COMMUNICATION

Immobilization of trypsin on graphene oxide for microwave-assisted on-plate proteolysis combined with MALDI-MS analysis†

Guobin Xu,‡a Xiaoyi Chen,‡a Jianhua Hu, Pengyuan Yang, Dong Yang,b and Liming Wei*a

Received 18th January 2012, Accepted 20th April 2012
DOI: 10.1039/c2an35093a

With an ultra-high surface area and abundant functional groups, graphene oxide (GO) provides an ideal substrate for the immobilization of trypsin. We demonstrated that trypsin could be immobilized on GO sheets assisted by polymers as molecular spacers to maintain the activity of the enzyme. And with the trypsin-linked GO as the enzyme immobilization probe, a novel microwave-assisted on-plate digestion method has been developed with subsequent analysis by MALDI-MS. The feasibility and performance of the digestion approach were demonstrated by the proteolysis of standard proteins. The results show that this novel approach substantially accelerated proteolysis and reduced the time required for traditional procedures involving on-plate enzymatic digestion and sample preparation prior to MALDI-MS analysis. The novel digestion approach is simple and efficient, offering great promise for high throughput protein identification.

Over the past decades, proteomics has drawn more and more research attention.1 One of its most important tasks is to develop efficient and rapid approaches to identifying various proteins. Matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a powerful tool for identification and characterization of proteins.2,3 Protein samples were usually separated and digested into peptides with proteases. Subsequently, the obtained digests were deposited on MALDI plates to perform MS measurements. To simplify the analysis process and analyze small quantities of protein sample, on-plate proteolysis approaches were developed by combining digestion and spotting into one procedure.4,5 Meanwhile, the on-plate digestion technique could be connected to the separation of sample by multidimensional liquid chromatography with the analyses of MALDI-MS.6 This association makes the identification of proteins more reliable and the assignment of multiple locations of post-translational modification or sequence variation to a single protein species less problematic. Furthermore, on-plate digestion has promise in biomarker discovery and MALDI imaging.7,8 However, the traditional on-plate digestion of proteins is time-consuming (typically 3–5 h at 37 °C) with a weight ratio typically 1 : 10–1 : 40 between protease and protein.9 And if a high concentration of protease is used, the digestion proceeds quicker but auto-proteolysis products become more abundant. Thus, it is a challenging task to enhance the digestion efficiency of the on-plate digestion.

Various methods have been proposed to increase the on-plate proteolysis efficiency, including the immobilization of the enzyme on various substrates and with other assisted radiation.10,11 For on-plate digestion, trypsin was immobilized on magnetic nanoparticles or carbon nanotubes which can be spotted on the MALDI plate and protein samples were then deposited on the trypsin-immobilized spots and were allowed to be digested in humidified enclosures with the aid of heat.12,13 Because a high amount of trypsin was immobilized on the plate, the typical time of on-plate digestion was significantly reduced to 5–30 min with no interference from enzyme-autolysis products. But a risk of sample loss could be associated with the above method assisted by magnetic nanoparticles, because the magnetic nanoparticles used as a magnet probe must be removed after digestion to dismiss the interference from nanoparticles on MS analysis. Meanwhile, the carbon nanotube immobilized with enzyme would interfere with the uneven crystal of matrix and peptides, and then reduce the signal of peptides. On-plate digestion with free protease could also be accelerated by infrared radiation and with the assistance of low-voltage AC at room temperature (25 °C).14 The digestion time was significantly reduced to 5 min compared to 12 h for conventional in-solution digestion. However, the method described in the latter publications is difficult to repeat and adapt in other laboratories since it is based on in-house equipment. Microwave irradiation was applied for the acceleration of the conventional in-solution proteolysis directly with an accessible instrument (microwave oven).15,16 In addition, a variety of trypsin-immobilized magnetic particles dispersed in protein solutions have been used as microwave absorbers to accelerate microwave-assisted enzymatic digestion and shortened the digestion process to 15 s. But until now it had not been applied directly in the on-plate digestion techniques.

Graphene, a single-layered graphite composed of a perfect honeycomb crystal lattice, has shown great potential for applications in nanoelectronic devices, transparent electrodes, and biosensors.17,18 And due to the property of the energy receptacle for laser radiation and homogeneous distribution, graphene was used as a matrix for MALDI-TOF MS analysis of small molecules.19,20 In addition to the
high matrix efficiency, graphene has an ultra-high specific surface area (theoretical value 2630 m$^2$ g$^{-1}$), making it a promising candidate for a sorption material.$^{21}$ Graphene oxide (GO), the oxidized counterpart of graphene, contains functional groups such as epoxide, carboxyl, and hydroxyl groups which can be functionalized with biomolecules and drugs for \textit{in vitro} and \textit{in vivo} biological applications.$^{22,23}$

To extend the application of GO, here we firstly modified it with poly-lysine (PL) and PEG-diglycolic acid (PEG), and then used the PEG–PL modified GO as a probe for immobilization of trypsin. Given in Scheme 1 is the immobilized approach of GO with PL, PEG, and trypsin. Trypsin, in principle, can be directly conjugated with the acid-treated GO \textit{via} carbodiimide chemistry.$^{24}$ Acid-treated GO has a huge surface area, but the degree of carboxylated environment is limited. To maximize the immobilization capacity, a poly-lysine layer was immobilized on the acid-treated GO surface to provide rich amino groups for further surface functionalization. However, the poly-lysine modified GO sheets are good adsorbents for proteins and peptides owing to the complex interplay of hydrogen-bonding, hydrophobic and electrostatic interactions between analytes and adsorbents.$^{25}$ Therefore, to suppress the adsorption of peptides and proteins onto PL-modified GO, the PEG-functionalization was further carried out after the PL coating.$^{26}$ PL and PEG layers both serve as molecular spacers to minimize the absorption of peptides and proteins onto the GO surface. In addition, the molecular spacers help the stability of the space structure of trypsin, keeping the activity of enzyme. Moreover, as aforementioned, PEG is present on the surface of GO, and makes GO strongly hydrophilic and is easily dispersible in liquid media as a true water solution. The enzyme immobilized GO can be distributed in sample solution evenly on the MALDI plate, which helps in accelerating on-plate digestion of protein.

The immobilization of trypsin onto GO can be confirmed by FTIR (Fig. 1). Fig. 1 shows the FTIR spectra of GO, trypsin, and trypsin-linked GO, respectively. The spectrum of GO showed the presence of O–H ($\nu_{\text{O-H}}$ at 3450 cm$^{-1}$), C==O ($\nu_{\text{C=O}}$ at 1736 cm$^{-1}$ in the carboxyl group), C==C ($\nu_{\text{C=C}}$ at 1612 cm$^{-1}$), C–O ($\nu_{\text{C-O}}$ at 1433 cm$^{-1}$ in carboxyl groups, and at 1228 and 1055 cm$^{-1}$ in epoxy and alkoxy groups, respectively) and the asymmetric and symmetric methylene stretching vibration (at 2924 and 2852 cm$^{-1}$) (Fig. 1a). After the functionalization of GO, the FTIR spectrum of trypsin-linked GO exhibits trypsin absorption features, such as N–H ($\nu_{\text{N-H}}$ at 3392 cm$^{-1}$) and C–O ($\nu_{\text{C=O}}$ at 1639 cm$^{-1}$). Especially, the peak of the C–N stretch mode in trypsin-linked GO appeared at 1366 cm$^{-1}$ ($\nu_{\text{C-N}}$ binding with an aromatic ring). The new peaks at 3275 and 1102 cm$^{-1}$ ascribed to O–H stretch modes and the stretching vibration of C–O–C also confirm the existence of PEG. We assign the absorption bands at 3066 and 2961 cm$^{-1}$ to the C–H stretching vibration of the polymer (PL and PEG). Furthermore, one can find that the peak at 1736 cm$^{-1}$ ascribed to C=O of –COOH totally disappeared in curve (c). And with the comparison FTIR of trypsin and trypsin-linked GO, there are more similarities in Fig. 1b and c. These results confirm
that trypsin has been successfully covalent bonded on GO surfaces via PEG and PL. The transmission electron microscopy (TEM) images obtained from GO before and after trypsin immobilization are compared in Fig. S1 (ESI†). As can be seen, GO has a nearly transparent flake-like shape with characteristic crumpled silk waves (Fig. S1a†). After it was immobilized by trypsin (Fig. S1b†), a blurry and loose layer was generated on GO sheets. In addition to silk-like thin parts, the re-stacked parts can also be seen. Besides, scrolling and corrugation are part of the intrinsic nature of trypsin-linked GO, which result from the fact that the two dimensional membrane structures become thermodynamically stable when bent. The immobilization ability of GO for trypsin by the above method was evaluated by measuring the UV absorption value of the supernatant trypsin solution after the immobilization procedure. The amount of trypsin immobilized on GO was calculated to be about 423 \( \mu \text{g mg}^{-1} \).

To evaluate the interference of trypsin-linked GO to MS analysis, digests of cytochrome C (Cyto C) were analyzed directly by MALDI-TOF mass spectrometry mixed with and without trypsin-linked GO. As shown in Fig. S2 (ESI†), there is little significant difference of signal intensity from the two spectra. This has indicated that the trypsin-linked GO does not interfere with the MS analysis at all. Thereby, in situ mass spectrometric measurements can be performed after on-plate proteolysis without any additional procedure to remove the trypsin-linked GO.

For the improvement of proteolysis efficiency and reducing the sample preparation steps, we applied the newly immobilized enzyme to perform microwave-assisted on-plate digestion followed by MALDI-TOF MS analysis. The suspension of trypsin-linked GO was first spotted on the MALDI plate, and then protein solution was spotted to the same spot of the MALDI plate and allowed to digest in a humidified enclosure under microwave irradiation. At the beginning, we decided to explore the feasibility and performance of microwave-assisted on-plate digestion with trypsin-linked GO using BSA, which had not been pretreated with any denaturing pretreatment. Surprisingly, we observed 31 and 4 tryptic peptides of BSA by 15 s on-plate digestion under microwave irradiation with trypsin-linked GO and with free trypsin (enzyme-to-protein ratio of 1 : 40 (w/w)), respectively, in Fig. 2a and b. BSA can be well digested and positively identified by using 15 s microwave-assisted on-plate proteolysis with trypsin-linked GO. Three replicate experiments were carried out for the performance of the novel proteolysis approach. The average sequence coverage found upon comparison with structures recorded in the Swiss-Prot database was 38% for BSA. The identified peptide residues obtained are presented in Table S1 (ESI†). However, with free trypsin, the protein molecules of BSA were not well digested even with microwave assistance for 15 s. It was found that 52 out of the 607 possible amino acids of BSA were matched with the corresponding amino acid sequence coverage of 9%. That is, the trypsin-linked GO accelerates the on-plate digestion under microwave irradiation. Following this observation, the same sample of BSA was proteolysed on-plate with trypsin-linked GO and with free trypsin (enzyme-to-protein ratio of 1 : 40 (w/w)) at 37 °C for 15 min and analyzed by MALDI-TOF mass spectrometry, as shown in Fig. 2c and d, respectively. It was found that 19 and 3 tryptic peptides of BSA were matched with the corresponding amino acid sequence coverage of 24% and 4%, respectively. It is noticeable that the protein in the sample solution was not digested completely in the absence of trypsin-linked GO and it could not give a credible identification protein score. Therefore, the efficiency of on-plate digestion was also substantially enhanced by trypsin-linked GO. By comparing the on-plate digestion of BSA by trypsin-linked GO with and without microwave assistance (Fig. 2a and c), the results show that the number of matched peptides and sequence coverage increased from

![Fig. 2](https://example.com/image2.png)

**Fig. 2** MALDI-TOF mass spectra of the digests of 40 ng \( \mu \text{L}^{-1} \) BSA obtained by microwave-assisted on-plate digestion with and without trypsin-linked GO for 15 s (power 850 W) (a and b), and on-plate digestion with and without trypsin-linked GO for 15 min (c and d), respectively. All matched peptides are marked with “*” and “U” represents unknown peptides.
19 to 31 and from 24 to 38% for BSA under microwave irradiation, respectively (Table S1†). Furthermore, microwave irradiation gives some accelerated effect in the traditional on-plate digestion by free trypsin (Fig. 2b and d). These results imply that the efficiency of on-plate digestion with trypsin-linked GO was also enhanced by microwave irradiation, which is attributed to the uniform and rapid heating digestion solution by microwave irradiation and the microwave energy absorption on trypsin-linked GO.† Besides BSA, Cyto C and horseradish peroxidase (HRP) were also digested by the microwave-assisted on-plate proteolysis with trypsin-GO. The obtained MALDI-TOF MS spectra (Fig. S3†) indicate that a total of 11 and 9 tryptic peptides were matched with the amino acid sequence coverage of 85% and 47% to Cyto C and HRP (Tables S2 and S3†), respectively. These results definitely show that the novel microwave assisted on-plate proteolysis method combined with mass spectrometric peptide mass analysis is amenable to explicit identification of protein.

Besides the advantages of integrating and reducing the sample preparation steps for on-plate proteolysis, it could be applicable to the identification of very small amounts of protein with low concentration. As before, no denaturation procedures were performed before protein and the trypsin-linked GO were mixed on-plate directly. The mass spectrum of the digests of BSA and Myo (1 ng μL⁻¹ each) obtained by using 15 s microwave-assisted on-plate proteolysis with trypsin-linked GO are shown in Fig. 3. It was found that 6 and 9 tryptic peptides were matched with the corresponding amino acid sequence coverage of 12% and 76% for BSA and Myo, respectively. All matched peptides are presented in Tables S4 and S5†. At the same time, the same sample was digested by on-plate proteolysis with and without trypsin-linked GO for 15 min and in-tube for 12 h with the enzyme-to-protein ratios (1 : 40) as comparison, respectively. In the absence of microwave irradiation, the trypsin-linked GO was incapable of producing sufficient peptides to allow protein identification (Fig. S4†). Meanwhile, as can be seen from the mass spectra of the digests of BSA and Myo obtained by 12 h conventional in-tube digestion, only a few peptides were observed with weak signal intensity in each sample (Fig. S5†). Furthermore, no signal peak was observed in the MALDI-TOF mass spectrum of the same sample on-plate digested with free trypsin for 15 min (data not shown). Thus, both proteins were negatively validated. The result of comparison clearly shows that this novel on-plate digestion approach can be well accommodated for the digestion of low amount samples.

In summary, due to the ultra-high surface area and more activated functional group of GO, the novel functionalized GO with trypsin was prepared for the first time. And we have demonstrated that microwave-assisted on-plate proteolysis with trypsin-linked GO followed by MALDI-TOF MS analysis is a promising strategy for efficient protein digestion and peptide mapping. The high digestion efficiency and miniaturizing sample preparation of this microwave-assisted on-plate proteolysis approach offer great promise for rapid and high-throughput protein identification because hundreds of samples could be digested simultaneously within a short time (15 s). Another advantage of the present approach is its minimal sample consumption. Therefore, the simplicity and efficiency of the novel proteolysis approach indicate that it may find further application in automated analysis of large sets of proteins combined with capillary LC or a capillary array.

Acknowledgements

This study was supported by National Science and Technology Key Project of China (2007CB914100, 2009CB825607, 2010CB91270 and 2011CB910600), National Natural Science Foundation of China (20735005, 20975024 and 30530040), Ministry of Education of China (20080246011 and NCET), Shanghai Leading Academic Discipline (B109) and Shanghai Projects (08DZ2293601).

References

7 D. S. Cornett, J. A. Moblely and E. C. Dias, et al. A novel histology-directed strategy for MALDI-MS tissue profiling that improves...


