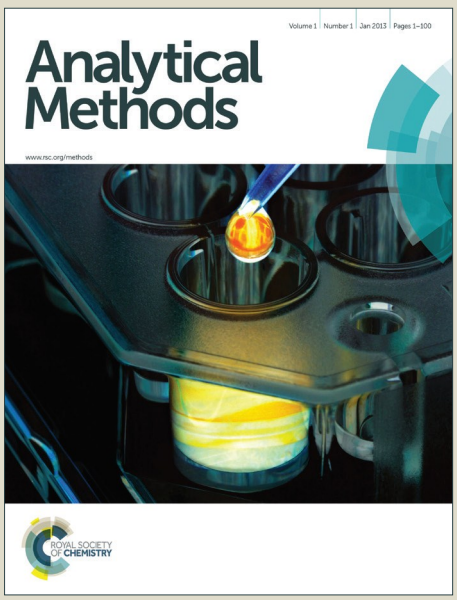
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

A trypsin aptamer modified silica particle was prepared by surface initiated atom transfer radical polymerization for proteome identification

Yun Wang, Xuefei Lv*, Yu Li, Guang Peng, Javed Iqbal and Yulin Deng

School of Life Science, Beijing Institute of Technology, No. 5 Zhongguancun Nandajie, Haidian District, Beijing 100081, People's Republic of China

*Corresponding author: Dr. Xuefei Lv

Tel: 0086-10-68914607

E-mail: xuefeilv@163.com

Address: School of Life Science and Technology, Beijing Institute of Technology, No. 5 Zhongguancun Nandajie, Haidian District, Beijing 100081, People's Republic of China

Abstract

Highly efficient and complete protein digestion is vital for achieving accurate protein quantification. However, the efficiency and completeness of the digestion obtained by conventional protease free digestion is not satisfactory for extremely complicated proteomic samples. In this study, a new method of trypsin immobilization based aptamer is developed using surface initiated atom transfer radical polymerization (ATRP) as linker (TAMSP-ATRP), and bovine serum albumin (BSA) was chosen as target to investigate the enzymatic performance of ATRP modified aptamer-silica. The digestion efficiency, repeatability and recovery of the TAMSP-ATRP were evaluated by mass spectrometry (MS) analysis. Highly efficient digestion was achieved by using TAMSP-ATRP only 2 min. Compared with traditional methods (glutaric dialdehyde as linker and free-trypsin), ATRP reaction as linker obtained BSA coverage of 62.77%, with 33 identified peptides with 0 miss cleavage, which is much better than that of glutaric dialdehyde modified trypsin digestion (sequence coverage of 33.8 % and identified 0 miss cleavage peptide number of 17) and the trypsin free digestion (coverage of 58.87% and identified 0 miss cleavage peptides of 30). In addition, the human serum was digested by TAMSP-ATRP in 2 min and free digestion in 16 h. For the TAMSP-ATRP method, 45 proteins were identified, compared to 34 proteins in free-digestion, indicating digestion efficiency improved. All these results demonstrated that aptamer could serve as a potential medium for the immobilization of trypsin to enhance protein digestion efficiency. Moreover, the TAMSP-ATRP was easily removed from the digestion solution. With further development,

TAMSP-ATRP can be used in ^{18}O labeling protein quantitation with the performance of suppressing the back-change of ^{18}O labeling and matrix regeneration, which will be huge potential advantage for online and high throughput proteome quantification.

Keywords: trypsin aptamer, surface initiated atom transfer radical polymerization(SI-ATRP), TAMSP-ATRP,immobilized eneyme, mass spectrometry

Abbreviation

TAMSP-ATRP: Trypsin aptamer modified silica particles using Surface Initiated Atom Transfer Radical Polymerization

TAMSP-Glut: Trypsin aptamer modified silica particles using glutaraldehyde as linkers

Trypsin aptamer: TApt.23

DTT: dithiothreitol

IAA: iodoacetamide

BSA: bovine serum albumin

GMA: glycidyl methacrylate

$NaCNBH_3$: sodium cyanoborohydride

1 Introduction

Proteomics is an important field in biological and biomedical research at present.[1] Identification and characterization of proteins, efficiently and rapidly, is still a challenge in the research of proteomics.[2-4] Proteolysis is one pivotal step for protein identification in proteomics research. Sample preparation including protein digestion need to spend a lot of time, and the durations are found to be from 16 to 20 h for maximum yield of peptides and reproducible digestion results.[5] These prolonged digestion times, however, limit sample turnover and then prevent results outcome within a short period of time. Recently, the immobilization of enzyme has played a key role in the process of proteins digestion. The immobilized enzyme could be isolated and removed from the protein digests prior to mass spectrometry (MS) easily, eliminating or reducing the influence of the enzyme fragments on MS results.[2, 6, 7] Additionally, the stability of enzyme toward chemical denaturants and organic solvents could be enhanced when immobilized on the solid matrix. However, the process of covalent immobilization was relatively complex in most cases, which might cause a decline of enzyme activity to some extent. In addition, the immobilization matrix cannot be reused resulting in higher experimental costs.

Keeping in view the above problem, aptamers received considerable attention in enzyme immobilization technique and proved as an excellent tool to act as a carrier.[8] Aptamers are single-stranded oligonucleotides included

DNA or RNA sequences, displaying high affinity and specificity towards protein targets.[9] Xiao peng et al. in our group obtained chymotrypsin aptamers by systemic evolution of ligands by exponential enrichment (SELEX) technology and an aptamer-based immobilized chymotrypsin microreactor was developed exhibiting excellent enzymatic capacity.[10] Moreover, in our previous work, aptamer for trypsin was selected and characterized in vitro. The aptamer-based trypsin reactor was developed for proteomic digestion using glutaraldehyde as linker.[11] We all know that protein digestion is greatly affected by the loading amount and accessibility of the immobilized trypsin. If glutaraldehyde acts as the linker, only a single layer of trypsin can be immobilized on the silica. Compared with glutaraldehyde as linker, surface-initiated atom transfer radical polymerization (SI-ATRP) method ("grafting from" method) gives significantly increased surface grafting density and well controlled polymer structure and thickness.[12-15] This is because in "grafting from", the polymer chains grow from the initiator-modified inorganic particle surface or initiator-functionalized self-assembled monolayer. Until now, this technique has been widely used in surface modification of various materials.[15-17] Xiaohong Qian et al. developed a new type of immobilized trypsin using SI-ATRP for highly efficient proteome digestion and ^{18}O labeling absolute protein quantification.[18]

In this study, "grafting from" technique was applied to link trypsin-aptameric-silica. First, aptamer was amino modified, and then was

covalently bound with amino-modified silica surface by ATRP reaction. After trypsin immobilization, standard protein bovine serum albumin (BSA) was mixed with lyophilized silica-trypsin to further examine the performance of the trypsin aptamer modified silica particles using Surface Initiated Atom Transfer Radical Polymerization (TAMSP-ATRP), which was compared with the results of free trypsin and glutaraldehyde as linker.

2 Experimental

2.1 Materials and chemicals

Dithiothreitol (DTT), urea, iodoacetamide (IAA), bovine serum albumin (BSA), TPCK-treated trypsin, glycidyl methacrylate (GMA), glutaraldehyde, and sodium cyanoborohydride, ($NaCNBH_3$) were obtained from Sigma (USA). Aminopropyl modified silica (VN850010-T) was obtained from Agela (Tianjin, China); trypsin aptamer (abbreviation TApt.23) is 5'-GACAG CCACA TGTAC TGAGG TAGAC TTGGG TGGGG GACAG-3' and a poly(T) at the end of 5' was synthesized from Invitrogen (Beijing, China). HPLC-grade formic acid (FA) and acetonitrile (ACN) were purchased from Fisher Scientific Canada (Edmonton, Canada). Water was prepared by Millipore purification system (USA).

2.2 Synthesis of 3-(2-Bromoisobutyramido) propyl silane

2-bromoisobutyryl bromide (0.5 mL, 50 mmol/L) was added dropwise to aminopropyl silica gel (9.4 mL, 40 mmol/L) and triethylamine (0.5 mL, 50 mmol/L) dissolved in anhydrous tetrahydrofuran (10 mL) under ice-cooling.

The solution was further stirred for 8 h and the precipitate (triethylammonium bromide) was collected after centrifugation at 16,500 g for 1min. The precipitate was washed three times with anhydrous methanol, deionized water and anhydrous alcohol, respectively, and finally dried under vacuum. The resultant bromine modified silica was stored at 37 ^{o} C.

2.3 ATRP reaction

The prepared bromine modified silica particles were initiator for immobilization. ATRP grafting was performed by adding nitrogen purged mixture of 2 M GMA, 0.01 M CuCl, 0.001 M CuCl2, and 0.015 M N, N, N', N'', N''-pentamethyldiethylenetriamin in anhydrous methylbenzene (16 mL) to the initiator immobilized silica particles, sealed and agitated at room temperature for 12 h. After reaction, excess chemical reagents were removed by washing with methanol repeatedly. Eventually, the epoxy groups on the side chains of the hairy polymer chains on silica particles were converted to aldehyde groups by 0.01 M sodium periodate solution for 4 h. And then the mixture was centrifuged at 12,000 g for 1 min and the precipitate was collected and was washed three times with deionized water. Then 500 pmol trypsin aptamer was added into the precipitate and the solution was stirred for 2 h, the 2500 pmol $NaCNBH_3$ was added into the solution and was further stirred for 1h at room temperature. Finally, the mixture was centrifuged at 12,000 g for 5 min and the precipitate was collected and was washed three times with deionized water and finally dried under vacuum.

2.4 Characterization of aptamer modified silica particles

Fourier Transform Infrared (FT-IR) spectra were obtained in transmission mode using a FTS 135 FT-IR spectrophotometer under ambient conditions. All samples were prepared including grounded, mixed with potassium bromide (KBr), and pressed to form pellets. The background spectrum was taken out from the sample spectrum. In addition, the surface of silica particles was observed by S-3400 scanning electron microscopy (SEM) (Hitachi, Japan).

2.5 Trypsin immobilization on silica particles

20 mg aptamer modified silica was added into 1 mL 1 mg/mL TPCK-treated trypsin solution and incubated in 50 mM ammonium bicarbonate (pH=8.0) at 37 ^{o}C for 1 h. In addition, the aptamer of glutaraldehyde as linker is prepared as previous reported[11]. After trypsin immobilization, the mixture was centrifuged at 12,000 g for 30 min and the sediment was collected. After washing with 50 mM ammonium bicarbonate buffer three times to remove excess reagents, the obtained TAMSP-ATRP was kept in 4 ^{o}C for further use.

2.6 Determination of loading amount of immobilized trypsin

First, a calibration curve was obtained by measuring the absorption value of a gradient concentration of BSA at 595 nm by Braford method. Next, 1 mg/mL trypsin solution (NH_4HCO_3, pH 8.0) was mixed with TAMSP-ATRP for trypsin immobilization. After incubation at 37 ^{o}C for 1 h, TAMSP-ATRP was retained after centrifugation at 12,000 g for 30 min and the absorption value of the supernatant at 595 nm was measured to calculate the amount of trypsin

immobilized on TAMSP-ATRP.

2.7 Digestion of BSA by TAMSP-ATRP and free trypsin

100 μg of BSA were dissolved in 50 mM ammonium bicarbonate containing 8 M urea for denaturation, followed by DTT reduction and IAA alkylation as reported in previous studies.[19, 20] For TAMSP-ATRP digestion, the protein solution was directly mixed with 10 μg of lyophilized TAMSP-ATRP and incubated at 37 ^{o}C for 2 min. Then the mixture was centrifuged at 12,000 g for 10 min. After centrifugation, the supernatant was collected for mass spectrometry analysis. As for free trypsin digestion, the protein solution was diluted with 50 mM ammonium bicarbonate to reduce urea concentration below 1 M before digestion. Next, free trypsin was added into the protein solution at substrate to trypsin ratios of 50 : 1 and incubated at 37 ^{o}C for 16 h. Finally, 3 % of formic acid was introduced to the solution to terminate the reaction.

2.8 Digestion completeness of TAMSP-ATRP

In order to evaluated the digestion completeness of TAMSP-ATRP, we analysed the tryptic digests of BSA using SDS-PAGE. Generated peptides of BSA by TAMSP-ATRP and BSA protein were separated using 10% SDS-PAGE gel accomplished with Bio-Rad Mini Protein III apparatus. Electrophoresis was run at constant 100 V. The overall running time was 90 min. The gels were stained by Coomassie Brilliant Blue Stain to evaluate the digestion completeness of TAMSP-ATRP.

2.9 Stability of TAMSP-ATRP

In order to evaluate the digestion stability of TAMSP-ATRP, the intra-day and inter-day stability were determined by continuous digestion of BSA on the same day and on five consecutive days, respectively.

2.10 Regeneration of TAMSP-ATRP

When trypsin lose digestion ability, it is necessary to regenerate it. Based on the nature of aptamer, the TAMSP-ATRP was mixed with 95 % ACN for 1 h at room temperature. Subsequently, the mixture was centrifuged at 16,500 g for 20 min, the sediment was saved and washed three times with pure H_2O. Then, the silica particles were mixed with 1 mg/mL trypsin for 1 h at 37 ^{o}C and centrifuged at 16,500 g for 20 min, after that the sediment was saved and washed three times. Then the performance of the regenerated matrix was investigated using BSA as target.

2.11 Digestion of human serum by TAMSP-ATRP and free trypsin

100 μg of human serum were dissolved in 50 mM ammonium bicarbonate containing 8 M urea for denaturation, followed by DTT reduction and IAA alkylation as reported in previous studies.[19, 20] For TAMSP-ATRP digestion, the protein solution was directly mixed with 10 ug of lyophilized TAMSP-ATRP and incubated at 37 ^{o}C for 2 min. Then the mixture was centrifuged at 12,000 g for 10 min. After centrifugation, the supernatant was collected for mass spectrometry analysis. As for free trypsin digestion, the protein solution was diluted with 50 mM ammonium bicarbonate to reduce urea concentration below 1 M before digestion. Next, free trypsin was added into the protein solution at

substrate to trypsin ratios of 50 : 1 and incubated at 37 ^{o}C for 16 h. Finally, 3 % of formic acid was introduced to the solution to terminate the reaction.

2.12 LC-MS/MS analysis

Sample analysis was performed on an Agilent 1100 series HPLC system with a DIKMA Bio-Bond C18 column (2.1×150 mm, 5 μm, 300 A) using a 60 min linear gradient of 3 – 40 % acetonitrile (ACN) in 0.1 % formic acid (FA) at a flow rate of 0.2 mL/min. Mass spectrum analysis was carried out in data-dependent analysis mode, in Agilent 1100 MSD Trap, where MS1 scanned full MS mass range from m/z 100 to 1800. A MS scan was conducted followed by MS/MS scan of the five most intense peaks with dynamic exclusion for 0.5 min of sequenced masses.

2.13 Data analysis

The data was searched against a SwissProt. Bovine database using Spectrum Mill search engine (version B04.00.127). The parameters were as follow: the trypsin as digestion enzyme, two missed cleavages allowed; fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 20 ppm was allowed. Carbamidomethyl as fixed modifications, oxidation of methionine as variable modifications. Spectrum Mill Peptide score above 6, and 60 % SPI(Scored Peak Intensity) are usually considered to be reliable for proteins.

3 Results and Discussion

3.1 Preparation and characterization of TAMSP-ATRP

First, initiators are required to be immobilized on the surface of the silica

particles and then ATRP reaction is introduced using glycidyl methacrylate (GMA) as the monomer to obtain a great number of GMA polymer chains. For the graft of amino modified aptamer on the obtained GMA polymer chains, epoxy groups are required to be converted into aldehyde groups.

In our study, two chemical crosslinking template-based approaches together with amino modifier silica and amino - poly(T) - modified TApt.23 (5'-H_2N-T10-TAPt.23-3') were used for the preparation of aptamer based silica matrix. Poly(T) is a conserved sequence, which guarantees the structural integrity of aptamer and was synthesized between amino and TApt.23.[11] In addition, it can provide distance and space between TAPt.23 and silica particles, which can reduce the impact of silica on the activity of immobilized TAPt.23. The surface of silica and the aptamer-silica particles was observed by SEM, and the results are shown in Figure 1. It can be seen that the silica surface began to get villous after ATRP treatment. Compared with glutaraldehyde as linker, SI-ATRP "grafting from" technique enhanced the surface grafting density and well controlled polymer structure and thickness. The grafting is highly controllable by in situ growth of polymer chains from the initiators immobilized surface via living/controlled polymerization.[18]

The prepared silica particles were also characterized by FT-IR spectroscopy to confirm successful growth of GMA polymer chains on the surface of silica. By comparing Figure 2A with Figure 2B, it can be found that after ATRP reaction of GMA on the surface of silica particles, strong absorption

peaks at 1730 cm^{-1} corresponding to C=O bonds in the ester group and peaks characteristic of epoxy ring at 901 and 845 $cm^{-1}$are observed, which did not exist in the amino propyl silica particles. Through the FT-IR spectra, the introduction of GMA polymer chains onto silica surface was validated.

The loading amount of trypsin on the silica particles by TAMSP-ATRP was determined by measuring the discrepancy of absorption values at 595 nm of the trypsin solution before and after immobilization. The results indicated that the amount of 20.7 μg trypsin/mg silica particle was obtained, which was higher than that of TAMSP-Glut with the amount of 11.1 μg trypsin/mg silica particle. The enhanced trypsin loading amount is attributed to the particular structure with numerous no cross-linked binding sites that work as scaffolds to support 3D trypsin immobilization. In contrast to this, just a single layer of trypsin can be immobilized on the surface using glutaraldehyde as linker. As a result, the surface area of microspheres limits the trypsin binding.

3.2 Evaluation of the digestion performance of silica-trypsin using standard protein BSA

The digestion efficiency was assessed by mixing 100 μg of BSA with 10 μg of silica-trypsin slurry. After incubation at 37 ^{o}C for 2 min, the mixture was centrifuged at 12,000 g for 10 min and the supernatant was separated for HPLC-Agilent 1100 Trap analysis. BSA digestion in solution with free trypsin using the reported method was used as the control.[19, 20] Agilent 1100 MSD Trap was used to analyze tryptic digests. In addition, the digestion completeness is

evaluated by SDS-PAGE. If the sample is digested completely, the band of the digested sample disappeared. Therefore, after digestion, SDS-PAGE was carried out to evaluate the digestion completeness. The SDS-PAGE result obtained in our study is shown in the following figure 3, almost no BSA band can be found when BSA sample was digested by the immobilized trypsin with ATRP, indicating the complete digestion.

A typical mass spectrum of silica –trypsin digestion of BSA is shown in Figure 4. The results showed that there was no significant difference on retention time between the TAMSP-ATRP and free digestion. The zero miss cleavage peptide is often chosen to quantitate ion to make sure the quantitative accuracy. Therefore, we compared the BSA coverage and identified peptide of the three methods according to the zero miss cleavages. The results indicated that TAMSP-Glut digestion of BSA obtained lower sequence coverage (33.8%), identified peptide number 17; while TAMSP-ATRP obtained BSA coverage of 62.77%, identified peptide number 33; while free trypsin digestion gets BSA lower coverage of 58.87%, and identified peptide number 30, through much longer incubation time was used(Table 1). One miss cleavages were allowed to cover BSA sequence of identified peptides as shown in Table 2. The results indicated that TAMSP-Glut digestion of BSA obtained lower sequence coverage (44%), identified peptide number 26; while TAMSP-ATRP obtained BSA coverage of 67%, identified peptide number 41; while free trypsin digestion gets BSA coverage of 67.6%, and identified peptide number 40 (Table 2). While, two miss cleavages were

allowed to cover BSA sequence of identified peptides as shown in Table 3. The results indicated that TAMSP-Glut digestion of BSA obtained lower sequence coverage (46.3%), identified peptide number 27; while TAMSP-ATRP obtained BSA coverage of 67.06%, identified peptide number 41; while free trypsin digestion gets BSA coverage of 67.5%, and identified peptide number 40 (Table 3). It can be concluded that TAMSP-ATRP digestion has a similar and good cleavage capacity as that of the traditional in-solution digestion. However, the free digestion suffers many drawbacks such as long incubation time, enzyme auto digestion, and non-reusability. The traditional digestion method often takes 12-20 h to digest the sample, which limits its high throughput analysis. At the end of the reaction, it is also required to add acid to terminate the reaction. However, the immobilized trypsin method need only centrifugation to finish the reaction. The immobilized trypsin can be used many times to digest the samples. All the results demonstrated that the new "graft from" method was preceded over another TAMSP-Glut.

Considering the nonspecific adsorption of protein on the aptamer modified particles, three peptides with 0 miss cleavage of BSA which have different hydrophobic were chosen to be quantified to evaluate the nonspecific adsorption of protein on the aptamer modified particles. The intensity of three peptides with 0 miss cleavage digested by the aptamer modified particles were compared with the results of the free digestion. When the intensity was submitted to a Student's two-sampled t-test analysis, all of the p values were greater than 0.05, indicating that there was no significant difference between the two methods. it means that there was no

nonspecific adsorption of protein on the aptamer modified particle (Table 5).

The stability of TAMSP-ATRP was investigated using BSA as the target as well. As for the sequence coverage, RSD value of 2.7 % with five consecutive runs in one day (intra-day) and RSD value of 4.14 % with five consecutive runs in five days (inter-day) were obtained (Table 2). And as for the matched peptide, RSD values of 4.7% and 5.73% for intra-day and inter-day were obtained, respectively (Table 4). Those results indicated that TAMSP-ATRP has a good stability.

3.3 Regeneration of TAMSP-ATRP

Based on the nature of aptamer, the aptamer modified silica particles can be regenerated. When the immobilized trypsin was inactivated, it was eluted and re-immobilized. BSA was also chosen as the target to assess the performance of the regenerated trypsin. The results demonstrated that after the TAMSP-ATRP regeneration, peptide sequence and coverage of BSA are the similar as before re-immobilization. TAMSP-ATRP after regeneration obtained BSA coverage of 66.03%, identified peptide number 38 (Table 3). These results indicated that TAMSP-ATRP regeneration was simple, successful, and feasible. In addition, the ability to regeneration ensures wider application of the TAMSP-ATRP for the quantitative analysis of protein and proteomic.

3.4 Digestion of Complex Protein Sample human serum using TAMSP-ATRP

To further demonstrate the digestion efficiency and potential of the TAMSP-ATRP, the human serum is digested by TAMSP-ATRP and

free-digestion. With no prefractionation treatment, the denatured proteins of human serum were incubated with TAMSP-ATRP for 2 min at 37^{o}C and went through RPLC-ESI-MS/MS with 90 min gradient elution. Free trypsin used the same LC-MS condition was performed as the control. For the TAMSP-ATRP method, 45 proteins were identified, while, 34 proteins were identified in the free-digestion method. It is very clear that TAMSP-ATRP has obvious advantages in digestion throughout and efficiency over conventional free trypsin (supplementary human serum sample).

4 Conclusions

The main purpose of the present study was to develop a new technique for higher amounts of trypsin immobilization based on the high specificity and affinity between aptamer and ligand. The hairy poly chain hybrid modified silica particles prepared by ATRP technique enhanced trypsin loading amount and accessibility compared with that of TAMSP-Glut. The results presented that TAMSP-ATRP has obvious advantages in digestion throughout and efficiency over traditional in-solution digestion. Moreover, compared with covalent immobilization approaches, aptamer based matrix performed a convenient immobilization process as well as a good regeneration capacity.

With further development, the developed enzyme immobilization approach can be readily expanded into automated-platform for high-throughput analysis of proteomics. Moreover, TAMSP-ATRP can be also used in ^{18}O labeling protein quantitation with the performance of suppressing the back-change of

^{18}O labeling and matrix regeneration, which will be huge potential advantage for online and high throughput proteome quantification.

Acknowledgements

This work was supported by the National Nature Science Foundation of China (21275019).

References

1. R. E. Banks, M. J. Dunn, D. F. Hochstrasser, J.-C. Sanchez, W. Blackstock, D. J. Pappin and P. J. Selby, *The Lancet*, 2000, **356**, 1749-1756.
2. J. Duan, L. Sun, Z. Liang, J. Zhang, H. Wang, L. Zhang, W. Zhang and Y. Zhang, *J Chromatogr A*, 2006, **1106**, 165-174.
3. R. J. Deshaies, J. H. Seol, W. H. McDonald, G. Cope, S. Lyapina, A. Shevchenko, A. Shevchenko, R. Verma and J. R. Yates, *Molecular & Cellular Proteomics*, 2002, **1**, 3-10.
4. M. Mann, R. C. Hendrickson and A. Pandey, *Annual review of biochemistry*, 2001, **70**, 437-473.
5. Y. Xiong, Y. Li, K. Liu, M. Ke, U. Awan and Y. Deng, *Anal. Methods*, 2013, **5**, 2892-2897.
6. J. Liu, S. Lin, D. Qi, C. Deng, P. Yang and X. Zhang, *J Chromatogr A*, 2007, **1176**, 169-177.
7. G. W. Slysz, D. F. Lewis and D. C. Schriemer, *Journal of proteome research*, 2006, **5**, 1959-1966.
8. P. Xiao, X. F. Lv, S. S. Wang, J. Iqbal, H. Qing, Q. Li and Y. L. Deng, *Anal Biochem*, 2013, **441**, 123-132.
9. J. Vivekananda and J. L. Kiel, *Lab Invest*, 2006, **86**, 610-618.
10. P. Xiao, X. Lv, Y. Man, H. Qing, Q. Li and Y. Deng, *Analytical Letters*, 2013, **46**, 868-878.
11. P. Xiao, X. Lv, S. Wang, J. Iqbal, H. Qing, Q. Li and Y. Deng, *Analytical biochemistry*, 2013, **441**, 123-132.
12. A. K. Mallik, M. Takafuji and H. Ihara, *European Polymer Journal*, 2009, **45**, 1811-1819.
13. C. Fan, Z. Shi, Y. Pan, Z. Song, W. Zhang, X. Zhao, F. Tian, B. Peng, W. Qin, Y. Cai and X. Qian, *Analytical Chemistry*, 2014, **86**, 1452-1458.
14. W. Zhang, B. Peng, F. Tian, W. Qin and X. Qian, *Analytical Chemistry*, 2014, **86**, 482-489.
15. A. K. Mallik, M. M. Rahman, M. Czaun, M. Takafuji and H. Ihara, *J Chromatogr A*, 2008, **1187**, 119-127.
16. W. Qin, Z. Song, C. Fan, W. Zhang, Y. Cai, Y. Zhang and X. Qian, *Analytical chemistry*, 2012, **84**, 3138-3144.
17. D. Antonow, T. Marrafa, I. Dawood, T. Ahmed, M. R. Haque, D. E. Thurston and G. Zinzalla, *Chemical communications*, 2010, **46**, 2289-2291.
18. W. Qin, Z. Song, C. Fan, W. Zhang, Y. Cai, Y. Zhang and X. Qian, *Anal Chem*, 2012, **84**, 3138-3144.
19. I. Javed, L. Wang, H. M, K. Liu, A. U, H. Qing and Y. Deng, *Proteomics*, **2014**, **14**, 1424**-**1433.
20. J. Iqbal, W. Li, K. Ullah, M. Hasan, G. Linna, U. Awan, Y. Zhang, S. Batool, H. Qing and Y. Deng, *Proteomics*, 2013, **13**, 2455-2468.

Figure legends:

Figure 1: Scanning electron microscopy (SEM) of the surface of aminopropyl silica particles (A) and the aptamer modified silica particles based on ATRP reaction (B).

Figure 2: Fourier Transform Infrared (FT-IR) spectra of aminopropyl silica particles (A) and GMA polymer chains modified silica particles (B).

Figure 3. The SDS-PAGE of BSA. 1.Protein marker. 2. The BSA after digested. 3 The BSA after digested.4 The BSA after digested. 5 The BSA before digested.6. The BSA before digested.7. The BSA before digested.

Figure 4: Comparison of HPLC peptide mapping of BSA digestion by free trypsin (A), immobilized trypsin by glutaraldehyde as linker (B), and immobilized trypsin by ATRP reaction as linker (C).

Table legends:

Table 1 Spectrum Mill searching results of BSA digestion by free trypsin, immobilized trypsin by glutaraldehyde as linker, and immobilized trypsin by ATRP reaction as linker (0 miss cleavage)

Table 2 Spectrum Mill searching results of BSA digestion by free trypsin, immobilized trypsin by glutaraldehyde as linker, and immobilized trypsin by ATRP reaction as linker (1 miss cleavage)

Table 3 Spectrum Mill searching results of BSA digestion by free trypsin, immobilized trypsin by glutaraldehyde as linker, and immobilized trypsin by ATRP reaction as linker (2 miss cleavage)

Table 4 .Stability of digestion BSA using TAMSP-ATRP

Table 5 Nonspecific adsorption of BSA peptides using TAMSP-ATRP

Table 1. Spectrum Mill searching results of BSA digestion by free trypsin, immobilized trypsin by glutaraldehyde as linker, and immobilized trypsin by ATRP reaction as linker (0 miss cleavage)

BSA	Protein Digestion approach			
	In-solution	TAMSP-Glut	TAMSP-ATRP before regeneration	TAMSP-ATRP after regeneration
Sequence coverage (%)	58.87±4.05	33.8±7.66	62.77±1.51	59.87±5.87
Peptide matched	30±1.53	17±2.89	33±1.15	30±3.61
Digestion time	16 h	30 min	2 min	2 min

Table 2. Spectrum Mill searching results of BSA digestion by free trypsin, immobilized trypsin by glutaraldehyde as linker, and immobilized trypsin by ATRP reaction as linker (1 miss cleavage)

BSA	Protein Digestion approach			
	In-solution	TAMSP-Glut	TAMSP-ATRP before regeneration	TAMSP-ATRP after regeneration
Sequence coverage (%)	67.6±1.10	44±3.90	67±0.46	64.9±3.80
Peptide matched	40±1.00	26±1.00	41±1.73	38±2.65
Digestion time	16 h	30 min	2 min	2 min

Table 3 Spectrum Mill searching results of BSA digestion by free trypsin, immobilized trypsin by glutaraldehyde as linker, and immobilized trypsin by ATRP reaction as linker (2 miss cleavage)

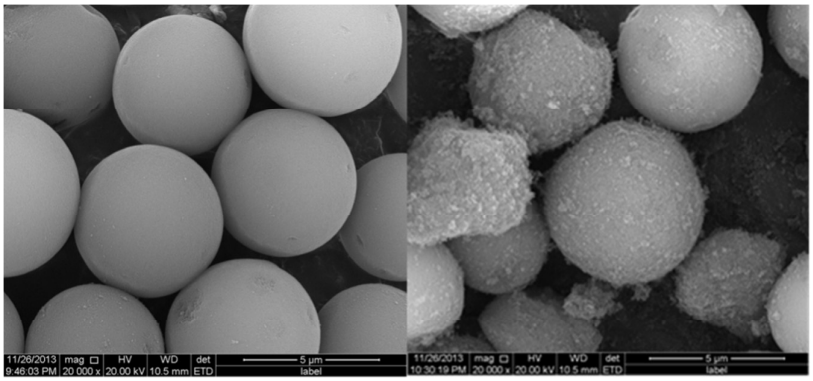
BSA	Protein Digestion approach			
	In-solution	TAMSP-Glut	TAMSP-ATRP before regeneration	TAMSP-ATRP after regeneration
Sequence coverage (%)	67.5±1.42	46.3±1.96	67.06±0.98	66.03±1.25
Peptide matched	40±1.15	27±3.21	41±1.53	38±1.00
Digestion time	16 h	30 min	2 min	2 min

Table 4.Stability of digestion BSA using TAMSP-ATRP

BSA	TAMSP-ATRP Stability (n=5)			
	Intra-day	Intra-day RSD	Inter-day	Inter-day RSD
Sequence coverage (%)	66.03	2.7	67.17	4.14
Peptide matched	38	4.7	39	5.73

Table 5.Nonspecific adsorption of BSA using TAMSP-ATRP

Hydrophobicity	Peptide Sequence	Peak Mean Intensity		P 值
		TAMSP	Free-trypsin	
8.47	(K)AEFVEVTK(L)	50400000	52900000	P >0.05
12.71	(K)LVNELTEFAK(T)	161000000	173000000	P >0.05
16.08	(K)LGEYGFQNALIVR(Y)	382000000	36500000	P >0.05



A B

Figure. 1 Scanning electron microscopy (SEM) of the surface of aminopropyl silica particles (A) and the aptamer modified silica particles based on ATRP reaction (B).

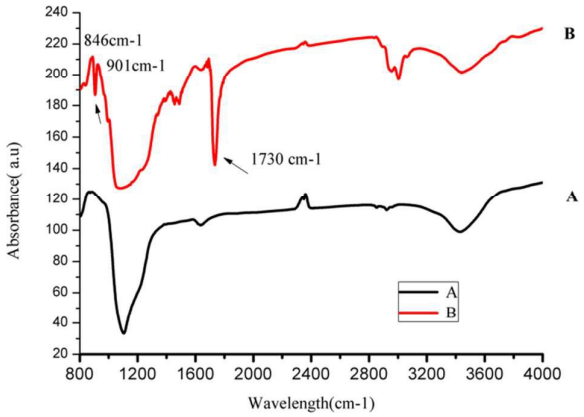


Figure. 2 Fourier Transform Infrared (FT-IR) spectra of aminopropyl silica particles (A) and GMA polymer chains modified silica particles (B).

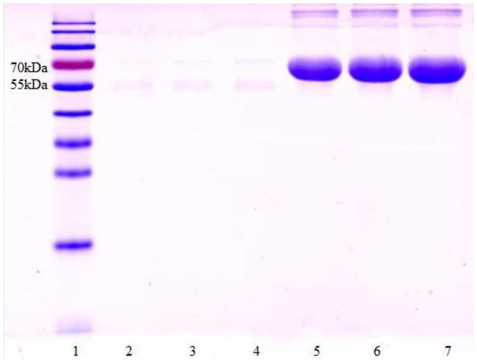


Figure 3. SDS-PAGE gel of BSA before and after TAMSP-ATRP. Lane 1:Protein marker. Lane 2: The BSA after digested. Lane 3: The BSA after digested. Lane 4 :The BSA after digested. Lane 5: The BSA before digested. Lane 6: The BSA before digested. Lane 7: The BSA before digested. 5 μg loading amount of digested or intact protein was used for lanes 2-7.

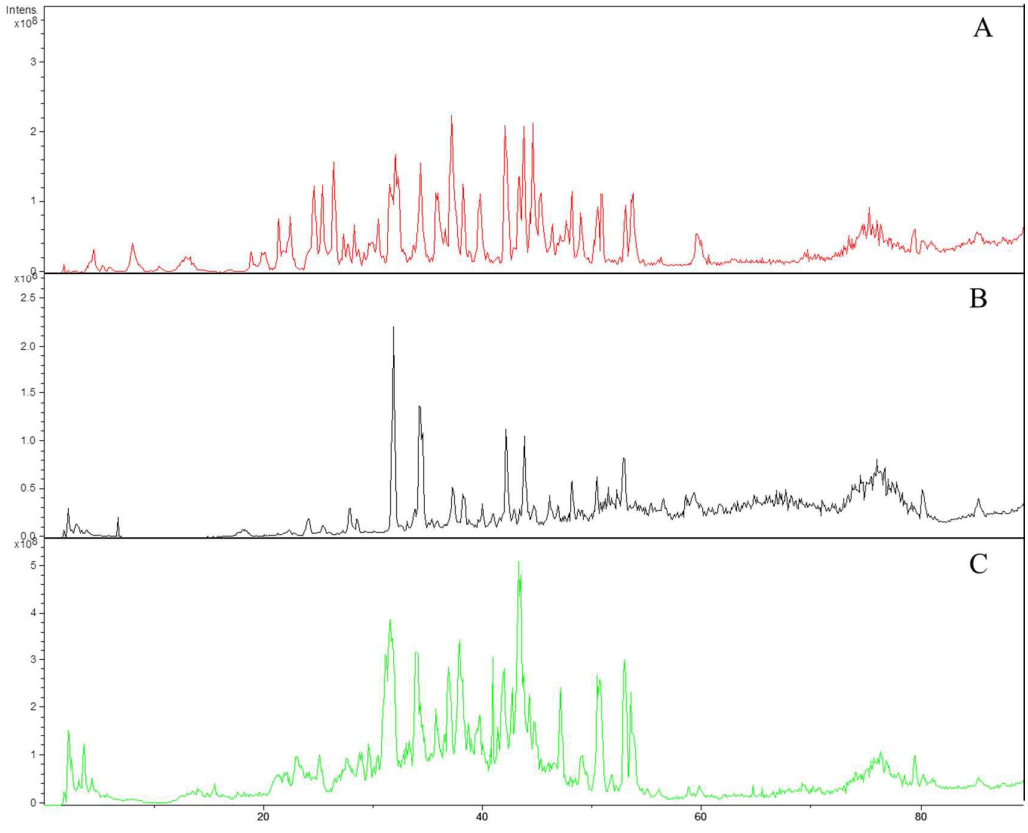
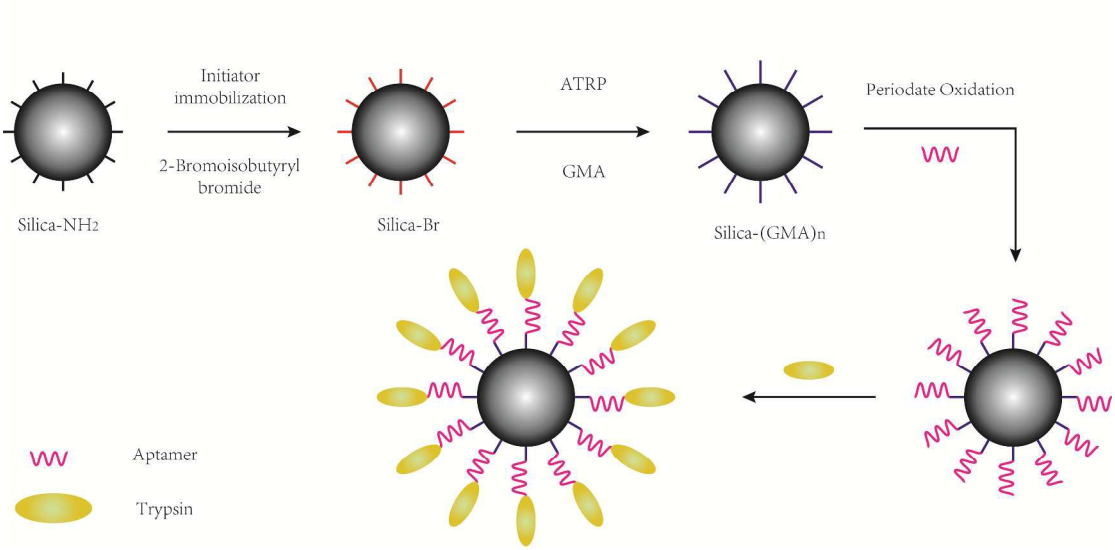


Figure. 4 Comparison of HPLC peptide mapping of BSA digestion by free trypsin (A), immobilized trypsin by glutaraldehyde as linker (B), and immobilized trypsin by ATRP reaction as linker (C).



Graphical abstract