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Functionalized magnetic carbonaceous microspheres for trypsin immobilization and the application to fast proteolysis

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ABSTRACT

In this study, magnetic carbonaceous (MC) microspheres prepared with a large-scale synthesis approach were developed as the novel substrate for enzyme immobilization, and the trypsin-immobilized MC microspheres were successfully applied to protein fast digestion. Firstly, MC microspheres with small size, strong magnetism, and biological compatibility were prepared through two-step solvothermal reactions. Secondly, MC microsphere surface was modified by 3-glycidoxypropyltrimethoxysilane (GLYMO). Finally, the enzyme was immobilized on the GLYMO-functionalized MC microspheres. The enzyme-immobilized magnetic microspheres were applied for fast protein digestion with microwave-assistance. Bovine serum albumin, myoglobin and cytochrome *c*, were used as model proteins to verify the digestion efficiency, and the digestion products were then characterized using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with sequence coverage of 43%, 90% and 77%, respectively. The enzyme-immobilized magnetic particles were also successfully applied to the analysis of human pituitary extract. After database search, 485 proteins ($p < 0.01$) were identified when the extract was digested by the microspheres. This opens a route for its future application in bottom-up proteomic analysis.

1. Introduction

In recent years, proteomics research has become one of the fastest developing areas of biological research. It aims to obtain the global perspective of changes in protein expression by characterizing a large number of proteins [1,2]. To obtain detailed structural information, proteins are selectively cleaved into smaller polypeptide fragments by chemical hydrolysis or enzymatic digestion, and the resulting mixture is then analyzed by mass spectrometry (MS). Thus, protein digestion is a crucial step in the identification of proteins.

It is well known that conventional in-solution digestion is prone to such intrinsic limitations as prolonged digestion time, autolysis and sample loss, resulting in negative effects on comprehensive proteomic profiling. To solve these problems, immobilized enzymes have been widely utilized owing to their advantage of allowing the use of higher enzyme concentrations that lead to shorter digestion time. Furthermore, immobilized molecules are more resistant to the unfolding of their native structure that may be caused by heat and pH changes, and consequently show better reproducibility. Additionally, immobilized enzyme avoids contamination of the digestion products by free enzyme molecules, peptides, which can be very detrimental to MS results [3]. Many efforts have been reported on the increase in tryptic digestion efficiency by enzyme immobilized on various substrates [4–18].

Comparing to other supports, magnetic spherical particles with micro- and nanometer size are gaining increasing attention [19–25]. Several groups have demonstrated that magnetic particles with different diameters, size distributions, and chemical properties of the surface [26–29] are available. With unique magnetic responsivity, they can be easily removed from the reaction mixture with the application of magnetic field, and can be used repeatedly. The storage properties and the pH stability of enzymes are often improved by immobilization [30–32]. Therefore, the application of magnetic microspheres as carriers facilitates the manipulation and recovery of the soluble enzymes, as well as the purification of product.

An alternative approach for improving the digestion efficiency is the use of microwave irradiation. Microwave reactions work on the principle of agitation of polar molecules that oscillate under the effect of the electromagnetic field. The three principle mechanisms including dipole rotation/polarization, conduction, and interfacial polarization amount to increased molecular movement and hence faster interactions between reactive entities whether it is via thermal mechanisms or otherwise [33]. More than two decades ago, the

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world of synthetic chemistry embraced microwave technology to increase reaction yields and decrease incubation times in their synthesis protocols. Just a few years later, microwave technology began to emerge in protocols of a more bio-analytical nature. Pramanik et al. [34] demonstrated that accelerated proteolytic cleavage of proteins under controlled microwave conditions (i.e., set temperature, pressure, and power) was achieved with endoproteinase Lys-C (Lys-C) and trypsin. Juan et al. [35] demonstrated that an optimum condition for in-gel microwave-assisted tryptic digestions was at 195 W for 5 min. In 2006, Sun et al. [36] combined microwave-assisted protein preparation and enzymatic digestion in proteomics protocol. More recently, Chen and Chen [37] found that microwave-assisted digestion could be further accelerated by magnetite beads, which proved to be excellent absorbers of microwave radiation. In our group, magnetic microspheres including magnetic particles have been used as the substrate for immobilized enzymes for protein digestion [21–25]. Recently, we demonstrated that it is feasible to develop microwave-assisted protein digestion method based on trypsin-immobilized magnetic silica microspheres with core–shell structure (Fe_3O_4@SiO_2) or magnetic mesoporous silica [27,28]. However, the synthesis protocol of Fe_3O_4@SiO_2 and magnetic mesoporous silica is very complex and time-consuming. Therefore, novel magnetic materials that can be obtained by a facile and large-scale synthesis approach, are very desirable for enzyme immobilization for fast protein digestion.

In our previous study [24], we have successfully applied hydrothermal reaction to large-scale synthesis of magnetic carbonaceous (MC) microspheres. In this report, we developed the MC microspheres as a new substrate for enzyme immobilization and applied them to protein fast digestion. Initially, MC microspheres were prepared by two-step solvothermal reaction. Then, 3-glycidoxypropyltrimethoxysilane (GLYMO) functionalized MC microspheres were prepared for enzyme immobilization. Finally, we applied these easily prepared trypsin-immobilized magnetic carbonaceous (TIMC) microspheres in microwave-assisted digestion. High digestion efficiency was observed for both standard proteins and human pituitary extract which demonstrates that this novel digestion method based on TIMC microspheres can speed up the bottom-up proteomic technique for batch analysis of biological and clinical samples.

2. Materials and methods

2.1. Materials and chemicals

3-Glycidoxypropyltrimethoxysilane (GLYMO), tosyl-phenylalanine chloromethyl-ketone (TPCK)-treated trypsin, cytochrome *c* (EC 232-700-9), bovine serum albumin (BSA), and myoglobin were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) was of HPLC grade from Fisher Scientific (Fairlawn, NJ, USA). Water was purified using a Milli-Q system (Millipore, Molsheim, France). All of the other chemicals were of analytical grade and were purchased from Shanghai Chemical Reagent Co. (Shanghai, China).

2.2. Preparation of GLYMO-functioned MC microspheres

The magnetic microspheres were synthesized through solvothermal reaction described as follows: 1.35 g of $FeCl_3 \cdot 6H_2O$ was first dissolved in 75 mL of ethylene glycol under magnetic stirring. A yellow clear solution was obtained after stirring for 0.5 h. Then 3.60 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, followed by washing with deionized water six times. The product was then ultrasonicated for 10 min in 0.1 M HNO_3, followed by two washes with deionized water. After introducing 80 mL of 0.5 M solution of glucose, the mixture was ultrasonicated for another 5 min and thereafter heated in the same autoclave at 180 °C for 6 h. The cooled MC microspheres were washed with deionized water five times and dried in vacuum at 40 °C for 4 h.

In the next step, 80 mg MC microspheres were resuspended 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 40 °C for 24 h.

2.3. Immobilization of trypsin onto MC microspheres

Five milligrams of prepared GLYMO-linked MC microspheres were transferred to a 1.5 mL Eppendorf tube to which 500 μL of 2 μg/μL TPCK-treated trypsin solution (25 mM ammonium hydrogencarbonate as the buffer, pH ~ 8) was added. The mixture was ultrasonicated for 1 min to form a unique suspension. Then the suspension was agitated at 37 °C for 2 h. After reaction, the supernatant solution was removed with the help of an external magnet. Retained magnetic microspheres were washed with deionized water three times.

The UV absorption value of the supernatant solution was measured at $\lambda = 280$ nm and compared to the UV absorption value of the trypsin solution before immobilization to calculate the amount of trypsin immobilized on the MC microspheres.

2.4. Extract protein from human pituitary tissue

Human pituitary was obtained from Pituitary Cancer Institute in Huashan Hospital, Fudan University. The pituitary tissue was cut into small pieces and cleaned with cold physiological saline solution (0.9% NaCl) to remove blood and other contaminants. Then, 0.18 g of tissue debris was transferred to a homogenizer and rapidly mixed with 1.2 mL lysis buffer, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM Na_3VO_4, 1 mM NaF, complete protease inhibitor cocktail (Roche, Basel, Switzerland), and 0.1% trifluoroacetic acid (TFA). The tissue sample was homogenized in an ice bath. The resulting homogenate was swirled for 30 min and centrifuged for 15 min at 18,000 × *g*. The supernatant was collected. The protein concentration of the sample was 20.0 mg/mL according to the modified Bradford method described by Qu et al. [38].

2.5. In-solution digestion of standard proteins and human pituitary extract

Three standard proteins, BSA, myoglobin and cytochrome *c* were dissolved in 25 mM ammonium hydrogencarbonate (pH 8) to a concentration of 2 μg/μL, followed by denaturing solely in a 95 °C water bath for 15 min. Low-concentration (0.2 μg/μL) protein solutions were prepared by dilution of the former solutions with the same buffer. Trypsin enzyme was added for a ratio of 1:40 enzyme/protein (w/w), and the mixtures were incubated overnight (12 h) with shaking at 37 °C. After digestion, 1 μL formic acid was added straight into 1 mL sample solution to stop the reaction.

For the human pituitary extract, an amount of 50 μL of the sample solution (20.0 μg/μL) was diluted to 1 mL with ammonium hydrogencarbonate (25 mM, pH 8). The solution was denatured in 95 °C water bath for 15 min, and digested by trypsin (1:20) at 37 °C for 16 h.