of all glycans within approximately 30 min. Mobile phase A contained 20 mM diethylamine (DEA) as ion pairing agent and 50 mM formic acid in water. Mobile phase B was 20 mM DEA and 50 mM formic acid in 25 % methanol and 75 % water. The gradient started with 10 % B, which was raised to 20 % B within 40 min. The flow rate was 350 µL/min. The column was maintained at 60 °C.

Porous graphitic carbon chromatography

A Thermo Hypercarb™ column (3 mm i.d., 100 mm length) packed with 3µm particles was used for PGC chromatography. Mobile phase A contained 100 mM trifluoracetic acid (TFA) and 50 mM

ammonia in water. Mobile phase B contained 100 mM TFA and 50 mM formic acid in 55.6 % acetonitrile and 44.4 % water. A linear gradient from 45 to 100 % B within 30 min was applied. The flow was 500 µL/min. The column compartment temperature was set to 75 °C.

Multiple linear regression analysis of retention data

The retention data obtained by HILIC, IP-RPC and PGC chromatography were analyzed by multiple linear regression (MLR) using the Microsoft Excel® *Data Analysis* add-in. For each method the retention times were correlated to the numbers of fucose (Fuc), N-acetylglucosamine (GlcNAc), galactose (Gal), mannose (Man) and sialic acid (N-acetylneuraminic acid – NANA), respectively, of the glycans. Thus the impact of different types of monosaccharides could be quantitatively evaluated. In these models high correlation is obtained for chromatography methods where retention is primarily determined by the monosaccharide composition, whereas low correlation indicates that significant factors, e.g. the three-dimensional structure, were not included.

Results and Discussion

For the unambiguous correlation of retention times of glycans on different columns mass spectrometric detection is insufficient, because isomeric glycans can not be differentiated by their mass. Hence, the glycan samples containing isomers in comparable amounts (fetuin, asialo fetuin, Rnase-B) were fractionated by HILIC, which is the standard chromatography method for fluorescently labeled glycans. Due to the high content of organic solvent in the running buffer, as well as the elevated column temperature the back pressure was comparably low allowing for the use of an ultra-high performance column with our HPLC system with a pressure limit of 600 bar. The glycans in the mAb sample (A2G0F, 1,3- A2G1F, 1,6- A2G1F, A2G2F) could be clearly differentiated by their peak areas. Hence, this sample needs not to be fractionated.

Table 1. Retention times of 2-AB-labeled N-glycans from different sources on HILIC, IP-RPC and PGC, respectively. For all methods a linear gradient was applied to achieve elution of all glycans within approximately 25 min. The monosaccharide composition was assigned based on exact mass. Isomeric glycans are indicated by the shading of the area.

Sample	Monosaccharide composition	t_R(HILIC) [min]	t_R(IP-RPC) [min]	t_R(PGC) [min]
Fetuin	$HexNAc_4Hex_5NeuAc_2$	16.12	16.06	15.76
	$HexNAc_4Hex_5NeuAc_2$	17.34	15.20	12.31
	$HexNAc_5Hex_6NeuAc_3$	21.50	21.84	24.45
	$HexNAc_5Hex_6NeuAc_3$	22.57	21.07	19.42
	$HexNAc_5Hex_6NeuAc_3$	22.57	21.07	19.65
	$HexNAc_5Hex_6NeuAc_4$	24.55	22.35	21.53
Asialo fetuin	$HexNAc_4Hex_5$	9.14	12.05	8.92
	$HexNAc_5Hex_6$	12.47	16.20	11.04
	$HexNAc_5Hex_6$	12.84	16.21	11.85
mAb	$HexNAc_4Hex_3dHex_1$	6.22	23.14	10.63
	$HexNAc_4Hex_4dHex_1$	8.46	24.34	11.10
	$HexNAc_4Hex_4dHex_1$	8.15	24.34	11.28
	$HexNAc_4Hex_5dHex_1$	10.72	25.62	11.80
RNase-B	$HexNAc_2Hex_5$	6.81	6.10	11.85
	$HexNAc_2Hex_6$	9.06	4.91	6.86
	$HexNAc_2Hex_7$	13.01	5.23	6.76
	$HexNAc_2Hex_7$	13.58	4.18	6.04
	$HexNAc_2Hex_7$	13.58	4.62	6.43
	$HexNAc_2Hex_8$	14.34	3.96	5.71
	$HexNAc_2Hex_9$	16.50	3.97	5.73

To attain comparability between the retention data of the three chromatography methods linear gradients were applied achieving elution within approximately 25 min. The mobile phases were composed of an aqueous buffer and an organic solvent. The determined retention times are shown in Table 1.

The reported peak capacities were calculated from the chromatographic data of the latest eluting glycan in the respective method.

The monosaccharide compositions of the glycans from fetuin, asialo fetuin and RNase-B were determined by infusion of the respective fraction into an Orbitrap® mass spectrometer. The mAb glycans were previously analyzed by HILIC-MS.

The glycans were termed according to [18].

HILIC

HILIC has become the method of choice for the analysis of oligosaccharides due to the high retentivity and selectivity for these hydrophilic compounds. The amide-based ligand of the stationary phase used in this study exhibited typical HILIC selectivities, i.e. glycans are retained according to their hydrophilicity. Hence, the retention times correlate to the number of hydroxyl groups of the compound (correlation coefficient 0.979) as illustrated in Figure 1. The peaks are evenly distributed within the elution range, thus minimizing the number of overlapping peaks. Since the glycans used in this study significantly differ in their polarity a steep gradient was applied to achieve elution within about 25 min. This steep gradient resulted in narrow peaks. The now available UHPLC material additionally provides superior peak capacity, which was determined to be 92 based on the last eluting peak (A3G3S4, t_R = 24.55 min).

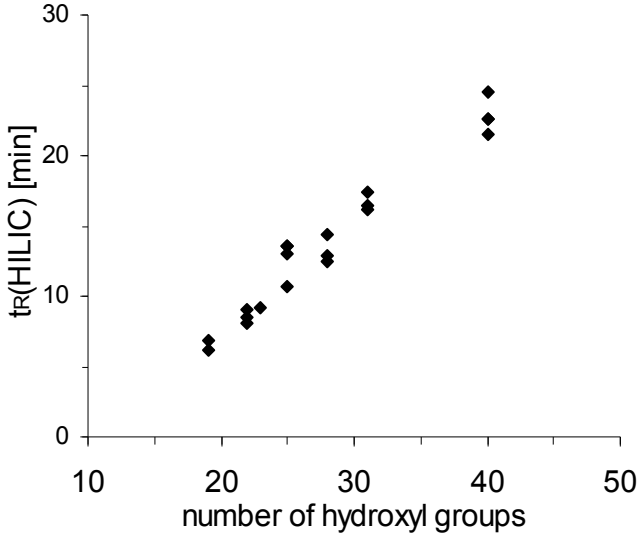

Figure 1. Retention time of glycans plotted against the number of hydroxyl groups.

Since every monosaccharide contributes to the hydrophilicity of a glycan one may anticipate that retention times in HILIC-mode are correlated to all types of monosaccharides. But the results of the MLR analysis as shown in Table 2 indicate, that the contribution of Fuc and GlcNAc are insignificant ($P > 0.05$), which may be explained by the comparably low hydrophilicity of these monosaccharides. A regression including the remaining monosaccharides produced a model with a regression coefficient of 0.987, which is comparable to the correlation of the retention times with the number of hydroxyl groups. Since the latter model bases upon only two parameters it offers a higher significance ($F = 167$). Nonetheless the number of hydroxyl groups provides a similar

correlation applying a simple linear regression and thus the highest significance (F = 413) and is thus the best model for HILIC.

Table 2. Results of the multiple linear regression analysis of the retention times of the glycans in HILIC, IP-RPC and PGC chromatography. For each parameter the confidence interval (95 %) is given. Furthermore the regression coefficient R^2, the significance value (F-value) and the number of data points (n) are reported for each model.

HILIC	Intercept	-5.4	+/-	6.3
	Fucose	1.1	+/-	2.1
	Mannose	2.3	+/-	0.55
	Galactose	2.3	+/-	1.5
	N-acetylglucosamine	0.87	+/-	1.7
	N-acetylneuraminic acid	3.2	+/-	0.5
	R^2	0.983		
	F	167		
	n	20		
IP-RPC	Intercept	2.8	+/-	3.6
	Fucose	12.9	+/-	1.2
	Mannose	-0.34	+/-	0.32
	Galactose	1.2	+/-	0.85
	N-acetylglucosamine	2.1	+/-	1.0
	N-acetylneuraminic acid	1.7	+/-	0.29
	R^2	0.997		
	F	1117		
	n	20		
PGC	Intercept	11	+/-	15
	Fucose	1.4	+/-	5.1
	Mannose	-0.57	+/-	1.4
	Galactose	0.58	+/-	3.6
	N-acetylglucosamine	0.11	+/-	4.3
	N-acetylneuraminic acid	2.9	+/-	1.2
	R^2	0.901		
	F	25		
	n	20		

IP-RPC

In reversed-phase chromatography (RPC) structurally related glycans elute in groups, whereas high-mannose type glycans exhibit the lowest retention. Man9 and Man8 eluted first with no significant difference in their retention times, which was already reported [10]. The chromatograms of both Man7 fractions separated on HILIC exhibited an additional peak, with a retention time between these two isomers. Deducing from the retention times on HILIC and on IP-RPC this peak was assigned to an additional Man7 isomer. Interestingly Man6 showed lower retention than the latest eluting Man7 isomer. Apart from that the retention of high-mannose glycans was inversely correlated to their size, as reported in literature [9, 10].

The mAb glycans A2G0F, A2G1F and A2G2F were baseline separated, whereas the method offered no selectivity for the A2G1F isomers. For complex glycans the core fucose exhibited the most decisive effect on the retention. While the fucosylated A2G2F offered the highest retention of all glycans the corresponding non-fucosylated glycan was the first eluting complex glycan. An additional antenna, as well as galactosylation resulted in increased retention times.

Sialylated glycans were well separated from corresponding neutral glycans by the addition of the ion pairing agent. In contrast to RP methods applying unbuffered acetic acid as additive in the mobile phase, peak tailing was not observed [9, 10]. Even though, two peaks corresponding to sialylated glycans were unambiguously not Gaussian shaped. Figure 2 shows the measured chromatograms and calculated chromatograms obtained by superposition of two Gaussian peaks. The excellent congruence of these data indicates the presence of isomeric glycans within the HILIC fractions, which are only partially separated by IP-RPC.

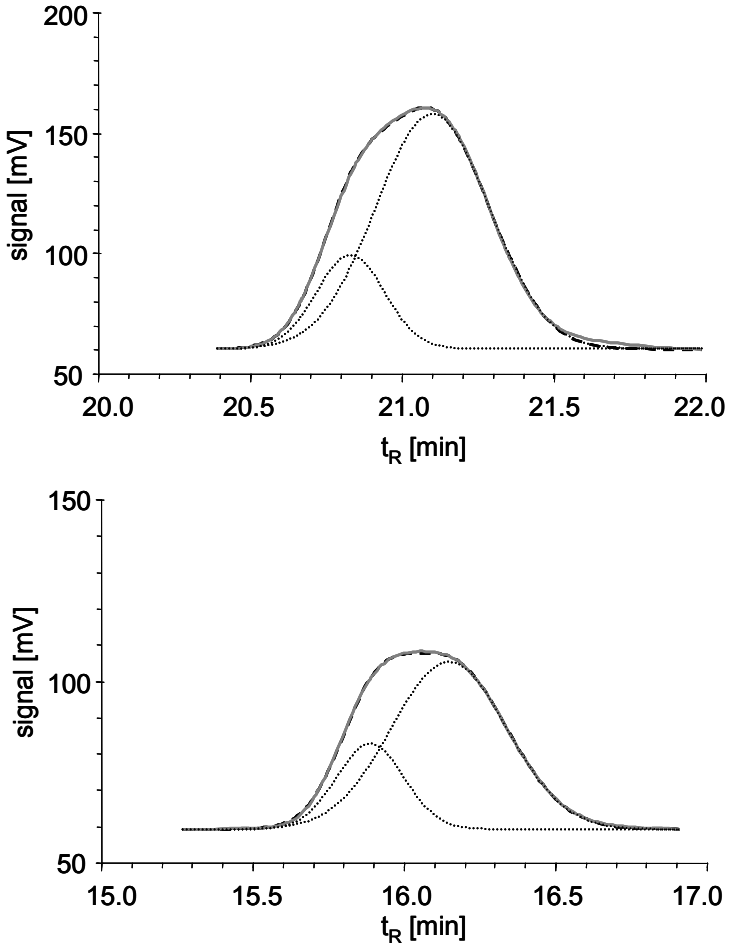

Figure 2. Non-Gaussian peaks from chromatograms of fetuin glycan fractions analyzed by IP-RPC (solid, grey). The shape of the peaks can be modeled by the sum (dashed, black) of two Gaussian peaks (dotted, black).

Despite the use of a UHPLC column the peak capacity of 50 calculated for the last eluting peak (A2G2F, t_R = 25.62 min) was the lowest for the tested methods, due to the shallow gradient applied. This may be a drawback for complex glycan samples, which necessitate a high peak capacity for resolution of all compounds for in-depth characterization. Nevertheless, due to the grouping of the glycans the IP-RPC method may be useful if only quality parameters e.g. the degree of galactosylation, sialylation, fucosylation and mannosylation are required [19].

The MLR analysis indicated statistical significance for all monosaccharides at the 95 % confidence level. The resulting model exhibited an excellent approximation with a R^2 of 0.997. The significance of the model could be further enhanced by summing up Gal, GlcNAc and NANA monosaccharide units giving a single parameter (F = 1911), because these monosaccharides increased retention times in a comparable magnitude. Thereby the number of parameters was reduced to four, while the correlation was not significantly affected. Hence the measured retention

times of 2-AB labeled glycans in IP-RPC could be described quite precisely by their monosaccharide composition.

PGC chromatography

PGC offers increased retention of hydrophilic analytes due to the polar retention effect on graphite (PREG) [20] compared to conventional reversed phases. Even reduced glycans are sufficiently retarded to allow direct analysis, while the rigid surface provides alternative selectivities .

Similar to the IP-RPC method, high-mannose glycans elute first with the bigger glycans exhibiting lower retention. Man5 is considerably stronger retarded on PGC, eluting in the range of fucosylated biantennary glycans. Core fucosylation and additional antenna result in stronger retention, but this increase is significantly lower than in IP-RPC. Sialic acids cause a substantial increase in retention with the highly sialylated glycans exhibiting the highest retention times on PGC. This remarkable selectivity for sialylation indicates extensive interactions of PGC with charged moieties. Hence, for chromatography of oligosaccharides PGC can be considered as highly retentive reversed-phase type stationary phase with additional Coulomb type interactions with acidic moieties. To enhance the elution of highly sialylated glycans TFA was used in the mobile phase, which offers high elution strength for anionic species [21].

The peak capacity was 64 for the last eluting peak (A3G3S3a; t_R = 24.45 min) outperforming the UHPLC ODS column used for IP-RPC. This was accomplished by the much steeper gradient, which could be applied due to higher selectivities on PGC.

The retention times on PGC could not be satisfactorily correlated to the monosaccharide compositions by MLR analysis (R^2 = 0.901). Solely the number of sialic acids exhibited a significant effect (P = 0.000 039), which again demonstrates the impact of Coulomb type interactions on the retention. A simple regression of the retention times with the number of sialic acids moderately improved the significance of the model (F = 65).

Even though hydrophobic interactions contribute to retention of oligosaccharides on PGC the retention mechanism is quite distinct from conventional reversed-phase chromatography. PGC offered major selectivities for isomeric glycans, which generally can not be predicted by the monosaccharide composition MLR model. Furthermore the effect on the retention resulting from the addition of a single monosaccharide unit differs for individual glycans, e.g. addition of a mannose moiety to Man8 has no effect on the retention, whereas addition to Man5 results in a significant decrease of the retention. Thus structural implications of attached monosaccharides need to be considered for estimation of the retention on PGC.

Comparison of chromatography systems

In the diagrams in Figure 3 the retention times in the respective methods are plotted against one another. Thus selectivities for structural elements, e.g. core-fucosylation or sialylation, as well as correlations between the methods become directly apparent.

The low correlation between HILIC and IP-RPC (correlation coefficient 0.17) demonstrates the orthogonality of the retention mechanisms. Selectivities for isomeric glycans were quite low in both, HILIC and IP-RPC limiting the usefulness of this combination for detailed characterization of complex glycan samples.

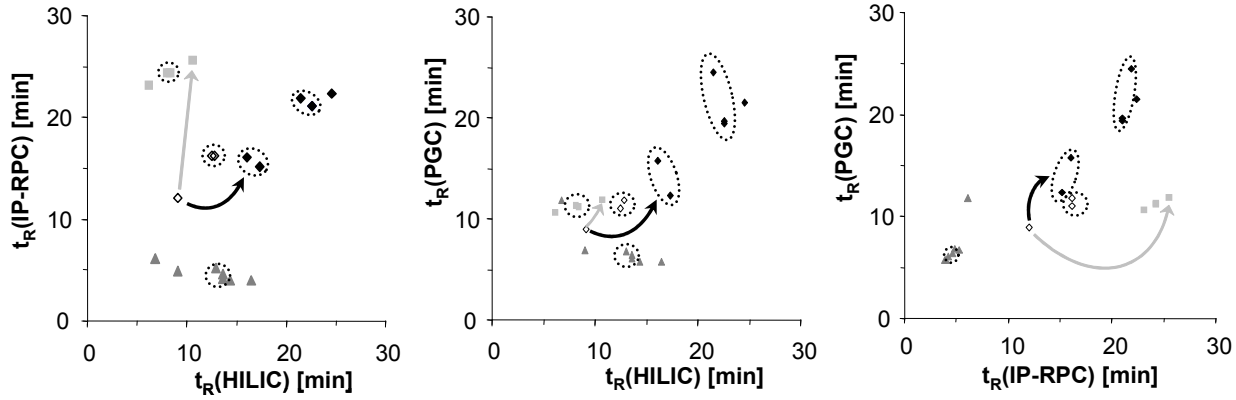

Figure 3. Retention times of high-mannose (▲), fucosylated complex (■), non-fucosylated complex (◇) and non-fucosylated complex sialo glycans (◆) in HILIC, IP-RPC and PGC plotted against one another, respectively. Light grey arrows indicate the effect of the addition of a core-fucose. The black arrows indicate the effect of the addition of two sialic acids. Isomers are marked by dotted ellipses.

In both, HILIC and PGC sialylated glycans are strongly retained due to their high hydrophilicity and their negative charges, respectively. Thus a certain correlation between these methods was observed, indicated by a correlation coefficient of 0.67. For neutral glycans the correlation coefficient was significantly lower (0.38).

Since PGC can be regarded as a reversed-phase with additional ionic interaction properties certain correlation to IP-RPC may be expected. Based on the whole glycan sample set the correlation coefficient of the retention times was 0.69 indicating moderate correlation of these chromatography methods. The selectivities for high-mannose glycans are similar on RPC and on PGC. In both methods three Man7 isomers were separated, while Man8 and Man9 could not be resolved on any of these columns. The increase of retention time caused by the core fucose was more prominent on RPC. Sialic acids exhibit a more prominent increase on the retention on PGC than in IP-RPC, demonstrating the impact of ionic interactions on PGC.

Conclusion

HILIC offered high selectivities for the tested glycans, thus allowing for a steep gradient to be applied. Furthermore, the UHPLC stationary phase provided a superior peak capacity. The high content of acetonitrile in the mobile phase allowed for the use of a long column on a HPLC system with a pressure limit of 600 bar. Thus HILIC is particularly useful for detailed analysis of complex glycan samples.

In RPC fluorescently labeled glycans interact with the stationary phase primarily via the aromatic label. Thus selectivities for glycans in RPC were generally low and only shallow gradients could be applied, which significantly reduced the peak capacity. DEA as an ion pairing agent increased the retention of sialylated glycans and enabled the separation of isomeric sialylated glycans by IP-RPC. Furthermore the peak shapes of sialylated glycans were improved compared to recently published methods employing un-buffered mobile phases. Due to the grouping of the glycans, reversed-phase chromatography may be a suitable method for quality control purposes, e.g. for determining the degree of galactosylation, sialylation, fucosylation and mannosylation of mAb glycan samples, respectively.

PGC interacts with hydrophilic analytes, e.g. with glycans, via induced dipoles, thus increasing retention. The polar retention effect accounts for increased selectivities, particularly for sialylated glycans. Furthermore the steeper gradient results in superior peak capacities compared to conventional reversed-phases. On the other hand the reproducibility and robustness of operation of the PGC column are somewhat limited [4, 22] hampering its application for routine analysis. The monosaccharide composition was found insufficient to explain the retention behavior of the glycans indicating the impact of the three-dimensional structure of the glycans to their retention on PGC.

For the comprehensive characterization of complex glycan samples a combination of these three chromatography techniques and hyphenation to MS is recommended, whereas specific questions may also be addressed by a single method, e.g. for the demonstration of batch-to-batch consistency.

Reference List

[1] H.Li, M.d'Anjou, Cur. Opin. in Biotechnol. 20 (2009) 678.

[2] R.Jefferis, Nat. Rev. Drug Discovery 8 (2009) 226.

[3] G.Walsh, R.Jefferis, Nat. Biotechnol. 24 (2006) 1241.

[4] M.Pabst, F.Altmann, Anal. Chem. 80 (2008) 7534.

[5] A.Tornkvist, S.Nilsson, A.Amirkhani, L.M.Nyholm, L.Nyholm, J. Mass Spectrom. 39 (2004) 216.

[6] J.Yuan, N.Hashii, N.Kawasaki, S.Itoh, T.Kawanishi, T.Hayakawa, J. Chromatogr. A 1067 (2005) 145.

[7] P.Hemstrom, K.Irgum, J. Sep. Sci. 29 (2006) 1784.

[8] D.C.A.Neville, R.A.Dwek, T.D.Butters, J. Proteome Res. 8 (2009) 681.

[9] X.Y.Chen, G.C.Flynn, Anal. Biochem. 370 (2007) 147.

[10] B.D.Prater, H.M.Connelly, Q.Qin, S.L.Cockrill, Anal. Biochem. 385 (2009) 69.

[11] S.Itoh, N.Kawasaki, M.Ohta, M.Hyuga, S.Hyuga, T.Hayakawa, J. Chromatogr. A 968 (2002) 89.

[12] J.Stadlmann, M.Pabst, D.Kolarich, R.Kunert, F.Altmann, Proteomics 8 (2008) 2858.

[13] L.R.Ruhaak, A.M.Deelder, M.Wuhrer, Anal. Bioanal. Chem. 394 (2009) 163.

[14] Y.Westphal, H.A.Schols, A.G.J.Voragen, H.Gruppen, J. Chromatogr. A 1217 (2010) 689.

[15] M.Melmer, T.Stangler, A.Premstaller, W.Lindner, J. Chromatogr. A (2010) accepted.

[16] M.Melmer, T.Stangler, A.Premstaller, W.Lindner, J. Chromatogr. A (2010) accepted.

[17] J.Ahn, J.Bones, Y.Q.Yu, P.M.Rudd, M.Gilar, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 878 (2010) 403.

[18] G.R.Guile, P.M.Rudd, D.R.Wing, S.B.Prime, R.A.Dwek, Anal. Biochem. 240 (1996) 210.

[19] Production and Quality Control of Monoclonal Antibodies and Related Substances (2009) European Medicines Agency, London, UK. Accessed 22 April 2010.

[20] L.Pereira, J. Liq. Chromatogr. Rel. Technol. 31 (2008) 1687.

[21] M.Michel, B.Buszewski, Adsorption 15 (2009) 193.

[22] R.S.Jansen, H.Rosing, J.H.M.Schellens, J.H.Beijnen, J. Chromatogr. A 1216 (2009) 3168.

Abstract of the thesis

Biopharmaceuticals are nowadays indispensable tools of the modern medicine. The production of basic biomolecules (e.g. insulin) counts as routine, but the expression of complex proteins is still a major challenge for biotechnology and the biopharmaceutical industry. Often post-translational modifications are a prerequisite for the proper function of the product.

Glycosylation is one of the most common post-translational modifications in eukaryotic cells. Several glycoproteins are being used as biopharmaceuticals, e.g. erythropoietin, interferons, fibrinogen and a number of monoclonal antibodies. The impact of the glycosylation on efficacy and safety is well documented in the literature.

Since the glycosylation of proteins is not driven by a template, but the result of the activities of many enzymes it typically exhibits a very heterogenic pattern. The individual glycan structures and their quantitative ratios are important parameters, which have to be determined to demonstrate the consistency of the production process, as well as the safety and efficacy of the final product.

Several methods are employed for the analysis of protein glycosylation. High-performance liquid chromatography (HPLC) of fluorescence labeled glycans serves as a reference method due to the straight-forward quantitation utilizing the fluorescence signal. Typically reversed-phase chromatography (RPC), *hydrophilic-interaction chromatography* (HILIC) or porous graphitic carbon (PGC) chromatography are applied for the separation of glycans. This thesis focuses on PGC.

Hence two publications investigate the retention mechanism of oligosaccharides on PGC. The experiments revealed that the nature of the organic solvent in the mobile phase significantly impacts the retention mechanism. Particularly acetonitrile offers unique properties for the separation of glycans on PGC. The column temperature was identified as important parameter for the selectivity. Furthermore the effect of oxidizing and reducing agents on the retention of neutral glycans were demonstrated. Oxidation of PGC impeded the elution of an acidic glycan, which indicates that the stationary phase was polarized. The phenomena generated by oxidation and reduction of PGC are thoroughly discussed in the second publication.

A further publication describes the development and evaluation of an analysis method for *monoclonal antibody* (mAb) glycans. The glycans were released from the protein by enzymatic means and subsequently labeled with a fluorescence dye. The glycans were separated on a HILIC

column and quantified by the fluorescence signal. The sample preparation procedure was optimized in respect to maintaining the original glycan pattern. The final analysis method was validated for the use in the biopharmaceutical industry. Reproducibility data exhibited only marginal differences between the results reported by different technicians from several laboratories. Furthermore the data were affirmed by an orthogonal method, namely RPC analysis of (glyco-)peptides (peptide mapping) with MS detection.

In a fourth publication the retention properties of RPC, HILIC and PGC in respect to fluorescence labeled glycans are compared. The retention times of a set of glycans covering high-mannose and complex type glycans were determined in all chromatography modes and subsequently compared. Furthermore the retention times were correlated to the respective monosaccharide composition by *multiple linear regression* (MLR). In contrast to RPC and HILIC no adequate model could be found for PGC, which indicates that retention of oligosaccharides in PGC chromatography is not determined by the monosaccharide composition.

Zusammenfassung der Dissertation

Biopharmazeutika sind aus einigen Bereichen der modernen Medizin kaum noch wegzudenken. Während die Herstellung vergleichsweise einfacher Biomoleküle, wie z.B. des Peptids Insulin, mittlerweile Routine ist, stellt die Expression komplexer Proteine immer noch eine große Herausforderung für die Biotechnologie bzw. die biopharmazeutische Industrie dar. Oft sind post-translationale Modifikationen der Proteine erforderlich, damit diese eine entsprechende Wirkung zeigen.

Glykosylierung ist eine der häufigsten post-translationalen Modifikationen in eukaryotischen Zellen. Deshalb ist es nicht überraschend, dass auch Glykoproteine als Biopharmazeutika Anwendung finden, wie z.B. Erythropoietin, Interferone, Fibrinogen, sowie eine Reihe von monoklonalen Antikörpern. Dass die Glykosylierung Einfluss auf die Wirksamkeit und auch die Verträglichkeit hat, ist gut dokumentiert.

Da die Glykosylierung von Proteinen im Gegensatz zur Protein- und zur Nukleinsäurebiosynthese nicht nach einer Vorlage erfolgt, sondern durch das Zusammenspiel einer Vielzahl an Enzymen zustande kommt, weist sie in der Regel eine recht heterogene Zusammensetzung auf. Die vorkommenden Glykane, sowie deren Verhältnisse sind wichtige Parameter, die analysiert werden müssen um die Stabilität des Herstellprozesses, sowie die Sicherheit und Wirksamkeit des Produkts sicherzustellen.

Für die Analyse der Glykosylierung kommen eine Reihe von Analysemethoden zur Anwendung, wobei die Analyse von freien, mit Fluoreszenzfarbstoff gelabeleten Glykanen mittels Hochleistungs-Flüssigchromatographie (*high-performance liquid chromatography*, HPLC) als Referenzmethode gilt. Als stationäre Phasen stehen Umkehrphasen (*reversed phase chromatography*, RPC), hydrophile Wechselwirkungsphasen (*hydrophilic interaction chromatography*, HILIC) und poröser, graphitähnlicher Kohlenstoff (*porous graphitic carbon*, PGC) zur Verfügung. Der Schwerpunkt der vorliegenden Arbeit lag auf der letztgenannten Option.

Zwei Publikationen befassen sich mit dem Retentionsmechanismus von Oligosacchariden auf PGC. Die Untersuchungen zeigen, dass das organische Lösungsmittel in der mobilen Phase den Wechselwirkungsmechanismusstark beeinflusst. Besondere Eigenschaften weist vor allem Acetonitril auf. Weiters wurde der Einfluss der Temperatur des Säulenofens als wichtiger Parameter für die Selektivität identifiziert. Schließlich wird der Effekt von Oxidations- und Reduktionsmitteln

auf die Retention von neutralen Glykanen gezeigt. Die Oxidation von PGC verhinderte außerdem die Elution eines sauren Glykans vollständig, was für eine Polarisation der Oberfläche durch das Reagens spricht. Die Auswirkungen der Reduktions- und Oxidationsmittel wird in der zweiten Publikation ausführlich diskutiert.

Eine weitere Publikation beschäftigt sich mit der Etablierung einer vollständigen Analysemethode für Glykane von therapeutischen Antikörpern. Die Glykane werden enzymatisch abgespalten, mit einem Fluoreszenzfarbstoff gelabelts, mittels HILIC getrennt und durch Fluoreszenzdetektion quantifiziert. Besondere Aufmerksamkeit lag auf der Probenvorbereitung, die mehrere Schritte umfasst und dahingehend optimiert wurde die Integrität der ursprünglichen Verhältnisse der Glykane zu erhalten. Die Methode wurde für den Einsatz in der pharmazeutischen Industrie validiert. Die Daten zur Reproduzierbarkeit der Methode zeigten nur unwesentliche Abweichungen zwischen unterschiedlichen Operatoren in verschiedenen Labors. Außerdem zeigte sich, dass die Daten dieser Analysemethode mit den Ergebnissen einer orthogonlen Analysemethode, nämlich der RPC-Analyse der (Glyko-)Peptide, übereinstimmen, was die Korrektheit der Quantifizierung bestätigt.

Schließlich werden in der vierten Publikation die Eigenschaften von RPC, HILIC und PGC-Chromatographie in Hinblick auf die Trennung von fluoreszenz-gelabelten Glykanen verglichen. Hierfür wurde ein breites Set von *high-mannose* und *complex* Glykanen auf diesen stationären Phasen chromatographiert und die generierten Retentionsdaten verglichen. Weiters wurden die Retentionszeiten mittels multipler linearer Regression (MLR) mit der Monosaccharidzusammensetzung korreliert. Im Gegensatz zu RPC und HILIC konnte für PGC-Chromatographie kein adäquates Modell gefunden werden, was dafür spricht, dass die Retentionszeiten von Glykanen auf PGC nicht allein durch deren Zusammensetzung determiniert sind.

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Scientific contributions

Talks	5. ASAC JunganalytikerInnen Forum 2009 Innsbruck, Austria *Development of protein glycan analysis methods implementing LC-fluorescence and LC-MS*
Posters	34th International Symposium on High-Performance Liquid Phase Separations and Related Techniques, HPLC 2009 Dresden, Germany *Orthogonality of Porous Graphitic Carbon to established HPLC stationary phases for the analysis of N-glycans* 34th International Symposium on High-Performance Liquid Phase Separations and Related Techniques, HPLC 2009 Dresden, Germany *Acetonitrile offers a unique retention mechanism for oligosaccharides on Porous Graphitic Carbon* (awarded top-twenty poster) Euroanalysis 2009 Innsbruck, Austria *Gaining a Representative Glycan Sample for HPLC-FL Analysis*
Publications	Foettinger, A., Melmer M., Leitner A., Lindner W. *Reaction of the indole group with malondialdehyde: Application for the derivatization of tryptophan residues in peptides* Bioconjugate Chemistry (2007) 18:1678