Published in final edited form as:
Anal Chem. 2009 May 15; 81(10): 3776–3783. doi:10.1021/ac900085k.

Enrichment of Glycoproteins using Nano-scale Chelating Con A Monolithic Capillary Chromatography

Shun Feng[1], **Na Yang**[1], **Subramaniam Pennathur**[3], **Steve Goodison**[4], and **David M. Lubman**[1,2,5,*]

[1] Department of Surgery, The University of Michigan Medical Center, Ann Arbor, MI 48109

[2] Department of Chemistry, The University of Michigan, Ann Arbor, MI 48109

[3] Department of Internal Medicine, Division of Nephrology, The University of Michigan, Ann Arbor, MI 48109

[4] Department of Surgery, The University of Florida, Jacksonville, FL 32009

[5] Comprehensive Cancer Center, The University of Michigan Medical Center, Ann Arbor, MI 48109

Abstract

Immobilized lectin chromatography can be employed for glycoprotein enrichment, but commonly used columns have limitations of yield and resolution. In order to improve efficiency and to make the technique applicable to minimal sample material, we have developed a nano-scale chelating Concanavalin A (Con A) monolithic capillary prepared using GMA-EDMA (glycidyl methacrylate–co-ethylene dimethacrylate) as polymeric support. Con A was immobilized on Cu(II)-charged iminodiacetic acid (IDA) regenerable sorbents by forming a IDA:Cu(II):Con A sandwich affinity structure that has high column capacity as well as stability. When compared with conventional Con A lectin chromatography, the monolithic capillary enabled the better reproducible detection of over double the number of unique N-glycoproteins in human urine samples. Utility for analysis of minimal biological samples was confirmed by the successful elucidation of glycoprotein profiles in mouse urine samples at the microliter scale. The improved efficiency of the nano-scale monolithic capillary will impact the analysis of glycoproteins in complex biological samples, especially where only limited material may be available.

Keywords

Nano-scale; glycoprotein enrichment; monolithic capillary; Con A; urine

Introduction

Glycosylation of proteins is one of the most ubiquitous post-translational modifications observed in eukaryotic organisms. On average, approximately half of the mammalian proteome is composed of glycoproteins.[1] Furthermore, glycoproteins are implicated in a multitude of cellular and biological functions, including immune defense, cell growth and differentiation and cell adhesion.[2] Aberrant glycosylation has been recognized to be associated with several disease states such as cancer, inflammatory diseases, and congenital disorders, *etc*.[3-6] Given the major roles glycoproteins play in cellular functions, numerous efforts have been focused on improving the efficiency of glycoprotein analysis. Although the glycoprotein abundance in

* To whom correspondence should be addressed. Phone: +1-734-647-8834, FAX: +1-734-615-2088, E-mail: dmlubman@umich.edu.

organisms is relatively high, it is difficult to analyze due to the presence of other high abundant proteins in complex biological samples. Consequently, enrichment of glycosylated proteins is generally required before analysis by RPLC-MS/MS.

Analytical tools developed for analysis of glycoproteins have been based on different mechanisms, including chemical reaction[7, 8], gel-binding[9-11], and lectin affinity chromatography[12-14]. Lectins, known as proteins with highly specific binding affinities for different sugar residues, have been used as molecular probes for the characterization of carbohydrates on the cell surface of cultured cells, or in tissues after embedding and sectioning the organ of interest.[15] The most widely used lectins are concanavalin A (Con A) and wheat germ agglutinin (WGA). They can be immobilized on different supports including agarose, polymers and silica. Polymeric supports are considered as an important alternative for silica supports by providing high protein recovery and a stable surface with low intrinsic activity. [16] Importantly, lectin binding does not interfere with the specific interaction of glycoprotein glycan structures, and so the bound proteins maintain their biological properties.[17-19] There are many formats for lectin affinity chromatography including tubes, packed columns[16], micro-chips[20] and membranes[21].

Monolithic columns have many advantages: low-pressure drop across the column, fast mass transfer kinetics and a high binding capacity. There have been numerous successful applications in proteomic analysis; C18 capillary column for highly efficient separation of peptide mixtures; immobilized enzyme microreactor for fast digestion of protein mixtures; and immobilized metal affinity chromatography (IMAC) for protein enrichment.[22-29] The most important feature of monolith columns is that they can be prepared in different volumes or desired sizes. Poly(glycidyl methacrylate–co- ethylene dimethacrylate) (GMA-EDMA) monoliths have been widely used because of their high bio-compatibility and low non-specific adsorption.[30]

In this work, a nano-scale Con A-chelating monolithic capillary is introduced to enrich glycoproteins from complex biological samples available only in minimal amounts. Con A was immobilized on a Cu (II)-charged iminodiacetic acid (IDA) regenerable GMA-EDMA monolith with a special sandwich structure. The versatility of capillary monoliths for analysis of glycoproteins from small amounts of sample was demonstrated by successful analysis of as little as 20 μL of biological fluid sample. When the monolithic capillary was used to enrich glycoproteins from human urine specimens it was shown that many more unique glycoproteins were detected than was possible using standard lectin affinity columns. Furthermore, this capillary was used to successfully capture glycoproteins in mouse urine samples, confirming its application potential for minimal sample material.

Experimental Section

Chemicals and Materials

Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). Glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA), cyclohexanol, 1-dodecanol and sodiumcyanoboro hydride were obtained from Aldrich (Milwaukee, WI, USA). Azobisisobutyronitrile (AIBN), BSA, trypsin γ-methacryloxypropyl trimethoxysilane (γ-MAPS), glutaraldehyde solution, IDA, sodium azide, iodoacetamide, and dithiothreitol (DTT) were from Sigma (St. Louis, MO, USA). Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Milford, MA. USA). All other chemicals and solvents were of analytical or HPLC grade.

Preparation of IDA-GMA-EDMA monolithic capillary

TremeSimple Nano-LC Pump system (Micro-Tech Scientific, Vista, CA) was used. The preparation of the monolith involved two steps. The first step was to prepare a monolithic capillary[31]. The capillary with inner diameter of 200 μm was filled with the polymerization reaction solution containing GMA, EDMA, cyclohexanol, dodecyl alcohol and AIBN and the polymerization was allowed to proceed at 50°C in a water bath for 12 h, then washed with methanol and water, respectively. The second step was to derivatize IDA onto the surface of the monolithic rod. 50 mM of IDA solution (pH 11, adjusted by NaOH) was pumped at a flow rate of 0.010 μL/min through the column, for 12 h at 70°C. Finally it was cut into 10 cm length with a volume of about 2.36 μL.

Urine collection

Human Urine samples were collected from the University of Florida urology clinic at Shands & UF hospital, Jacksonville, FL. Samples were collected from patients cystoscopically confirmed to have bladder cancer and asymptomatic volunteers according to University of Florida IRB informed consent protocols. Each sample consisted of 15-50 mL of midstream urine collected in a sterile cup. All specimens were processed in the same way, and as previously described.[32] For mouse urine samples, male $LDLr^{-/-}$ mice backcrossed onto a C57Bl/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). They were housed in a temperature-controlled facility with a 12:12 hour of light-dark cycle. The mice urine were collected and immediately frozen to -80°C until analysis.

Sample preparation

For human urine, cells and debris were removed from urine samples by centrifugation at 500 *g* and 5000 *g* for 5 min and 10 min respectively at 4 C. The supernatant was subjected to total protein precipitation by adding four times the sample volumes of cold acetone (-20°C) or stored at -80°C until analysis. The sample was left at -20°C for 1 h followed by centrifugation at 12000 *g* for 15 min at 4°C. The supernatant was removed, and the centrifuge tube was left open in the fume hood to remove the remaining solvent. The pellet was resuspended in binding buffer (20 mM Tris, 0.15 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.4). The sample was vortexed vigorously to completely dissolve the pellet. The protein concentration in the sample was determined by the Bradford protein assay (Bio-Rad, Hercules, CA) For mouse urine, 20 μL of sample were lyophilized to dryness directly, and redissolved in 20 μL binding buffer.

Con A lectin affinity chromatography

A 200 μL resin slurry of agarose-bound concanavalin A (Con A) (Vector Laboratories, Burlingame, CA) was packed into a disposable snap cap spin column (Thermo Scientific, Rockford, IL). Storage buffer was removed by centrifugation at 500 *g* for 30 sec. The resin was first washed three times by binding buffer. About 10 μg diluted protein sample (1:4) was then added to the column and incubated for 30 min. The flow-through was discarded using centrifugation at 500 *g* for 30 sec. The nonspecific binding was removed by washing resin four times with 400 μL of binding buffer. 200 μL of elution buffer (20 mM Tris, 0.5 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, 0.4 M methyl-α-D-mannopyroside pH 7.0) was added and mixed for 10 min. Eluate was collected after centrifugation and the elution step was repeated once. The pooled eluate was denatured with 100mM DTT, and then digested using trypsin. Finally it was lyophilized to dryness and redissolved in 65 μL of 0.1% acetic acid.

Glycoprotein enrichment using nano-scale Con A-chelating monolithic capillary

A TremeSimple Nano-LC Pump system with a 20 μL sample loop was used. The monolithic capillary was directly connected to the pump, the binding buffer was used as mobile phase, and flow rate was set as 1 μL/min. The whole procedure of enrichment is illustrated in figure

1. (1) 20 μL of 50 mM EDTA was used to remove metal ions in IDA-monolith; (2) 20 μL of 50 mM $CuSO_4$ solution was eluted through the monolithic capillary, Cu^{2+} was chelated with IDA; (3) 20 μL of 1 μg/μL diluted Con A solution was pumped through, where Con A was immobilized on the monolithic capillary through Cu^{2+} ion where the sandwich structure was constituted; (4) 20 μL of 10 μg diluted urine protein mouse urine or 20 μL of 1 μg/μL diluted ovalbumin solution was injected and pushed through the capillary, and an additional 10 min was needed to thoroughly wash out unbound proteins; (5) here two methods were conducted to elute the captured glycoproteins: method 1, 20 μL of ammoniated water (pH 10.3); method 2, 20 μL of elution buffer: 25 mM Tris, 0.5 M NaCl, 0.5 M methyl-α-mannopyranoside, was used to elute the captured glycoproteins for 20 min. The pooled eluate was denatured with DTT and then digested using trypsin. Finally it was lyophilized to dryness and redissolved in 65 μL of 0.1% acetic acid.

Nano-RPLC-ESI MS Instruments

A Paradigm MG4 micropump (Michrom Biosciences Inc., Auburn, CA) was used to deliver the mobile phase. The pump flow rate was split by a cross to achieve a column flow rate of about 300 nL/min. The separation column (0.1mm×150mm, C18 AQ particles, 5 μm, 120 Å) was from Michrom BioResources (Auburn, CA, USA). The resolved peptides were analyzed on a linear ion trap mass spectrometer with a nano-LC-ESI source (LTQ, Thermo Finnigan, San Jose, CA). A mobile phase system of two solvents was used, where solvents A and B were composed of 0.1% formic acid and 2% acetonitrile or water in HPLC-grade water and acetonitrile, respectively. A 60 min acetonitrile/water gradient method was used, starting with 5% acetonitrile which was ramped to 40% in 55 min and to 95% in another 5min. The LTQ instrument was operated in positive ion mode. The capillary transfer tube was set at 200 °C, the ESI spray voltage at 2.5 kV, and the capillary voltage at 30 V. Ion activation was achieved by utilizing helium at a normalized collision energy of 35%. The data acquisition and generation of peak list files were automatically done by Xcaliber software. For each cycle of one full mass scan (range of m/z 400-2000), the three most intense ions in the spectrum were selected for tandem MS analysis, unless they appeared in the dynamic or mass exclusion lists.

Database Searching and Manual Validation

All MS/MS spectra were analyzed by TurboSequest of Bioworks software, version 3.1 SR1 (Thermo Finnigan). Peptide fragment lists were generated and submitted to Swiss-Prot database searching. The search parameters were as follows: (1) protein database, Uniprot_Sprot_Human_121307.fasta downloaded from NCBI and non-redundant mouse protein database from the Mouse International Protein Index (ipi_mouse.v3.36.fasta); (2) allowing two missed cleavages; (3) possible modifications, oxidation of M; (4) peptide ion mass tolerance 1.50 Da; (5) fragment ion mass tolerance 0.0 Da; (6) peptide charges +1, +2, and +3. All SEQUEST search parameters and data filtering were the same in all digest fractions, except the modification of asparagine was allowed by +1 Da in the tryptic digest fractions. The +1 Da was allowed owing to hydrolysis of the amide of the asparagine side chain to release the asparagine-linked oligosaccharides from glycopeptides. To further validate data obtained from SEQUEST, Trans-Proteomic Pipeline (TPP) software was used[33] where the threshold was set at 0.9 for protein probability, only those that passed threshold and contained NXS/T motif in their peptide sequences were sorted as glycoproteins.

Results and discussion

The GMA-EDMA monolithic polymer has a neutral hydrophilic surface and its non-specific adsorption of biomolecules is very weak. The surface of GMA-EDMA beads possess chemically active sites, i.e. epoxide groups, for chemical modification, which makes it easy to derivatize other functional groups. The GMA-EDMA monolithic polymer has been applied in

affinity chromatography and enzyme reactors in various formats.[30] Luo *etc al* prepared GMA-EDMA monoliths in a HPLC column with Cu^{2+} ions being immobilized through iminodiacetic acid (IDA) for the purification of lysozyme from egg white.[34] Anspach and Altmann-Haase investigated the stability of the IDA:Cu(II):Con A sandwich structure, and demonstrated that such structures behave very stably. With the sandwich affinity sorbent of Con A, twice the capacity for ovalbumin was obtained compared to covalently bound Con A.[35] These reports suggested that Con A-Cu-IDA-GMA-EDMA could become a promising candidate for glycoprotein enrichment.

The current work is based on the use of the IMAC mechanism to prepare the nano-scale Con A-chelating monolithic capillary and applying it to glycoprotein capture. Con A was immobilized on the surface of GMA-EDMA monolith utilizing a chelating bond instead of covalent bond in the conventional immobilized lectin method, by which not only the number of active sites for glycoproteins may double,[35] but also the eluted fractions can be managed by change of pH value. These factors may result in the improved performance of the Con A-chelating monolithic capillaries compared to conventional lectin chromatography. Another disadvantage of conventional ConA purification has been the use of high-concentration sugar-based buffers (generally containing 0.5 M α-D-mannopyranoside), which interfere with the performance of downstream separation because the sugars are difficult to remove from C18 RP separation systems. There are existing standard protocols for removing α-D-mannopyranoside including trichloroacetic acid precipitation[36] or acetone precipitation[37], repeated buffer exchange by ultracentrifugation[38] and dialysis of eluted fraction with Milli Q water[12]. Nevertheless, these methods are time-consuming and they involve either multi-step or extensive wash cycles, which lead to loss of glycoprotein yield and potential sample contamination. Thus, they are not applicable where minute quantities of sample are available.

Other advantages of the monolithic column using our methods are that the captured glycoprotein together with Con A can be co-eluted using ammoniated water instead of the sugar-based elution buffer. This allows the improved recovery of captured glycoproteins and provides a rapid and efficient alternative to conventional Con A affinity chromatography. An additional advantage of Con A-chelating monolithic capillary is the stability. The capillary can be stable for its consecutive use for ten times without losing capacity in our experiments. In both monoliths and standard lectin columns frequently biologically complex samples result in blocking or clogging of the columns during the experiment. However, the Con A capillary column can be continued to be used by cutting the capillary by approximately 2 mm at the end, whereas in the conventional Con A column it may not be reusable. Additionally, the GMA-EDMA monolithic capillary has a special bimodal pore sketch structure, relatively large specific surface areas, and fast mass transfer kinetics, all of which also contribute to improved capture of glycoproteins by the immobilized Con A.

One standard glycoprotein ovalbumin was used to evaluate the performance of this Con A-monolithic capillary. As described in the experimental section, the collected fractions were denatured, digested, and then analyzed using LTQ MS. Four experiments were performed and no Con A was identified in 3 of the samples. Only in 1 experiment were 4 unique peptides derived from Con A with a coverage of 13.1% identified in the collected fraction of the loading step (Supplementary File: Loading_Step_Protein.xls). These results supported Anspach and Altmann-Haase's conclusion that the IDA:Cu(II):Con A sandwich structure is very stable.[35]

The two elution buffers, ammonium vs. conventional sugar-based buffers, were used to elute the captured ovalbumin for comparison. The collected fractions in the elution step were also denatured, digested and then analyzed using the LTQ MS. After data analysis using SEQUEST and TPP for filtering the data, the average protein coverage using sugar-based buffers is greater than that using ammonium (32.1% vs 27.1%, Supplementary File:

Elution_Fraction_Protein.xls). Although only 20 μL elution buffer was used, there was still white precipitate observed in the bottom of the tube due to the high concentration of α-D-mannopyranoside in the elution buffer. In our experience, there is a layer deposited on the surface of the skimmer of the LTQ after using sugar-based buffers even for a short time, which can block the skimmer. However, this problem can be avoided when using ammonium as elution buffer.

To further evaluate the capability of this Con A-monolithic capillary, analysis of urine samples from normal and bladder cancer patients were performed by both standard Con A lectin affinity chromatography and nano-scale Con A-chelating monolithic capillary. With 10 μg initial protein, the collected fraction was lyophilized, denatured, and digested using trypsin and analyzed by LTQ-MS/MS. Figure 2 is the typical base peak chromatogram of processed urine samples. From the spectra, it can be seen that there appear more peaks in figure 2-B and 2-D for both normal and bladder cancer samples using the nanoscale monoliths compared to figure 2-A and 2-C where the conventional Con A lectin approach was used.

Furthermore, the impact of co-eluted ConA on the elution system was investigated in this study. The raw files from LTQ were searched against one combined database of Con A and human, and an average protein sequence coverage of Con A was obtained (13.1%, Supplementary File: Elution_Fraction_Protein.xls). The greatest number of unique peptides detected for Con A is 5, and the spectral counting number of Con A is 50. In a 55-min gradient elution, there are approximately 15000 scans in total. By comparison, the effect of Con A on the whole experiment is negligible.

Using the nano-scale Con A-chelating monolithic capillary, an average of 36 N-linked glycoproteins out of 45 total proteins were identified in the normal sample and 37 N-linked glycoproteins out of 52 proteins were identified in the bladder cancer sample. In comparison, an average of only 13 N-linked glycoproteins out of 16 proteins in the normal sample, and only 10 N-linked glycoproteins out of 13 proteins in the bladder cancer sample were identified using conventional Con A lectin affinity chromatography. The number of identified glyproteins is listed in Table 1 and the identified glyprotein IDs are listed in Supplementary Tables 1-4. Starting with the same limited amount of protein, the nano-scale monolithic capillary demonstrated a higher capability for enriching glycoproteins than conventional lectin columns. Further, from the analysis of the percentage of the common identified proteins in both methods, nano-scale monolithic capillary revealed its superiority over conventional Con A lectin column. The percentage of the common identified proteins of replicate normal urine samples was 56.52%, and 60.87% for replicate cancer samples (Table 1). When using the conventional Con A lectin method, the reproducibility was 52.79% for the normal replicate samples and only 25.00% for the cancer replicate samples (Table 1), where the raw data produced by TPP are attached in the Supplementary Files: Human_Urine_Beads.xls and Human_Urine_Monolith.xls.

Figure 3 shows representative MS/MS spectra of two peptide sequences from α-1-microglobulin (AMBP), one of the most frequent positively identified proteins[31] and it was identified in all analyses of urine samples by the nano-scale Con A-chelating monolithic capillary. However, it was identified only once out of four analyses using conventional affinity Con A lectin chromatography with 10 μg initial protein (Supplementary Table 1-4). From the MS/MS spectra, it can be evidently seen that b-ion and y-ion peaks are consistent with theoretically predicted peaks. Also, there are in total 13 out of 14, and 19 out of 22 ion peaks detected for two peptides respectively, which were both assigned to glycoprotein AMBP with high confidence.

In a previous study on urinary proteomic profiling by our group, Con A affinity chromatography coupled to nano-flow liquid chromatography was utilized to profile the N-linked glycoprotein component in naturally micturated human urine specimens.[31] The initial protein amount in this study was more than 100 μg, where an average of 26 N-linked glycoproteins out of 35 proteins was identified in each run. The average number of identified proteins was less than that detected using nano-scale Con A-chelating monolithic capillaries in this study, even though the initial amount of protein was 10 times greater, thus indicating the potential advantage of monolithic capillaries. To test the monolithic capillary on real, minimal samples, the method was employed to profile glycoproteins in urine samples collected from 3 mice. The number of glycoproteins identified from these 3 samples were 11, 7, and 7 respectively and a total of 18 unique glycoproteins were identified (Table 2, The result of TPP listed in Supplementary Files, Mouse_Urine.xls). This is the first report to describe the application of nano-scale monolithic capillary analysis to glycoprotein capture from mouse urine. This new technique will greatly enable efficient analysis of glycoproteins in complex, minimal biological samples.

Conclusion

A nano-scale Con A-chelating monolithic capillary was prepared and applied to glycoprotein enrichment based on an IMAC mechanism for biological samples available in small amounts. The capillary was used to enrich glycoproteins in human urine specimens. Compared with conventional Con A lectin chromatography, nano-scale Con A-chelating monolithic capillaries displayed much higher efficiency for enriching glycoproteins.

Automation in proteomic analysis is certainly a current trend. The most popular technique for high-throughput proteomic analysis is nano-RPLC-MS/MS where separation columns with 75 μm i.d. are typically used. Based on the capillary LC column capacity, the nano-scale Con A-chelating monolithic capillary is therefore preferable to conventional lectin columns when only limited sample is available, and it is also readily coupled with nano-RPLC-ESI MS. Furthermore, the alternative elution method simplifies the separation by eliminating the need to clean the column of sugar impurities. These improvements and advantages will enable the development of automated analyses.

In recent years, multi-lectin affinity chromatography adsorbents have been widely used to enrich glycoproteins from complex bio-samples[16, 37], and the results showed they are more efficient than using a single lectin. As one of the most widely used lectins, Con A was reasonably selected to be the first candidate for immobilization on the capillary. Con A was immobilized based on an IMAC mechanism as discussed herein. Theoretically, if one lectin contains functional groups that can chelate with Cu^{2+} ion, it can be immobilized on the capillary as well. Therefore we plan in the next step to immobilize multi-lectins on the monolithic capillary to evaluate its performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded under the National Institutes of Health Grant No. R01 49500(D.M.L.) and also received partial support under the National Cancer Institute under Grants R01CA100104(D.M.L.), R21CA124441(D.M.L.), R21CA134623 (D.M.L.), R01CA116161 (S. G.), and R21HL092237-02(S.P.).

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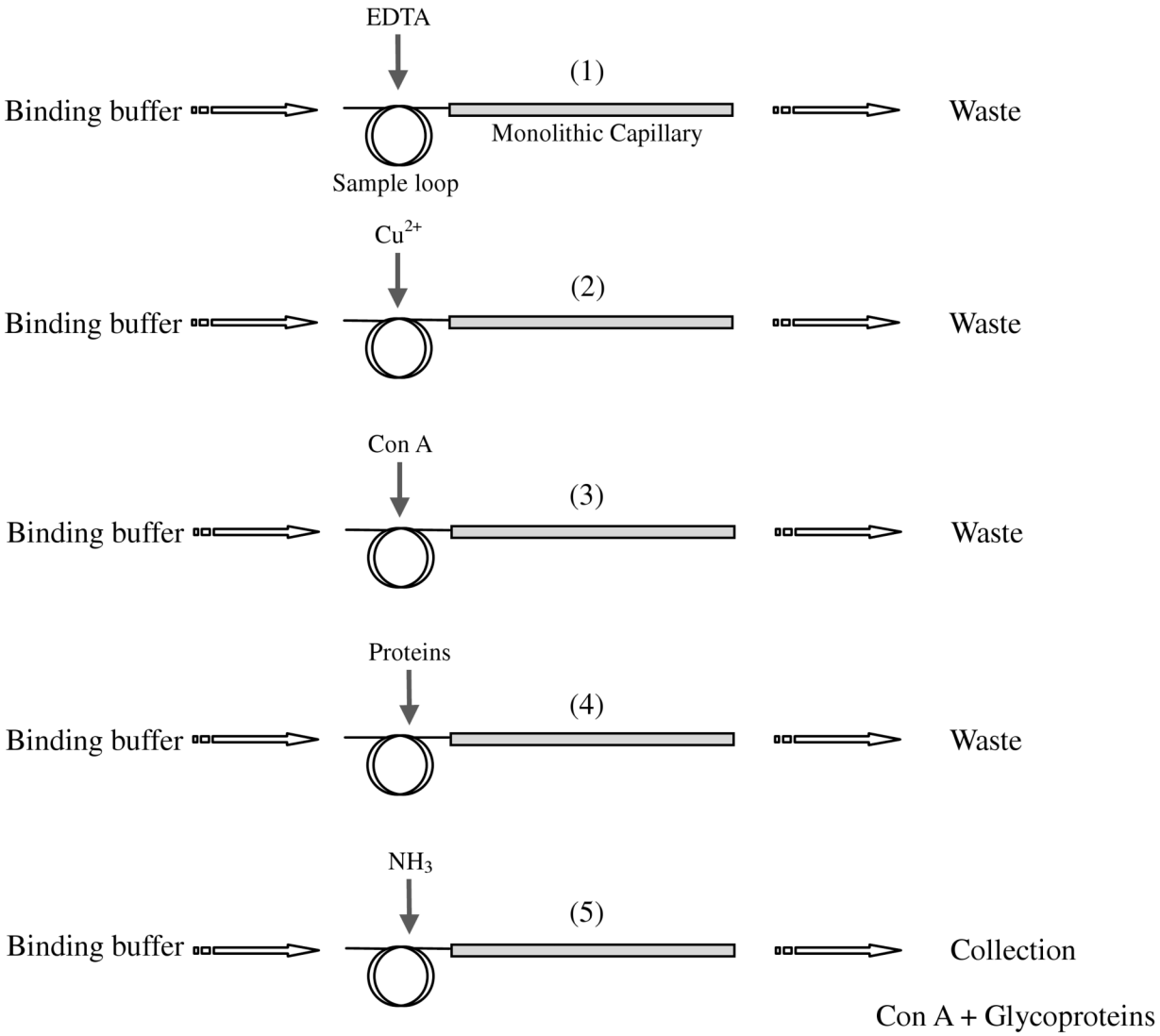

Figure 1.
Schematic diagram of the procedure of enrichment using nano-scale chelating Con A monolithic capillary. The formats in monolithic capillary are (1) IDA-monolith; (2) Cu^{2+}-IDA-monolith; (3) Con A-Cu-EDA-monolith; (4) Glycoproteins-Con A-Cu-IDA-monolith; (5) IDA-monolith.

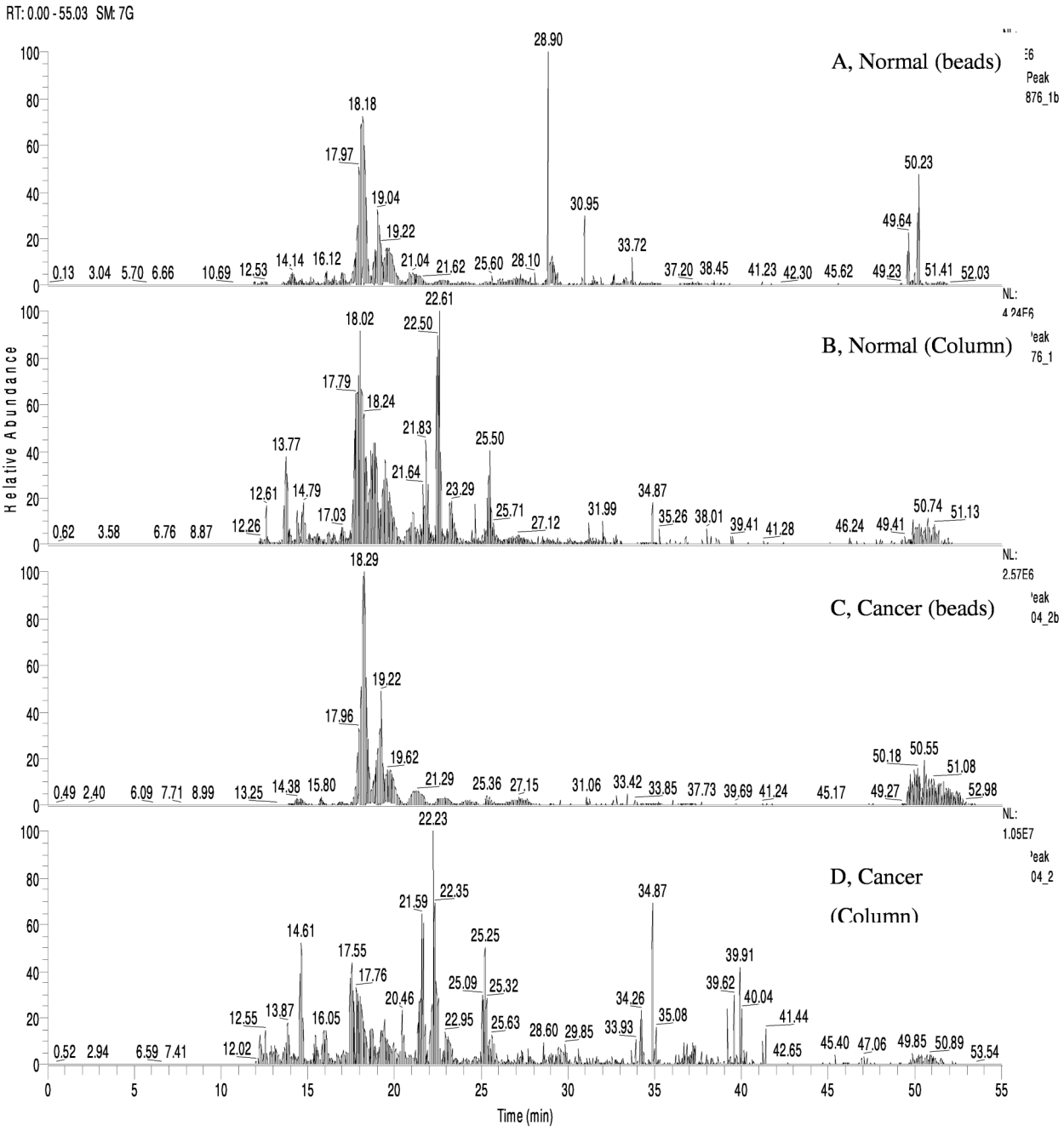

Figure 2.

Base peak chromatogram of the glycoprotein enrichment of urine specimen from bladder cancer patient, the initial protein amounts of sample are 10 μg. A and B, normal sample, enriching using Con A beads and monolith, respectively; C and D, bladder cancer sample, enriching using Con A beads and monolith, respectively.

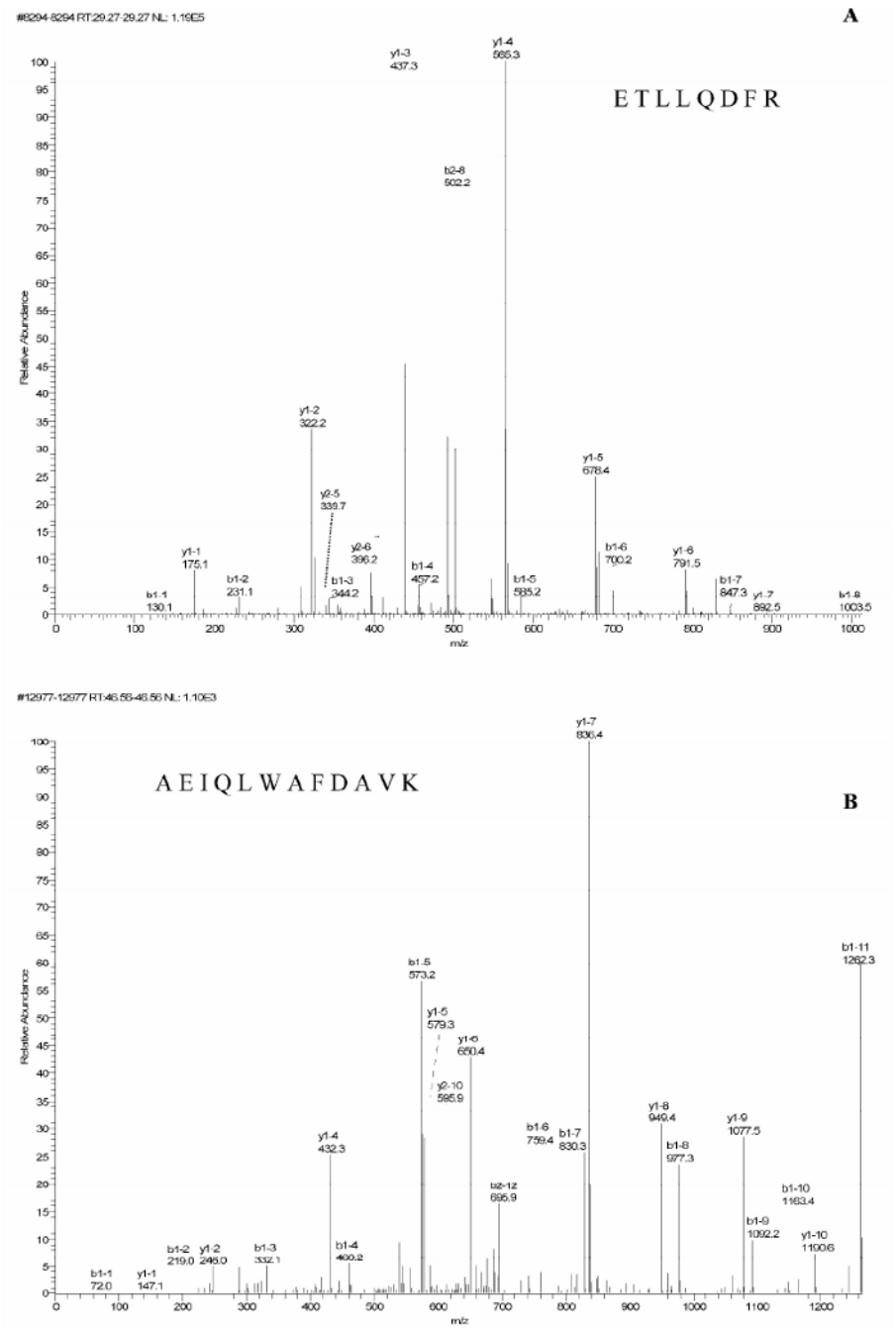

Figure 3.

MS/MS spectra of peptides from α-1-microglobulin (AMBP), identified from urinary samples by nano-scale Con A-chelating monolithic capillary. A, spectrum of peptide: ETLLQDFR; B, spectrum of peptide: AEIQLWAFDAVK

Table 1

The number of identified proteins and glycoproteins in experiments

Method	Sample	Total Number of Identified Glycoprotein	Number of Identified Glycoprotein/ Protein		Number of Common identified Glycoprotein	Reproducibility*
			Run 1	Run 2		
1	normal	46	35/45	38/45	28	56.52%
	cancer	46	33/43	41/61	26	60.87%
2	normal	17	16/19	11/14	9	52.94%
	cancer	16	8/10	12/15	4	25.00%

Method 1: Enrichment using nano-scale Con A-chelating monolithic capillary

Method 2: Enrichment using conventional Con A lectin affinity chromatography

Initial protein amount: 10 μg

*, percentage of the common glycoproteins over the total glycoproteins identified in two runs for each sample

Anal Chem. Author manuscript; available in PMC 2010 May 15.

Table 2

The total identified glycoproteins in mouse urine samples

Accession Number	Identified Glycoprotein Name
IPI00116945	ISOFORM 1 OF COMPLEMENT FACTOR D
IPI00128334	UROMODULIN
IPI00323869	KALLIKREIN
IPI00330913	MAJOR URINARY PROTEIN 26
IPI00112580	ISOFORM 2 OF UNCHARACTERIZED PROTEIN C6ORF182 HOMOLOG
IPI00129572	PROTOCADHERIN GAMMA SUBFAMILY C, 5
IPI00604958	KIF14 PROTEIN
IPI00129509	GLYCOSYLATION-DEPENDENT CELL ADHESION MOLECULE 1
IPI00120832	MAJOR URINARY PROTEIN 3
IPI00123351	NUCLEAR RECEPTOR COACTIVATOR 6
IPI00114065	COMPLEMENT COMPONENT 2
IPI00114252	PROSTAGLANDIN-H2 D-ISOMERASE
IPI00118130	ALPHA-1-ACID GLYCOPROTEIN 1
IPI00129250	LEUCINE-RICH ALPHA-2-GLYCOPROTEIN 1
IPI00132121	PRO-EPIDERMAL GROWTH FACTOR
IPI00314042	MEPRIN 1 ALPHA
IPI00323869	KALLIKREIN-1
IPI00378551	PEPTIDASE INHIBITOR R3HDML

The number of mouse urine samples: 3

The volume of each sample: 20 μL