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Affinity chromatography with monolithic capillary columns II. Polymethacrylate monoliths with immobilized lectins for the separation of glycoconjugates by nano-liquid affinity chromatography

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Abstract

Monolithic capillary columns with surface bound lectin affinity ligands were introduced for performing lectin affinity chromatography (LAC) by nano-liquid chromatography (nano-LC). Two kinds of polymethacrylate monoliths were prepared, namely poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) and poly(glycidyl methacrylate-*co*-ethylene dimethacrylate-*co*-[2-(methacryloyloxy)ethyl]trimethyl ammonium chloride) to yield neutral and cationic macroporous polymer, respectively. Two lectins including concanavalin (Con A) and wheat germ agglutinin (WGA) were immobilized onto the monolithic capillary columns. The neutral monoliths with immobilized lectins exhibited lower permeability under pressure driven flow than the cationic monoliths indicating that the latter had wider flow-through pores than the former. Both types of monoliths with immobilized lectins exhibited strong affinity toward particular glycoproteins and their oligosaccharide chains (i.e., glycans) having sugar sequences recognizable by the lectin. Due to the strong binding affinity, the monoliths with surface bound lectins allowed the injection of relatively large volume (i.e., several column volumes) of dilute samples of glycoproteins and glycans thus allowing the concentration of the glycoconjugates and their subsequent isolation and detection at low levels ($\sim 10^{-8}$ M). To further exploit the lectin monoliths in the isolation of glycoconjugates, two-dimensional separation schemes involving LAC in the first dimension and reversed-phase nano-LC in the second dimension were introduced. The various interrelated methods established in this investigation are expected to play a major role in advancing the sciences of "nano-glycomics".

Keywords: Monolithic capillary; Immobilized lectins; Nano-liquid affinity chromatography; Glycomics; Glycoproteins; Glycans

1. Introduction

Affinity chromatography has been employed for many years for the analytical and preparative separations of biological samples. It is well established that monolithic stationary phases exhibit improved mass-transfer properties compared to conventional chromatographic media, and this fact has been attributed to reduced diffusional length and convective transport [1,2]. The porous structure of monoliths can be designed such that the mobile phase flows unobstructed through gigapores while smaller pores provide the surface area necessary for the separation of different analytes. Despite these sound features, monolithic capillary columns with immobilized affinity ligands for performing affinity-based separations in nano-LC and CEC are still lagging behind traditional affinity media (i.e., microparticulate affinity phases), and only two attempts have been made to show the potentials of affinity monoliths in nano-LC by Pan et al. [3] and more recently in nano-LC and CEC by Bedair and El Rassi [4]. Furthermore, affinity monolithic capillary columns are the ideal column configuration for the analysis of small sample size and for the determination of species at trace levels simply because micro-bore columns offer higher sensitivity, i.e., lower mass limit of detection.

This article is concerned with the development of monolithic capillary columns with immobilized lectins, including *Canavalia ensiformis*, concanavalin A (Con A),

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from jack bean seeds and *Triticum vulgare*, also known as wheat germ agglutinin (WGA) for achieving nano lectin affinity chromatography (nano-LAC) of glycoconjugates. This investigation is related to our previous contribution [4] in the sense that it extends the utility of polymethacrylate monoliths to nano-LAC of glycoconjugates (e.g., glycoproteins and glycans). Glycosylation of proteins represents a critically important post-translational modification (PTM) reaction. In fact, glycans are changed during carcinogenesis and development as a result of alterations in the level of glycosyl transferases, which are implicated in biosynthesis and trimming of the glycoforms. The functional studies on the glycans (i.e., glycomics) are expected to provide fundamental answers in the area of functional genomics research. This is because advances in genomics can only provide the sequence of hundred of thousands of "naked proteins" and does not allow the efficient prediction of all PTM (particularly glycosylation) of which the majority of proteins are the target [5]. Thus, any approach to proteomics, which fails to address the analysis of glycans is necessarily incomplete. Currently, the general consensus is to keep up efforts to increase further the sensitivity and specificity of PTM analysis methodologies. This is due to the fact that the glycoproteins of interest might not be available in large quantities. Therefore, developments of sensitive microcolumn separation methods as the ones described within the framework of this investigation are still in full development and should contribute greatly to the quest for higher sensitivity for glycan and glycopeptide analyses.

Lectins are sugar binding proteins or glycoproteins of non-immune origin, which agglutinate cells and/or precipitate glycoconjugates [6,7]. They possess at least two sugar-binding sites, the presence of which explains the agglutination and precipitation ability of lectins towards cells and glycoconjugates [8]. As these interactions can often be reversed by monosaccharides (hapten sugars), immobilized lectins are widely used to fractionate glycoconjugates, e.g., glycoproteins, glycopeptides and glycans. Very often, lectins are classified according to the monosaccharide (hapten sugar) which inhibits the interaction between a lectin and a glycoconjugate or which allow the specific elution of a bound glycoconjugate from an immobilized lectin column. However, the interaction between lectins and glycoconjugates are much more complex since (i) lectins can interact with internal sequences of an oligosaccharide, (ii) lectins identical in terms of monosaccharide specificity can present different specificity toward glycans and glycopeptides, and (iii) for many lectins, the spatial conformation of glycans may affect the interaction with lectins (for a review see Ref. [8]).

A sugar chain must have at least two non-substituted or C-2 substituted α-mannosyl residues to be recognized and retained on the immobilized Con A column. This explain the fact that high mannose- and hybrid-type glycans, which have many non-reducing terminal mannose residues, can bind more strongly to the column than biantennary complex-type carbohydrates and are eluted with 200 mM methyl-α-D-mannopyranoside instead of 5 mM methyl-α-D-glucopyranoside [9,10], see Fig. 1. The binding constant of high mannose-type glycans to Con A is on the order of 2×10^3 to $8 \times 10^5\ M^{-1}$ [11,12], and that of hybrid-type glycans to Con A is in the range 1.8×10^3 to $4 \times 10^5\ M^{-1}$ [12].

Wheat germ agglutinin has strong affinity for *N*,*N*′-diacetylchitobiose, the tetrasaccharide GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc and bisected hybrid-type glycans [8,10], see Fig. 1. The binding constant of the bisected hybrid glycans was reported to be in the range of 5.8×10^3 to $7.1 \times 10^5\ M^{-1}$ [12] and that of *N*,*N*′-diacetylchitobiose was found to be $5.3 \times 10^3\ M^{-1}$ [13]. Also, glycoconjugates with clustered sialic acid residues have strong affinity to immobilized WGA [14]. In fact, the binding of WGA to glycopeptides has been shown to decrease after treatment with neuraminidase [15]. Oligosaccharides with poly-*N*-acetyllactosamine chains have weak affinity and are retarded in the WGA column [10] (i.e., elute with the binding mobile phase).

It is important to emphasize that LAC is an "all or nothing" interaction exactly as in all other biospecific interactions (i.e., affinity interactions). In other words, a glycoconjugate that has no affinity ("nothing" interaction) towards a given lectin will pass through the column unretained thus eluting at the dead time of the column. Conversely, a glycoconjugate that exhibits affinity towards the immobilized lectin ("all" interaction) will be strongly retained by the lectin affinity ligand and will only elute from the column by a step gradient with the eluting mobile phase containing a haptenic sugar (i.e., inhibitor) thus exiting the column at exactly the dead time of the column.

This investigation evaluated Con A and WGA affinity monoliths in nano-LAC of glycoproteins and glycans including their isolation and preconcentration from dilute samples. Also, this study introduced two-dimensional (2D) separation schemes incorporating nano-LAC in the first dimension and nano-reversed-phase liquid chromatography (nano-RPLC) in the second dimension. The nature of the monolith affected largely the affinity of the immobilized lectins towards glycoconjugates as well as the porosity and consequently the flow characteristic of the monolithic columns.

2. Experimental

2.1. Instrumentation

The instrument used was an HP^{3D}CE system from Hewlett-Packard (Waldbronn, Germany) equipped with a photodiode array detector. Chromatograms were recorded with a personal computer running an HP^{3D}CE ChemStation. The chromatograms shown in this report are representative of the data, which in most cases involved two or more replicate measurements. Column temperature was held constant at 25 °C. An external pressure of 1.0 MPa (i.e., 10 bar) was applied to the column inlet for sample injection and separation.

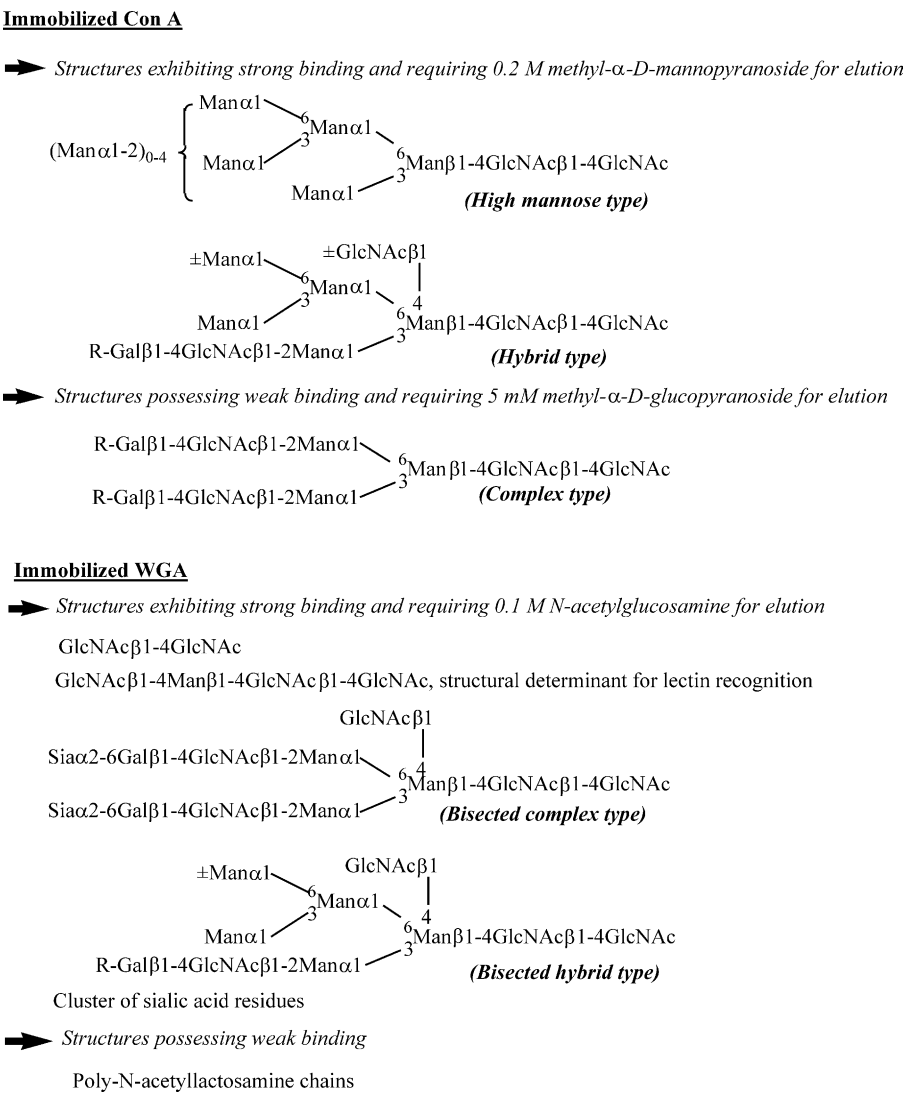


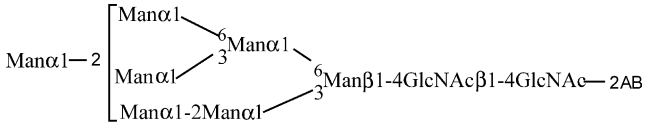
Fig. 1. Specificity of Con A and WGA toward oligosaccharides belonging to *N*-glycosylproteins. R = H or sugar residue.

2.2. Reagents and materials

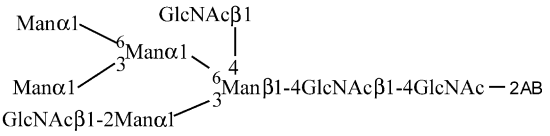
Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EDMA), [2-(methacryloyloxy)ethyl]trimethyl ammonium chloride (MAETA), pentaerythritol diacrylate monostearate (PEDAS), 2,2′-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)propyl methacrylate and 1-dodecanol were purchased from Aldrich (Milwaukee, WI, USA). Cyclohexanol, ethylene glycol and acetonitrile (HPLC grade) were from Fisher Scientific (Fair Lawn, NJ, USA). Wheat germ agglutinin (WGA) was purchased from Vector Labs. (Burlingame, CA, USA). *C. ensiformis*, also known as concanavalin A (Con A), from jack bean seeds, horse skeletal muscle myoglobin, bovine milk β-lactoglobulin B, horseradish peroxidase (HRP), ovalbumin, human and bovine α_1-acid glycoprotein, bovine milk α-lactalbumin (α-Lac), human transferrin (HT) glucose oxidase (GO) from *Aspergillus niger*, conalbumin from chicken egg white (an ovotransferrin OT), trypsinogen (TR) and ribonuclease B (RNase B) both from bovine pancreas, glycophorin A (GPA) from human blood, pNP-α-D-mannopyranoside (pNP-α-D-Man), pNP-β-D-mannopyranoside (pNP-β-D-Man), pNP-α-D-glucopyranoside (pNP-α-D-Glc), pNP-*N*-acetyl-β-D-glucosaminide (pNP-β-D-GlcNAc) and pNP-*N*-acetyl-α-D-glucosaminide (pNP-α-D-GlcNAc) were purchased from Sigma (St. Louis, MO, USA). High mannose-type and hybrid-type *N*-glycans derivatized with 2-aminobenzaminide (2AB) referred to as Man 7-2AB and Hybrid-2AB, respectively were purchased form Prozyme (San Leandro, CA, USA). The structures of the two glycans are shown in Fig. 2. Fused-silica capillaries with an internal diameter of 100 μm and an outer diameter of 360 μm were from Polymicro Technology (Phoenix, AZ, USA).

2.3. Column pretreatment

The inner wall of the fused-silica capillary was treated with 1.0 M sodium hydroxide for 30 min, flushed with 0.10 M



2-Aminobenzamide high mannose-type (oligomannose 7) (Man 7-2AB)



2-Aminobenzamide hybrid-type with bisecting GlcNAc (Hybrid-2AB)

Fig. 2. Structures of Man 7-2AB and Hybrid-2AB oligosaccharides.

hydrochloric acid for 30 min, and then rinsed with water for 30 min. The capillary inner wall was then allowed to react with a 50% (v/v) solution of 3-(trimethoxysilyl)propyl methacrylate in acetone for 12 h to vinylize the inner wall of the capillary. Thereafter, the capillary was first rinsed with methanol and then with water and dried under a stream of nitrogen.

2.4. In situ polymerization

2.4.1. Affinity columns

The polymethacrylate monoliths were prepared following the procedure described earlier [4]. For the fabrication of neutral monoliths, polymerization solutions weighing 2 g each were prepared from GMA 18% (w/w), EDMA 12% (w/w), cyclohexanol 58.8% (w/w) and dodecanol 11.2% (w/w). For the preparation of positively charged monoliths, MAETA 1% (w/w) was added to the polymerization solution (2 g) consisting of GMA 17% (w/w), EDMA 12% (w/w), cyclohexanol 59.5% (w/w) and dodecanol 10.5% (w/w). In all cases, AIBN (1.0%, w/w, with respect to monomers) was added to the polymerization solution as initiator. The solution was then degassed by purging with nitrogen for 5 min.

A 40 cm of the pretreated capillary was filled with the polymerization solution up to 30 cm by immersing the inlet of the capillary in the solution vial and applying vacuum to the outlet. The capillary ends were then plugged with GC septum, and the capillary submerged in a 50 °C water bath for 24 h. The resulting monolithic column was washed with acetonitrile and then with water using an HPLC pump.

2.4.2. RPLC monolithic columns

Polymerization solution weighing 1.0 g was prepared from PEDAS 30% (w/w), cyclohexanol 55.5% (w/w), ethylene glycol 12.0% (w/w) and water 2.5% (w/w). The solution was then sonicated to obtain a clear solution, and purged with nitrogen for 5 min [16].

A 40 cm of the pretreated capillary was filled with the polymerization solution up to 30 cm by immersing the inlet of the capillary in the solution vial and applying vacuum to the outlet. The capillary ends were then plugged with GC septum, and the capillary submerged in a 60 °C water bath for 18 h.

The resulting monolithic capillary column was washed with acetonitrile mixture using an HPLC pump. A detection window was created at 1–2 mm after the end of the polymer bed using thermal wire stripper. Finally, the column was cut to a total length of 33.5 cm with an effective length of 25 cm.

2.5. Immobilization of lectins

The glycidyl methacrylate monolithic column was first rinsed thoroughly with water and then filled with 0.1 M HCl solution and heated at 50 °C for 12 h to hydrolyze the epoxy groups. The column was then rinsed with water followed by a freshly prepared solution of 0.1 M sodium periodate for 1 h to oxidize the ethylene glycol on the surface of the column to an aldehyde. Lectin was immobilized to the monolithic column by pumping a solution of Con A at 5 mg/mL in 0.1 M sodium acetate, pH 6.4, containing 1 mM of $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 M methyl α-D-mannopyranoside and 50 mM sodium cyanoborohydride through the column for overnight at room temperature. WGA was immobilized by using the same procedure of pumping a solution of 5 mg WGA/mL in 0.1 M sodium acetate, pH 6.4, and containing 0.1 M *N*-acetyl-D-glucosamine and 50 mM sodium cyanoborohydride. The resulting column was then rinsed for 3 h at room temperature with a solution of 0.4 M Tris–HCl, pH 7.2, and containing 50 mM sodium cyanoborohydride to react with any unreacted aldehyde group. The resulting column was then rinsed with water and cut to an effective length of 25 cm and a total length of 33.5 cm.

3. Results and discussion

3.1. Monolithic columns with immobilized Con A

3.1.1. Neutral Con A column

The specificity of a lectin is often expressed in terms of the best monosaccharide inhibitor, i.e., hapten sugar. The monosaccharide specificity of Con A is α-D-mannose (α-D-Man) and to a lesser extent α-D-glucose (α-D-Glc). In fact, the binding constant of Con A to α-D-Man is $3.74 \times 10^3\ M^{-1}$ as compared to $0.82 \times 10^3\ M^{-1}$ for the association of α-D-Glc–Con A [17]. Therefore, α-D-Man and its glycoside methyl-α-D-mannopyrannoside (Me-α-D-Man) are more effective hapten sugars and are usually added to the eluting mobile phase to reverse the interactions of immobilized Con A with glycosylated solutes and bring about their elution form a Con A column. In addition, α-D-Man and Me-α-D-Man are UV transparent and do not interfere with the detection of the eluted glycoconjugates over a wide range of the UV spectrum. However, the binding constant of Me-α-D-Man ($K = 17 \times 10^3\ M^{-1}$) is ∼4.5 times higher than that of

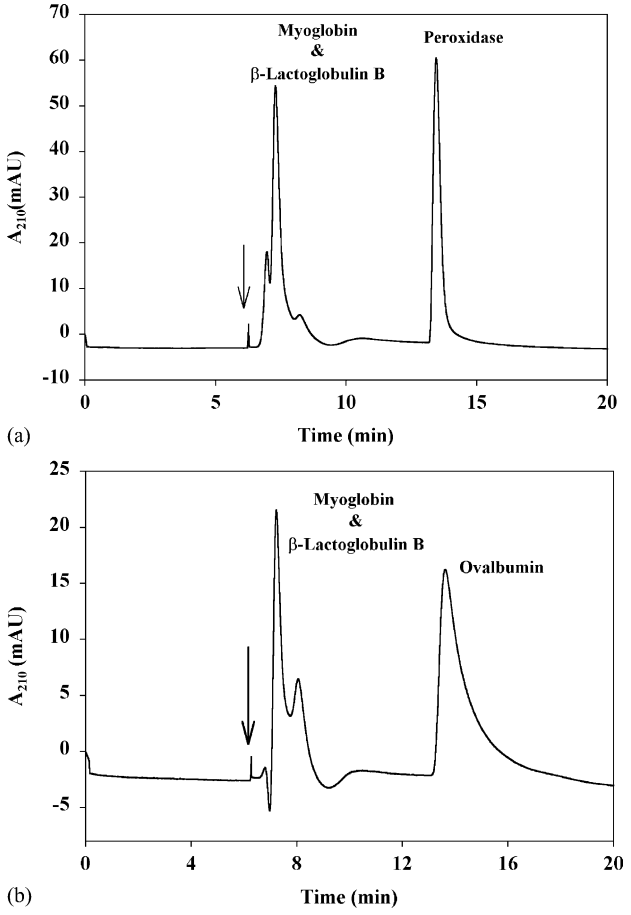


Fig. 3. Chromatograms of horseradish peroxidase in (a) and ovalbumin in (b) in the presence of non-glycosylated myoglobin and β-lactoglobulin B, using Con A immobilized on a neutral monolithic column, with 25 cm effective length, 33.5 cm total length × 100 μm I.D. Binding mobile phase: 20 mM BisTris, pH 6.0, containing100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and1 mM $MgCl_2$; eluting mobile phase: 0.2 M Me-α-D-Man in the binding mobile phase, introduced at 6.0 min as indicated by the arrow. Pressure drop: 1.0 MPa for both running mobile phase and sample injection. Sample injection: 12 s.

α-D-Man, thus favoring Me-α-D-Man to be the most effective hapten sugar at relatively low concentration in the eluting mobile phase. On the other hand, pNP-α-D-mannopyranoside (pNP-α-D-Man) with its strong pNP chromophore and strong binding specificity to immobilized Con A constitutes the test solute of choice to assess the bio-specificity and activity of Con A columns. In fact, the binding constant of pNP-α-D-Man is twice that of Me-α-D-Man [12] and ∼10 times that of α-D-Man.

The neutral monolithic column with immobilized Con A was evaluated with a mixture of pNP-α-D-Man and pNP-β-D-Man. The Con A column showed strong affinity to pNP-α-D-Man (high k') and the lack of affinity to pNP-β-D-Man ($k' = 0$) as proof of binding specificity. The elution of pNP-α-D-Man necessitated the use of hapten sugar Me-α-D-Man in the mobile phase.

Fig. 3a shows the affinity of horseradish peroxidase to the Con A column in the presence of non-glycosylated proteins, e.g., myoglobin and β-lactoglobulin B. The non-glycosylated proteins were eluted in the dead volume of the column ($k' = 0$ or pass through fraction) with the binding mobile phase, while the glycoprotein (HRP) was very strongly retained ($k' \sim \infty$) by the Con A column and its elution necessitated the use of a mobile containing the hapten sugar Me-α-D-Man. This reveals the absence of non-specific interaction between the Con A column and the non-glycosylated proteins and the high specific interactions of the immobilized Con A with HRP, which has glycans with mannose core (i.e., Con A reactive glycans) as most glycans in plant glycoproteins [18]. In fact, HRP has four glycans that are (Xyl)Man_3(Fuc)$GlcNAc_2$ (major species), (Xyl)Man_2(Fuc)$GlcNAc_2$, (Xyl)$Man_3GlcNAc_2$ and Man_3(Fuc)$GlcNAc_2$ (minor species), which account for greater than 95% of the carbohydrate. Other members of this glycan family, namely $(Xyl)_xMan_m(Fuc)_fGlcNAc_2$ ($x = 0$ or 1, $f = 0$ or 1, $m = 4$, 5, 6 or 7), account for the rest of glycans. Only traces of high mannose type glycans were detected [19,20]. It is well established that HRP has strong binding affinity to immobilized Con A [21,22]. The flow velocity at 1.0 MPa (i.e., 10 bar) inlet pressure is 0.6 mm/s using the mobile phase conditions of Fig. 3a.

Fig. 3b shows the affinity of the Con A column to another glycoprotein, ovalbumin, in the presence of two non-glycosylated proteins, myoglobin and β-lactoglobulin B using the same conditions as in Fig. 3a. Ovalbumin has a single *N*-glycosylation site (Asn 292) to which various high mannose type or hybrid type *N*-glycans are attached [23,24]. This explains the tight binding of ovalbumin to the Con A column, the elution of which necessitated an eluting mobile phase containing 0.2 M Me-α-D-Man. When compared to HRP, the peak of ovalbumin is broader indicating slower sorption kinetics for the latter than the former. The flow velocity is the same as that in Fig. 3a.

Other glycoproteins, including ribonuclease B (RNase B), human transferrin, *A. niger* glucose oxidase, bovine and human α_1-acid glycoprotein (AGP) exhibited affinity for the Con A column while conalbumin (or ovotransferrin OT) passed through the column with no retention, see Table 1. It is well established that RNase B has one glycosylation site at Asn 60 to which various high mannose type *N*-glycans (Man 5 to Man 9) are attached with the relative molar proportion of 57, 31, 4, 7 and 1% for Man 5, Man 6, Man 7, Man 8 and Man 9, respectively [23,25]. Such *N*-glycans are Con A reactive. HT is known to possess bi-antennary and in smaller amounts tri-antennary complex type *N*-glycans at different degree of sialylation [26,27]. Of these *N*-glycans, the bi-antennary ones have been reported to be Con A reactive [27]. The carbohydrate content of the enzyme GO from *A. niger* consists mainly of D-mannose (about 14% of the enzyme by mass) but it also contains D-glucosamine (2.3%) and D-galactose (0.3%) [28,29]. The high mannose content would explain the affinity of GO for Con A. α_1-Acid glycoprotein has five *N*-glycosylation sites at asparagine residues in the positions 15, 38, 54, 75 and 85 in the 181 amino acid long polypeptide chains. To these five glycosylation sites various sialylated

Table 1
Affinity of some glycoproteins toward Con A and WGA

	Neutral monolithic Con A	Positively charged monolithic Con A	Positively charged monolithic WGA
Horseradish peroxidase	(+)	(+)	(−)
Bovine pancreas ribonuclease B	(+)	(+)	(−)
Ovalbumin	(+)	(+)	(−)
Human transferrin	(+)	(−)	(+)
A. niger glucose oxidase	(+)	NM	(−)
Bovine α_1-acid glycoprotein	(+)	NM	(+)
Human α_1-acid glycoprotein	(+)	NM	(+)
Glycophorin A	NM	NM	(+)
Conalbumin	(−)	(−)	(−)

Capillary column, 33.5 cm total length, 25 cm effective length × 100 μm I.D. (+), bound protein; (−), passed through protein; NM, not measured. Con A binding mobile phase: 20 mM BisTris, pH 6.0, containing 100 mM NaCl, 1mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$. Con A eluting mobile phase: 0.2 M methyl α-D-mannopyranoside in the binding mobile phase. WGA binding mobile phase: 20 mM BisTris, pH 6.0, containing 100 mM NaCl. WGA eluting mobile phase: 0.2 M *N*-acetyl-D-glucosamine in binding mobile phase. Pressure applied for mobile phase flow and sample injection was 1.0 MPa (10 bar).

bi-, tri- and tetra-antennary complex type *N*-glycans as well as tetra-antennary chains with repeating lactosamine block are attached [30]. Most likely the bi-antennary *N*-glycan structures are the Con A reactive oligosaccharides [31]. Conalbumin (an ovotransferrin) form chicken egg white contains only 2.65% carbohydrate most of it consists of tri-antennary *N*-glycans [26], which has no affinity towards Con A.

3.1.2. Positively charged Con A column

Recently, we have designed affinity monoliths with immobilized mannan for the isolation of mannose binding proteins [4]. In this contribution to affinity nano-LC and CEC, we have found that the inclusion of small amount of the positively charged monomers MAETA in the polymerization solution yielded monoliths, which exhibited high permeability with excellent flow characteristics and improved mass transfer properties in nano-LC as compared to the neutral monoliths. In fact, Fig. 4 that shows the affinity of HRP to the immobilized Con A yielded much sharper peak than that produced by the neutral Con A column using the same elution conditions. The peak width at base of HRP on neutral Con A column is about 1 min with noticeable tailing (see Fig. 3a) as opposed to a peak width at base of about 20 s on the positively charged Con A column (see Fig. 4). The positively charged Con A produced a linear flow velocity of 1.67 mm/s as opposed to 0.6 mm/s for the neutral Con A monolith at constant pressure of 1.0 MPa. This represents a three-fold higher flow at constant pressure, which is a major improvement. Also, the positively charged Con A column did not exhibit non-specific interactions with the non-glycosylated proteins, myoglobin and β-lactoglobulin B. However, the positively charged Con A column did not show affinity towards HT and pNP-α-D-Man probably due to its macroporous nature and consequently smaller surface area than the neutral Con A monolith. This is an indication that the density of Con A coupled to the macroporous, positively charged monolith is lower than that coupled to the neutral monolith. This agrees with earlier observation on non-porous silica microparticles with immobilized Con A [32] and Sepharose 4B with immobilized WGA [33].

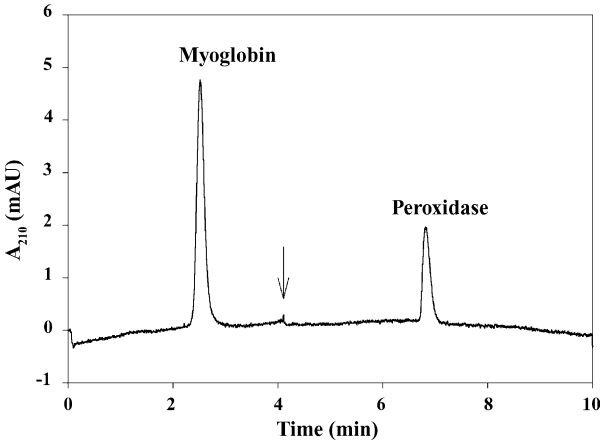


Fig. 4. Chromatogram of horseradish peroxidase in presence of non-glycosylated myoglobin using Con A immobilized on a positively charged monolithic column. Sample injection: 3 s. Other experimental conditions are the same as in Fig. 3.

3.2. Monolithic columns with immobilized WGA

Considering the high permeability of the Con A column based on the positively charged monolith, the same monolith was also used for coupling WGA. The monosaccharide specificity of WGA is *N*-acetyl-D-glucosamine (GlcNAc) [8,10,33]. The binding constant of GlcNAc to WGA is $4 \times 10^2\ M^{-1}$ [13,34], and therefore GlcNAc is an effective hapten sugar additive to the eluting mobile phase to displace tightly bound glycoconjugates from immobilized WGA column. pNP-*N*-acetyl-D-glucosaminide (pNP-GlcNAc) has a higher binding constant to WGA than GlcNAc and possesses a strong chromophore (pNP residue) for UV detection, thus serving as a probe solute for testing the activity and bio-specificity of immobilized WGA.

Both α and β anomers of pNP-GlcNAc exhibited strong affinity toward the WGA-monolithic column equilibrated with the binding mobile phase, and eluted form the column with the eluting mobile phase containing the hapten sugar GlcNAc. Fig. 6 shows further the binding specificity of the

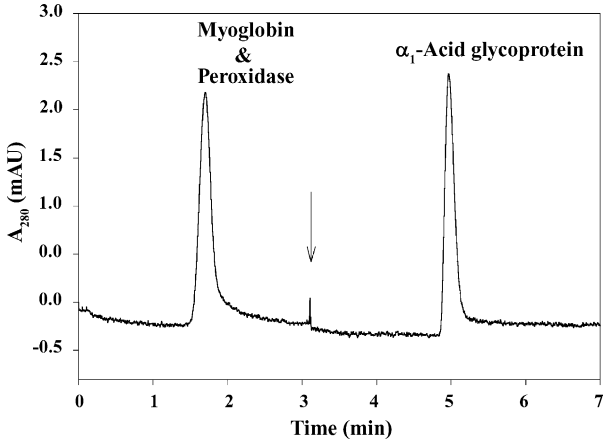


Fig. 5. Chromatogram of human α_1-acid glycoprotein in presence of non-glycosylated myoglobin and non retained glycoprotein horseradish peroxidase, using WGA immobilized on a positively charged monolithic column, with 25 cm effective length, 33.5 cm total length × 100 μm I.D. Binding mobile phase: 20 mM BisTris, pH 6.0, containing 100 mM NaCl; eluting mobile phase: 0.2 M GlcNAc in the binding mobile phase, introduced at 3.0 min as indicated by the arrow. Pressure drop: 1.0 MPa for both running mobile phase and sample injection. Sample injection: 6 s.

lectin column. Two glycoproteins (HRP and human AGP) and a non-glycosylated protein (myoglobin) were separated on the WGA column. While AGP was strongly retained ($k' \sim \infty$) and eluted with the hapten sugar (GlcNAc), HRP, which is another glycoprotein had no affinity ($k' = 0$) for WGA and eluted in the dead volume of the column with the binding mobile phase, as did myoglobin. This example shows the potential of lectin affinity chromatography (LAC) with immobilized WGA in the selective separation of pure glycoproteins/glycoconjugates form a biological mixture containing other glycoproteins and non-glycosylated proteins. As stated above AGP has five glycosylation sites to

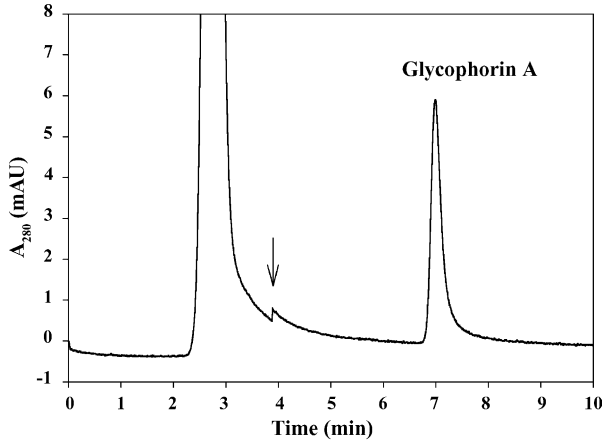


Fig. 6. Chromatogram of human erythrocyte glycophorin A using WGA immobilized on a positively charged monolithic column, with 25 cm effective length, 33.5 cm total length × 100 μm I.D. Binding mobile phase is 20 mM BisTris, pH 6.0, 100 mM NaCl, 0.05% SDS. Eluting buffer is 0.2 M *N*-acetyl-D-glucosamine in the binding mobile phase, and is introduced at 4.0 min as indicated by the arrow. Pressure drop: 1.0 MPa for both running mobile phase flow and sample injection. Sample injection: 30 s.

which various sialylated bi-, tri- and tetra-antennary complex type *N*-glycans as well as tetra-antennary chains with repeating lactosamine block are attached [30]. Most likely, the high degree of sialylation of the *N*-glycans as well as the presence of repeating lactosamine blocks are responsible for the affinity of AGP to the immobilized WGA. Similarly, bovine AGP exhibited affinity to the WGA column under otherwise the same running conditions. The WGA-monolithic column yielded a linear flow velocity of 2.9 mm/s with the binding mobile phase at 1.0 MPa inlet pressure. This is about 1.7-fold higher flow velocity than that obtained on the positively charged Con A monolithic column despite the fact that both columns are based on the same positively charged monolithic backbone. This difference in flow velocity at constant inlet pressure may be attributed to the slight difference in the nature of the mobile phase and the injected sample as well as to the difference in the size of the immobilized affinity ligand. Con A has a molecular weight ~3 times higher than that of WGA.

Lectin columns are effective tools for the isolation of small amounts of pure glycoproteins as shown in Fig. 6, where one can see the contaminants eluting in the dead volume of the column with the binding mobile phase while the glycoprotein (here glycophorin A) eluting with the hapten sugar by stepwise elution. In this example, a crude extract of blood glycophorin A, a membrane protein, has been purified. This is a relatively large injection (lasted 30 s) representing 0.17 column volume (4.4 cm long) giving rise to a large breakthrough band. Glycophorin A, the erythrocyte membrane sialoglycoproteins, contains an extensive mucin-like *O*-glycosylation domain carrying 25 *O*-glycans [35,36], of which as many as five consecutive serine or threonine residues are *O*-glycosylated. The most common *O*-glycan structure is the tetrasaccharide NeuAcα2-3Galβ1-(NeuAcα2-6)GalNAc. Smaller *O*-glycans (e.g., lacking one of the NeuAc residues) or more complex chains (e.g., containing one or more Galβ1-4GlcNAc units) also are present, reflecting inter- and intra-molecule heterogeneity of glycosylation [37]. In the case of glycophorin (Fig. 6), the flow velocity at constant inlet pressure (1.0 MPa) obtained with the binding mobile phase is about 1.45 mm/s, which is much slower than that in Fig. 5. The binding mobile phase for glycophorin A sample contains sodium dodecyl sulfate, and it is likely that the membrane protein extract did not dissolve well contributing to possible partial plugging of the column and consequently reduced flow velocity.

3.3. Selective on-line pre-concentration of dilute samples by nano-lectin affinity chromatography

3.3.1. Glycoproteins

In order to show the effectiveness of lectin monolithic capillary columns in on-line pre-concentration of dilute glycoconjugate samples at the nano-scale level, a dilute solution of 5×10^{-8} M human AGP was fed through the WGA-monolithic column for 30 min. This sample injection time corresponds to introducing 15.8 column volumes or

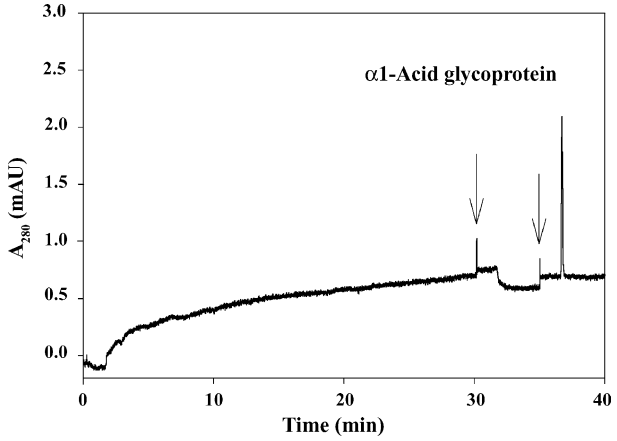


Fig. 7. Chromatogram showing the isolation and concentration of 5×10^{-8} M human α_1-acid glycoprotein using WGA immobilized on a positively charged monolithic column, with 25 cm effective length, 33.5 cm total length × 100 μm I.D. The α_1-acid glycoprotein is injected for 30 min into the column equilibrated with the binding mobile phase. At 30 min, the column is washed with the binding mobile phase for 5 min as indicated by the first arrow. At 35 min, the captured α_1-acid glycoprotein is eluted from the lectin column by using the eluting mobile phase as indicated by the second arrow. Binding mobile phase: 20 mM BisTris, pH 6.0, containing 100 mM NaCl; eluting mobile phase: 0.2 M GlcNAc in the binding mobile phase. Pressure drop: 1.0 MPa for both running mobile phase and sample injection.

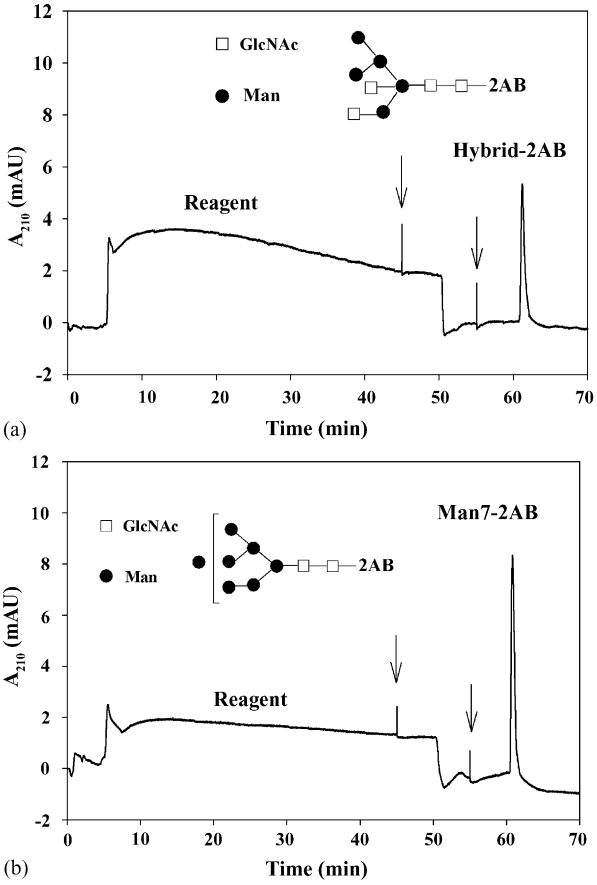


Fig. 8. Chromatograms showing the isolation and concentration of 5×10^{-7} M Hybrid-2AB in (a) and 5×10^{-7} M Man 7-2AB in (b) using Con A immobilized on a neutral monolithic column, with 25 cm effective length, 33.5 cm total length × 100 μm I.D. The Hybrid-2AB is injected for 45 min into the column equilibrated with the binding mobile phase. At 45 min, the column is washed with the binding mobile phase for 10 min as indicated by the first arrow. At 55 min, the captured Hybrid-2AB is eluted form the lectin column by using the eluting mobile phase as indicated by the second arrow. Binding mobile phase: 20 mM BisTris, pH 6.0, containing 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$; eluting mobile phase: 0.2 M Me-α-D-Man in the binding mobile phase. Pressure drop: 1.0 MPa for both running mobile phase and sample injection.

~21.7 μL, which represent ~43 ng protein or ~1.08 pmol of the glycoprotein accumulated at the inlet end of the column. Thereafter, the column was eluted with the eluting mobile phase containing 0.2 M GlcNAc as the hapten sugar (Fig. 7). In this experiment, the concentration factor is about 153, which is defined as the ratio of the concentration in the collected fraction to that in the feed solution (i.e., the dilute sample). A much larger enrichment factor can be readily achieved since a more dilute sample (i.e., at trace concentration) can be concentrated for longer injection time. In other words, there is no limit on the volume injected. As can be seen in Fig. 7, and for the same injection time, at least five-fold more diluted samples can be determined by the lectin pre-concentration scheme, which is about 10^{-8} M protein using UV detection.

3.3.2. Glycans

To demonstrate the usefulness of lectin affinity monoliths in the on-line preconcentration of glycans from dilute samples, a small amount of a high mannose glycan (Man 7) derivatized with 2-AB (Man 7-2AB) and hybrid glycan derivatized with 2-AB (Hybrid-2AB), at low concentration of ~5×10^{-7} M were introduced separately into a Con A monolithic column for 45 min. This corresponds to introducing ~8.1 column volumes or ~11.3 μL at 1.0 MPa inlet pressure. Thereafter, the column was washed with the binding mobile phase for 10 min until the baseline reached the original value (see Fig. 8a and b). Finally, the column was eluted stepwise with a mobile phase containing 0.2 M Me-α-D-Man. An enrichment factor of ~400-fold was achieved. This experiment demonstrates the potentials of affinity nano-LC in isolating small amount of derivatized glycan and detecting very dilute glycan samples (at the 10^{-8} M level or lower) even with a UV detector. In fact, intensities of the eluted glycan peaks, as well as the ability to increase the sample loading time show that much lower amount of the glycans can be recovered and concentrated in very small volumes.

3.4. Two-dimensional chromatography using tandem lectin affinity and RPLC columns

As can be seen from the above discussion, LAC generally results in an enrichment of classes of different glycoproteins (see Table 1), possessing similar carbohydrate determinants recognized by the immobilized lectin and called "lectin receptors". As different lectins are able to recognize different saccharidic sequences belonging to the same glycan class and as these glycans are likely to be common to numerous

glycoproteins, the different lectins interact in fact with a broad spectrum of glycoproteins. This may explain why immobilized Con A and WGA are most often sufficient to fractionate complex mixtures of glycoproteins into classes either sequentially [33] or by tandem columns (i.e., connected in series) [22]. Therefore, LAC is an efficient first step in the purification process of glycoproteins, which should be followed by another chromatographic method to purify individual glycoproteins. The second chromatographic method should be based on non-specific interactions such as RPLC or ion-exchange chromatography in order to further fractionate glycoproteins on the basis of hydrophobicity or ionic character, respectively. This calls for a 2D separation approach whereby LAC will occupy the first dimension and the other non-specific interactive chromatographic method will constitute the second dimension. Accordingly, this section describes the operation of 2D separation based on nano-LAC and nano-RPLC. Nano-LAC and nano-RPLC are truly orthogonal separation techniques based on uncorrelated separation mechanisms thus allowing improved resolution of complex mixtures. This is because peak capacity in a multidimensional system is approximately equivalent to the product of the peak capacities of each individual system. A band form a lectin column may consist of a discrete set of glycoproteins. Therefore, this band must be fractionated further by another separation dimension, e.g., RPLC. The 2D separation approach developed here is an important undertaking for "nano-glycomics/nano-proteomics."

For the 2D separation, the experimental set-up consisted of connecting a relatively short lectin-monolithic capillary (12 cm × 100 μm I.D.) to a longer C17 monolithic capillary column (25 cm to detection point, 35 cm total length × 100 μm I.D.) via a zero dead volume Upchurch Nanotight union. The two capillary columns connected in series were installed in the capillary cartridge holder of the HP^{3D}CE system and all the nano-LC runs were carried out at a constant column inlet pressure of 1.0 MPa. In this 2D platform, the nano-RPLC step must be rapid to reduce the total time needed for the 2D separation and not for any other reason as in other 2D separation since the band captured by the lectin column will only be available for analysis by the second dimension once the exact hapten sugar has been applied to the lectin column connected in series to the C17 monolithic column. The 2D platform combining nano-LAC and nano-RPLC has an additional advantage in terms of solvent compatibility of the two modes when LAC is used as the first dimension as it is the case. The LAC mobile phase used during adsorption and elution of glycoconjugates (salted aqueous buffer containing a hapten sugar) is a weak eluent for RPLC thus allowing the adsorption of the eluted glycoprotein/glycoconjugate band on the top of the RPLC capillary column. After rinsing out the hapten sugar from the RPLC capillary column, the organic-rich mobile phase can be applied to the RPLC column to promote elution and separation of the bound glycoconjugates, which was previously eluted form the lectin column.

3.4.1. *Two-dimensional Con A ⇒ RPLC*

To test the novel nano-scale 2D concept, we have selected a mixture of five standard proteins that are glucose oxidase, human transferrin, conalbumin (an ovotransferrin), trypsinogen and α-lactalbumin. Three of the proteins are glycoproteins (i.e., GO, HT and OT) and the remaining two are ordinary proteins (i.e., TR and α-Lac). The mixture was introduced into the 2D system containing a short Con A column connected to a C17 monolith. While GO and HT have affinity to Con A, OT does not. Under this condition, GO and HT were captured by the Con A, while OT, TR and α-Lac passed through the Con A column and accumulated on the top of the C17 monolith. After this sample introduction step, the two columns were disconnected, and the C17 monolithic column was kept in the cartridge holder while the lectin column with the adsorbed glycoproteins was taken out and let aside for further use. The elution of the C17 monolith by step gradients of increased percent of acetonitrile in the mobile phase

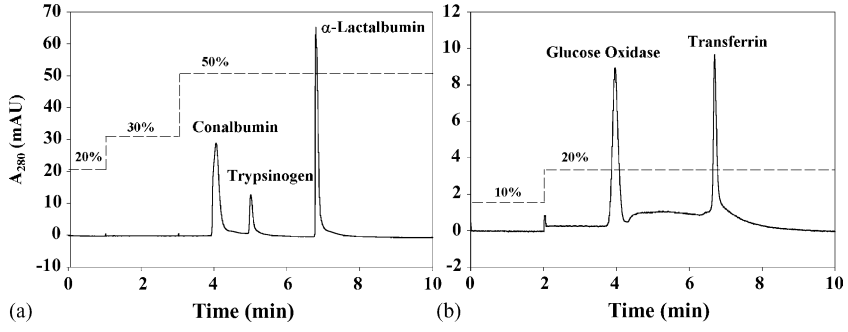


Fig. 9. Two dimensional separations of a mixture of some proteins using a Con A column (12 cm × 100 μm I.D.) in the first dimension followed by RPLC on a neutral C17 capillary column (25 cm effective length, 33.5 cm total length × 100 μm I.D.) in the second dimension. (a) Chromatogram obtained on the C17 column for the unretained proteins on the Con A column (passed through fraction); mobile phase used was 20 mM BisTris, pH 6.0, at 20% (v/v) acetonitrile for 1 min and then the acetonitrile content was increased stepwise to 30% at 1 min, and to 50% at 3.0 min. (b) Chromatogram obtained on the C17 column for the two glycoproteins transferred form the Con A column (i.e., previously captured by Con A column); mobile phase used was 20 mM BisTris, pH 6.0, at 10 % (v/v) for 2 min and then the acetonitrile content was increased to 20% at 2.0 min. Con A column conditions are as follows. Binding mobile phase: 20 mM BisTris, pH 6.0, containing 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$; eluting mobile phase: 0.2 M Me-α-D-Man in the binding mobile phase. Pressure drop: 1.0 MPa for both running mobile phase and sample injection. Sample injection: 12 s.

allowed the separation of OT, TR and α-Lac (Fig. 9a), meanwhile the two glycoproteins (GO and HT) are "parked" on the Con A monolith. In a second step, the Con A column was reconnected to the C17 monolithic column and subsequently eluted with the hapten sugar Me-α-D-Man, a condition that moved GO and HT to the C17 monolith. Thereafter, the Con A column was disconnected again and taken out from the capillary cartridge holder, and the C17 monolith was eluted by step gradients, which resulted in separating the two glycoproteins GO and HT (Fig. 9b). These 2D separation schemes are very advantageous specially for small amounts of glycoproteins/glycoconjugates, since the separated analytes stay in the liquid phase and are transferred form column-to-column without sample loss and finally are eluted with a volatile hydro-organic mobile phase typical of RPLC.

4. Conclusions

This investigation has demonstrated the suitability of monolithic capillaries with immobilized lectins for the nano-LAC of glycoconjugates. Nano-LAC not only offers the best column format for dealing with small sample size but also allows the achievement of important tasks in glycomics such as (i) the isolation of small amount of pure glycoconjugates from complex proteins extract, (ii) the preconcentration of dilute samples of glycoconjugates thus allowing their direct UV detection at low levels and (iii) the establishment of 2D separation schemes for the fractionation and separation of complex glycoconjugates mixtures. The 2D separations presented here are initial studies and in-depth studies are still underway.

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