

Synthesis of Thermally Switchable Chromatographic Materials with Immobilized Ti^{4+} for Enrichment of Phosphopeptides by Reversible Addition Fragmentation Chain Transfer Polymerization

Di Wang¹, Zhihan Cao¹, Xinzhu Pang¹, Yulin Deng¹, Bo Li², Rongji Dai^{1,*}

¹Beijing Key Laboratory for Separation and Analysis in Biomedicine and Pharmaceuticals, School of Life Science, Beijing Institute of Technology, Beijing 100081, PR China

²Advanced Research Institute of Multidisciplinary Science, Beijing Institute of Technology, Beijing 100081, PR China

*Corresponding author e-mail: wangdi19920719@163.com

Abstract. Reversible phosphorylation of proteins is one of the most crucial types of post-translational modifications (PTMs). And it shows significant work in diversified biological processes. However, the separation technology of phosphorylated peptides is still an analytical challenge in phosphoproteomics, because phosphopeptides are always in low stoichiometry. Thus, enrichment of phosphopeptides before detection is indispensable. In this study, a novel temperature regulated separation protocol was developed. Silica@p (NIPAAm-co-IPPA)- Ti^{4+} , a new Ti(IV)-IMAC (Immobilized Metal Affinity chromatography) material was synthesized by reversible addition fragmentation chain transfer polymerization (RAFT). By the unique thermally responsive properties of poly(N-isopropylacrylamide) (PNIPAAm), the captured phosphorylated peptides could be released by changing temperature only without applying any other eluent which could damage the phosphopeptides. We employed isopropanol phosphonic acid (IPPA) as an IMAC ligand for the immobilization of Ti(IV) which could increase the specific adsorption of phosphopeptides. The enrichment and release properties were examined by treatment with pyridoxal 5'-phosphate (PLP) and casein phosphopeptides (CPP). Two phosphorylated compounds above have temperature-stimulated binding to Ti^{4+} . Finally, silica@p (NIPAAm-co-IPPA)- Ti^{4+} was successfully employed in pretreatment of phosphopeptides in a tryptic digest of α -casein and human serum albumin (HSA). The results indicated a great potential of this new temperature-responsive material in phosphoproteomics study.

1. Introduction

Protein phosphorylation is a reversible post-translational modification. And it plays important roles in different cellular processes and development, such as cell cycle control, signaling intermediates, division and so forth. [1-4]. Separation, identification and characterization of phosphorylated peptide/properties and phosphorylation sites are essential to phosphoproteomic studies [5]. Technique



based on liquid chromatography mass spectrometry (LC-MS) has become an major method in phosphoproteomic analysis [6,7]. However, phosphopeptides are always in low stoichiometry. And large quantities of unphosphorylated peptides and complex impurities exist in bio-samples, which interfere the detection of phosphopeptides greatly [8]. Therefore, direct analysis of phosphopeptides using LC-MS is not possible without pre-enrichment.

A great many approaches for highly efficient phosphopeptide enrichment have been developed, such as immobilized metal affinity chromatography (IMAC) [9,10], metal oxide affinity chromatography (MOAC) [11], ion exchange chromatography (IEC) [12] and so on. Among them, IMAC is the most frequently used because of its excellent performance [13]. IMAC is composed of substrate, chelating agent and metal ions. Metal ions like Ti^{4+} , Zr^{4+} could coordinate to phosphate groups specificity due to metal(IV) phosphonate chemistry [14]. More than one phosphate molecule are coordinated to one metal ion and more than one metal ion are coordinated with one phosphate group, which result in stable binding between metal(IV) ions and phosphate molecules [15]. Metal ions are immobilized on various substrates through chelating agents and chelating agents effect the performance of IMAC greatly. Nitriilotriacetic acid (NTA) and iminodiacetic acid (IDA) are traditional chelating agents. However, they will cause non-specific adsorption especially the acidic amino acid residues. To promote selectively isolation of phosphopeptides and decrease the non-specific adsorption, chelating agents with phosphate groups has been developed [16]. Phosphate groups in chelating agents could attract to phosphopeptides due to the affinity force. Zhang [17] immobilized Ti^{4+} to magnetic nanoparticles (MNPs) by adenosine triphosphate (ATP). There are three phosphate groups in ATP, which resulting in the increase of the biological compatibility and hydrophilicity. Because more phosphate groups provide more active crosslinked metal phosphonate sites. Therefore, were increased. Although more phosphate groups in chelating agents showing better specificity, multiple synthesis steps and stirring time in immobilization of ATP leading to the destruction of substrates and the decrease of metal irons. Herein, isopropenyl phosphonic acid (IPPA) was chosen as chelating agents. The copolymerization of IPPA and poly(N-isopropylacrylamide) (PNIPAAm) is easy commonly, and could be finished within five hours in one step. Silica substrates used in our experiments is not hard as MNPs, shorter stirring time is benefit to the protection of silica shape.

However, the release of adsorbed proteins require the changing of mobile phases or using eluent. The mobile phase and eluent are always harsh denaturants or contain high concentration of organic solvents sometimes, which lead to the damagement of the activity of analyte and the efficiency of separation [18]. Therefore, altering the mobile phase or using eluent is need to be changed. To solve the above-mentioned problems, we employed a simple approach to release targeted proteins by changing the stationary phase temperature of HPLC only. PNIPAAm polymer plays an significant role in protocol due to its unique thermal-properties. PNIPAAm is a typical temperature-responsive polymer and it has been commonly applied in biomedical applications [19-21] as drug carriers [22,23]. The lower critical solution temperature (LSCT) of PNIPAAm is $32.5^{\circ}C$ and it is similar to physiological temperature. In our protocol, PNIPAAm act as an temperature switch during the release and capture of phosphopeptides. Molecular conformation and hydrophilic/hydrophobic properties of PNIPAAm have a sharp change around LCST [24]. When the temperature is lower than LCST, the polymer chain will be extend. Therefore, the functional groups modified on the polymer chains will be exposed and reacted with target proteins sufficiently to capture the target proteins. However, when temperature is higher than LCST, polymer chain will have transformed from stretch to curl up shape in aqueous media. And the space around the silica surface will reduced. Captured proteins will be released by the extrusion of polymer chains. Recently, our group have synthesized a series of switchable materials based on PNIPAAm for capture, separation and enrichment of proteins/peptides by changing temperature only [25-28].

Herein, a novel modified temperature-responsive chromatographic stationary material silica@p (NIPAAm-co-IPPA)- Ti^{4+} was synthesized by reversible addition fragmentation chain transfer polymerization (RAFT). IPPA was chosen as the chelating agents for the highly efficient enrichment of phosphopeptides. The copolymerization of IPPA and NIPAAm is easy and timesaving leading to an

unbroken shape of silica. To obtain a better temperature-sensitivity, grafting conformations and detection pH were optimized. Efficiency of protein enrichment was examined by using pyridoxal 5'-phosphate (PLP) and casein phosphopeptides (CPP) as model phosphonate molecule. To further evaluate their performance on the enrichment of phosphopeptides in complex sample matrices, it was applied for enrichment of a tryptic digest of HSA and a-casein. This was the first time that thermal-responsive chromatography materials was used in enrichment of phosphopeptides.

2. Materials and methods

2.1. Reagents and materials

CPP, a-casein and HSA were purchased from Sigma-Aldrich. N-isopropylacrylamide (NIPAAm) was obtained from TCI (shanghai, China). Azodiisobutyronitrile (AIBN), (4-pyridyl) dimethylamine (DMAP), N, N'-diisopropylcarbodiimide (DIC), formic acid (FA) and trypsin were obtained from J&K Chemical (Beijing, China). 3-Aminopropyl silica (silica@NH₂, diameter, 5 μm; pore size, 100 Å) was purchased from Born-Again technologies (Tianjin, China). Hydrocortisone, prednisolone acetate, dexamethasone, hydrocortisone butyrate was purchased from National Institute for Food and Drug Control (Beijing, China). Urea ammonium bicarbonate, Dithiothreitol (DTT) and Indole-3-acetic acid (IAA) were purchased from Sinopharm Chemical Reagent Co. Ltd (Beijing, China). Adenosine monophosphate (AMP) and N-Acryloxysuccinimide (NAS) were purchased from Alfa Aesar Chemical (Tianjin, China).

2.2. Synthesis of silica@CTA

3-Aminopropyl silica (1.5 g), N-acetyl glycine (0.809 g, 0.55 mmol), S-1-Dodecyl-S'-(α,α'-dimethyl-α''-acetic acid) dithiocarbonate (CTA) [18] (0.486 g, 1.34 mmol), DMAP (0.6 g, 4.9 mmol) were mixed in dichloromethane (60 mL) in a round bottom flask. Grafting density of polymers were controlled by the molar ratio of CTA and DMAP, and it was marked after the name of silica, e.g. silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ 50% means the density of material is 50%. Reaction mixture was cooled to 10 °C under nitrogen. After vacuuming and purging the reaction mixture with nitrogen three times, DIC (6 mL) was then added over 30 mins. The reaction was stirred for 48 hrs at room temperature and then exposed to air to stop the reaction. The products were washed with dichloromethane, methanol and ethanol three times. Silica@CTA was then generated after drying 8 hrs at 60 °C.

2.3. Synthesis of synthesis of silica@p (NIPAAm-co-IPPA)-Ti⁴⁺

Silica@CTA (0.3 g), NIPAAm (2.165g, 19.13 mmol), IPPA (0.240g, 1.92 mmol) and DMF(12 mL) were mixed in a round bottom flask with three necks. The reaction mixture was cooled to 10 °C under nitrogen. After vacuuming and purging the reaction mixture with nitrogen three times, AIBN (25.0 mg, 0.088 mmol) was then added. The reaction was stirred for 5 hrs at 70 °C and then exposed to the air to stop the reaction. The products were washed with DMF, ethanol and deionized water several times. Silica@p (NIPAAm-co-IPPA) was then dried 8 hrs at 40 °C.

Silica@p (NIPAAm-co-IPPA) were incubated in an 100 mM Ti(SO₄)₂ solution for 4 h at room temperature to immobilize Ti⁴⁺ cations. Finally, the prepared silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ were washed with 0.1% (v/v) formic acid (FA) three times and storage in 0.1% (v/v) FA.

2.4. Sample preparation

HSA and a-casein (1mg) were dissolved in 1 mL denaturing buffer and incubated for 4 h. The denaturing buffer was composed of 50 mM ammonium bicarbonate and 8 M urea. After that, 20 μL DTT (10 mM) was added and the solution was incubated for 2 hrs at 37 °C. Then, 10 μL IAA (100 mM) was added and the obtained solution was incubated for one hour in the dark at 30 °C. Then, the mixture was diluted by 8-fold ammonium bicarbonate (50 mM) and incubated for one day at 37 °C

with trypsin and the ratio of enzyme and substrate was 1:50 (w/w). After the desalinization by SPE column, we lyophilized the peptide solution and stored the obtained peptide at -40°C .

2.5. Property examination of the thermal-responsive materials

Chromatographic stationary phase columns was made by our own. Silica materials synthesized as the above-mentioned methods were pushed into the Stainless-Steel column ($2.1\text{ mm} \times 50\text{ mm i.d}$) with methanol as the solvent under the high-pressure pump. Properties of the materials are examined as HPLC columns. The columns were connected with HPLC with UV detector and column temperature controller. The ability of the columns to retain four steroids at different temperatures were approved. Phosphorylated small molecules (PLP) and macromolecules (CPP) were employed to investigate the temperature-controlled capture and release properties of silica@*p* (NIPAAm-co-IPPA)-Ti⁴⁺.

2.6. Instrumentation

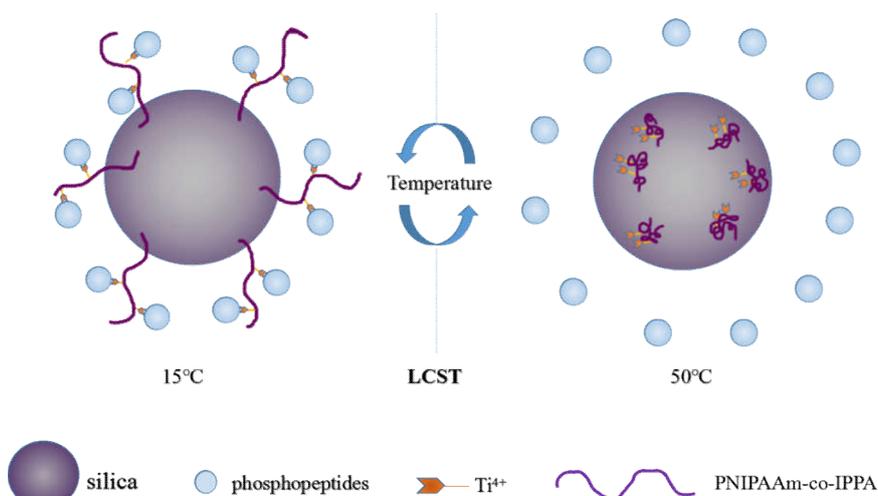
Fourier transform infrared (FTIR) spectra were obtained on PerkinElmer (Boston, MA) FTIR spectrometer. All HPLC spectra were obtained on Shimadzu LC-20AT HPLC with Shimadzu SPD-20A UV detector. Temperature was controlled by CTO-20AC column controller (Shimadzu, Japan). X-ray photo electron spectroscopy (XPS) spectra were obtained using PHI Quantera II (Ulvac-PHI). Scanning electron microscope (SEM) results were obtained using JSM-5600LV (Agilent Technologies, USA). Thermal Gravimetric Analyzer results were obtained using DISCOVERY (TA, USA).

3. Results and discussion

3.1. Preparation and characterization of materials

In phosphoproteomics analysis, HPLC/MS methods have been widely used to discovered and identified phosphopeptides. However, phosphopeptides were sub stoichiometry and their signals were always disturbed by abundant proteins. Therefore, a great number of enrichment methods and materials have been developed. Traditionally, the enriched phosphopeptides are released by changing mobile phase or eluant. However, they always damage the activity of phosphopeptides. This is one of the major hinderance in phosphoproteomics analysis. Therefore, a switchable chromatographic materials were synthesized for selectively capture and release of phosphopeptides with simpler changing temperature instead of the conventional changing of mobile phase.

Silica@*p* (NIPAAm-co-IPPA)-Ti⁴⁺ was prepared via surface initiated Reversible Addition-Fragmentation Chain Transfer Polymerization (RAFT) according to the procedures shown scheme 1. The shape of silica was protected well and there was no difference of silica before (figure 2a) and after (figure 2b) synthesized. The modified polymers have been successfully grafted onto silica surfaces and it was confirmed by FTIR (Figure 3) and XPS (Table 1) spectra of the materials. The ratio of modified polymer P(NIPAAm-co-IPPA) on silica was further determined using TGA in a quantitatively way. And the principle of capture and release was shown in figure 1.



Scheme 1. Synthesis of thermal-responsive polymer modified silica. The yellow dot represents silica.

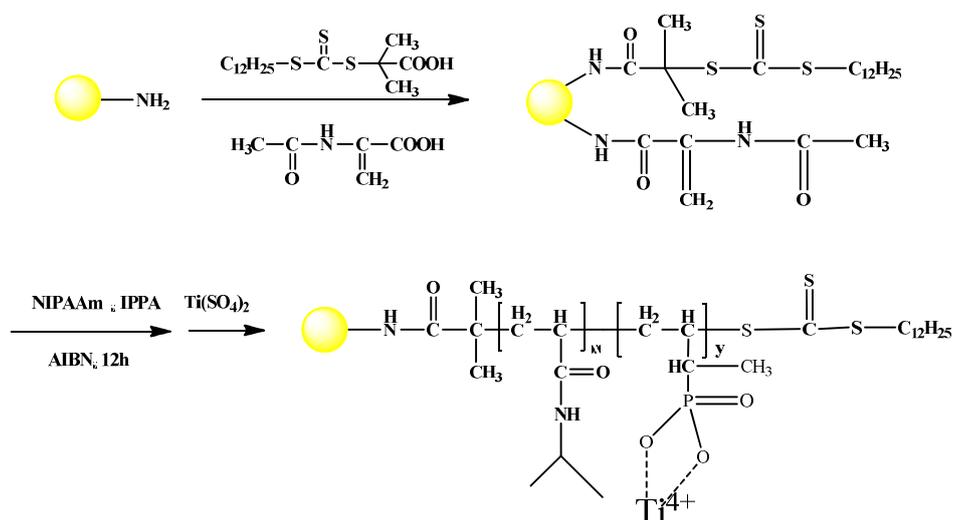


Figure 1. The working principle of silica@p(NIPAAm-co-IPPA)- Ti^{4+} .

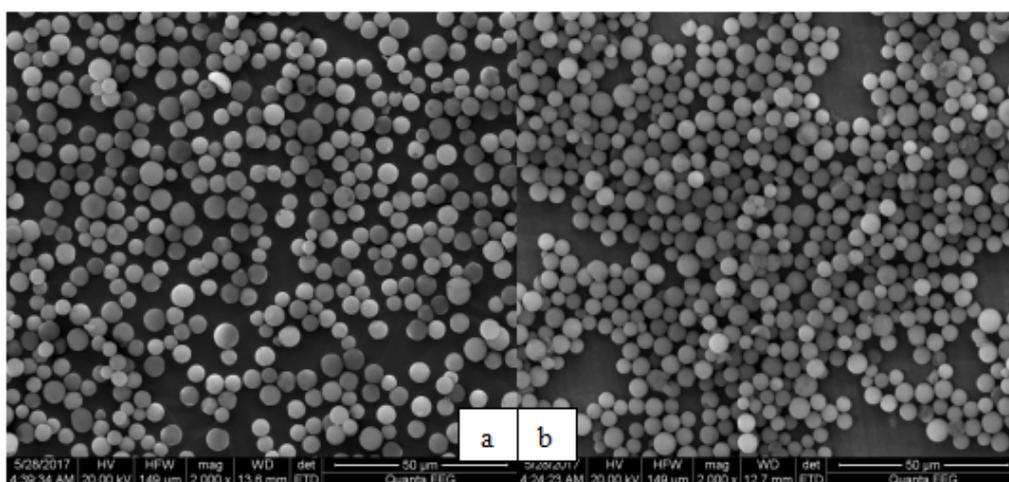


Figure 2. SEM of silica@ NH_2 (a) and silica@p(NIPAAm-co-IPPA)- Ti^{4+} (b).

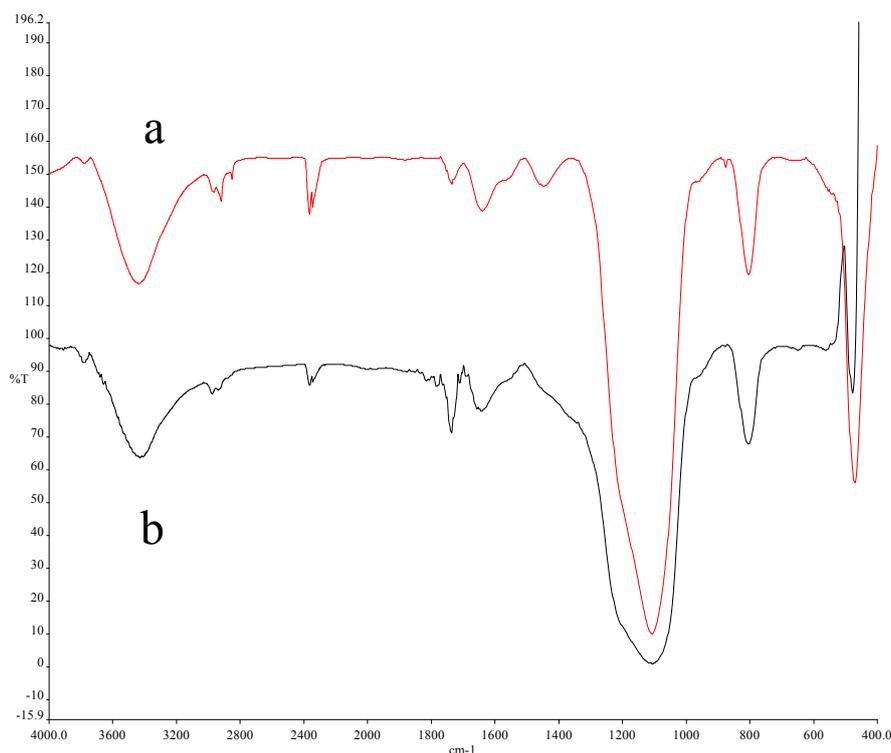


Figure 3. FTIR of silica@PNIPAAm and silica@p(NIPAAm-co-IPPA). a: silica@p(NIPAAm-co-IPPA). b: silica@PNIPAAm.

Table 1. The XPS results of silica@NH₂, silica @CTA50% and silica@p(NIPAAm-co-IPPA)-Ti⁴⁺ 50%

Element	C1s (%)	O1s (%)	N1s (%)	S2p (%)	P2p (%)	Ti 2p (%)
silica@NH ₂	39.61	58.94	1.45	0.00	0.00	0.00
silica @CTA 50%	46.50	49.68	2.37	1.44	0.00	0.00
silica@p(NIPAAm-co-IPPA)-Ti ⁴⁺ 50%	39.67	51.90	4.14	1.83	0.62	1.83

3.2. Temperature-responsive chromatography for steroids separation

We selected four steroids Hydrocortisone, dexamethasone, hydrocortisone butyrate, and prednisolone acetate as analytes to evaluate the separation and thermal-responsive performance of the silica materials. Silica@p(NIPAAm-co-IPPA)-Ti⁴⁺ was packed into stainless steel column [29,30]. The separation was investigated at 10 °C, 30 °C and 50 °C (Figure 4). Retention time of the steroids was prolonged as the temperature increased. As shown in figure 4, the number of peaks were increased with the increasement of temperature. In figure 4a, steroids had no retention on the column and there was only one peak at 10 °C. Four steroids were totally separated and there were 4 peaks occurred at 50°C. Therefore, four steroids were separated effectively by changing the stationary phase temperature only. The results indicated an excellent thermal-responsive ability of silica@p(NIPAAm-co-IPPA)-Ti⁴⁺.

The separation properties of materials was resulted from the configuration change of polymer chains mentioned above. And the configuration was related to the hydrogen bonds which generated between H₂O and amide group of PNIPAAm [27]. However, as for silica@p(NIPAAm-co-IPPA)-Ti⁴⁺, there were metal ions modified on the polymer chains which damage the hydrogen bonds. And the thermal-responsive properties would get worse at the present of Ti⁴⁺. As shown in figure 5, there were

chromatograms of steroids separated by materials with different Ti^{4+} content. By the increase of Ti^{4+} , the retention time of steroids was shorter. Therefore, the grafting density and ratio of silica@p (NIPAAm-co-IPPA)- Ti^{4+} should be optimized before other detection. And the results was shown in figure 6 and 7. The best grafting condition was around 50% (grafting density) and 21.8% (grafting ratio) (Figure 7c) and it was different form our previous studies [31].

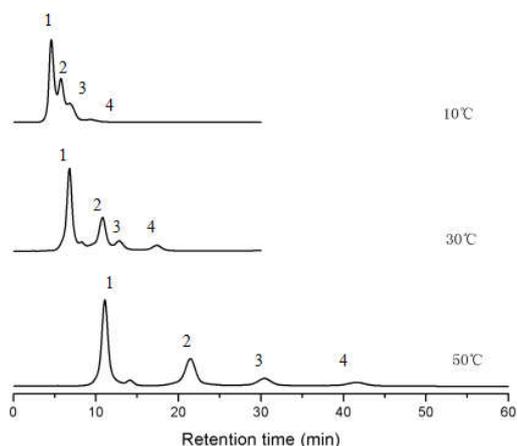


Figure 4. Chromatogram of steroids separated on silica@p (NIPAAm-co-IPPA)- Ti^{4+} columns. Mobile phase used is water; flow rate, 0.1 mL/min; Peaks: 1, hydrocortisone; 2, dexamethasone; 3, hydrocortisone butyrate; 4, prednisolone acetate. detection wavelength, 254 nm.

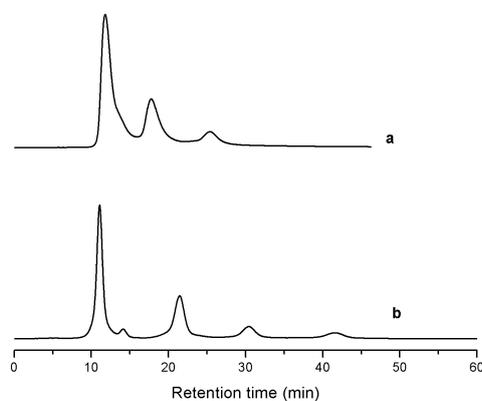


Figure 5. Chromatogram of steroids separated on polymer modified silica columns. a: silica@p (NIPAAm-co-IPPA)- Ti^{4+} ; b: silica@p (NIPAAm-co-IPPA). The temperature is 50°C. Mobile phase used is water; flow rate, 0.1 mL/min; detection wavelength, 254 nm. Peaks: 1, hydrocortisone; 2, dexamethasone; 3, hydrocortisone butyrate; 4, prednisolone acetate.

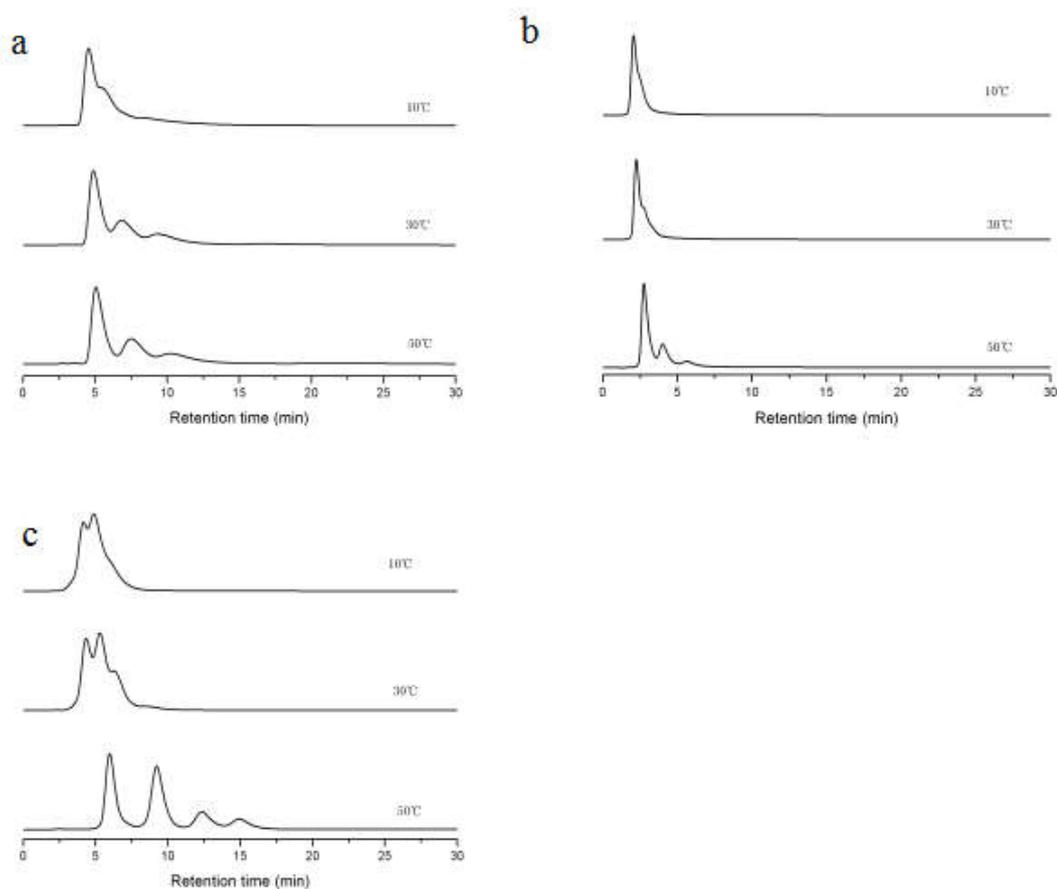


Figure 6. Chromatogram of steroids separated on silica@*p*(NIPAAm-*co*-IPPA)-Ti⁴⁺ columns. The grafting density is 30%, the grafting ratio is a.7.4%; b.15.2%; c.18.0%. Mobile phase used is water; flow rate, 0.1 mL/min; detection wavelength, 254 nm. Peaks: 1, hydrocortisone; 2, dexamethasone; 3, hydrocortisone butyrate; 4, prednisolone acetate.

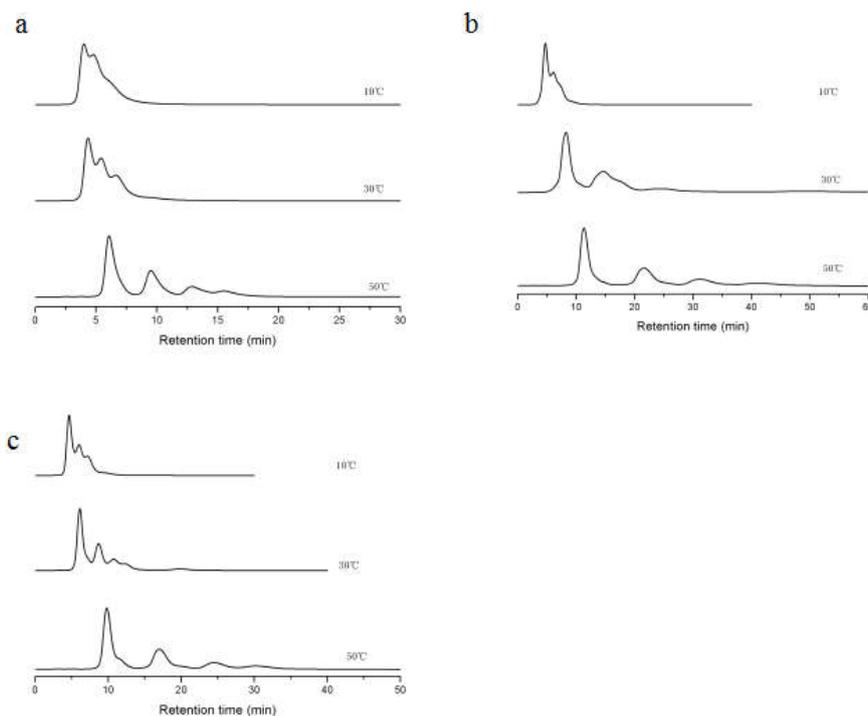


Figure 7. Chromatogram of steroids separated on silica@p(NIPAAm-co-IPPA)-Ti⁴⁺ columns. The grafting density is 50%, the grafting ratio is a.8.0%; b.14.1%; c.21.8%. Mobile phase used is water; flow rate, 0.1 mL/min; detection wavelength, 254 nm. Peaks: 1, hydrocortisone; 2, dexamethasone; 3, hydrocortisone butyrate; 4, prednisolone acetate.

3.3. Separation of adenosine monophosphate (AMP) and *N*-Acryloxysuccinimide (NAS)

After the optimization of grafting conformation, silica@p(NIPAAm-co-IPPA)-Ti⁴⁺ was used in separation of phosphate compounds. Adenosine monophosphate (AMP) and *N*-Acryloxysuccinimide (NAS) were chosen as analytes. AMP, a common biochemical reagent with a phosphate group was widely used in biochemistry. And NAS was chosen as a contrast agent without phosphate group. As shown in figure 8, there was only one peak in chromatogram at 50°C which indicated that AMP and NAS were not separated. However, as the decrease of column temperature, two compounds were separated totally and shown as two peaks in chromatogram at 10°C. The results could be explained by the configuration changed at different temperature. The polymer chains were crimp at 50°C and the Ti⁴⁺ modified on chains was masked. Therefore, AMP could not react with Ti⁴⁺. However, the situation was totally changed at 10°C. The polymer chains were stretched and Ti⁴⁺ exposed sufficiently when the temperature lower than LCST, which resulted in the retention of AMP. Therefore, AMP was separated from NAS. The results shown that silica@p(NIPAAm-co-IPPA)-Ti⁴⁺ could separate phosphate compounds by changing temperature.

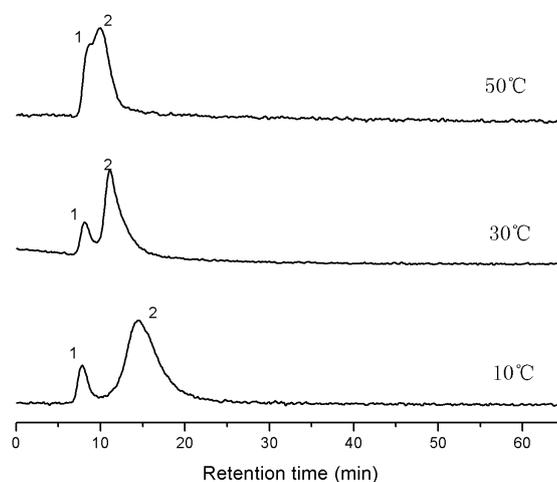


Figure 8. Chromatogram of NAS and AMP. Peak 1: NAS. Peak 2: AMP. From top to bottom, the column temperature is 50°C, 30°C, 10°C. Mobile phase: pH 3.2, ACN 50% and AC.

3.4. Optimization of detection condition

The pH of mobile phase in separation of adenosine monophosphate (AMP) and N-Acryloxysuccinimide (NAS) is 3.2 (50%ACN and AC), which was the best pH in previous study about Ti^{4+} -IMAC. However, as for the temperature-responsive materials, the pH influenced the properties of capture and release. Therefore, pH of mobile phase must be optimized before employing. As shown in figure 9, same volume pyridoxal 5'-phosphate (PLP) was injecting at very beginning at different pH, and it shown different retention time. From pH 7 to 3, the retention time was getting longer which is similar to previous study. However, PLP shown no retention when pH was lower to 1. This could be explained by acid-base principle. When the mobile phase is extremely acidity, the protonation degree of the phosphate group is enhanced and the hydroxyl group will be present in the form of H^+ . Therefore, phosphate group could be captured by Ti^{4+} . The result indicated that the best capture pH of mobile phase was 3.

However, the final performance of silica@p (NIPAAm-co-IPPA)- Ti^{4+} column was determined by not only capture ability but also the release ability. And figure 10 shown the release ability of column. As shown in figure 10d, NAS and PLP were injected at beginning together when the temperature was 10°C. The NAS shown no retention and it was rapidly eluted from the column and shown as a single peak at about 5min (figure 10d). There was no peak of PLP before 20min which indicated that PLP was captured by column at 10°C. When we are altering pH from 7 to 9 at 20min. The captured PLP was eluted and shown as a peak from 40 to 50 min. However, when we released PLP by increasing temperature from 10°C to 50°C after 30 minutes (figure 10 a,b,c), released quantity of PLP (peak area) was related to pH greatly. And the best released pH was 7 because the peak area of PLP was the biggest among figure 10a, b, c. Although PLP was captured greatly at pH 3, there shown no peak when we release the PLP by changing temperature (figure 10a). Considering all these two effects, the optimized pH was 7 and we chose water as the mobile phase of silica@p (NIPAAm-co-IPPA)- Ti^{4+} .

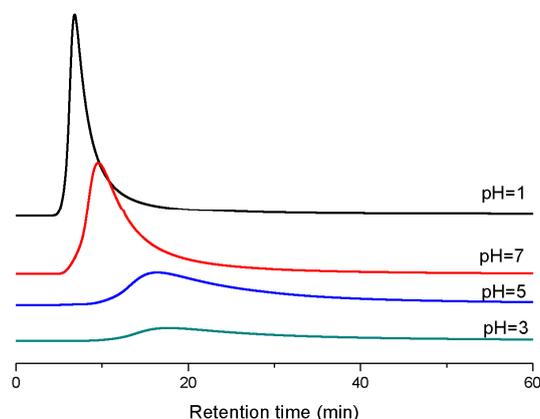


Figure 9. Chromatogram of PLP separated on polymer modified silica columns at different pH. flow rate, 0.1 mL/min; detection wavelength, 290nm.

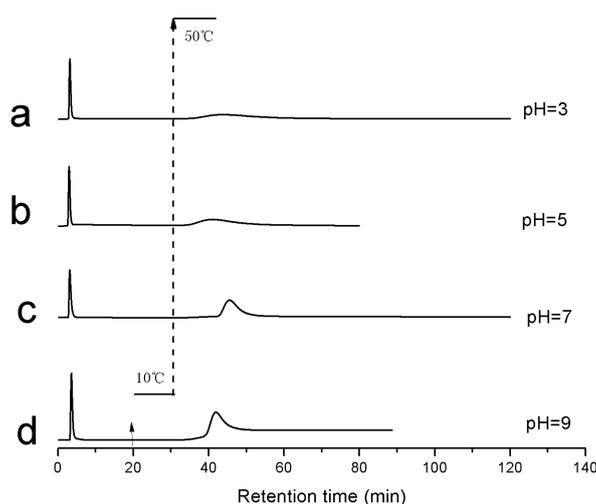


Figure 10. Chromatogram of NAS and PLP separated on polymer modified silica columns at different pH. flow rate, 0.1 mL/min; detection wavelength, 290 nm.

3.5. Separation of casein phosphopeptides (CPP) using silica@p (NIPAAm-co-IPPA)-Ti⁴⁺

As for silica@p (NIPAAm-co-IPPA)-Ti⁴⁺, Ti⁴⁺ could capture phosphopeptides due to the coordination chemistry. CPP, a typical phosphopeptides, was widely used as the analyte in phosphonate proteomics and chose as a model protein here. We chose 10 °C to 50 °C as the temperature range, which will not damage the proteins. As shown in Figure 11a, CPP was injected at the very beginning. There was no peak observed within 120 mins when the sample was injected at 10 °C. This indicated that CPP was captured completely by the column at 10 °C. However, when the sample was injected at 10 °C and temperature was increased to 50 °C after 30 minutes, there was one sharp peak observed between 30 to 40 mins (Figure 11b). There was no peak before 30 min which indicated CPP was captured completely. When temperature was increased to 50 °C, CPP was released rapidly and shown as a sharp peak. The release and capture of CPP was controlled by temperature only in above, and temperature seem to act as a switchable signal. These results indicated that silica@p (NIPAAm-co-IPPA)-Ti⁴⁺, the polymer modified silica with Ti⁴⁺, showed thermally controlled properties to efficiently capture and rapidly release phosphopeptides without changing the constitute of the mobile phase.

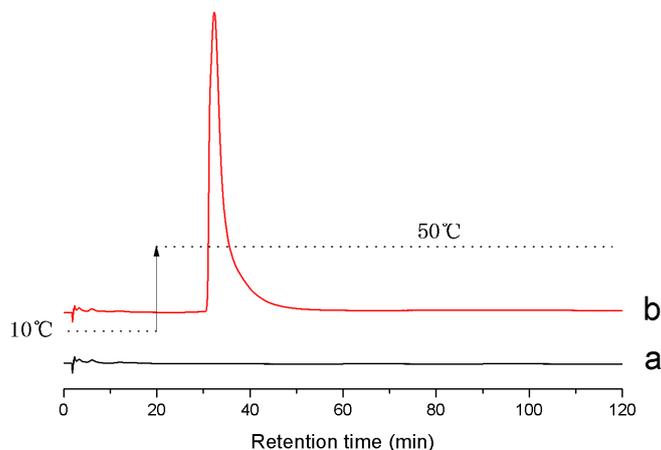


Figure 11. Chromatogram of CPP on silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ column. a: injecting the sample at 10 °C and increasing temperature to 50 °C at 30 min. b: injecting the sample at 10 °C and stay in the temperature 2 hours. Flow rate, 0.1 mL/min; detection wavelength, 254 nm. mobile phase: water.

The enrichment efficiency of CPP on silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ column was further examined. As shown in figure 12b, we injected CPP solution three times into the column at 10 °C. And the impurities eluted quickly and shown as three small peaks around 0,20 and 40 min. However, there were no peaks of CPP at 10 °C which indicated that it was retained by silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ column. And when we rose temperature to 50 °C, the captured CPP was released rapidly with an increased peak area (Fig. 12b). The release peak in figure 12b is much bigger than that in figure 12a which was injected only one time. The results shown that it was possible to enrich phosphopeptides via a thermally controlled capture and release mechanism.

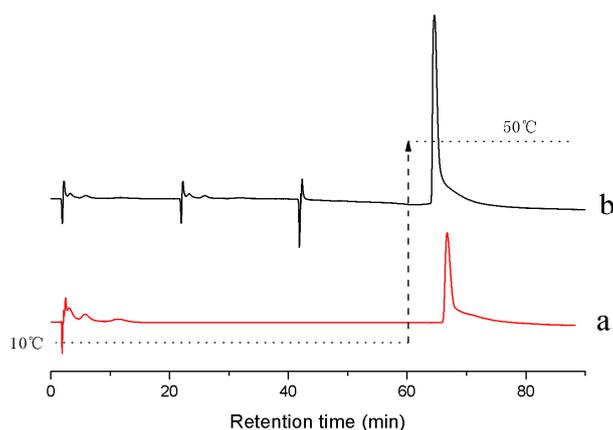


Figure 12. Chromatogram of CPP on silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ column at different sample size. Sample size: a:3uL, one injection (red line); b:8uL, three injections (black line). flow rate, 0.1 mL/min; mobile phase: water. detection wavelength, 254 nm.

3.6. Separation of tryptic digest of α -casein and human serum albumin (HSA)

As for the detection of phosphopeptides, it is important to removal the abundant proteins from the complex biological matrices. The human serum is one of the most complex bio-sample and HSA is the most abundant proteins in it. To simulate the detection of phosphopeptides in human serum. HSA and

α -casein were mixed together at a concentration ratio of 1:5. The silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ column was employed for separation of phosphopeptides in figure 13a, there was no retention at 50 °C and the sample was rapidly eluted from the column as a single peak. And the same trend was observed in figure 13c which the detection temperature was 10°C. However, the peak area at 10°C (figure 13c) was smaller than that in 50°C (figure 13a) and this indicated that the phosphopeptides were captured in the column. When we injected the sample at 10°C and raised the temperature to 50°C at 25min, the captured phosphopeptides was released and shown as a peak occurred between 30 to 32min (figure 13b). The results shown that the capture and release of the phosphopeptides using silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ column could be done by changing temperature simply without changing the mobile phase or using another eluent. It was a green and facile protocol in proteomics.

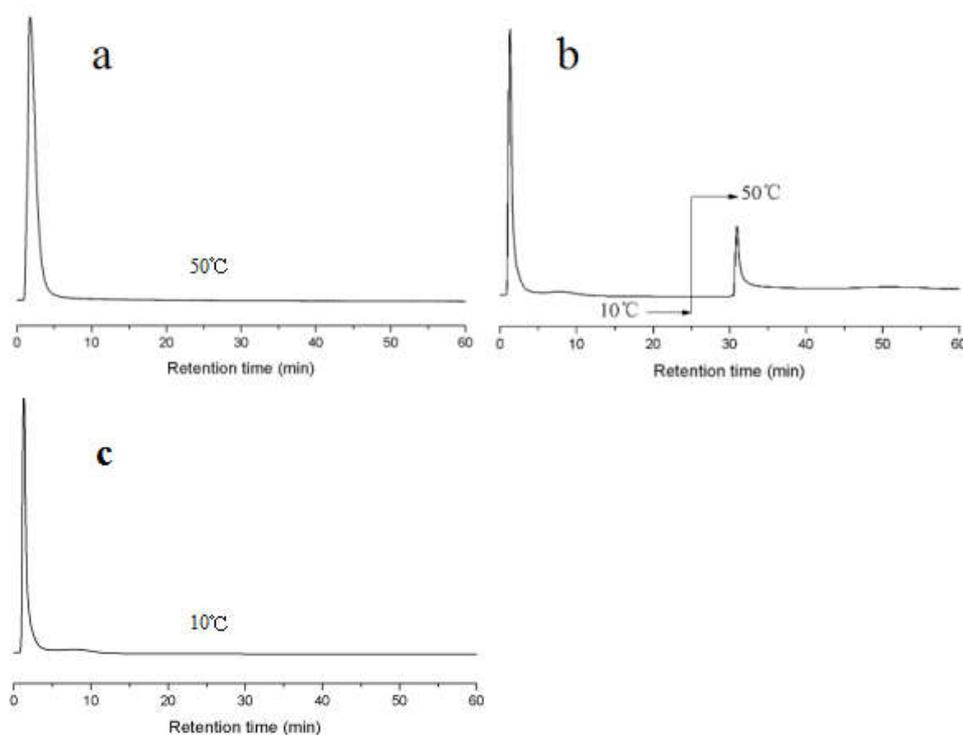


Figure 13. Chromatogram of CPP and HSA on silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ column. a: 50°C; b: from 10°C to 50°C; c:10°C. flow rate, 0.1 mL/min; detection wavelength, 254 nm. mobile phase: water.

4. Conclusion

In conclusion, we prepared a novel temperature-responsive chromatographic material silica@p (NIPAAm-co-IPPA)-Ti⁴⁺. The capture and release of phosphopeptides is achieved by changing temperature only. This is a simpler way compared to altering the mobile phase or using eluent. The grafting configuration and the pH of mobile phase were optimized to achieve better temperature-dependent retention changes for steroids, pyridoxal 5'-phosphate (PLP) and casein phosphopeptides (CPP). The mobile phase was water in our experiments which is an environment-friendly reagent. Finally, the column was tested for a bio-sample analysis of a tryptic digest of HSA and α -casein. The phosphopeptides were captured efficiently and released rapidly by changing temperature only. The results indicated that silica@p (NIPAAm-co-IPPA)-Ti⁴⁺ have great potential for the enrichment of phosphorylated proteins from biological samples in a temperature-regulated way.

Acknowledgments

The authors acknowledge the financial support from National Natural Science Foundation of China (81503353) and National Special Instrument and Equipment Development (2013YQ03059514).

References

- [1] J. Ptacek, G. Devgan, G. Michaud, H. Zhu, X. Zhu, J. Fasolo, H. Guo, G. Jona, A. Bretkreutz, R. Sopko, R.R. McCartney, M.C. Schmidt, N. Rachidi, S.J. Lee, A.S. Mah, L. Meng, M.J.R. Stark, D. Stern, C.D. Virgilio, M. Tyers, B. Andrews, M. Gerstein, B. Schweitzer, P.F. Predki, M. Snyder, Global analysis of protein phosphorylation in yeast, *Nature* 438 (2005) 679–684.
- [2] S. Morandell, T. Stasyk, K. Grosstessner-Hain, E. Roitinger, K. Mechtler, G.K. Bonn, L.A. Huber, Phosphoproteomics strategies for the functional analysis of signal transduction, *Proteomics* 6 (2006) 4047–4056.
- [3] K. Schmelzle, F.M. White, Phosphoproteomic approaches to elucidate cellular signaling networks, *Curr. Opin. Biotechnol.* 17 (2006) 406–414.
- [4] J.G. Abelin, P.D. Trantham, S.A. Penny, A.M. Patterson, S.T. Ward, W.H. Hildebrand, M. Cobbold, D.L. Bai, J. Shabanowitz, D.F. Hunt, Complementary IMAC enrichment methods for HLA-associated phosphopeptide identification by mass spectrometry, *Nat. Protoc.* 10 (2015) 1308–1318.
- [5] T.E. Thingholm, O.N. Jensen, M.R. Larsen, Analytical strategies for phosphoproteomics, *Proteomics* 9 (2009) 1451–1468.
- [6] Ficarro, S. B., et al. Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nature Biotechnology* 20 (2002) 301–5.
- [7] Tao, W. A., et al. Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. *Nature Methods* 2 (2005) 591–597.
- [8] J.D. Dunn, G.E. Reid, M.L. Bruening, Techniques for phosphopeptide enrichment prior to analysis by mass spectrometry, *Mass Spectrom. Rev.* 29 (2010) 29–54.
- [9] Nühse, Thomas S., et al. Large-scale Analysis of in Vivo Phosphorylated Membrane Proteins by Immobilized Metal Ion Affinity Chromatography and Mass Spectrometry. *Molecular & Cellular Proteomics* 2 (2003) 1234–1243.
- [10] Gaberc-Porekar, V., et al. Perspectives of immobilized-metal affinity chromatography. *Journal of Biochemical & Biophysical Methods* 49 (2001) 335–340.
- [11] Yang D S, Ding X Y, Min H P, et al. Design and synthesis of an immobilized metal affinity chromatography and metal oxide affinity chromatography hybrid material for improved phosphopeptide enrichment. *Journal of Chromatography A*, 7 (2017) 56–62.
- [12] Alpert, A. J., O. Hudecz, and K. Mechtler. Anion-exchange chromatography of phosphopeptides: weak anion exchange versus strong anion exchange and anion-exchange chromatography versus electrostatic repulsion-hydrophilic interaction chromatography. *Analytical Chemistry*. 87 (2015) 4704–11.
- [13] C.L. Nilsson, Advances in quantitative phosphoproteomics, *Anal. Chem.* 84 (2012) 735–746.
- [14] Nonglaton, G, et al. New approach to oligonucleotide microarrays using zirconium phosphonate-modified surfaces. *Journal of the American Chemical Society*. 126 (2004) 1497–502.
- [15] Zhou, H., et al. Specific phosphopeptide enrichment with immobilized titanium ion affinity chromatography adsorbent for phosphoproteome analysis. *Journal of Proteome Research*. 7 (2008) 3957.
- [16] Wang, Qian, et al. Pyridoxal 5'-phosphate mediated preparation of immobilized metal affinity material for highly selective and sensitive enrichment of phosphopeptides. *Journal of Chromatography A*. 1499 (2017) 30–37.
- [17] Zhang, L., et al. "Synthesis of adenosine functionalized metal immobilized magnetic nanoparticles for highly selective and sensitive enrichment of phosphopeptides." *Chemical Communications*. 48 (2012) 6274–6279.

- [18] Z. Deng, M. Dong, Y. Wang, H.F. Zhou, M.L. Ye, Biphasic Affinity Chromatographic Approach for Deep Tyrosine Phosphoproteome Analysis, *Analytical chemistry*. 89 (2017) 2405-2410.
- [19] Q. Zhang, N Tang, J. Brock, Enrichment and analysis of nonenzymatically glycosylated peptides: Boronate affinity chromatography coupled with electron-transfer dissociation mass spectrometry, *Journal of Proteome Research* 6 (2007) 2323-2330.
- [20] E.S. Gil, S.M. Hudson, Stimuli-responsive polymers and their bioconjugates, *Progress in Polymer Science*. 29 (2004) 1173-1222.
- [21] H. Kanazawa, T. Okano, Temperature-responsive chromatography for the separation of biomolecules, *Journal of Chromatography A*. 1218 (2011) 8738-8744.
- [22] M.T. Calejo, S.A. Sande, N.Bo, Thermoresponsive polymers as gene and drug delivery vectors: architecture and mechanism of action, *Expert Opin Drug Deliv*. 10 (2013) 1669-86.
- [23] H. Betre, W. Liu, M.R. Zalutsky, A thermally responsive biopolymer for intra-articular drug delivery, *Journal of Controlled Release*. 115 (2006) 175-182.
- [24] Gil, Eun Seok, and S. M. Hudson. "Stimuli-responsive polymers and their bioconjugates." *Progress in Polymer Science*. 29 (2004) 1173-1222.
- [25] Z. Liu, F. Geng, X. Ha, Evaluation of Temperature-Responsive Open Tubular Capillary Electrochromatographic Column Modified with Poly(N-isopropylacrylamide), *Chromatographia*. 76 (2013) 201-206.
- [26] Z. Liu, K. Ullah, L. Su, Switchable boronate affinity materials for thermally modulated capture, separation and enrichment of cis-diol biomolecules, *Journal of Materials Chemistry*. 22 (2012) 18753-18756.
- [27] Z. Liu, R. Su, X. Liang, A thermally switchable chromatographic material for selective capture and rapid release of proteins and nucleotides, *Rsc Advances*. 4 (2014) 15830-15834.
- [28] R.J. Dai, C. Lei, Z. Liu, Preparation and characterization of temperature-responsive chromatographic column containing poly(N-isopropylacrylamide) and poly([2-(methacryloyloxy)-ethyl] trimethylammonium chloride), *Journal of Applied Polymer Science*. 121 (2011) 2233-2238.
- [29] K. Nagase, A.M. Akimoto, J. Kobayashi, A. Kikuchi, Y. Akiyama, H. Kanazawa, T. Okano, Effect of reaction solvent on the preparation of thermoresponsive stationary phase through a surface initiated atom transfer radical polymerization, *Journal Chromatogr A*. 1218 (2011) 8617-8628.
- [30] M. Seino, K. Yokomachi, T. Hayakawa, R. Kikuchi, M.A. Kakimoto, S. Horiuchi, Preparation of poly(N-isopropylacrylamide) grafted silica bead using hyperbranched polysiloxysilane as polymer brush and application to temperature-responsive HPLC, *Polymer*. 47 (2006) 1946-1952.
- [31] Y.Y. Shen, L.L. Dong, Y.L. Liang, Z.J. Liu, R.J. Dai, W.W. Meng, Y.L. Deng, Effect of the grafting ratio of poly(N-isopropylacrylamide) on thermally responsive polymer brush surfaces, *Journal of Separation Science*. 40 (2016) 254-531.