Development of Core-shell Magnetic Mesoporous SiO₂ Microspheres for the Immobilization of Trypsin for Fast Protein Digestion

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Abstract

In the work, we developed glycidoxypropyltrimethoxysilane (GLYMO)-modified Fe₃O₄@SiO₂ core and perpendicularly aligned mesoporous SiO₂ shell (designated Fe₃O₄@nSiO₂@mSiO₂) as the novel substrate for the immobilization of large amount of trypsin and applied it for fast protein digestion.

Firstly, Fe₃O₄@nSiO₂@mSiO₂ microspheres were synthesized. Then, the surface of the microspheres was functionalized with GLYMO for enzyme immobilization. The amount of trypsin immobilized on Fe₃O₄@nSiO₂@mSiO₂ was about 188 μg/mg, which was much more than that on the previous magnetic materials. Using the trypsin-immobilized magnetic mesoporous SiO₂ microspheres, proteins in samples were fast digested with microwave irradiation. The efficacy of this technique for protein mapping was demonstrated by the mass spectral analysis of the peptide fragmentation of three standard proteins, including cytochrome c (Cyt-c), myoglobin (MYG), and bovine serum albumin (BSA). The functionalized magnetic microspheres served not only as substrate for enzyme immobilization, but also as excellent microwave absorbers, thus greatly improved the efficiency of protein digestion. It is also worth noting that by using this novel approach, the protein can be effectively digested within seconds, in contrast to hours required by conventional methods. Moreover, the trypsin-immobilized magnetic mesoporous SiO₂ microspheres exhibit better stability than conventional methods. Furthermore, the feasibility of using this novel strategy for real sample analysis was demonstrated by applying it to the analysis of human pituitary extraction which opens a route for its further application in large-scale proteomic analysis.

Keywords: Mesoporous SiO₂ microspheres; Peptide mapping analysis; Microwave-assisted digestion; MALDI-TOF MS

Introduction

Proteomic analysis of complex protein mixtures usually proceed along with either bottom-up or top-down approach. In both approaches, to obtain detailed structural information, proteins are selectively cleaved into smaller polypeptide fragments by controlled chemical or enzymatic reactions (Washburn et al., 2001; Wolters et al., 2001; Zhu et al., 2003). The resulting mixture is then analyzed by MALDI-MS or LC-ESI-MS. This protein analysis method is known as peptide mapping. With the progress of mass spectrometry, proteins can be rapidly identified. However, the conventional digestion method, solution based digestion, is considered to be slowly (more than hours), and presents a number of problems that may limit the speed of large scale protein identification. On the contrary, several reports have demonstrated the feasibility of protein digestion using immobilized enzyme in recent years. Immobilized enzyme is more resistant to the unfolding of their native structure that may be caused by heat and pH changes. Furthermore, they avoid contamination of the digestion products by free enzyme molecules, peptides, which can be very detrimental.
to the analysis (Nalivaeva and Turner, 2001). The immobilized enzyme has been adopted to characterize the proteins with benefits from the reusability and stability of enzyme, the higher digestion efficiency of protein analytes, and no enzyme autolysis products (Dogruel et al., 1995; Nelson, 1997; Gobom et al., 1997; Jiang et al., 2000; Ekstrom et al., 2000; Peterson et al., 2002; Licklider et al., 1995; Ma et al., 2007; Svec, 2006). The main approach of enzyme immobilization is covalent binding. Epoxide is a classical tool for protein immobilization due to its versatile chemistry (Tischer and Wedekind, 1999; Petro et al., 1996). Petro and coworkers made the first attempt to immobilize trypsin on organic monoliths with epoxide groups in the late 1990s (Kvenková et al., 2005). And in many other reported approaches (Kvenková et al., 2005; Luo et al., 2002), the epoxide functional groups for enzyme immobilization are applied. As the authors demonstrated, the organic monoliths functionalized with epoxide can immobilize enzyme efficiently, but the procedure that modified the capillary is complicated. The most important one is that the amount of enzyme located on organic monoliths is limited, so the digestion efficiency is not satisfactory.

Recently, several approaches have been developed for fast protein digestion. One promising approach is microwave-assisted protein enzymatic digestion (MAPED). The primary advantages of MAPED are the speed and convenience. Microwave irradiation gives an acceleration of enzymatic digestion of proteins (Bose et al., 2002; Pramanik et al., 2002; Sun et al., 2006). Juan et al., (2005) used microwave technology to digest several known proteins in gel with trypsin in 5 min (Juan et al., 2005). Pramanik et al. also applied microwave technology to digest known proteins in solution or gel with trypsin in 10 min including one protein that was tightly folded and extremely resistant to denaturation (bovine ubiquitin) (Pramanik et al., 2002). More recently, Chen et al., (2007) found that MAPED could be further accelerated by magnetite microspheres, which had been proved to be excellent absorbers of microwave radiation (Chen et al., 2007). Magnetic microspheres possess the unique magnetic responsivity, which means that the magnetic microspheres are not only available as highly dispersed suspensions in a wide range of sizes (50 nm to 5 mm) and permit modification on their surface, but also offer the advantage of straightforward and fast handling with the help of an applied magnetic field. With all these advantages, magnetic microspheres can be significant substrate to immobilize enzyme.

Mesoporous silica SiO₂ shows great advantages as a substratum for enzyme immobilization due to its sufficient functional groups for further grafting or attachment, special surface hydrophilic/hydrophobic microregion distribution as well as stable colloidal properties. Another advantage which makes the mesoporous SiO₂ more attractive for enzyme immobilization is that it possesses high specific surface area and capability of absorbing high amount of enzyme with the retention of physiological function. Recently, Zhao and Yang have successfully fabricated enzymatic reactors based on mesoporous SiO₂ for proteome analysis (Fan et al., 2005; Qiao et al., 2008). In our previous work (Lin et al., 2008a; Lin et al., 2008b; Li et al., 2007a; Li et al., 2007b; Li et al., 2007c; Li et al., 2007d), we have successfully immobilized enzyme onto various kinds of magnetic microspheres and utilized them for facile and quick protein digestion such as on-plate digestion, in-microchip digestion and microwave-assisted digestion. However, the enzyme amount (20-70 mg/ g) on these magnetic microspheres is low. New magnetic microsphere with the ability of large amount of enzyme immobilization is desirable.

More recently, a composite microsphere consisting of FeO₃@SiO₂ core and perpendicularly aligned mesoporous SiO₂ shell (designated FeO₃@nSiO₂@mSiO₂) with large surface areas was successfully synthesized and applied in environmental analysis (Deng et al., 2008). Herein, we report the feasibility of combining the advantages of mesoporous SiO₂, magnetic microspheres and microwave-assisted protein enzymatic digestion to develop a facile and highly efficient proteolysis strategy. Glycidoxypropyl-trimethoxysilane (GLYMO) functionalized FeO₃@mSiO₂ was successfully synthesized for trypsin immobilization and fast microwave assisted digestion. High digest efficiency can be achieved in 20 sec for both standard proteins and human pituitary extract. This approach greatly shortens and simplifies the digestion process and provides a promising way to facilitate the complete automation of top-down proteomic approaches for large-scale analysis.

Experiment

Materials and Methods

TFA was purchased from Merck (Darmstadt, Germany). ACN was HPLC grade from Fisher Scientific (Fairlawn, NJ, USA). Bovine serum albumin (BSA, fraction V) was obtained from Bio Basic Inc (Toronto, Canada). (TPCK)-treated trypsins, cytochrome c (EC 232-700-9), myoglobin were purchased from Sigma Chemical (St. Louis, MO). Sinapinic acid and cyano-4-hydroxycinnamic 6 acid (CHCA) were purchased from Sigma (St. Louis, MO, USA). Water was purified using a Milli Q system (Millipore, USA). Water was purified using a Milli Q system (Millipore, USA).
Molsheim, France). All of the other chemicals were of analytical grade and used as received.

**Synthesis of GLYMO-functionalized Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ magnetic mesoporous SiO$_2$ microspheres**

The magnetic microspheres were synthesized through solvothermal reaction as described in our previous work with some modification (Xu et al., 2006). Briefly, 2.70 g of FeCl$_3$·6H$_2$O was first dissolved in 100 mL of ethylene glycol under magnetic stirring. A yellow clear solution was obtained after stirring for 0.5 h. Then 7.20 g of NaAc (sodium acetate) was added to this solution. After being stirred for another 0.5 h, the resultant solution was transferred into a Teflon-lined stainless-steel autoclave with capacity of 200 mL. The autoclave was sealed and heated at 200°C for 16 h and cooled to room temperature. The black magnetic microspheres were collected with the help of a magnet filed, followed by washing with recycle of ethanol and deionized water for six times. The product was then dried in vacuum at 60°C for 12 h.

In order to obtain core-shell magnetic silica microspheres with narrow size distribution and uniform thickness of silica via sol-gel approach, magnetic microspheres (0.01 g) were first treated in HCl aqueous solution (1.0 g, 28 wt %) with the help of tetraethyl orthosilicate (TEOS) (0.05 g) was added to the above dispersion under mechanistic stirring, and a stable dispersion was obtained. Subsequently, tetraethyl orthosilicate (TEOS) (0.05 g) was added to the above dispersion under mechanistic stirring, and the reaction was allowed to proceed for 12 h. Finally, by the use of a magnet, the product was separated, washed with ethanol and water, and then vacuum dried at 60°C for 24 h.

**Synthesis of Fe$_3$O$_4$@nSiO$_2$ magnetic microspheres**

According to our previous method (Deng et al., 2008), the synthesis of Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ magnetic mesoporous SiO$_2$ microspheres were performed. At first, the Fe$_3$O$_4$@nSiO$_2$ microspheres were redispersed in a mixed solution containing cetyl trimethyl ammonium bromide (CTAB) (0.30 g, 0.823 mmol) deionized water (80 mL), concentrated ammonia aqueous solution (1.00 g, 28 wt %) and ethanol (60 mL). The mixed solution was homogenized for 0.5 h to form a uniform dispersion. 0.40 g of TEOS (1.90 mmol) was added dropwise to the dispersion with continuous stirring. After the reaction for 6 h, the product was collected with a magnet and washed repeatedly with ethanol and water to remove nonmagnetic by-products. Finally, the purified microspheres were redispersed in 60 mL of acetone and refluxed at 80°C for 48 h to remove the template CTAB. The extraction was repeated for three times, and the microspheres were washed with deionized water, and Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$-microspheres were finally produced.

**Synthesis of GLYMO-functionalized Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ magnetic mesoporous SiO$_2$ microspheres**

80 mg of Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ microspheres were redispersed in 20 ml methylbenzene containing 0.35 ml GLYMO with the help of ultrasonication. Subsequently, the suspension was refluxed at 80°C for 12 h. Finally, the microspheres were washed with ethanol three times, and then vacuum dried at 60°C for 24 h.

**Immobilization of trypsin to the GLYMO-functionalized Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$**

For enzyme immobilization (Scheme 1), 1.0 mg of GLYMO-functionalized Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ microspheres was incubated with TPCK-treated trypsin (0.1 mL; 5 mg/mL) for 1 h under gentle rotation. After removal of the excess trypsin solution, the trypsin immobilized GLYMO-functionalized Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ microspheres were washed with 25 mM NH$_4$HCO$_3$ (4 × 200 µL). The final product was stored in 25 mM NH$_4$HCO$_3$ at 4°C before use.

After the trypsin immobilization, the microspheres were retained by a magnet, and the UV absorption value of the supernatant solution was measured at $\lambda = 280$ nm to calculate the amount of trypsin immobilized on the GLYMO-functionalized Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ microspheres.

**Microwave-assisted protein digestion**

The procedure of tryptic digestion using trypsin-immobilized Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ magnetic microspheres is shown in Scheme S2. Three standard proteins, cytochrome c (Cyt-c), myoglobin (MYG) and bovine serum albumin (BSA), in 25 mM NH$_4$HCO$_3$ buffer solution (pH 7.7), were used as model substrate to evaluate the digestion performance. The trypsin-immobilized microspheres were transferred into 40 µL protein solution (0.20 µg/µL) in a 0.6mL Eppendorf tube. A domestic microwave oven (output power 700 W)
was used to conduct the microwave-assisted protein digestion process. After microwave irradiation, using an external magnet to retain the magnetic microspheres, the supernatant was deposited onto a MALDI plate directly.

**In-solution enzymatic digestion**

For comparison, the digestions of Cyt-C, MYG and BSA were also performed by free trypsin in solution according to the conventional procedure. The standard proteins were firstly denatured in 25 mM NH₄HCO₃ buffer containing 8 M urea for 1 h at 37°C, followed by dilution with 25 mM NH₄HCO₃ (pH 7.7) buffer to the concentration of urea below 1 M. The in-solution digestion was performed by adding trypsin into the protein solution at a substrate-to-enzyme ratio of 40:1, and the solution was incubated at 37°C for 12 h. After digestion, 1.0 µL of formic acid was added into the solution to stop the reaction.

**Extraction of human pituitary**

According to the references (Li et al., 2007a; Che et al., 2005; Zhan et al., 2006; Liu et al., 2006), the extraction of proteins in human pituitary was performed as the following protocol. The human pituitary tissue was cleaned with Milli-Q water to remove some possible contaminants, cut into small pieces, and homogenized in water containing 9.0 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) using a glass vessel in an ice bath. The resulting homogenate was swirled for 30 min and centrifuged for 20 min at 18000 g. The supernatant was collected, fractionated in aliquots, and stored at -80°C till further analysis. Protein concentration was measured using the Bradford assay using BSA as standard, 20 µg/µL for human pituitary tissue.

**Mass Spectrometry and Database Searching**

MALDI-TOF MS Analysis

Sample solutions were deposited on the MALDI target using the dried droplet method. An amount of 1 µL of sample solution was spotted onto the MALDI plate, and then another 0.5 µL of CHCA matrix solution (5 mg/mL, 0.1% TFA in 50% ACN/H₂O solution) was introduced. Positive ion MALDI-TOF-MS spectra were acquired on a 4700 Proteomics Analyzer (Applied Biosystems). The sample was excited using an Nd:YAG laser (355 nm) operated at a repetition rate of 200 Hz and acceleration voltage of 20 kV. Before identifying the samples, the MS instrument was calibrated by an internal calibration with tryptic peptides of myoglobin. The MASCOT server was used to interpret the MALDI-TOF MS data by searching the species of Mammals from sprot-horse for identification of three standard proteins with peptide fingerprint mass spectra.

**LC-ESI-MS/MS Process**

The elution gradient for the RPLC column was from 5 to 90% buffer B (0.1% formic acid, 95% ACN). Eluted peptides were detected in a survey scan from 400 to 1800 amu (1 microscan) followed by 8 data-dependant MS/MS scans in a completely automated fashion on an LTQ-Orbitrap ESI mass spectrometer. According to Washburn's method (Washburn et al., 2001), the filtering criteria was calculated through a reverse database searching and the Xcorr value vs charges was obtained as following: p< 0.01, >2.78(+3), >2.10(+2), >2.0(+1), ≥ Cn> 0.1 and peptide length > 7 were also applied.

Scheme 1: Illustration of synthesis of trypsin-immobilized Fe₃O₄@nSiO₂@mSiO₂ magnetic microspheres.
Figure 2: MALDI-TOF MS spectra of tryptic peptides originated from cytochrome c, myoglobin, and bovine serum albumin resulted from microwave-assisted digestion by trypsin-immobilized Fe₃O₄@nSiO₂@mSiO₂ magnetic microspheres.*, peptide from standard proteins.
Result and Discussion

Preparation of GLYMO-functionalized Fe$_3$O$_4$ @nSiO$_2$ @mSiO$_2$ Microspheres

The Fe$_3$O$_4$ @nSiO$_2$ @mSiO$_2$ microspheres were synthesized according to previously reported approach (Scheme S1) which involves first coating Fe3O4 particles (~300 nm in diameter) with nonporous silica layer and then with mesoporous silica layer by using organic surfactant as the templates. Transmission electron microscopy (TEM) image shows that the obtained Fe$_3$O$_4$ @nSiO$_2$ @mSiO$_2$ microspheres have a mean diameter of about 500 nm and possess well-defined silica-coated magnetite core and mesoporous silica shell (Figure S1a). The nonporous silica layer is 20 nm in thickness, which can serve as protective coating for magnetite, and the mesoporous silica layer is 70 nm in thickness, which can provide the microspheres with high surface area for derivation of numerous functional groups. Notably, the mesopores (~2.0 nm in diameter) in the shell were found to be cylindrical and perpendicular to the microsphere surface, which provide good accessibility for reactants. Scanning electron microscopy (SEM) images show that the microspheres are very uniform both in size and shape (Figure S1b). The unique microstructure of the obtained microspheres would be very useful for many applications. According to our method (Lin et al., 2008a, 2008b, Li et al., 2007a, 2007b; Li et al., 2007c, 2007d), the protein to enzyme ratio is about 1:5 as compared to 40:1 in in-solution digestion procedure.

Immobilization of Trypsin

The most important factor of conventional enzymatic proteolysis is the enzyme-substrate ratio, and the digestion efficiency increases observably with high enzyme-substrate ratio. Nevertheless, if a high concentration of free enzyme is used, the digestion proceeds quicker but autolysis products become more abundant, and enzyme autoproteolysis would impair signal interpretation, especially for low amounts of analytes (Bonnie et al., 2000). However, the immobilization can improve the enzyme stability, and retain its activity. The mesoporous SiO$_2$ with high specific surface can provide sufficient functional groups such as hydroxyl group for further modification, so more enzyme can be immobilized onto the channels of the microspheres. The elevated enzyme-substrate ratio on the surface leads to enhanced digestion efficiency. In the work, trypsin can be immobilized onto the functionalized magnetic microspheres only through a one-step reaction of its amine group with GLYMO group. As the procedures that mentioned above the amount of trypsin immobilized on the magnetic microspheres was about 188 µg/mg, which was much more than that on the previous magnetic materials including commercial magnetic materials (Lin et al., 2008a; Lin et al., 2008b; Li et al., 2007a; Li et al., 2007b; Li et al., 2007c; Li et al., 2007d). The protein to enzyme ratio is about 1:5 as compared to 40:1 in in-solution digestion procedure.

Microwave-assisted Protein Enzymatic Digestion

The procedure of tryptic digestion using trypsin-immobilized Fe$_3$O$_4$ @nSiO$_2$ @mSiO$_2$ magnetic microspheres is shown in Scheme S2. As we know, in many references (Juan et al., 2005, Chen et al., 2007, Fan et al., 2005, Qiao et al., 2008), MYG (MW 16 700) or Cyt-C (MW 12 384) and BSA (MW 66 000) was used as model proteins to test the digestion efficiency. In our work, the three proteins were also used. For comparison, the digestions were also performed by free trypsin in solution for 12 h. Cyt-C and MYG is small molecule proteins which would be proteolysis easily, and were used to investigate the feasibility of our new method. The peptide mass mapping of Cyt-C and MYG from microwave-assisted digestion were displayed in Figure 1. Many digest fragments were observed from the MS spectra suggesting a highly proteolytic efficiency for the Fe$_3$O$_4$ @nSiO$_2$ @mSiO$_2$ microspheres. Notably, there are no distinct peaks with m/z > 2500, indicating virtually complete digestion. The proteolytic results were listed in Table 1 in detail. The observation corresponded to the detection of fragments containing 81 out of 104 possible amino acids of Cyt-C, 137 out of 153 possible amino acids of MYG. The sequence coverage obtained from the database is 77% for Cyt-C, 89% for MYG. Therefore, we can confirm that our new approach is feasible for fast protein digestion. Then BSA with larger molecule weight was used to do further investigation of our method. The observation corresponded to the detection of fragments containing 263 out of 607 possible amino acids of BSA, and the sequence coverage is 45% for BSA. The results indicate that BSA has been digested completely, which means that the new method is suitable for large protein molecule digestion. The identification results are comparative with those by in solution digestion that required a reaction time of 12 h (Table 1). Meanwhile, the sample volume is only 40 µL per analysis. Moreover, no trypsin autolysis peaks were observed from mass spectra in the microwave-assisted digestion method, which demonstrated that enzyme immobilization technique can overcome the trypsin autolysis (Dogruel et al., 1995, Nelson, 1997, Gobom et al., 1997, Jiang et al., 2000, Ekstrom et al., 2000, Peterson et al., 2002, Licklinder et al., 1995, Ma et al., 2007, Svec, 2006). Since the magnetic microspheres are excellent microwave absorbers; and thus
greatly improve the efficiency of protein digestion. We compared the digestion efficiency of our protocol with other reported methods (Chen et al., 2007; Fan et al., 2005; Zhang et al., 2006; Guo et al., 2003), and the results were listed in Table S2 (see supporting information). The digestion time spanned from 20 s to 15 min with different digestion method, and the sequence coverage ranges from 77% to 89% of cytochrome c and 44% to 89% of myoglobin. This demonstrates that our method shows comparable digestion efficiency with other methods, and our method takes short digestion time (only 20s).

The effect of different addition amounts of trypsin-immobilized Fe$_3$O$_4$@nSiO$_2@mSiO$_2$ magnetic microspheres on the sequence coverage of MYG is shown in Figure 3. The sequence coverage of MYG increases slowly when the addition amounts are less than 200 µg. Increasing the amounts of microspheres does not change the sequence coverage distinctly. It suggests that the optimal addition amounts of the microspheres are 200 µg. Figure 4 shows the influence of incubation time on sequence coverage of MYG obtained from MALDI-TOF MS analysis. When the incubation time increases from 5 to 20 sec, the sequence coverage accordingly increases from 78% to 89%. With further increment of the time, no significant change on sequence coverage is observed, suggesting that 20s is enough for efficient microwave-assisted digestion. The digestion time

| protein | Cyt-c | BSA | MYG |
|---------|-------|-----|-----|
|         | microwave | In-solution | microwave | In-solution | microwave | In-solution |
| amino acids identified | 81 | 79 | 263 | 213 | 137 | 107 |
| sequence coverage (%) | 77 | 75 | 45 | 35 | 89 | 69 |
| peptides matched | 12 | 10 | 27 | 18 | 13 | 10 |
| accession no. | P00004 | P00004 | P02769 | P02769 | P68083 | P68083 |
| protein MW | 11694.1 | 11694.1 | 69248.4 | 69248.4 | 16941 | 16941 |

Table 1: MALDI-TOF/TOF MS data of digestion products by microwave-assisted digestion using trypsin-immobilized Fe$_3$O$_4$@nSiO$_2@mSiO$_2$ magnetic microspheres

* Three spot replicates were taken in the experiments.

**Figure 3:** Effect of microspheres amount on sequence coverage of MYG digests resulted from microwave-assisted digestion by trypsin-immobilized Fe$_3$O$_4$@nSiO$_2@mSiO$_2$ magnetic microspheres.

**Figure 4:** Effect of incubation time on sequence coverage of MYG digests resulted from microwave-assisted digestion by trypsin-immobilized Fe$_3$O$_4$@nSiO$_2@mSiO$_2$ magnetic microspheres.
| Cyt-c | MYG | BSA |
|-------|-----|-----|
| 9-22  | 1-31| 25-34|
| IFVQKCAQCHTVEK | GLSDGEWQQVLNVWGKVE | DTHKSEIAHR |
| 26-38 | 17-31| 35-44|
| HKTGPNLHGLFGR | ADIAGHGQEVLR | FDKLGEHFK |
| 28-39 | 17-31| 65-75|
| TGPNLHGLFGR | VEADIAGHGQEVLR | LONELTEFAK |
| 28-39 | 32-42| 89-105|
| TGPNLHGLFGRK | LGTGHPETLEK | SLHTLFGDELCKVSLR |
| 39-53 | 32-45| 157-167|
| KTGQAPGFTYTDANK | LGTGHPETLEKFDK | FWGKYLYEIAR |
| 40-53 | 48-56| 161-167|
| TGQAPGFTYTDANK | 64-77| YLYEIAR |
| 40-55 | HGTVVTLALGGILK | 161-168|
| TGQAPGFTYTDANKKNK | 79-96| YLYEIARR |
| 56-72 | KGHHEAELKPLAQSHATK | 168-183|
| GITWKEETLMLEYLENPK | 80-96| RHPYYFAPELYLYANK |
| 61-72 | GHHEAELKPLAQSHATK | 198-209|
| EEITMEYLENPK | 103-118| GACLIPKETMR |
| 61-73 | YLEFISDAIHLHSK | 221-228|
| EEITMEYLENPPK | 79-133| LRCASIQK |
| 80-87 | YLEFISDAIHLHSKHPGDF | 341-359|
| MIFAGIKK | GADAQGA MTK | NYQEAKDAFLGSLYE |
| 89-99 | 119-133| YSR |
| TEREDLIAYLK | HPGDFGADAGAMTK | 347-359|
|       | 134-145| DAFLSFLYEYSR |
|       | ALELFNRDMAAK | 347-360|
|       | 146-153| DAFLSFLYEYSRR |
|       | YKELGFQG | 360-371|
|       |       | RHPEYAVSVLLR |
|       |       | 361-371|
|       |       | HPEYAVSVLLR |
|       |       | 402-412|
|       |       | HLVDEPQLNIK |
|       |       | 421-433|
|       |       | LGEYGFQNALIVR |
|       |       | 437-451|
|       |       | KVQVSTPTLVEVSR |
|       |       | 438-451|
|       |       | VPQVSTPTLVEVSR |
|       |       | 456-468|
|       |       | VGRTRCCTKPESE |
|       |       | 469-482|
|       |       | MPCTEDYLSLILNR |
|       |       | 508-523|
|       |       | RPCFSAALTPOETYVPK |
|       |       | 529-544|
|       |       | LFFTHADICTLPDETEK |
|       |       | 569-580|
|       |       | TVMENFVAFVDK |
|       |       | 588-597|
|       |       | EACFAVEGPK |

**Table 2:** Detail identified fragments of Cyt-C, MYG and BSA by MALDI-TOF/TOF MS
by the microwave-assisted digestion was much less than that (5 min) in trypsin-immobilized magnetic microspheres without microwave assistance (Li et al., 2007). Figure 5 distinctly. Therefore, we concluded that 200 µg microspheres with microwave incubation at 700 W for 20 sec is the optimal condition for effective protein digestion. This shows that microwave-assisted digestion by using trypsin-immobilized Fe₃O₄@nSiO₂@mSiO₂ magnetic microspheres has very high digestion efficiency.

The high digestion efficiency of the procedure may mostly count on two reasons: firstly, the mesoporous SiO₂ with high specific surface can provide sufficient functional groups such as hydroxyl group for further modification, so more enzyme can be immobilized onto the channels of the microspheres. The elevated enzyme-substrate ratio on the surface leads to enhanced digestion efficiency. Secondly, the functionalized magnetic microspheres served not only as substrate for enzyme immobilization, but also as excellent microwave absorbers, thus greatly improved the efficiency of protein digestion.

To investigate dynamic range of protein by the proposed microwave-assisted digestion, 200 mg of trypsin-immobilized magnetic microspheres were added into 40 µl of MYG solution with the concentration of 100 ng/µl, 50 ng/µl, 20 ng/µl, 10 ng/µl respectively. The microwave-assisted digestion was conducted in the same conditions described as above. The protein sequence coverage of the four protein concentration (10 to 100 ng/µl) is 73%, 79%, 79%, 83%, respectively. The results indicated that the proposed approach can be used for fast digestion of low concentration of proteins.

To test the stability, seven consecutive operations for MYG with incubation for 20 sec using the trypsin-immobilized microspheres were conducted. As shown in Figure 6, no obvious decrease is observed in the runs, suggesting that the activity of the enzyme immobilized is not destroyed apparently.

Furthermore, we studied the longevity of the enzyme-immobilized microspheres, we used 200 mg of the enzyme-immobilized magnetic microspheres for microwave-assisted digestion in the same conditions described as above. Then, the microspheres were washed with 25 mM NH₄HCO₃ (4×200 µl), and resuspended in 200 µl 25 mM NH₄HCO₃ for further use. The same digestion procedure was conducted every other 24 h with the same microreactor. After the experiment ran four times, the protein sequence coverage didn't change obviously. It means that the activity of the protein microreactor didn't minish after 96 h. This shows that the microreactor has the longevity of more than 4 days.

**Application of microwave-assisted protein enzymatic digestion**

In recent decades, there has been an explosion of interest in the identification and characterization of proteins, using the techniques of mass spectrometry and database searching,
Figure 7: (A) The total ion chromatogram for the separation of human pituitary extract digests by microwave-assisted digestion. (B) The mass spectrum scan at 13.22 min in LC-ESI-MS for human pituitary digests. The ms/ms spectrum of 1095.46 m/z peak in figure 7B.
with the aim of establishing links to pathological conditions. A formal step in identification of proteins is protein digestion prior to mass spectrometry analysis. Our approach provides a facile and low-cost way to produce protein fragmentations, which generate sequence information and ultimately identification.

Here, to further confirm the feasibility of microreactor for the analysis of complex protein mixtures, it was applied to human pituitary extract. Without any preparation and prefractionation procedure, the entire proteome was digested for only 1 min and went through LC-ESI-MS/MS directly. Figure 7A was the total ion chromatogram acquired from the microwave-assisted protein digestion. After a database search according to the SEQUEST criteria set above (Experimental Section), 951 peptides were identified, 589 proteins were identified with p < 0.01 (Table S1). Figure 7B is the precursor mass scan at 13.22 min and Figure 7C is the corresponding MS/MS spectrum of the m/z 1095.46 in Figure 7C. Most y-ions together with b-ions produced from the precursor ion matched together and resulted in the high reliability for peptide sequence of R.NMGGPYGGGNYGPGGSGGSGGYGGR.S. These results clearly show that this novel digestion approach can be used for large scale proteomic analysis.

Conclusions

In this study, we successfully developed GLYMO-modified Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ magnetic mesoporous silica microspheres as a new substrate for immobilization of large amount of trypsin, and applied it to microwave-assisted protein digestion. Compared with conventional solution digestion, microwave-assisted protein digestion based on Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ can show similar identification results with much shorter incubation time. The excellent efficiency of trypsin-immobilized microspheres can be also verified when it is applied to real proteome, human pituitary extract. At the same time, the process of digestion is very facile due to the easy manipulation of magnetic microspheres and microwave processing. Considering the combination with diversely high automated separation techniques, the novel microwave-assisted protein digestion method with trypsin-immobilized magnetic mesoporous silica microspheres developed here would hasten high-throughput proteome analysis.

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