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DEVELOPMENT OF Ti^{4+}-IMMOBILIZED NANOPOROUS MONOLITHIC POLYMER FOR SELECTIVE SEPARATION AND DETECTION OF PHOSPHOPEPTIDES

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ABSTRACT
Phosphopeptides present in a large number of biological regulating mechanisms. It plays a significant role in gene regulation, eukaryotic signal transduction, and metabolic control in a cell. Abnormal phosphorylation can cause various diseases, including cancer. However, phosphoprotein abundances are very low, only 1-2%. In this work, the nanoporous monolith columns were developed for separation and detection of phosphopeptides in phosphoproteome analysis. The nanoporous monolith-based column is prepared inside a silicosteel tubing (100 x 1.02 mm i.d.) by in-situ copolymerization reaction of glycidyl methacrylate (GMA) with ethylene dimethacrylate (EDMA). This monolith was further chemically modified by introducing aminomethyl phosphonic acid (AMPA) before immobilization of Ti^{4+} ion (Ti^{4+}-immobilized). The monolithic column properties, such as morphology, elemental analysis, surface area analysis, permeability and pore distribution were characterized in detail. Such a Ti^{4+}-immobilized nanoporous monolith-based column with immobilization time 3 hours of $TiCl_4$ without glutaraldehyde addition was further applied to separation and detection of phosphopeptides from digested proteins (β-casein and cytochrome-c), and tyrosine phosphorylated peptide samples. The result demonstrated that Ti^{4+}-immobilized nanoporous monolith-based provide higher selectivity and efficiency for selective detection of phosphopeptides using liquid chromatography.
Keywords: Chromatography; Monolith; Phosphopeptide; Protein; Ti^{4+}-immobilized.

INTRODUCTION

High-performance liquid chromatography (HPLC) is widely used in analytical and bioanalytical chemistry.[1-3] As the heart of chromatographic method, the development of HPLC columns are always becoming great of interest. Monolithic columns have rapidly grown for several decades and received wide acceptance in separation techniques. Monolithic columns provide low pressure and excellent mass transfer due to their skeletal structure that allows sufficient surface area. Thus, monolithic-based columns hold influential positions in chromatographic separation as an alternative to conventional packaging columns due to their less time-consuming and rapid dynamics.[4-6] There are many reports of monolithic columns using silica, organic polymer, and hybrid inorganic-organic materials.[7-13] The application of monoliths has been extended from the original separation media to microreactors and solid phase extractors.[14-16]

The utilization of monolithic capillary column with inner diameters of 75-250 μm is more preferred in miniaturized system and analysis, because such capillary columns are well-suited in the field of life sciences, which the amount of sample is limited.[15,17] It also requires minimum consumption of reagents and samples, and contribute sensitivity of the limited sample. Nevertheless, this technique demanded high skills and dedicated HPLC equipment. Therefore a larger-diameter of monolith column is more desirable regarding ease of use for general HPLC users. Development of microbore column with inner diameter of

0.5 – 1.5 mm has been reported.[18-20] This kind of column easily utilized to standard HPLC system without any modification.[19,21] In term of applicability, the benefit of microbore column can be used for analysis and also for preparative purposes. Environmentally friendly due to low reagent and sample consumption, and shorter analysis could be attributed to this column as other advantages in comparison to a conventional column.

Recently, research trend of phosphopeptides enrichment is using pipette tips. ZipTip pipette tips modified with polydopamine for Ti^{4+} immobilization have been developed, and subsequently applied to isolation and phosphopeptide enrichment.[22,23] Other research reported a Ti(IV) monolithic spin tip that enabled efficient and fast capture of phosphopeptides. This tip demonstrated a good selectivity and high enrichment recovery by using β-casein and BSA tryptic digest as samples of phosphopeptide enrichment experiments.[24] Although a monolithic tip offered easiness for sample preparation, tedious work could not be avoided due to a batch-wise preparation. Additionally, stringent control is required to prevent contamination as well as sample instability caused by direct contact with open air. Due to the stability of the phosphopeptides matter and to provide automation of sample preparation, the development of an alternative technique remains challenging in phosphoproteomics analysis. To address these issues, Ti^{4+}-immobilized monolith inside a microbore chromatographic column is conducted.

In the present work, we have been developed Ti^{4+}-immobilized organic-based monolith with three-step of synthesis as follows: (a) synthesis of poly-(GMA-co-EDMA) monolith using glycidyl methacrylate (GMA) and ethylene dimethacrylate as monomers in the presence of a porogenic mixture of 1-propanol, 1,4-butanediol, and water inside the silicosteel tubing, (b) introduction of aminomethyl phosphonic acid (AMPA) to the monolith through the opening-ring reaction of epoxide group of GMA with amino group of AMPA, and (c) immobilization of Ti^{4+} ion to the monolith via phosphonate group of AMPA. Optimation of Ti^{4+}-immobilized monolithic column was carried out by varying the addition of glutaraldehyde and the immobilization time of Ti^{4+} to produce monolith with excellent characteristics. The morphology and elemental analysis of the monolithic column were studied by scanning electron microscopy-electron dispersive x-ray (SEM-EDX). Other properties such as total surface area, permeability and pore size distribution behavior were also characterized. Monolith prepared in this study, provide a promising tool in phosphoproteome analysis for liquid chromatography.

EXPERIMENTAL

Materials

All chemicals were of analytical grade and used without further purification. Glycidyl methacrylate (GMA), acetonitrile (ACN), ethylene dimethacrylate (EDMA), 1-propanol, 1,4-butanediol, 3-methacryloxypropyl-trimethoxysilane (MAPS), aminomethyl phosphonic acid (AMPA), ammonium hydroxide (NH_4OH), glutaraldehyde, ammonium hydrogen carbonate (NH_4HCO_3), acetone, calcium chloride ($CaCl_2$), pyridine, trifluoroacetic acid (TFA), tetrahydrofuran (THF), trypsin, β-casein, cytochrome-c and polystyrene standard set (Mw 500-2,000,000) were purchased from Sigma-Aldrich (Singapore). Titanium tetrachloride ($TiCl_4$) were obtained from Tokyo Chemical Industries, Co. Ltd (Chuo-ku, Japan). Tyrosine phosphorylated peptides sequence TSTEPQ[pY]QPGENL were purchased from GenScript (Carlsbad, USA). Acetone, ethanol, sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), and toluene were from Merck KGaA (Darmstadt, Germany). Silicon steel column (1.02 mm i.d.1/16 inch o.d) and 2,2'-azobisisobutyronitrile (AIBN) were obtained from Supelco (Bellefonte, Pennsylvania, USA) and Himedia (Mumbai, India), respectively.

HPLC Instrumentation and Phosphopeptides Measurement

All liquid chromatography experiments were performed using a Shimadzu Prominence HPLC system 20 (Japan). This system consists of an LC-20AD HPLC pump, a CTO-20AC column oven, an SPD-20A UV-Vis Detector, and a Rheodyne 8125 injector, and a-2 μL sample loop. A CBM-20A communication bus module and LabSolutions software existing in the system were used for system control and data acquisition.

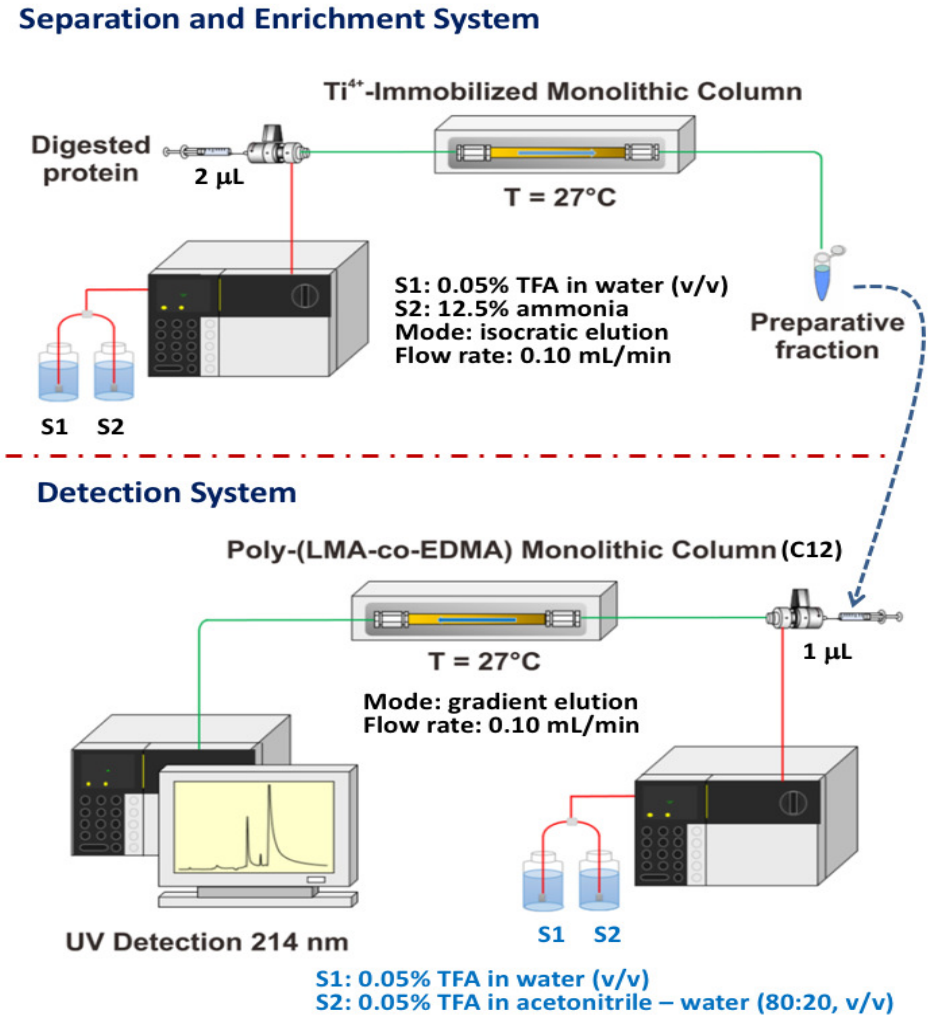

Fig.-1: Experimental set up for separation and detection of phosphopeptide

The experimental set up for separation and detection of phosphopeptides was shown in Figure-1. It consists of two steps, such as the separation-enrichment system and the detection system. In the first system, the digested protein or peptide samples were passed through the Ti^{4+}-immobilized monolithic column using a mobile phase of 0.05% TFA in water. Then, the adsorbed and enriched phosphopeptides in the column were eluted with ammonia solution. In the second system, the enriched phosphopeptides were detected according to their sizes by employing poly-(LMA-co-EDMA) monolithic column (C12).

Pretreatment of Silicosteel Tubing and Preparation of Ti^{4+}-immobilized Monolithic Column

Before the polymerization, the inner wall of silicon steel tubing was pretreated using MAPS to anchor the polymer into the inner wall column using the procedure as described by Shu, *et al*.[25] First, silicon steel tubing washed with water, filled with 0.2 M NaOH and 0.2 M HCl for 30 min twice respectively, rinsed with water and then with acetone. The mixture of MAPS, acetone, and pyridine with the ratio of 30: 65: % (v/v) was used for surface modification of the inner wall of the silicon steel tubing. The mixture was filled inside the tubing and placed at room temperature with both ends closed for 12 h. This procedure

was repeated twice, and finally the column was washed with acetone and cut into the desired length (10 cm).

Table-1: Preparation of Ti^{4+}-immobilized Monolithic Columns

Monolith	GMA (mL)	EDMA (mL)	Porogen Ratio (v/v)[a]	Immobilization Time (h)		
				Glutaraldehyde[b]	AMPA[c]	$TiCl_4$[d]
I	0.6	0.2	7:4:1	-	6	3
II	0.6	0.2	7:4:1	-	6	6
III	0.6	0.2	7:4:1	-	6	9
IV	0.6	0.2	7:4:1	6	6	3
V	0.6	0.2	7:4:1	6	6	6
VI	0.6	0.2	7:4:1	6	6	9

Total volume of polymer solution is 2 mL.
[a] Mixture of ternary porogen of 1-propanol (0.7 mL), 1,4-butanediol (0.4 mL) and water (0.1 mL)
[b] Glutaraldehyde (10%) in 100 mM carbonate buffer pH 8.0
[c] AMPA (2 mg mL^{-1}) in 100 mM 100 mM carbonate buffer pH 8.0
[d] $TiCl_4$ (100 mM) in ethanol

The poly-(GMA-co-EDMA) monolith was synthesized by in-situ copolymerization reaction inside a silicon steel tubing (1.02 mm i.d., 1/16 in o.d.) using the procedure described by Sabarudin, *et al*.[18] A mixture of GMA, EDMA, 1-propanol, 1,4-butanediol, water, and AIBN (1% w/v of the total monomer amount) was homogenized using ultrasonication for 10 min and injected into a silanized silicon steel tubing by mean of a-5 mL syringe. Both ends column was sealed, and the polymerization proceeded in the oven at 60°C for 12 h. The resulted monolithic column was then washed with ethanol and water to remove residual reagents. In this work, the optimized composition of total monomer (%T) and crosslinker (%C) was adopted from Tasfiyati *et al*.[26]

The Ti^{4+}-immobilized monolithic column prepared by attaching AMPA to the poly-(GMA-co-EDMA) monolith through ring-opening reaction of epoxy groups. The solution of 2 mg mL^{-1} AMPA in 100 mM carbonate buffer (pH 8.0) was pumped into the monolith column at flow rate of 0.05 mL min^{-1} for 6 h. Then, a-100 mM $TiCl_4$ in ethanol was passed through the column for immobilization of Ti^{4+} ion. Another work was performed by flowing a-10% glutaraldehyde in 100 mM carbonate buffer (pH 8.0) through the monolithic column for 6 h prior to modification with AMPA and $TiCl_4$. Six batch columns were prepared to examine the effect of glutaraldehyde immobilization and $TiCl_4$ immobilization time as displayed in Table-1. All procedures were conducted at room temperature. Finally, all modified monolithic column was washed with water for 1 h. The whole procedures of preparation of Ti^{4+}-immobilized monolithic column were illustrated in Fig.-2.

Pressure drop measurements

The pressure drop measurements were aimed to investigate the permeability and mechanical stability of monolithic columns. This work was performed using the HPLC pump bypassing ethanol through the columns at flow rates ranging from 0.01 to 0.1 mL min^{-1} for mechanical stability study, whereas permeability test was examined at a constant flow rate of 0.05 mL min^{-1}. Then, the back pressure of the monolithic column, which was shown in the monitor of the HPLC pump, was recorded. This procedure was done at room temperature. The permeability (K, m^2) was calculated according to Darcy's Law as the following equation:

$$K = \frac{\eta . L . u}{\Delta p} = \frac{\eta . L . F_m}{\Delta p . \pi . r^2} \qquad (1)$$

Where, η is the velocity of mobile phase (1.095 x 10^{-13} Pa s at 20°C for ethanol as used in this work), L is the monolithic column length (m), u is the linear velocity of mobile phase (m s^{-1}), and Δp is the pressure drop over the monolithic column. The permeability unit was then converted into Darcy (1 darcy = 9.9 x 10^{-13} m^2).

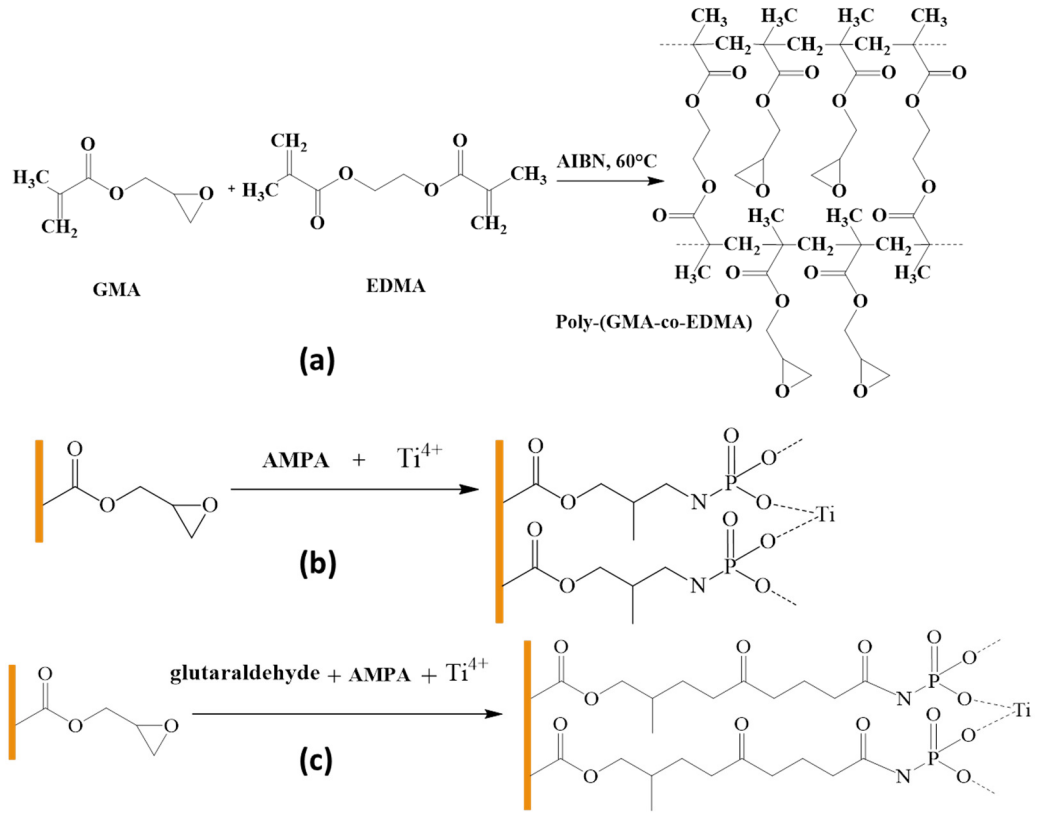

Fig.-2: Preparation of Ti^{4+}-immobilized monolithic column consists of polymerization of poly-(GMA-co-EDMA) monolith (a), preparation of Ti^{4+}-IMAC monolithic column without the addition of glutaraldehyde (b), and preparation of Ti^{4+}-IMAC monolithic column with the addition of glutaraldehyde (c).

Inverse Size Exclusion Chromatography (ISEC), Morphology and Elemental Analysis

Determination of monoliths porosity and pore size distribution were examined by inverse size exclusion chromatography method as described by Al-Bokari, *et al.*[27] A set of polystyrene standards (Mw 500-2,000,000) and toluene were used in this work. This examination was performed using the HPLC system with the sample size of 2 µL-polystyrene standards, the mobile phase of THF, and UV detection at 254 nm. Morphology observation and elemental analysis of the monolithic column were determined by the scanning electron microscopy equipped with energy dispersive X-ray spectroscopy (SEM-EDX) FEI Inspect S50 (USA).

RESULTS AND DISCUSSION

Preparation and Characterization of Ti^{4+}-immobilized Nanoporous Monolithic Column

Organic polymer monolith in this study was produced by the optimized mixture of 600 µL GMA (monomer) and 200 µL EDMA (crosslinker) with the porogenic solvents of 100 µL water, 700 µL 1-propanol and 400 µL 1,4-butanediol. The polymerization reaction of GMA with EDMA to form poly-(GMA-co-EDMA) occurred via a chain-growth reaction (addition polymerization) with AIBN as the free radical initiator. In the polymer mixtures, GMA and EDMA were directly mixed with porogen and initiator so that the alleged structure was random copolymers. The resulted monolithic columns were washed with ethanol and water by employing the HPLC pump at the flow rate of 0.05 mL min^{-1} for 1 h to

remove residual monomers and porogen. Then, the resulted poly-(GMA-co-EDMA) monolith columns were ready for further post-modification.

Ti^{4+}-immobilized monolith columns were made by further modification of the poly-(GMA-co-EDMA) monolith produced in previous treatment. Post-modification was performed by flushing 10% glutaraldehyde through the poly-(GMA-co-EDMA) monolithic column to provide suitable a space arm as a chelating ligand. Then, it was followed by flowing 2 mg mL^{-1} AMPA in 100 mM ammonium bicarbonate buffer (pH 8.0) through the monolithic column. AMPA is required to provide a functional group of PO_3^{2-} on the polymeric-support material. In this case, the monolith was used as the supporting material. As the final step, a further modification of Ti^{4+}-immobilized monolith was carried out by flowing 100 mM $TiCl_4$ (in ethanol) to provide Ti^{4+} ions binding to PO_3^{2-} groups on the poly-(GMA-co-EDMA) monolithic column. The procedures of preparation of Ti^{4+}-immobilized monolithic column were given in Fig.-2.

The morphology of monolithic columns was assessed SEM. From Figure-3, the porous network with globular structure of the produced monolith was observed clearly. Furthermore, the continuous porous channels, which were formed by flow through pores of monoliths, could also be found. The existence of flow through pores and their high connectivity indicated high permeability as well as the low-flow resistance of the monolith characters. The produced Ti^{4+}-immobilized monolith with the addition of glutaraldehyde formed dense and rigid along with the increasing the $TiCl_4$ immobilization. It also leads to monolith cracking with the presence of higher amount of Ti, which confirmed by the EDX data of the Ti^{4+}-immobilized monolithic column in Table-2. The permeability of monolithic columns was examined through measurement of their back pressure using ethanol at a constant flow rate. Column permeability of the prepared monolithic columns as shown in Table 2 was high with relatively low back pressure, demonstrating excellent permeability. The mechanical stability of the monolithic column was evaluated by measurement of the pressure drop versus flow rate. Good linear responses between back pressure and flow rate were observed with a correlation coefficient (R^2) of 0.994. This result indicated good mechanical stability of the prepared monolithic columns.

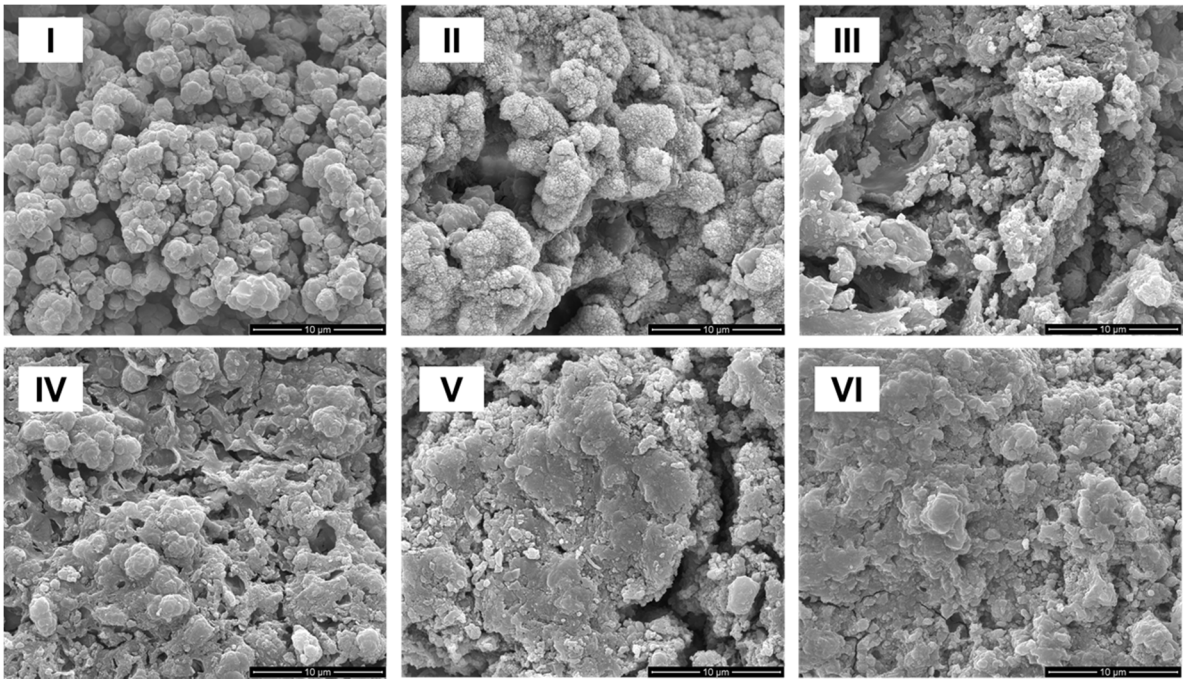

Fig.-3: Scanning electron microphotographs of Ti^{4+}-immobilized monolith at 5000x magnification. The composition of monolithic support consists of 40%T and 25%C.

The pore size distribution of the prepared monolithic columns was assessed using ISEC method. The ISEC method provides a proper way to determine pore size and pore distribution of the resulted monoliths as it works under similar conditions to those used in the real HPLC applications.[25] As shown in Figure-4(a), total porosity (εt) of monolith V calculated from the retention volume of the tracer compound (toluene, Vt) was 0.66. This value is commensurate with its porogen fraction (0.60). The interstitial/external (εe) porosity derived from the retention volume of the exclude molecular mass (Ve) and internal (εi) porosity obtained by subtraction of εt with εe of this monolith were 0.23 and 0.42, respectively. The monolith possesses larger internal porosities than their external porosities, implying predominant mesopore characters in these stationary phases. From the ISEC plot shown in Figure-4(b), the largest volume fraction (66.57%) could be attributed to the mesopore size (2-50 nm), whereas the volume fraction of flow-through pores/macropores (>50 nm) was found to be 27.50%. Micropore character (<2 nm), which is undesired in term of mass transfer point of view, was found to be the lowest volume fraction as it was estimated about 5.93%. A high surface area for chromatographic interaction, shown by high mesopores characters, is required to obtain sufficient functional groups for post monolith modification and to attain acceptable binding capacity. Therefore, monolith should gain an appropriate proportion between macropores for efficient convective mass transport and mesopores for sufficient surface area as well as excellent binding capacity. In this work, this monolith was applied for separation and detection of phosphopeptides.

Table-2: Pressure drop, permeability and elemental composition of Ti^{4+}-immobilized monolithic columns

Monolith	Pressure drop (MPa)	Permeability (Darcy)	Elemental Composition (% Wt)*				
			C	N	O	P	Ti
I	0.8	0.1405	44.58	15.36	28.01	5.8	6.26
II	0.6	0.1873	11.62	7.22	36.16	5.44	42.19
III	0.6	0.1873	29.44	8.52	29.83	5.13	27.09
IV	1.1	0.1022	36.89	12.52	27.17	4.76	18.67
V	0.7	0.1606	25.14	11.37	26.39	5.41	31.71
VI	0.9	0.1249	9.66	5.54	38.28	2.2	50.23

*Elemental composition was obtained from EDX data.

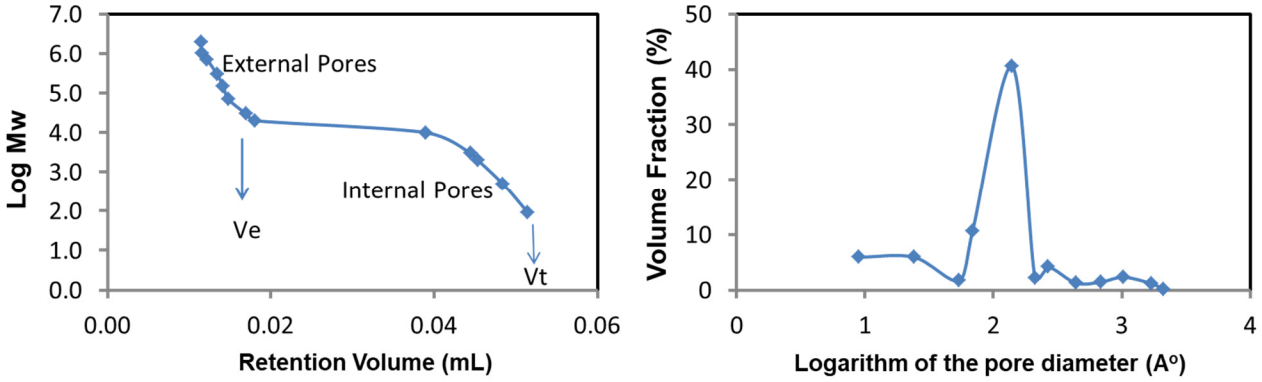

Fig.-4: Plot of the log of the polystyrene standards molecular masses (MW) versus their elution volume (a). The plot of the pore size distribution of Ti^{4+}-immobilized monolithic column (b).

Separation and Detection of Phosphopeptides

Separation of phosphopeptides samples using Ti^{4+}-immobilized monolithic column was based on the existing amounts of negatively charged phosphate groups of the phosphopeptides or phosphorylated

peptides. The longer amino acid sequence of phosphopeptide, the higher amount of the phosphate groups, resulting in stronger interaction between phosphopeptides and the positively charged Ti^{4+}-immobilized monolithic stationary phase.

The peptide sample with the sequence of TSTEPQ[pY]QPGENL was used to examine the selectivity of the Ti^{4+}-immobilized monolithic column. The obtained results can be seen in the Figure-5. It was found that before treated with the Ti^{4+}-immobilized monolithic column, several nonphosphopeptide peaks were observed. A high intensity of nonphosphopeptide peaks inhibits the detection of phosphopeptides. However, a peak of phosphopeptide in the peptide sample was selectively separated from non-phosphopeptides after treated with the Ti^{4+}-immobilized monolithic column. By comparing the peak height as well as peak area before and after treated with the Ti^{4+}-immobilized monolith, recovery of the phosphopeptide can be expected almost 100%.

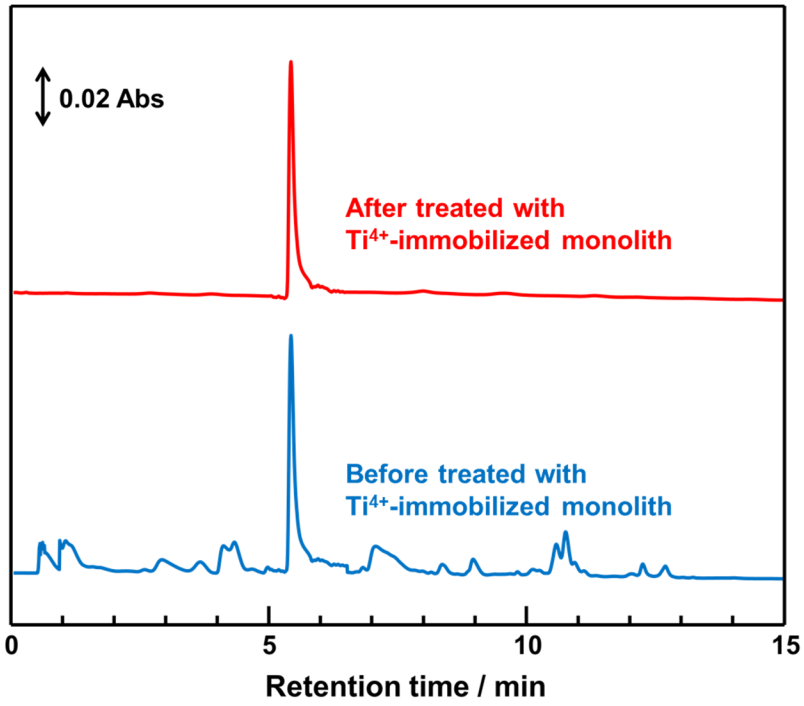

Fig.-5: Chromatogram of phosphopeptides sequence TSTEPQ[PY]QPGENL. Mobile phase (A) : 0.065% TFA in water (v/v), mobile phase (B) : 0.05% TFA in ACN (v/v), gradient elution : 5-65% B (0-12.5 min), 56-95% B (12.5-15.5 min), 95-5% B (15.5-20 min), flow rate : 0.10 mL/min, injection volume : 1 µL, wavelength : 214 nm.

To evaluate the selectivity of the Ti^{4+}-immobilized monolithic column, digested protein samples of cytochrome-c and β-casein were investigated with such column. The obtained results were shown in Figure-6. It can be seen in Figure-6(a) that no phosphopeptide was found in the digested protein sample of cytochrome-c. The presence of phosphopeptides in the digested protein of β-casein was recorded, as shown in Figure-6(b); three phosphopeptide peaks (β1, β2, and β3) were identified with few nonphosphopeptide peaks. Those peaks might represent two singly phosphorylated peptides, FQ[pS]EEQQQTEDELQDK (β1), and FQ[pS]EEQQQTEDELQDKIHPF (β2) along with a triply phosphorylated peptide, RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR (β3). These results demonstrated the high selectivity of the Ti^{4+}-immobilized monolithic column towards phosphopeptides. Possibly, it was due to a strong interaction between phosphate groups in phosphopeptides with immobilized Ti^{4+}ions onto the monolith.

CONCLUSION

A Ti^{4+}-immobilized monolithic column was successfully produced by a three-step procedure, involving the synthesis of poly-(GMA-co-EDMA) monolithic column and post-modification with aminomethyl

phosphonic acid and titanium tetrachloride via ring opening reaction of epoxy groups. Based on the characterization results of the Ti^{4+}-immobilized monolithic column, immobilization time of 3 h of $TiCl_4$ without the addition of glutaraldehyde was found to be an ideal monolithic column. This monolith has the characteristics of a large surface area, high permeability, accompanied by the dominant proportion of mesopores (66.57%). Such result demonstrated that Ti^{4+}-immobilized monolithic column is very effective and efficient for separation, enrichment, and detection of phosphopeptides using liquid chromatography.

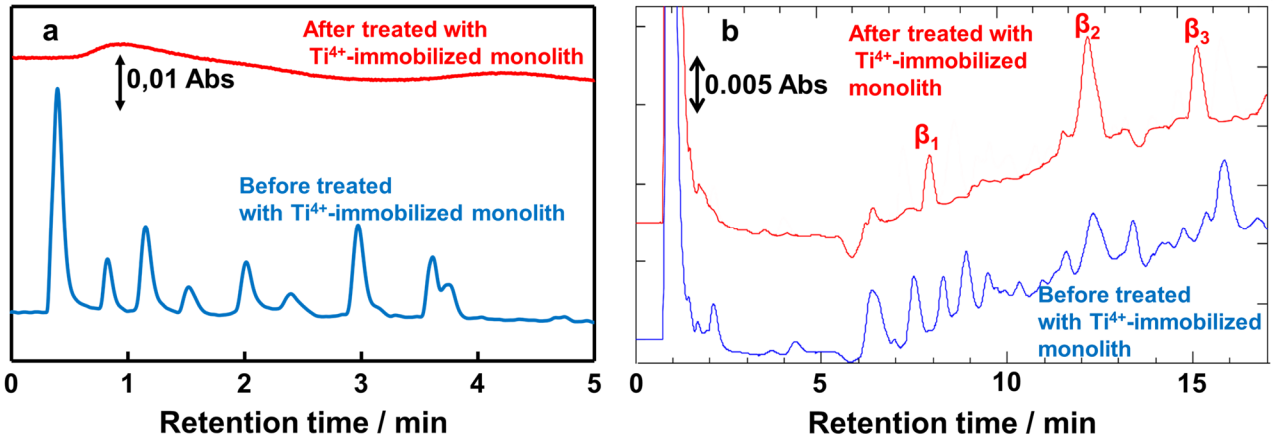

Fig.-6: Chromatogram of separation of cytochrome-c peptides. Mobile phase (A) : 0.065% TFA in water (v/v), mobile phase (B) : 0.05% TFA in ACN : water (4 : 1 v/v), gradient elution : 2.5-80% B (0-10 min), flow rate : 0.20 mL/min, injection volume : 1 µL, wavelength : 214 nm (a). Chromatogram of separation of peptides β-casein. Mobile phase (A) : 0.05% TFA in water (v/v), mobile phase (B) : 0.05% TFA in ACN : water (4 : 1 v/v), gradient elution: 0-53% B (0-10 min), flow rate : 0.10 mL/min, injection volume : 1 µL, wavelength: 214 nm (b).

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