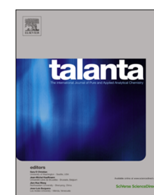
Talanta 122 (2014) 278–284

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta



Dendrimer-grafted graphene oxide nanosheets as novel support for trypsin immobilization to achieve fast on-plate digestion of proteins ☆

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ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form
22 January 2014
Accepted 25 January 2014
Available online 1 February 2014

Keywords:
Graphene oxide
Dendrimer
On-plate digestion
Proteomics
IMER

ABSTRACT

In this study, dendrimer grafted graphene oxide nanosheets (dGO) were prepared by covalent reaction. The successful synthesis of dGO was confirmed by Fourier-transform infrared spectra, Raman spectra, Thermo gravimetric analysis and *Zeta* potential. Taking advantages of large surface area, excellent biocompatibility and abundant functional groups, dGO provided an ideal substrate for trypsin immobilization. Trypsin-linked dGO was synthesized through covalent bonding using glutaraldehyde as coupling agents. The amount of trypsin immobilized on dGO nanosheets was calculated to be about 649 ± 20 mg/g. The activity of immobilized trypsin could be maintained for over 10 days at 4 °C. On-plate proteolysis could be performed without removing trypsin-linked dGO, because dGO did not interfere with matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry analysis. By such an immobilized enzymatic reactor, standard proteins could be efficiently digested within 15 min, with sequence coverages comparable or better than those obtained by conventional over-night in-solution digestion. Furthermore, trypsin-linked dGO showed high sensitivity when applied to trace samples analysis. All these results demonstrated that the developed dGO based enzymatic reactor might provide a promising tool for high throughput proteome identification.

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1. Introduction

One of the most important tasks of proteomics is to develop efficient and rapid approaches to identify various proteins [1,2]. Enzymatic cleavage coupled with matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF MS) is a powerful technique for effective protein identification [3,4]. For MALDI-TOF MS detection, the rapid and complete digestion of proteins is crucial to achieve the high throughput and accurate protein identification [5]. However, sample pretreatment prior to MALDI-TOF MS analysis is often time-consuming, resulting in low analytical throughput. Furthermore, sample loss during the multi-step transfer is inevitable, which could lead to the low identification efficiency for proteins [6]. Therefore, the successful MALDI-TOF MS analysis requires the acceleration of enzymatic digestion and the simplification of sample preparation procedures. Immobilized enzyme reactors (IMER), as an alternative to traditional in-solution digestion, possess the advantage of tolerance for high enzyme to protein ratio, resulting in short digestion time and low risk for enzyme autolysis [7,8]. Furthermore, on-plate proteolysis could simplify sample preparation procedure and reduce sample loss [9]. Therefore, IMER coupled with on-plate proteolysis has been considered as a potential solution to solve the above-mentioned problems [10,11].

For on-plate digestion, the support used for enzyme immobilization plays a key role. To date, several matrixes have been successfully used for on-plate digestion. Li et al. developed a novel on-plate digestion method using magnetic nanospheres as support for trypsin immobilization. Compared to traditional in solution digestion, the digestion time was significantly reduced to 5 min [9]. However, the magnetic nanoparticles must be removed from MALDI plate before MS analysis, to eliminate the interference on MS signals of peptides, which inevitably increased the risk of sample loss. Although with carbon nanotube as the support, the immobilized enzyme amount could be increased, the poor dispersibility would interfere the crystal formation of matrix and peptides, which might significantly decrease MS signals [10]. Therefore, it is urgently required to develop novel supports which could simultaneously provide high enzyme immobilization

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http://dx.doi.org/10.1016/j.talanta.2014.01.056

amount, excellent dispersibility and non-interference with MALDI analysis.

Graphene oxide (GO), one of the most important derivatives of graphene, has recently attracted increasing attention in biological fields, such as biological imaging [12,13], drug/gene delivery [14–16], and biosensing [17–19], due to its large surface area and good biocompatibility. Furthermore, GO possesses abundant functional groups on its surface, such as carboxylic acids, hydroxyl, carbonyl and epoxy groups, facilitating chemical modifications [20–22]. GO has been successfully used as MALDI-TOF MS matrix for the analysis of small molecules [23], indicating no interference with MALDI analysis. The above-mentioned advantages make GO of great superiority as the candidate for enzyme immobilization to achieve on-plate digestion.

Up till now, several GO-based IMER were successfully prepared for rapid and efficient proteolysis. Chen et al. developed an efficient microchip proteolysis by immobilizing trypsin in the layer-by-layer coating of GO and chitosan on in-channel glass fiber [24]. We also prepared magnetic GO based IMER, in which trypsin was immobilized via π–π stacking and hydrogen bonding interaction [25]. Although high efficient digestion was obtained in both cases, trypsin desorption occurred inevitably because trypsin was attached on GO via non-covalent bonding. Recently, trypsin was further covalently bonded on polymers functionalized GO [26] and microchip channel with GO coating [27]. However, the active sites of trypsin might be well exposed with fragile cross-linkers introduced between GO and enzymes.

Recently, dendrimer has received more and more attention in biological fields due to its defined hyperbranched nanoarchitecture, excellent biocompatibility and large amount of functional groups at the periphery [28–31]. Furthermore, well-organized and close-packed arrays of dendrimer could be formed on GO surface, leading to the improved dispersibility of GO in water [32]. Due to the hyperbranched nanoarchitecture of dendrimer, enzyme could not be wrapped by the fragile chains, beneficial to avoid the mask of its active sites and decrease the risk of enzyme autolysis [33]. Besides, with abundant functional groups at the periphery of dendrimer, the immobilized amount of enzyme could be obviously improved [34]. Therefore, it could be envisaged that dendrimer-grafted GO (dGO) provides superiority of good dispersibility, hyperbranched nanoarchitecture and high capacity for enzyme immobilization. Herein, to the best of our knowledge, for the first time, dGO was prepared by covalent bonding and used as probe for trypsin immobilization with glutaraldehyde as coupling agents. The prepared trypsin-linked dGO was successfully applied to on-plate protein digestion, and showed great potential in large-scale proteome analysis.

2. Experimental

2.1. Chemicals and materials

GO was obtained from Xianfeng Nanotech Port (Nanjing, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysulfosuccinimide (NHS), 2-morpholino-ethanesulfonic acid (MES), TPCK-treated trypsin, dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA), bovine serum albumin (BSA), myoglobin (Myo), cytochrome *c* (Cyt-*c*), sodium cyanoborohydride ($NaCNBH_3$), glutaraldehyde (GA, 50 wt%), manganese chloride ($MnCl_2$), dendrimer (G2.0) and trifluoroacetic acid (TFA) were purchased from Sigma Chemical (St. Louis, MO, USA). Acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Enhanced BCA protein assay kit was provided by Beyotime Biotech (Nantong, China). Α-Cyano-4-hydroxy-cinnamic acid (CHCA) was obtained from Bruker (Daltonios, Germany).Water was purified using a Milli-Q system (Millipore, Molsheim, France). Other chemicals and analytically pure reagents were used as received.

2.2. Preparation of dGO

Under optimal conditions, 10 mg GO in 10 mL of 2-morpholino-ethanesulfonic acid solution (MES, 0.1 M, pH 5.6) was ultrasonicated for 3 h to obtain uniform dispersion. Then, 5 mg of EDC and 4 mg of NHS were added, and stirred for another 30 min at room temperature to activate carboxyl groups of GO. Next, 200 μL methanol solution of dendrimer with an ethylenediamine core was continuously dropped within 30 min. Afterwards, the mixture was stirred vigorously for another 2 h at room temperature to accelerate the reaction. Finally, dGO nanosheets were purified with a dialyzer (10 K molecular weight cutoff) using coupling buffer (50 mM NH_4OAc, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH ~ 8.3) to remove the excessive dendrimer.

2.3. Preparation of trypsin-linked dGO

One milligram dGO was transferred to a 1.5 mL Eppendorf tube, and the amino group of dGO was activated at room temperature under gentle rotation for 1.5 h by adding 1 mL 10 wt% GA solutions in coupling buffer. GA modified dGO was then collected by centrifugation at 20,000 rpm. Followed by four times washing with coupling buffer, GA modified dGO was incubated with 1 mg TPCK-treated trypsin, dissolved in 1 mL coupling buffer containing 1 wt% $NaCNBH_3$, for 3 h under rotation. After the removal of the excessive trypsin solution, the product was incubated for 1 h with 1 mL 0.75 wt% glycine/1 wt% $NaCNBH_3$ in coupling buffer. Finally, the obtained trypsin-linked dGO was washed four times with coupling buffer, followed by dissolving in 50 mM NH_4HCO_3 (pH 8.0), and storing at 4 °C as the stocking solution.

2.4. Standard protein preparation

Standard proteins (BSA, Cyt-*c*, Myo) dissolved in 1 mL 50 mM NH_4HCO_3 (pH 8.0) were denatured at 90 °C for 20 min, and then reduced in 10 mM DTT for 2 h at 56 °C. After cooled to room temperature, cysteines were alkylated in the dark in 20 mM IAA for 1 h at 37 °C. The obtained protein solution was used for on-plate and in-solution digestion, respectively.

2.5. On-plate and in-solution digestion

Each diluted protein solution (0.5 μL) was deposited on the plate of a MALDI-TOF MS (Bruker Ultraflex III). After that, 0.5 μL of trypsin-linked dGO stock solution was spotted, aspirated and dispensed for 5–10 cycles with pipette tip. The MALDI plate was then placed in a home-built humidity chamber, and incubated at 37 °C for 15 min. Subsequently 0.5 μL CHCA (7 mg/mL) was added to the digests. For comparison, in-solution digestion was performed in NH_4HCO_3 buffer (50 mM, pH 8.0) with enzyme to protein ratio (m/m) at 1:40 at 37 °C for 12 h, before 2 μL FA was added to terminate the digestion.

2.6. Human plasma preparation and digestion

The human plasma sample was thawed at −20 °C and ultrafiltrated at 134,000*g* for 60 min. A multiple affinity removal column (4.6 × 50 mm, Hu-14) was used to remove 14 high-abundant proteins. The obtained low-abundant proteins were desalted by a C8 trap column, and dissolved in 50 mM NH_4HCO_3 (pH 8.0) containing 8 M urea. The concentration of the low-abundant proteins was determined as 1 mg/mL by BCA reagents.

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