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Affinity chromatography with monolithic capillary columns I. Polymethacrylate monoliths with immobilized mannan for the separation of mannose-binding proteins by capillary electrochromatography and nano-scale liquid chromatography

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Abstract

Monolithic capillary columns with surface-immobilized mannan have been introduced for affinity-based micro-column separations by nano-liquid chromatography (nano-LC) and capillary electrochromatography (CEC). 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. While neutral monoliths with immobilized mannan were only useful for affinity nano-LC, the cationic monoliths with surface-bound mannan were useful in both affinity nano-LC and affinity CEC. The cationic monoliths allowed a relatively high electro-osmotic flow (EOF) when mannan was immobilized to the epoxy monolith via a positively charged spacer arm, triethylenetetramine. The neutral monoliths exhibited lower permeability under pressure-driven flow (PDF) than the cationic monoliths indicating that the latter had wider flow-through pores than the former. Both types of monoliths with immobilized mannan exhibited strong affinity toward mannose-binding proteins (MBP) such as the plant lectins concanavalin A and *Lens culinaris* agglutinin and a mammalian lectin (e.g. rabbit serum mannose-binding protein). Due to the strong binding affinity, the monoliths with surface bound mannan allowed the injection of large volume of rabbit serum and to isolate in a single run the mannose-binding protein in an amount sufficient to run with it sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), thus demonstrating their capability in "nano-proteomics".

Keywords: Affinity capillary electrochromatography; Electrochromatography; Affinity chromatography; Nano-scale liquid chromatography; Monolithic columns; Immobilized carbohydrates; Proteins; Carbohydrates; Mannan

1. Introduction

1.1. Background and rationale of the study

Over the last 15 years, monolithic stationary phases have been introduced to column- [1,2] and disk-based [3,4] separation approaches, and are now increasingly employed in many applications [5–8]. This is primarily due to: (i) the various chemistries available for the fabrication of widely differing monoliths; (ii) the ease with which the monoliths can be produced in situ and confined in large diameter columns, capillaries and channels of microfluidic devices; (iii) the macroporous structure of monoliths that allows improved mass transport; and (iv) the ability to operate monoliths at high flow velocity without loosing resolution. The favorable mass transport is very suitable for all kinds of chromatographic modalities, and in particular for affinity chromatography, which is characterized by its slow adsorption/desorption kinetics involving key-and-lock recognition. Slow adsorption/desorption kinetics usually leads to band broadening, which can become excessive when compounded with unfavorable pore mass transport. Therefore, the improved pore mass transport of monoliths constitutes an advantage for performing affinity chromatography at reduced band broadening.

Affinity chromatography using monolithic supports has involved immobilized low-molecular-mass ligands (e.g. dyes, inhibitors, co-enzymes, chelating species) [8–11],

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combinatorial ligands [12,13], and high-molecular-mass ligands (e.g. proteins A and G, antibodies, receptors and other proteins with miscellaneous biological functions) [7,14–19]. Despite the major advances made in monolithic-based affinity phases, the exploitation of their full potentials is yet to come. In fact, monolithic capillary columns with immobilized affinity ligands for performing affinity-based separations in nano-liquid chromatography (nano-LC) and capillary electrochromatography (CEC) have not witnessed yet the development stage they deserve, and only one attempt has been made to show their potential in nano-LC [18]. Thus far, affinity CEC has involved either open tubular fused-silica capillaries with immobilized ligands (e.g. aptamers) on the capillary walls [20,21] or packed columns with silica microparticles having immobilized antibodies [22] and other immobilized proteins [23,24]. Thus, it is the aim of this investigation to further the development of monolithic affinity phases at the nano-scale levels.

Typically, monolithic stationary phases for affinity separations are polymerized using ethylene dimethacrylate (EDMA) [18,25,26] or trimethylolpropane trimethacrylate [18] as the cross-linking monomer and glycidyl methacrylate (GMA) as the active monomer for subsequent ligand attachment. The macroporous poly(glycidyl methacrylate–*co*-ethylene dimethacrylate) monoliths have been also used for the immobilization of enzymes for the production of bioreactors [26].

This article is concerned with the development of monolithic capillary columns for their consecutive use in affinity-based separation in both nano-LC and CEC modes. More specifically, monoliths based essentially on the polymerization of ethylene dimethacrylate and glycidyl methacrylate monomers have been fine-tuned with a positively charged monomer, [2-(methacryloyloxy)ethyl]trimethyl ammonium chloride (MAETA), and other surface functionalities to allow their subsequent use in both affinity nano-LC and CEC. These monoliths were further functionalized with yeast *Saccharomyces cerevisiae* mannan in the aim of providing affinity capillary columns for the isolation and separation of mannose (or mannan)-binding proteins (MBPs) or lectins.

Mannose-binding proteins belong to the family of lectins, which are proteins of non-immune origin produced by living organisms (from microbes to mammals) and are known to react with sugar residues of carbohydrates [27,28]. Mammalian MBPs, which belong to the family of animal lectins, are usually found in serum and liver of rabbits, humans and rodents [29,30]. The two related MBPs, the serum- and liver-type are composed of monomers of about $M_r \approx 26\,000$–$32\,000$, which oligomerize to form large protein entities of M_r 200 000 and 600 000 in liver and serum, respectively [29]. Although relatively little is known about the roles of mammalian MBPs in vivo, there are some supporting evidence that MBPs are involved in the intracellular transport of glycoproteins, the activation and elimination of pathogens [31,32] and the inhibition of influenza A viruses [33]. Also, MBPs have been reported to be present in tumors, forming a new class of tumors markers and targets for therapy [33] and are involved in the pathogenesis and spread of cancer at different stages [34]. These unique biological activities of MBPs are associated with their binding to mannose containing proteins on the surface of the target cells and microorganisms. MBPs are usually present at low level in serum (0.1–2 mg/ml) [29], liver tissue (0.03–1.25 mg/5 g) and tumor tissue (0.01–0.2 mg/5 g) [34] and require Ca^{2+} for binding to mannan. This relatively low level of MBPs calls for the development of nano-scale liquid phase separation methods such as the ones proposed in the present article, i.e. nano-LC and CEC-based affinity approaches. These two nano-scale separation approaches are ideal for relatively small sample volumes since they allow relatively low mass detection limits and offer nano-scale preparative capabilities for subsequent identification by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).

Mannose-binding proteins have been previously isolated by affinity chromatography using Sepharose 4B with immobilized mannan [29,34,35] or immobilized mannose [30,34] as well as other supports such as alumina with immobilized yeast mannan [36] and AKTA FPLC with immobilized mannose [37]. The isolation of MBPs has been carried out for preparing sufficient amounts of the MBPs to purify IgM by affinity chromatography with immobilized MBP [35,36], study ligand binding characteristics of MBP [30] and to identify and assign a preliminary functional annotation of a large number of both known and novel MBPs, e.g. from rice [37].

The present article involving nano-scale affinity LC and CEC constitutes an initial study to demonstrate the potentials of these two methods in the area of “nano-proteomics”. This will be shown by the nano-preparative capability of the monolithic capillary columns with immobilized mannan to capture a sufficient amount of MBP from rabbit serum (few hundred nanograms) in a single chromatographic run, which is readily trypsin digested and consequently measured for its peptide content by MALDI-MS.

1.2. Overview of the structural characteristics of yeast mannan

To better understand the interaction of the affinity ligand (i.e. mannan) with MBPs and the LC and CEC behavior of the monoliths with immobilized mannan, an overview of the structural features of mannan must be provided. Yeast mannan is a covalently linked polysaccharide–protein complex (i.e. mannoprotein) in which the protein contributes 5–50% of the total mass, depending on the source of mannan and the purification/extraction method [38]. The yeast mannoprotein is a complex of *N*- and *O*-linked glycopeptides. The yeast *S. cerevisiae* (from which the mannan used in this study originates) extends the *N*-linked core oligosaccharide (Man_8-$GlcNAc_2$) to yield a highly branched

homopolysaccharide consisting of a long α1,6-linked mannose backbone which is further modified by numerous α1,2- and α1,3-linked mannose side chains [39,40]. This outer chain of the *N*-linked oligosaccharide (linked to asparagines) is composed of 150 or more mannose units where 65% of the mannose residue is in the side chains [38]. The *O*-linked oligosaccharides (linked to serine and methionine) are much shorter chains than the *N*-linked ones consisting of one to four mannose residues [39]. A further modification of the *N*-linked oligosaccharides with mannosylphosphate was observed in several yeasts including *S. cerevisiae*. Mannosylphosphate is found at four positions in the *N*-linked oligosaccharides from *S. cerevisiae*, namely at two sites in the polymannose outer chain portion (non-reducing end and branches of mannose outer chain) and two sites in the core oligosaccharide portion [39]. The *S. cerevisiae* mannan used in this study was obtained from Sigma. According to the supplier, the *S. cerevisiae* mannan was extracted by hot alkali using the method of Haworth et al. [41], which is known to extensively disrupt the alkali-labile bonds in the mannan including the glycosyl–serine and glycosyl–threonine linkages, phosphodiester bonds, some peptide and disulfide bonds, and acyl ester linkages [42]. Therefore, the preparation of mannan used in this study is essentially free of protein and consists of the polysaccharide portion of mannan.

2. Experimental

2.1. Instrumentation

The instrument used was a HP^{3D}CE system from Hewlett-Packard (Waldbronn, Germany) equipped with a photodiode array detector. Electrochromatograms were recorded with a personal computer running an HP^{3D}CE ChemStation. Column temperature was held constant at 25 °C. An external pressure of 1.0 MPa (i.e. 10 bar) was applied to column inlet for injection and separation in nano-LC mode. The same pressure (i.e. 10 bar) was applied to both column ends in CEC mode to minimize bubble formation. Samples were injected either electrokinetically or by pressure for various times, voltage and pressure, which are stated in figure captions.

2.2. Reagents and materials

Glycidyl methacrylate, ethylene glycol dimethacrylate, [2-(methacryloyloxy)ethyl]trimethyl ammonium chloride, 2,2′-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)propyl methacrylate, 1-dodecanol, ethylenediamine (EDA), polyethylene glycol (PEG) of average M_r 200 (PEG200), 1,4-butanediol diglycidyl ether (BDDGE) and triethylenetetramine tetrahydrochloride (TETA) were purchased from Aldrich (Milwaukee, WI, USA). Cyclohexanol and HPLC grade acetonitrile were from Fisher Scientific (Fair Lawn, NJ, USA). Poly(ethylene glycol 200) diglycidyl ether (PEG-DGE) was from Polysciences (Warrington, PA, USA). *Lens culinaris* agglutinin (lectin lentil, LCA) was purchased from Calbiochem (San Diego, CA, USA). Yeast mannan (from *S. cerevisiae*), methyl-α-D-mannopyranoside, 2-*O*-α-D-mannopyranosyl-D-mannopyranose (2α-mannobiose), concanavalin A (Con A) from *Canavalia ensiformis*, horse skeletal muscle myoglobin, bovine pancreas α-chymotrypsinogen A, bovine milk β-lactoglobulin B, chicken egg white conalbumin and rabbit serum were purchased from Sigma (St. Louis, MO, USA). Sequencing grade-modified trypsin was obtained from Promega (Madison, WI, USA). Fused-silica capillaries with an internal diameter (i.d.) of 100 μm and an outer diameter (o.d.) 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 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 successively rinsed with methanol water and then dried under a stream of nitrogen.

2.4. In situ polymerization

Polymerization solutions weighing 2 g each were prepared from monomers the GMA and EDMA with or without the addition of MAETA (Table 1) and a porogenic solvent in ratios of 30:70 (w/w) monomers/solvents. The mixtures of monomers were dissolved in a porogenic solvent consisting of 85% (w/w) cyclohexanol, 15 wt.% dodecanol. AIBN (1.0% (w/w) with respect to monomers) was added to the solution as initiator. The polymerization solution was then degassed by a stream of 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 successively

Table 1
Composition of monomers in polymerization solutions used in the preparation of the different monolithic columns

Column designation	Monomer composition (% (w/w))		
	GMA	EDMA	MAETA
Neutral column (A)	60	40	
Positively charged column (B)	58	40	2

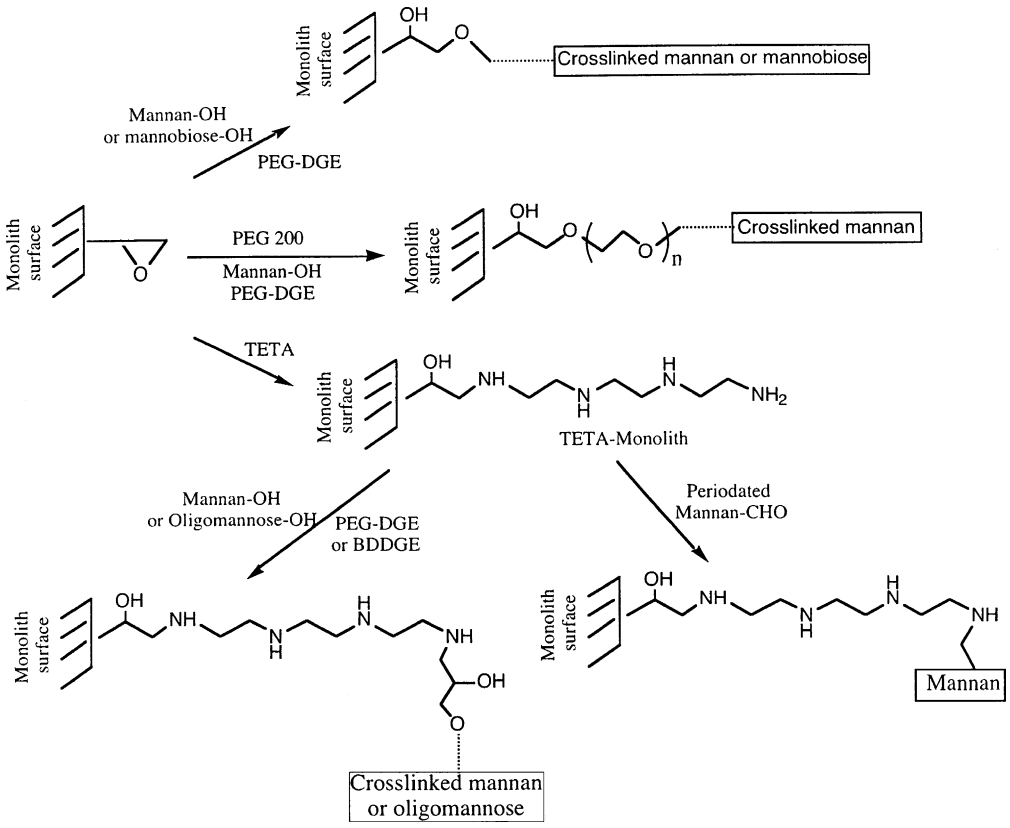

Fig. 1. Schematic illustrations of the various immobilization strategies of mannan and oligomannose on the surface of polymethacrylate glycidyl monolithic columns. The notations mannan-OH, mannan-CHO, mannobiose-OH, oligomannose-OH are used to indicate the nature of the sites involved in the attachment of the affinity ligands to the surface of the monoliths.

washed with acetonitrile and with water using an HPLC pump.

As shown in Table 1, columns made out of GMA and EDMA are labeled as Column A (neutral monoliths), whereas columns prepared from GMA, EDMA and MAETA are called Column B (cationic monoliths).

2.5. Immobilization of 2α-mannobiose and mannan

2α-Mannobiose and mannan, in various forms, were immobilized on the monolithic capillary column directly or through a spacer arm by nucleophilic attack on the epoxide ring of the monolith in basic media [35,43]. Triethylenetetramine was used as the spacer arm in the immobilization of the affinity ligand on the positively charged columns while PEG200 was used as the spacer arm for affinity ligand immobilization on neutral columns (Fig. 1).

2.5.1. Intact mannan

When a spacer arm (e.g. PEG200 or TETA) was used in the immobilization of intact mannan (otherwise mannan was immobilized directly), the epoxide monolithic column (i.e. polyglycidylmethacrylate column) was rinsed with 0.10 M of the spacer arm dissolved in 0.10 M sodium carbonate solution, pH 10.5 and allowed to react at 45 °C for 6 h. Thereafter, the resulting column was first rinsed with water, and consequently mannan was immobilized by continuously rinsing the column overnight with a 200 μl solution of mannan (1% (w/v)) and PEG-DGE (1% (w/v)) in 0.10 M sodium carbonate pH 10.5 (Fig. 1). Following, the resulting column having surface immobilized mannan was washed with water, and 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.

2.5.2. Acid hydrolyzed mannan

Mannan (1% (w/v)) was hydrolyzed in 0.10 M sulfuric acid by heating the solution at 98 °C for 3 h following the procedure described in Ref. [44] with slight modifications. The resulting solution consisting of short oligomannoses [44] was cooled and excess sulfate ions were precipitated by adding 0.10 M barium hydroxide solution to a pH of 5–7. The solution was then membrane filtered and the filtrate was evaporated to dryness at low temperature using a Speed Vac. The hydrolyzed mannan was re-dissolved in 0.10 M sodium carbonate pH 10.5 containing 1% (w/v) BDDGE to form a 1% (w/v) hydrolyzed mannan solution. The immobilization of acid hydrolyzed mannan to the column was carried out as described in Section 2.5.1.

2.5.3. Periodated mannan

Mannan (1% (w/v)) was oxidized in a solution of 1.25 mM sodium periodate and 20 mM sodium acetate, pH 5.0, for 30 min at room temperature [43]. A volume of 200 μl of this solution was acidified with 20 μl of glacial acetic acid, and subsequently 1.4 mg of sodium cyanoborohydride was dissolved in it. This solution was then pushed through a monolithic column modified with a TETA spacer arm to immobilize the periodated mannan (see Fig. 1) via a reductive amination reaction [45]. The column was left to react overnight and then washed with water and cut to the required length after making the window as described above.

2.6. MALDI-MS measurements

The monolithic capillary column with surface-immobilized mannan, was used to capture the mannose-binding protein from rabbit serum. An aliquot of the collected fraction was analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and yielded a band at $M_r \sim 30\,000$. The collected fraction was also digested with trypsin at 37 °C overnight, and the resulting digest was extracted with 50% acetonitrile: 0.1% trifluoroacetic acid (v/v), mixed with the matrix α-cyano-4-hydroxycinnamic (purchased from Sigma and re-crystalized from 33% ethanol) and spotted on the MALDI plate. A standard peptide solution was mixed with the matrix and spotted on the plate for external calibration of the masses. A Voyager DE PRO MALDI time-of-flight (TOF) mass spectrometer (PerSeptive Biosystems, Foster City, CA, USA) was used in positive reflectron mode to obtain the mass spectra. The tryptic monoisotopic peptide masses were searched against the MASCOT database for identification using parameters set for mass tolerance of 100 ppm, one missed cleavage, oxidized M and propionamide C modifications.

3. Results and discussion

3.1. Strategies for ligand immobilization: column flow velocity and affinity

Since our aim was to develop monolithic capillary columns with immobilized affinity ligand for mannose/mannan-binding proteins, we have examined the immobilization of 2α-mannobiose and various forms of mannan (e.g. native, acid hydrolyzed, periodated mannan). Since the columns were to be used in affinity nano-LC as well as in affinity CEC, various strategies for affinity ligand immobilization were tested to yield capillary columns having both relatively strong EOF and high permeability in pressure-driven flow (PDF) to achieve rapid CEC and nano-LC, respectively. Of course, and in all cases, the column must have sufficient affinity toward mannose-binding proteins or lectins. Otherwise, the column was not considered further in the study.

Table 1 summarizes the composition of the polymerization solutions used in the preparation of the various monolithic columns. Essentially, two types of epoxy-monolithic columns were prepared and subsequently used for affinity ligand immobilization. Neutral and positively charged monolithic columns designated by letters A and B, respectively, were used for mannan and 2α-mannobiose immobilization. The data on the flow velocity in nano-LC and CEC and the presence and absence of affinity toward mannose-binding lectins, e.g. Con A and LCA, are summarized in Table 2.

3.1.1. Electro-osmotic flow (EOF) and pressure-driven flow

In the class of neutral monoliths, only Column A3 exhibited a weak electro-osmotic flow (EOF) (0.57 mm/s at an applied voltage of −25 kV). Although Column A3 with immobilized 2α-mannobiose has no fixed charges on its surface, the binding of ions from the running electrolyte can

Table 2
Various strategies examined for column construction and mannan immobilization

Column code	Spacer arm	Immobilization approach	LC		CEC	
			Affinity	Flow velocity (mm/s)	Affinity	EOF (mm/s)
A1		Mannan/PEG-DGE	Yes	0.69	Yes, CNE	∼0
A2	PEG200	Mannan/PEG-DGE	Yes	0.76	Yes, CNE	∼0
A3		Mannobiose/PEG-DGE	WA	0.75	WA	0.57
B1		Mannan/PEG-DGE	Yes	1.83	Yes, CNE	∼0
B2	TETA	Mannan/PEG-DGE	Yes	2.0	Yes	2.1
B3	TETA	Periodated mannan	Yes	1.64	Yes, CNE	∼0
B4	TETA	Acid hydrolyzed mannan/BDDGE	Yes	1.81	Yes	3.1

Binding mobile phase for nano-LC: 10 mM EDA/HCl, pH 7.0, containing 100 mM NaCl and 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$. Eluting mobile phase: 100 mM methyl-α-D-mannopyranoside in the binding mobile phase for nano-LC and 100 mM methyl-α-D-mannopyranoside in 5 mM EDA/HCl, pH 7.0, for CEC. In nano-LC, the flow velocity is measured at a constant pressure of 10 bar while in CEC, the EOF is measured at a constant applied voltage of 25 kV (± polarity for neutral monoliths and − polarity for positively charged monoliths). Column lengths: in all cases, total column length is 33.5 cm having a 25 cm segment filled with the monolith while the remaining 8.5 cm segment is empty. Uracil was used as the inert tracer for measuring the dead time in both nano-LC and CEC. CNE: could not be eluted; WA: weak affinity, i.e. lectins elute with the binding mobile phase.

provide a zeta potential for the surface, and consequently generate an EOF [46]. The absence of a noticeable EOF in the other columns of type A can be explained as follows. According to the supplier (i.e. Sigma), the *S. cerevisiae* mannan preparation used in this study is to a large extent free of protein [36]. Also, since the *S. cerevisiae* mannan obtained from Sigma was extracted by hot alkali using the method of Haworth et al. [41], this mannan may have lost the majority of its phosphate moieties. In fact, and as mentioned above, the extraction by hot alkali is known to extensively disrupt the alkali-labile bonds in the mannan including the glycosyl–serine and glycosyl–threonine linkages, phosphodiester bonds, some peptide and disulfide bonds, and acyl ester linkages [42]. Under these conditions (i.e. absence of covalently bound protein and disruption of phosphodiester bonds), the mannan used in this study does not have sufficient amount of charges to support a noticeable EOF. Also, the presence of immobilized mannan in the porous structure may have inhibited any EOF that can arise from adsorption of ions to the monolith backbone due to the shielding effect of the large mannan entity. In the nano-LC mode, and operating the column at 10 bar, the various A-type columns exhibited moderate permeability with a flow velocity in the range 0.69–0.76 mm/s (Table 2).

In general, the incorporation of MAETA monomer in the monolith (Column B-type) provided larger perfusive pores (i.e. flow-through pores) as manifested by the large increase in flow velocity in nano-LC as compared to neutral monoliths (Column A-type). Among the three different positively charged monoliths (Column B-type) with immobilized mannan (Table 2), only Column B2, which has a positively charged spacer arm (i.e. TETA), produced both a good PDF in nano-LC and a relatively strong EOF in CEC while Columns B1 and B3 exhibited only a good PDF in nano-LC. Of course, the column with immobilized acid-hydrolyzed mannan (Column B4) produced the highest EOF velocity. The partial acid hydrolysis of *S. cerevisia* mannan was reported to yield a mixture of mannobiose, mannotriose and mannotetraose [44]. These short oligomannoses do not shield the positively charged amino groups in the TETA spacer arm as much as the intact mannan does. Periodated mannan seems to inhibit the EOF (Column B3) may be due to multiple point attachment of the periodated mannan to the TETA spacer arm and consequently a stronger shielding of the charged surface.

3.1.2. Affinity of monoliths to mannose/mannan binding proteins

As shown in Table 2, the various affinity monoliths showed relatively strong affinity towards Con A and LCA except the one based on immobilized 2α-mannobiose, i.e. Column A3, which exhibited weak affinity (WA). Weak affinity refers to a weak retention and elution with the binding mobile phase.

3.1.2.1. Neutral monoliths. Immobilization of mannan onto the neutral monolith directly (Column A1) or through a spacer-arm of polyethylene glycol 200 (Column A2) has yielded capillary columns having strong affinity toward Con A and LCA. The two lectins adsorbed strongly to the two columns (in nano-LC) and were eluted stepwise by applying 100 mM methyl-α-D-mannopyranoside in the binding mobile phase. Other proteins with no affinity to mannose (or mannan) such as conalbumin (isoelectric point, pI = 5.9), myoglobin (pI = 6.8) and α-chymotrypsinogen A (pI = 8.8) eluted in the column void volume with the binding mobile phase which was composed of 10 mM EDA/HCl, pH 7.0, containing 100 mM NaCl and 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$. The fact that these three proteins of different pI values did not show retention is an indication of absence of non-specific interactions with the monolithic affinity columns. More specifically, the elution of the basic protein α-chymotrypsinogen A with no retention form the Columns A1 and A2 is an indication of the low phosphorylation level of the mannan preparation used in this study.

The immobilization of 2α-mannobiose (Column A3) instead of mannan was tried to evaluate its level of affinity toward mannose-binding lectins. Column A3 showed small EOF (0.57 mm/s) using 5 mM EDA/HCl, pH 7.0, at −25 kV. Column A3 also showed weak affinity toward Con A and LCA as they were slightly retained on the column and eluted off the column with the binding mobile phase.

3.1.2.2. Cationic monoliths. In this part of the investigation, the major aim was to produce monoliths having relatively strong EOF and affinity towards MBPs and little or no non-specific interaction. This calls to introduce fixed positive charges into the monolith backbone (i.e. cationic monoliths) via a positively charged monomer during the polymerization step and a positively charged spacer arm that will attach mannan to the monolithic surface during the immobilization step. Cationic monolithic columns (Column B) were based on the same monomers as the type A columns but incorporating 2% of the monomer MAETA.

The immobilization of mannan onto the cationic monolith yielded an affinity column (Column B1) that showed relatively strong affinity towards Con A and LCA in the nano-LC mode but showed no EOF, a fact that did not allow the use of the column in the affinity CEC mode. Although Column B1 has a cationic monolith backbone, it is believed that the immobilization of mannan prohibits the production of EOF due to its shielding effect of the positive charges of the monolith and/or to the presence of small amount of phosphate groups in mannan, which can neutralize the positive charges in the monolith backbone. The immobilization of mannan via triethylenetetramine as the spacer arm yielded Column B2. This column showed affinity toward the lectins in the nano-LC mode and the CEC mode. This column has an EOF of 2.1 mm/s. Fig. 2 is a typical example of affinity CEC showing the basic three-step operations. After injecting Con A for 12 s into Column B2 equilibrated with the

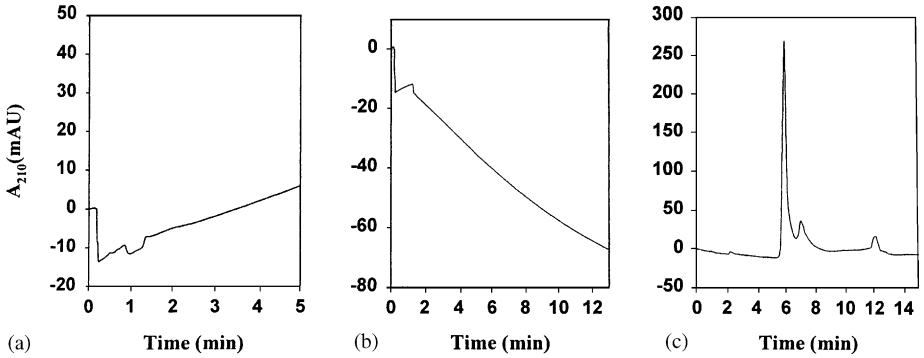

Fig. 2. Typical example of affinity CEC showing the basic three-step operation: (a) first step, injection of Con A for 12 s at 10 bar into the column equilibrated with the binding mobile phase followed by washing for 5 min with the binding mobile phase at 10 bar by nano-LC; (b) second step, washing the column by nano-LC at 10 bar for 12 min using a mobile phase of lower ionic strength; (c) third step, CEC with eluting mobile phase. Column B2, 25 cm effective length, 33.5 cm total length, 100 μm i.d. Binding mobile phase: 10 mM EDA/HCl, pH 7.0, containing 100 mM NaCl, 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$; washing mobile phase, 5 mM EDA/HCl, pH 7.0; CEC eluting mobile phase: 100 mM methyl-α-D-mannopyranoside in the washing mobile phase. Running voltage: −25 kV.

binding mobile phase at 10 bar (nano-LC step), the column is then washed with 5 mM EDA/HCl, pH 7.0, at 10 bar (a second nano-LC step) in order to prepare the column for elution by CEC with a mobile phase containing 100 mM methyl-α-D-mannopyranoside in the washing buffer (i.e. the elution mobile phase). This figure shows the unique potential of affinity CEC in the sense that the affinity-sorbed band can be separated into its components as the band is desorbed from the stationary phase with the haptenic sugar during the CEC step. In fact, Con A has been reported to exist as monomer, dimer and tetramer of respective M_r of 27 000, 55 000 and 110 000 [47].

The immobilization of periodated mannan through the TETA spacer arm by reductive amination onto the cationic monolith yielded Column B3. This column showed affinity toward Con A and LCA in the nano-LC mode, but produced no EOF in the CEC mode. Fig. 3 shows a nano-LC chromatogram of LCA and three other proteins namely conalbumin, myoglobin and α-chymotrypsinogen A. While the three proteins exhibited no affinity to the mannan column and eluted in the void volume with the binding mobile phase, Con A was eluted stepwise with the haptenic sugar.

Acid hydrolyzed mannan immobilized onto the cationic monolithic column through TETA and cross-linked via BDDGE yielded Column B4. This column showed a strong EOF of 3.1 mm/s and a relatively strong affinity toward Con A and LCA in both nano-LC and CEC modes. Fig. 4 shows a typical electrochromatogram for Con A obtained by affinity CEC on Column B4. Since the amount of injected Con A is much less in Fig. 3 than that in Fig. 2, only the major peak of Con A was observed in Fig. 3 and the two minor forms (seen in Fig. 2) could not be detected.

Under the mobile phase conditions, the affinity monoliths did not exhibit non-specific interactions (i.e. electrostatic attractions) as was manifested by the elution of conalbumin, myoglobin and α-chymotrypsinogen A in the column void volume with the binding mobile phase. However, more acidic proteins such as β-lactoglobulin B (pI = 5.1) exhibited relatively small retention factor ($k' \sim 1.1$) in nano-LC on the monolith. This non-specific interaction was totally eliminated and the β-lactoglobulin B eluted in the column void volume with a mobile phase of 0.20 M NaCl. At any rate, the interfering electrostatic interactions are not a major issue since the column is usually washed extensively with the loading mobile phase thus allowing interfering sample components to elute from the column before the stepwise elution step aiming at eluting the affinity retained species.

In summary, while affinity nano-LC does not allow the separation of the components of the retained band (i.e. components having the same affinity to the immobilized ligand) upon elution with the hapten sugar, CEC does allow the

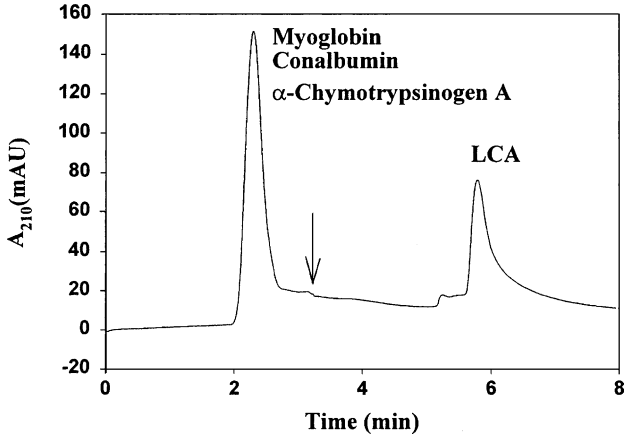

Fig. 3. Typical chromatogram of LCA and three other proteins having no affinity to immobilized mannan. Column B3: 25 cm effective length, 33.5 cm total length, 100 μm i.d. Injection for 12 s at 10 bar into the column equilibrated with the binding mobile phase followed by first washing the column with the binding mobile phase for 3 min and then a stepwise elution at 3 min with eluting mobile phase (indicated by an arrow) using constant inlet pressure of 10 bar. Binding mobile phase: 10 mM EDA/HCl, pH 7.0, containing 100 mM NaCl, 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$; eluting mobile phase: 100 mM methyl-α-D-mannopyranoside in the binding mobile phase.

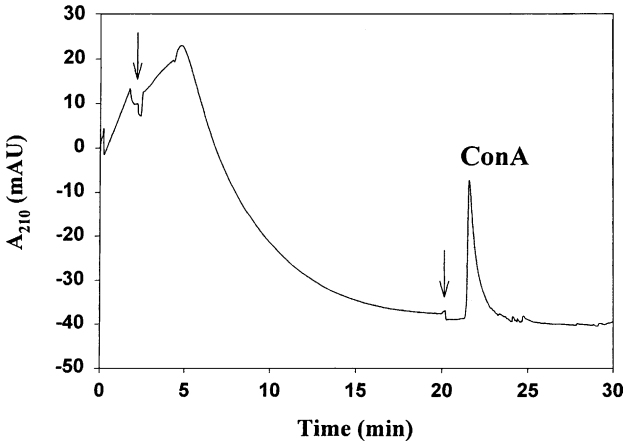

Fig. 4. Typical example of affinity CEC showing continuous recording of the basic three-step operation as in Fig. 2 with the difference in the duration of the first and second step. Column B4: 25 cm effective length, 33.5 cm total length, 100 μm i.d. Two arrows at 2.5 and 20 min indicate the stepwise change to the washing and eluting mobile phase. Binding mobile phase: 10 mM EDA/HCl, pH 7.0, containing 100 mM NaCl, 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$; washing mobile phase, 5 mM EDA/HCl, pH 7.0; CEC eluting mobile phase: 100 mM methyl-α-D-mannopyranoside in the washing mobile phase. Running voltage: −25 kV.

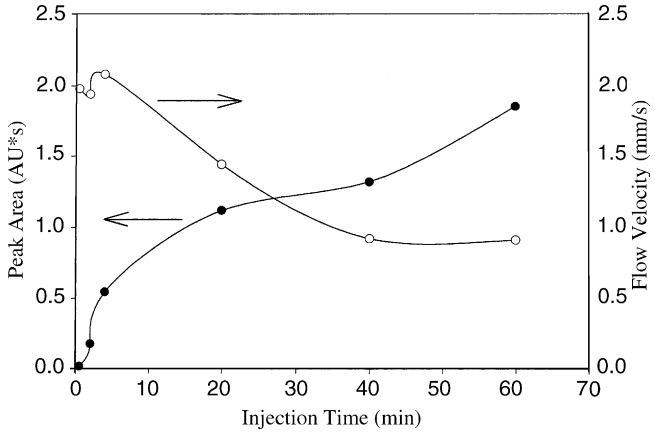

Fig. 5. Plot of peak area of the MBP vs. the injection time of rabbit serum in nano-LC at constant pressure of 10 bar. Column B2: 25 cm effective length, 33.5 cm total length, 100 μm i.d. Binding mobile phase: 10 mM EDA/HCl, pH 7.0, containing 100 mM NaCl, 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$; eluting mobile phase: 100 mM methyl-α-D-mannopyranoside in the binding mobile phase.

components of the retained band to be separated upon elution by virtue of different interactions of the components with the applied electric field (see Fig. 2). As expected, another advantage of CEC over nano-LC is the sharper band. However, CEC requires washing the affinity column by pressure-driven flow from the high ionic strength binding mobile phase before applying the eluting mobile phase of lower ionic strength to avoid excessive current and subsequently Joule heating (refers to conditions in Figs. 2 and 3). This may lead to some non-specific interactions of the retained analyte with the affinity column at low ionic strength.

3.2. Application to the analysis of mannan/mannose-binding protein in rabbit serum

Mannan/mannose-binding protein is present at relatively low levels in serum [29]. Affinity chromatography is a convenient approach to selectively concentrate MBP and consequently detect and identify this protein especially when the exact affinity ligand (e.g. mannan) is immobilized on the surface of a suitable support. Under this condition, the analyte (i.e. MBP) should exhibit very strong binding to the affinity column thus allowing the accumulation of sufficient amount of that analyte from a large sample volume of serum without overloading the column and causing band broadening. Fig. 5 shows the dependence of the peak area of MBP on the injection time of rabbit serum. In this set of experiments, the serum was directly injected into the column after simply adding the components of the binding mobile phase and filtering it through a micro-filter and without other sample cleanup steps. As can be seen the peak area of the eluted MBP is a non-linear function of the injection time due primarily to the variation in the flow velocity since the injection and elution were carried out at constant pressure of 10 bar. The column used in this study is of the B2-type, which should allow a flow velocity of 2.1 mm/s as shown in Table 2. This flow velocity is more or less conserved up to 4 min injection time, which indicates that the viscosity of the serum is very close to that of the binding mobile phase. Thereafter, the flow velocity decreases gradually as the injection time is prolonged to 20 min and greater. The drop in flow velocity at injection time of 20 min and greater is most likely due to partial plugging of the column inlet by small particulate material which could not be removed by micro-filtering of the serum. An injection of 0.5, 2 and 4 min has resulted in introducing ∼0.24, 0.93 and 2 column volumes, respectively, at the observed flow velocities. An injection time of 20 min corresponds to introducing ∼6.9 column volumes while 40 and 60 min lead to introducing ∼8.8 and ∼13.0 column volumes, respectively, at the observed flow velocities.

Fig. 6 shows a nano-preparative injection for 40 min into Column B2 previously equilibrated with the binding mobile phase. The 40 min injection time corresponds to introducing ∼8.8 column volumes or ∼13 μl of rabbit serum into the column. Thereafter, the column was first washed with the binding mobile phase for 15 min until absorbance decreased substantially and reached a value very close to the original baseline reading, and then the column was eluted at 65 min with eluting mobile phase containing 100 mM methyl-α-D mannopyranoside. As can be seen in Fig. 6, the peak that eluted with the eluting mobile phase emerged at ∼80 min. Considering that the rabbit serum has been found to contain 0.020 mg/ml [29,36], the introduction of 13 μl of serum corresponds to offering the column 260 ng of MBP.

The eluted peak, which was collected in a micro-vial, was subjected to SDS–PAGE showing a faint band at M_r ∼

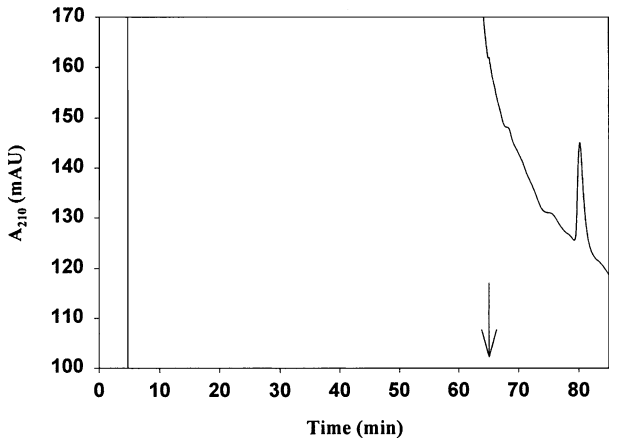

Fig. 6. Nano-preparative LC of MBP from rabbit serum. Column B2: 25 cm effective length, 33.5 cm total length, 100 μm i.d. Binding mobile phase: 10 mM EDA/HCl, pH 7.0, containing 100 mM NaCl, 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$; eluting mobile phase: 100 mM methyl-α-D-mannopyranoside in the binding mobile phase. Arrow indicates the change to eluting mobile phase at 65 min.

30 000. This protein band was excised from gel, diced, and digested with trypsin (see Section 2). The protein digest was analyzed by MALDI-MS, which showed 20 peptide fragments the total molecular mass of which was 33 600. The MBP from rabbit is not yet in the database, a fact that did not allow the confirmation of the nature of the isolated protein. Due to the fact that the protein was captured by mannan, eluted with mannose-rich mobile phase and had a molecular mass very close to what is reported in the literature for MBP [29,48,49], the protein is tentatively identified as the rabbit serum MBP.

4. Conclusions

This investigation has shown that monolithic capillary columns with immobilized mannan are viable stationary phases for performing the selective isolation and concentration of mannose/mannan binding proteins in dilute samples by affinity nano-LC and affinity CEC. The concentrating capability of affinity monoliths generates nanogram quantities of pure proteins, which are sufficient for achieving "nano-proteomics".

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