Design and evaluation of synthetic silica-based monolithic materials in shrinkable tube for efficient protein extraction

Eman Alzahrani* and Kevin Welham*

Sample pretreatment is a required step in proteomics in order to remove interferences and preconcentrate the samples. Much research in recent years has focused on porous monolithic materials since they are highly permeable to liquid flow and show high mass transport compared with more common packed beds. These features are due to the micro-structure within the monolithic silica column which contains both macropores that reduce the back pressure, and mesopores that give good interaction with analytes. The aim of this work was to fabricate a continuous porous silica monolithic rod inside a heat shrinkable tube and to compare this with the same material whose surface has been modified with a C_{18} phase, in order to use them for preconcentration/extraction of proteins. The performance of the silica-based monolithic rod was evaluated using eight proteins; insulin, cytochrome C, lysozyme, myoglobin, β-lactoglobulin, ovalbumin, hemoglobin, and bovine serum albumin at a concentration of 60 μM. The results show that recovery of the proteins was achieved by both columns with variable yields; however, the C_{18} modified silica monolith gave higher recoveries (92.7 to 109.7%) than the non-modified silica monolith (25.5 to 97.9%). Both silica monoliths can be used with very low back pressure indicating a promising approach for future fabrication of the silica monolith inside a microfluidic device for the extraction of proteins from biological media.

1. Introduction

Proteomics deals with the large scale determination of gene and cellular function directly at the protein level. It is increasingly important in the development of new medicines and is becoming increasingly important as a tool for identifying proteins implicated in disease pathways. As the search for novel molecules to tackle diseases increases, the need to identify proteins as biological targets becomes more urgent.[1–3] The main steps in proteomics are sample preparation, extraction, digestion, separation, and detection. Efficient extraction of proteins is the most critical step for proteomics in order to remove the interfering materials such as salts, buffer, and detergents. In addition, analysis of proteins at low concentrations in complex matrices requires SPE techniques to preconcentrate the sample, and improve the detection sensitivity.[4]

The sorbent materials can be used for desalting, concentrating sample from dilute solution, and removing interferences by adsorbing the samples on the porous matrix with appropriate chemistry to effect preconcentration and then later release them using a stronger eluent.[5–7]

The recently invented monolithic materials are highly permeable to liquid flow and have high mass transport compared with the packed bed. Moreover, the monolithic stationary phase does not need frits, which can cause air bubbles to form and proteins to be adsorbed into the frits and remain trapped inside the capillary.[8] Based on the nature of their construction materials, monoliths can be divided into polymer- and silica-based monoliths.[9,10] The main advantages of the polymer-based monoliths are their excellent biocompatibility, the extended pH range above pH 8, and they can be washed with caustic mobile phase. However, it can be difficult to ensure the pores are large enough to reduce the back pressure and the mesopores are distributed over the desired size range. In addition, organic monolithic materials are not mechanically stable since they are affected by temperature and/or organic solvents causing shrinking or swelling, and this can affect the performance of the monolith. With polymer monoliths, the majoring of pores are micropores which results in low protein binding efficiency.[11,12] The porous inorganic monolithic materials can overcome these drawbacks since they are tolerant of organic solvents and they contain a distribution of both macropores that can reduce the backpressure, and mesopores that can increase the surface area giving a good interaction with analyte and maximising loadability of the column.[13,14]

The typical manufacturing of the silica-based monoliths is based on the sol–gel approach with phase separation, which can fabricate a uniform structure of monolith.[15] The first step in the preparation of the monolithic silica material is hydrolytic polycondensation carried out in a sol solution that consists of an

Analytical Chemistry Group, Department of Chemistry, University of Hull, Hull, HU6 7RX, UK. E-mail: em-s-z@hotmail.com; k.j.welham@hull.ac.uk

alkoxy silicon derivative in the presence of water-soluble polymer (such as polyethylene oxide) acting as porogen, and a catalyst that can be an acid catalyst (such as acetic acid or nitric acid)[16] or a base catalyst (such as N- methylimidazole or dimethylaminopyridine)[17] or a binary catalyst, acid and base in sequence.[18,19] The hydrolysis of the alkoxysilane precursor (or its alkyl/aryl derivative) produces the silanol groups. This is followed by water or alcohol condensation to produce polycondensed species containing siloxane linkages between two silane molecules, forming a three-dimensional network of sol–gel polymer.[20] This is followed by thermal decomposition of the monolith in the presence of urea or ammonium hydroxide to form mesopores. The surface of the silica monolith can be easily derivatised with many functional moieties leading to additional efficiency and selectivity.[21]

The synthetic silica-based monolithic materials have been introduced as porous monolithic separation media in high performance liquid chromatography (HPLC), gas chromatography, and capillary eletrochromatography (CEC).[18,22] In addition, they have been used as immobilized enzymatic reactors[23] and as sorbent in solid phase extraction.[24] The main drawback of the silica monolith is the shrinkage of the silica skeleton during the silica preparation. This problem has been minimised by treating the inner walls of the tube with 1 mol L^{-1} NaOH solution to attach the silica skeleton to the walls of the tube; however, this can cause a problem by forming large interstitial voids and the shrinkage cannot be completely avoided during fabrication of the silica monolith.[19,25]

The aim of this contribution is to investigate the fabrication of an inorganic silica-based monolith for preconcentration/extraction of proteins using a heat shrinkable tube. By using shrinkable tube, the effects of shrinkage can be avoided, moreover, it is relatively cheap to make and can be subsequently modified by chemically bonding with octadecyl ligands, to make a hydrophobic surface. The extraction recovery of the non-modified silica and the C_{18} stationary phase are then compared.

2. Materials and methods

2.1. Chemicals and materials

Polyethylene oxide (PEO) with average relative molecular mass MW = 10,000 Da, trimethylchlorosilane, tetramethylorthosilicate 99% (TMOS), chloro(dimethyl)octadecylsilane 95%, 2,6-lutidine 99%, ammonium hydrogen carbonate buffer, NaCl and Tris-HCl were purchased from Sigma-Aldrich (Poole, UK) and used as received without any further purification. Nitric acid, ammonia, toluene, HPLC grade acetonitrile (ACN), and trifluoro acetic acid (TFA) was obtained from Fisher Scientific (Loughborough, UK). Water used for preparing the solution was deionised in the laboratory using Elgastat Prima 3 reverse osmosis water system from Elga Ltd. (High Wycombe, UK). Bovine pancreas insulin, bovine heart cytochrome C, chicken egg white lysozyme, myoglobin from horse heart, β-lactoglobulin from milk bovine, oval albumin from chicken egg white, human hemoglobin and bovine serum albumin were purchased from Sigma-Aldrich (Poole, UK). Heat shrinkable sleeving PTFE, shrink ratio 2 : 1, internal diameter shrinks from 4.8 mm to 2.8 mm, fully recovered wall thickness: 0.30 mm was purchased from Adtech Polymer Engineering Ltd. (Stroud, UK). Adapter straight/standard bore 1.5 mm was purchased from Kinesis (Cambs, UK). PTFE thread seal tape was purchased from ARCO Ltd. (Hull, UK). Disposable plastic syringe (1ml) was purchased from Scientific Laboratory Supplies (Nottingham, UK). Borosilicate tube with an internal diameter of 2.10 mm and an outer diameter of 3.90 mm was purchased from Smith Scientific (Kent, UK).

2.2. Instrumentation

Baby bee syringe pump from Bioanalytical System Inc. (West Lafayette, USA). The instrument used for detection was HPLC-UV detection: 785A UV/Visible Detector from PerkinElmer (California, USA). Symmetry C_8 column, 4.6 mm × 250 mm packed with silica particles (size 5 μm) from Thermo Fisher Scientific (Loughborough, UK). Hot plate-stirrer (VWR International, LLC, West Chester, PA, USA). pH meter (Fisherman hydrus 300, thermo Orion, Beverly, MA, USA). Scanning electron microscope (SEM) (EVO 60. Manufacturer: Carl Zeiss Ltd. (Welwyn Garden City, UK). Brunauer-Emmett-Teller (BET) model using a Surface Area and Porosity Analyser from Micromeritics Ltd. (Dunstable, UK).

2.3. Fabrication of the silica based monolith

The monolithic silica rod was fabricated following previously reported procedure[26] with some modifications in the fabrication conditions. The porous silica rod was fabricated by adding 0.282 g of polyethylene oxide (PEO) to 2.537 ml of 1 M nitric acid solution and 0.291 ml of distilled water. The solution was violently agitated to promote a hydrolytic reaction for 20 min while immersed in ice bath. Then, 2.256 ml of tetramethyl orthosilicate (TMOS) was added to the cooled transparent solution and the solution was mixed for 30 min until the two-phase mixture gradually became a homogeneous solution. When the mixture was homogeneous, it was left to settle for 2 min to remove any bubbles that may have formed during mixing. The resulting homogeneous mixture was left in the ice. The resulting solution (the sol) was poured slowly down inside a 1 ml disposable plastic syringe which acted as a mould (internal diameter 4.5 mm). When the mixture was in the syringe, it was shaken carefully to remove any air bubbles. The thin end of the syringe was sealed using PTFE thread seal tape. The syringe was placed in an oven at 40 °C. Gelation occurred within 2 h, and subsequently the gelled sample was aged for 24 h at the same temperature to give a white solid rod. Some shrinkage occurred and the wet silica monolith rod was released slowly from the plastic syringe. The silica rod was soaked in a water bath for 2 h at room temperature.

The resulting monolithic silica rod was treated with a basic environment, produced by thermal decomposition of 1 M aqueous ammonia solution at elevated temperature (85 °C) for 24 h, to form mesopores. Then the rod was washed with distilled water. The monolithic silica rod was placed in an oven for 24 h at 40 °C, followed by a further 24 h at 100 °C. For heat treatment, the rod was placed in an oven at 500 °C for 2 h. After preparation, the silica rod was cut to a desired length, which was around 4 mm. The silica rod was connected to the borosilicate tube (o.d. 3.90 mm) *via* the poly (tetrafluoroethylene) (PTFE) shrinkable

tube by placing both the silica rod and the borosilicate tube inside the shrinkable tube and then placed them in the furnace at 330 °C for 2 h to seal the heat shrinkable tube around the tube and monolithic rod. After this step, the resulting monolithic silica was ready to use or for the surface of the monolithic silica to be modified.

2.4. Derivatisation of the silica-based monolith with C_{18}

The silica rod was modified by a method similar to the one previously described by ref. 27. The surface of monolithic silica was chemically modified by C_{18}. The derivatisation reagent was 1 g chloro(dimethyl)octadecylsilane as the silanisation reagent in 10 ml toluene and 10 drops 2,6-lutidine. The derivatisation was done on column by continuous flow from a syringe pump at a flow rate of 30 μl min^{-1} for 6 h at 80 °C. The end capping procedure used 1 g trimethylchlorosilane in 10 ml toluene for another 6 h in order to block unreacted silanol moieties. After derivatisation, the monolith was flushed with toluene and then with methanol using a syringe pump for 2 h and finally the derivatised silica column was placed in an oven for 24 h at 40 °C prior to use.

2.5. Monolithic material characterization

2.5.1. Pore structure by scanning electron microscopy (SEM). The morphology of the monolith was characterized by scanning electron microscopy (SEM). Images were obtained using an accelerating voltage of 20 KV and a probe current of 100 pA in high vacuum mode using a Cambridge S360 scanning electron microscope. The samples were coated with a thin layer of gold-platinum (thickness around 2 nm) using a SEMPREP 2 Sputter Coater from Nanotechnology Ltd. (Sandy, UK).

2.5.2. BET analysis. The physical properties of the bulk monolith (surface area, average pore diameter, and the pore volume) were studied using BET model. The porous monolith was fabricated inside a 1 ml disposable syringe using the same polymerisation mixture. Then, the monolith rod was removed from the syringe and the unreacted materials were extracted *via* a soxhlet extractor with methanol for 24 h. The polymer rod was dried using N_2 gas. The porous properties of the monoliths were determined using the BET isotherms of nitrogen adsorption and desorption at 77 K. The isotherms were analysed to get the surface area according to the (BET) model. The pore volume and pore size distribution of pores within the monoliths were measured from the isotherms using the BJH (Barett-Joyner-Halenda) model.

2.5.3. Measuring porosity. The total porosity ($\varnothing_t$) equals the fraction by volume of pores in the cylindrical monoliths. They were measured by the following equation:[28]

$$\varnothing_t = \frac{W_M - W_T}{dLR^2\pi} \quad (1)$$

Where W_T and W_M are the weights of the monolith when dried and when filled with water respectively, d is the density of water (at 23 °C = 0.9975 g cm^{-3}), and L and R are the whole length and radius of the cylindrical monolith, respectively. The measurement was repeated five times and the average was taken.

2.6. Using the silica based monolith for extraction

The standard proteins used in extraction were insulin, cytochrome C, lysozyme, myoglobin, β-lactoglobulin, albumin, hemoglobin, and bovine serum albumin. They were dissolved individually in 5 mM ammonium hydrogen carbonate buffer solution (pH 8.0) for non-modified silica monolith and in 50 mM Tris-HCl buffer solution (pH 7.0) containing 10 mM NaCl the performance examination of the C_{18}-bonded silica. The concentration of proteins was 60 μM and all experiments were carried out at ambient temperature around 23 °C. The proteins were extracted following the procedure described by ref. 29 with some modifications. All solutions were injected using a syringe pump *via* the borosilicate tube. The purification profile of proteins on non-modified monolithic silica extraction was measured, using a syringe pump at flow rate 10 μl min^{-1} for all steps except for loading the sample (5 μl min^{-1}). The proteins were extracted individually to calculate the recovery of each protein. The non-modified sorbent was conditioned with 400 μl acetonitrile and the solvent was discarded. Then, the sorbent was equilibrated using 400 μl 20 mM ammonium hydrogen carbonate buffer (pH 8.0) and the buffer was discarded. After that 1 ml of the sample solution was applied. After loading of the sample solution through the extraction monolith, the sorbent was rinsed with the washing solvent, which was 200 μl 20 mM ammonium hydrogen carbonate buffer solution (pH 8.0). Finally, proteins were eluted from the sorbent using an elution solvent, which was 500 μl 20% ACN (0.1% TFA) solution and collected into the eppendorf tube. For C_{18}-bonded modified silica monolith, the sample was adjusted with 0.1% TFA. The sorbent was cleaned with 400 μl ACN (0.1% TFA) solution and then equilibrated with 400 μl ACN. After sample application (1 ml sample), the monolith was washed with 200 μl ACN (0.1% TFA) solution. Finally, the sample was eluted using 500 μl 60% ACN (0.1% TFA) solution and dispense into eppendorf tube.

A sample of the eluent was injected directly into the HPLC-UV detector to study the peak area obtained for the proteins and compared them with the peak areas of the protein standard solutions to calculate the efficiency of extraction. The mobile phase was acetonitrile-water (50 : 50) in the presence of 0.1% trifluoroacetic acid (TFA) under isocratic conditions and the detection wavelength was adjusted to 210 nm and the injection volume was 20 μl. In order to evaluate the extraction efficiency of proteins, the enrichment factor (EF) and extraction recovery (ER) were calculated.

The enrichment factor[30–32] is the ratio between the protein concentration in eluent (C_{elu}) and the initial concentration of the protein (C_o) within the sample:

$$EF = \frac{C_{elu}}{C_o} \quad (2)$$

Where C_{elu} and C_o were obtained from the peak area obtained with the solid phase extraction and without purification.

The extraction recovery (ER)[30–32] was defined as the percentage of the total proteins amount (n_o) that was extracted to the eluent (n_{elu})

$$ER = \frac{n_{elu}}{n_o} \times 100 = \left[\frac{C_{elu} \times V_{elu}}{C_o \times V_{aq}}\right] \times 100 = EF \times \left(\frac{V_{elu}}{V_{aq}}\right) \times 100 \quad (3)$$

Where V_{elu} and V_{aq} are the volumes of eluent and sample solution, respectively.

3. Results and discussion

3.1. Preparation of the monolithic silica rods

This work involved fabrication of an effective monolith to use as a solid phase extractor. Silica based monoliths were investigated since these types of monolith contain both micro- and nano-scale pores.[33,34] The monolithic silica columns were prepared by a sol–gel method. The composition of the starting mixtures were an alkoxy silicon derivative, which was tetramethyl orthosilicate (TMOS), undergoing hydrolytic polymerisation reaction in the presence of water-soluble organic polymer, which was polyethylene oxide (PEO). TMOS was chosen as the alkoxy silicon derivative because it was easier to hydrolyse than tetraethyl orthosilicate (TEOS). PEO was used as a porogen to form the through pores and the micropores in the silica gel. Nitric acid was used as a catalyst to start the hydrolysis and condensation reactions.[35] A 1 ml disposable plastic syringe was used as a mould for preparation the monolithic silica rod. It was observed that the monolith was white, and crack-free. In addition, the preparation of the silica monolith was accomplished with the volume reduction of the whole structure and around 20% shrinkage occurred during the gel formation without the cracking in the monolith that was observed by other groups.[36–38] The percentage of shrinkage was calculated based on the size of the cylindrical monoliths. Shrinkage in the sol–gel skeletons helped to remove the silica rods from the mould.

After formation of the network structure of silica skeletons, the internal pore structure of the monolith was tailored by solvent exchange and aging in order to form mesopores on the skeletons by using a basic environment that can increase the surface area of the monolith by converting the micropores, which have low surface area, to mesopores (high surface area) within the monolithic silica skeletons.[39] This pore tailoring process involved treating the wet gel from thermal decomposition of 1 M aqueous ammonia solution producing an alkaline pH environment homogeneously around the whole monolithic structure at elevated temperature (85 °C). After the thermal decomposition step, the monolith was calcinated at 500 °C in order to decompose the organic residues and remaining polymer in the rod with no serious deformation of gel specimens.

3.2. Silica monolith after derivatisation with C_{18}

The surface of the silica rod was chemically modified with C_{18} in order to make the sorbent hydrophobic. The modification of the silica monolith was done after placing the bare silica monolith inside the shrinkable tube. The derivatisation was done by continuous flow of the derivatisation reagent through the porous monolithic rod.[20] After derivatisation, the end capping was carried out after bonding using trimethylchlorosilane. This was done to decrease the adsorption of proteins, since the OH groups can interact with proteins, especially at high pH, by blocking the unreacted silanol groups. It was observed that the colour of the non-modified silica rod was bright white while the modified silica was translucent as can be seen in Fig. 1, which shows the silica rods before and after modification with octadecyl ligands.

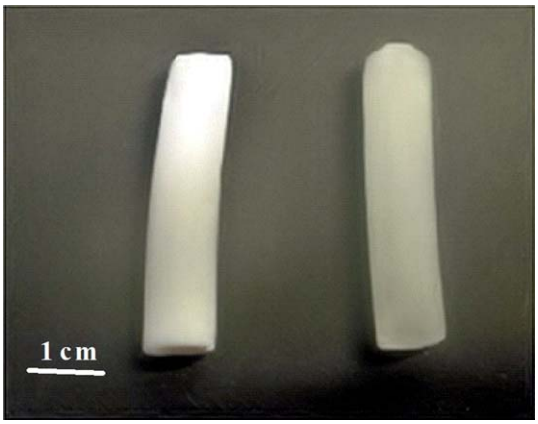

Fig. 1 The porous silica monolith before (left) and after modification with C_{18} (right). The colour of the rod was changed from bright white monolith to translucent. The sol–gel precursor was 0.282 g PEO (MW = 10,000 Da), 2.537 ml of 1 M HNO_3 and 0.291 ml of distilled H_2O. The derivatisation reagent was 1 g chloro(dimethyl)octadecylsilane in 10 ml toluene and 10 drops 2,6-lutidine, the end capping reagent was 1 g trimethylchlorosilane in 10 ml toluene.

The structural morphology of the monolithic silica was examined by scanning electron microscopy (SEM). It can be

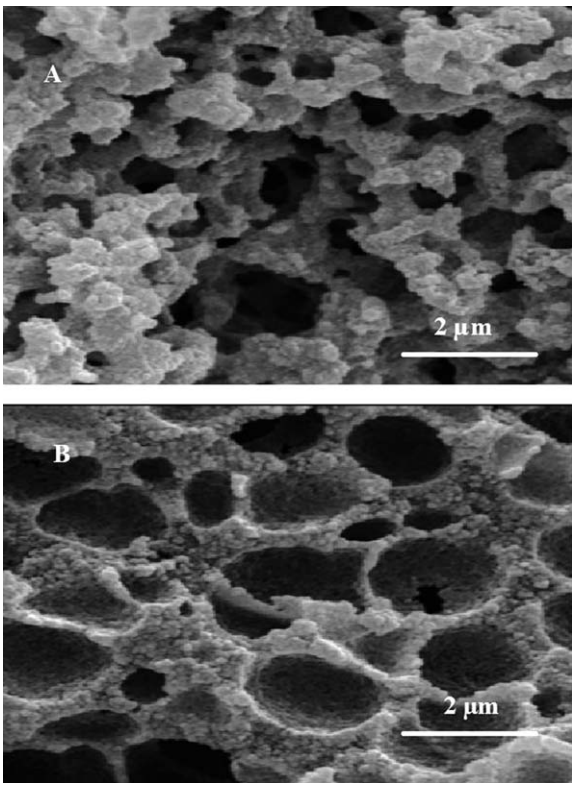

Fig. 2 (A) SEM micrographs showing the main structure of the silica based monolith rods without modification prepared at 40 °C for 24 h (B) after modification with C_{18}.

Table 1 The surface area, total pore volume, and average pore diameter for bared silica based monolith before and after modification with C_{18}, calculated using the BJH method, the porosity was calculated using eqn (1), and RSD (n = 3)

Type of silica monolith	Surface area (m^2 g^{-1}) ± RSD (%)	Total pore volume (cm^3 g^{-1}) ± RSD (%)	Average pore diameter (μm) ± RSD (%)	Porosity (nm) ± RSD (%)
Non-modified	173.31 ± 3.7	0.40 ± 2.7	13.58 ± 3.8	0.51 ± 3.9
Modified with C_{18}	154.29 ± 5.2	0.38 ± 6.5	12.22 ± 4.2	0.23 ± 3.1

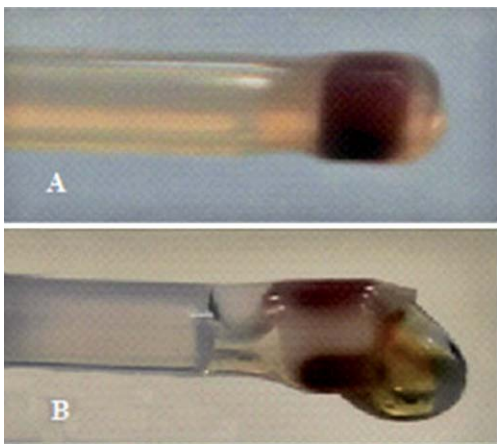

Fig. 3 (A) Loading cytochrome C solution using non-modified silica monolith using syringe pump at flow rate 5 μl min^{-1}, and (B) eluting cytochrome C using 500 μl 20% ACN (0.1% TFA), solutions at flow rate 10 μl min^{-1} and dispensed into eppendorf tube.

observed from Fig. 2 that the SEM micrographs of silica-based monolith before and after modification with C_{18}. In general, SEM photographs indicate that the monolithic silica has high homogeneity and a spongy structure as characterized by through-pores penetrating several layers of these skeletons and a network structure of skeletons of different sizes. It can be seen that there is a difference in the size of the through-pores before and after the derivatisation. Fig. 2(B) displays the shape of the through-pores in the modified silica rod was relatively round and the size of the macropores is smaller than that of the non-modified silica monoliths. The reproducibility in the fabrication of the monolithic materials was assessed by checking the morphology of the scanning electron microscopy (SEM) for three different batches of the silica monolith before and after modification with C_{18} and it was found that there was no difference in the morphology of the monolith.

The physical properties of the silica monolith were studied using nitrogen adsorption isotherms BET measurement. This was done before and after the derivatisation reaction. As can be seen in Table 1, it was found that the BET surface area of the porous silica monolith rods in this study decreased slightly from 173.31 to 154.29 m^2 g^{-1} after derivatisation with alkyl chains (C_{18}). The total pore volume was calculated to be 0.40 cm^3 g^{-1} for non-modified silica and 0.38 cm^3 g^{-1} for modified silica. The average diameter of the pores for both monoliths are in the mesopores range, 13.58 and 12.22 nm for bare silica and C_{18} silica monolith, respectively. The porosity of the silica monolith decreased from 0.51 to 0.23 after derivatisation (Table 1). The reason for the decrease in the surface area, pore volume, average pore diameter, and porosity after derivatisation with C_{18} group is that the micropores were blocked by the bonded phase, the alkyl chains attached to the silica surface. In addition, surface silanol groups are replaced by larger chemical ligands, resulting in the decrease in the pore size.[21,40]

3.3. Evaluation of silica monoliths for protein extraction

As previously mentioned the objective of this study was to compare bare silica monolith and modified silica with C_{18} in terms of their use in the extraction of proteins. The monolithic column was used for off-line preconcentration of proteins. The non-modified silica sorbent with polar functional groups was conditioned with 400 μl ACN to remove impurities and then it was displaced with 400 μl 20 mM ammonium hydrogen carbonate buffer solution (pH 8.0) to equilibrate the monolith so it can react with the analyte. Although the permeability of the silica monolith was high and high flow rate (30 μl min^{-1}) can be used without leakage, all solutions were injected using a syringe pump at a flow rate of 10 μl min^{-1} for all steps through the SPE column, except applying the sample, in order to make sure that the sorbent was well cleaned and conditioned. After equilibrating

Table 2 Comparison of the extraction recovery of proteins purified with bare silica, and with C_{18}-bonded silica monolith

Protein	Molecular weight (Da)	Isoelectric point(pI)	Bare silica monolith		Modified silica monolith with C_{18}	
			Extraction recovery (ER) (%)	RSD (%) (n = 3)	Extraction recovery (ER) (%)	RSD (%) (n = 3)
Insulin	5,800	5.6	88.2	6.10	105.2	6.10
Cytochrome C	12,327	10.0	97.9	2.97	109.7	2.03
Lysozyme	14,300	11.0	92.1	5.88	96.9	3.88
Myoglobin	16,951	6.8	65.2	4.63	95.6	4.50
β-Lactoglobulin	36,000	5.2	29.4	7.42	94.8	5.42
Albumin	44,287	4.6	45.9	4.79	92.7	5.79
Hemoglobin	64,500	7.4	96.8	5.39	99.6	3.79
BSA	66,382	4.7	25.5	3.12	95.4	2.12

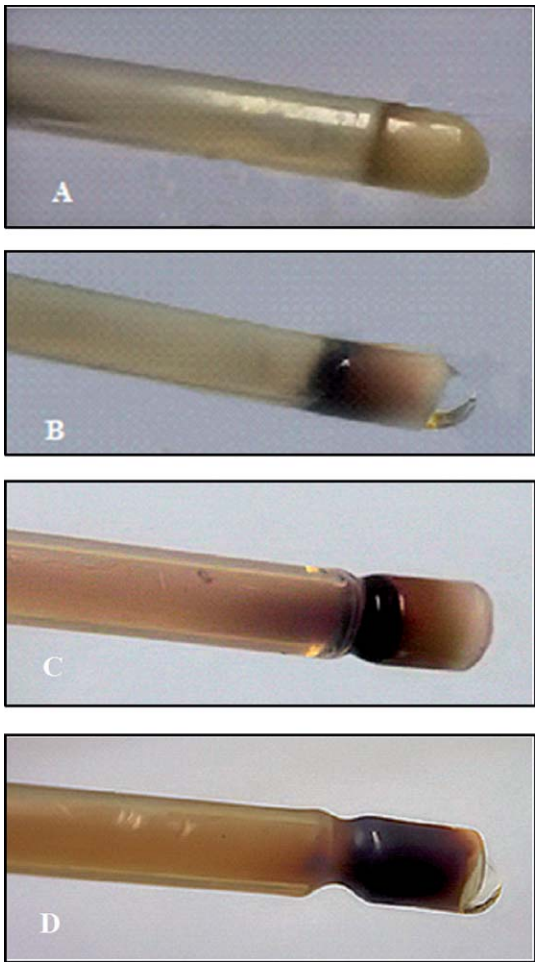

Fig. 4 Loading myoglobin using (A) non-modified silica and (B) modified silica with C_{18}. Loading hemoglobin using (C) non-modified silica and (D) modified silica with C_{18}. The sample was injected using syringe pump at flow rate 5 μl min^{-1}. Concentration of both proteins was 60 μM.

the sorbent, the sample was applied at low flow rate (5 μl min^{-1}) in order to obtain good percolation between the analyte and the sorbent. Fig. 3(A) shows binding of cytochrome C (12,327 Da) in the non-modified silica sorbent as confirmed by the change of the colour of the sorbent from white to red (the colour of cytochrome C is red). During application of the sample, the proteins had affinity to the polar sorbent while the interfering materials were not retained to the monolith.

Before elution of the analyte, the sorbent was rinsed with 200 μl 20 mM ammonium hydrogen carbonate buffer solution (pH 8.0) to remove interferences without losing the analytes. It was found that 200 μl of the buffer did not decrease the extraction recovery of the analytes by checking the wash solution for breakthrough of the proteins. Finally, the protein was released by 500 μl of 20% ACN (0.1%TFA) solution and the eluent was collected in a 1.5 ml eppendorf tube. As can be seen in Fig. 3(B), cytochrome C was eluted successfully from the monolithic surface and the elution solvent was able to elute the target protein from the sorbent in a minimum volume that was confirmed by the colour of the sorbent returning white.

For the modified silica sorbent, it was wetted and equilibrated using 400 μl ACN, to ensure optimum binding to proteins. Then, 1 ml protein sample was loaded. The monolithic column was conditioned with 200 μl ACN (0.1% TFA). The residual matrix components that were weakly bound to the sorbent were rinsed from the sorbent using 200 μl ACN solution and the solvent was discarded. The proteins were concentrated and purified using a slightly acidic aqueous-organic solvent, which was 500 μl 60% ACN (0.1% TFA) solution and the eluent was dispensed into the eppendorf tube then injected into the HPLC-UV instrument to study the extraction efficiency. It was found that the recovery of cytochrome C was increased slightly when using modified silica monolith from 97.9% to 109.7%, as can be seen in Table 2. It was found that the modified sorbent with C_{18} has the ability to purify and preconcentrate both myoglobin (16,951 Da), and hemoglobin (64,500 Da) better than bare silica, confirmed by Fig. 4.

The extraction recovery (ER) of proteins was calculated from the chromatogram by comparing the peak area of extracted sample to non-processed sample solution. Fig. 5 shows a huge difference in the peak areas of lysozyme between the direct using non-modified silica monolith, and with purification using C_{18}-bonded sorbent. From Fig. 5, it can be seen that lysozyme was concentrated after extraction since the concentration of analyte is proportional to the peak area. In addition, there is a huge

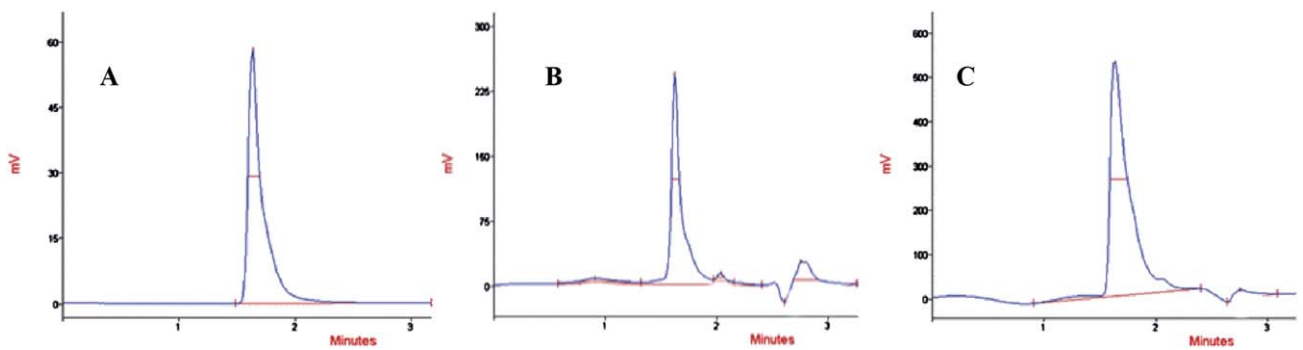

Fig. 5 The comparison of lysozyme, (A) without purification, (B) with purification by bare silica, and (C) with purification by modified silica with C_{18}. Experimental condition: the mobile phase was composed of ACN and distilled water (50 : 50) containing 0.1% TFA, flow rate 1 ml min^{-1}. The separation column was Symmetry C_8, 4.6 mm × 250 mm packed with silica (size 5 μm). Between consecutive analyses, a needle for the automated injector was washed with 70% aqueous methanol. Detection: UV at 210 nm, injected sample volume: 20 μl.

increase in the peak area after purification with the chemically-modified silica monolith with C_{18} compared with bare silica. As a result, the sensitivity of the detection was enhanced.

The recovery of protein purified with non-modified monolithic extraction and with a C_{18} phase was calculated. The comparison in Table 2 indicates that a huge increase in the extraction recoveries of proteins when using modified silica monolith even for large protein, BSA (66,382 Da) and different isoelectric point (pI), ranged from 92.7 to 109.7% while the range of recovery using bare silica was 25.4–97.9%. This means efficient protein extraction was achieved using modified silica.

The reproducibility of the performance of the bare silica and modified silica monolith were evaluated by calculating the RSD in the peak area. The relative standard deviations (RSD) for peak area counts was calculated to be 2.97–7.42% for bare silica monolith and 2.03– 6.10% for modified silica monolith. There were no memory effect or deterioration in performance of the device was observed indicating that the silica monolith had a long lifetime and the coating was not affected.

Conclusions

The aim of this work was to investigate the fabrication of crack-free monolithic silica in shrinkable tube. It was evaluated for its use in performing solid phase extraction (SPE) for preconcentration of proteins. The bare silica monolith and modified monolith had low flow resistance and high surface area. This study shows that the octadecylsilated porous silica rod was much better for proteins of different molecular weights and isoelectric points. Since a satisfactory extraction recovery of protein was achieved using C_{18}-bonded monolithic silica, fabrication of the monolithic silica inside the microchip rather than shrinkable tube in order to speed the analysis, reduce the volume of the analyte and the reagents, and integrate it with other microfluidic devices will be attempted by this process.

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