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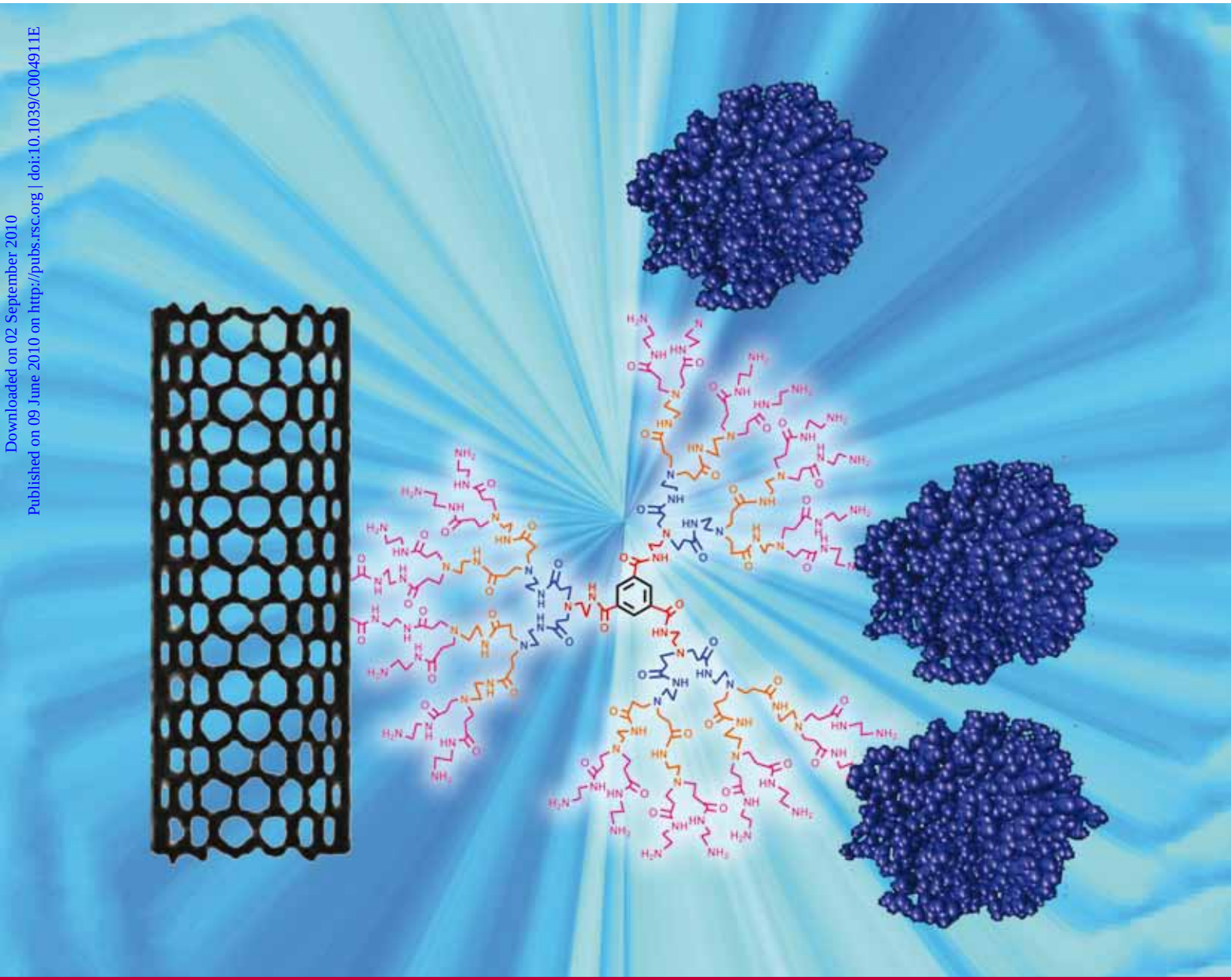

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Immobilization of trypsin on water-soluble dendrimer-modified carbon nanotubes for on-plate proteolysis combined with MALDI-MS analysis†

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A novel on-plate digestion method combined with MALDI-MS analysis is reported, using trypsin-linked dendrimer-modified carbon nanotubes (dCNTs) as the enzyme immobilization probe. Excellent digestion performance was achieved in a short time without any complicated reduction and alkylation procedures.

Protein separation by gel or liquid chromatography combined with enzymatic cleavage of the intact protein by proteases (for example, trypsin), followed by mass spectrometry determination of the generated peptides and subsequent database searching has become a standard technique for many applications in proteomics. However, stages of current protein identification strategies based on peptide formation and designated peptide mass fingerprint (PMF) are rather complex. Especially, the proteolysis procedure is the key process of protein sequencing in proteome research. But conventional digestion of proteins in Eppendorf tubes offers limited sensitivity and is time-consuming which should always take as long as 4–12 h.[1] In some cases, when the sample was at extremely low concentration or low amount, the proteolysis was hard to realize, because the digestion efficiency is known to be significantly hampered as the concentration of protein is reduced below micromolar levels.[2] In order to solve this problem, it is of high importance to develop efficient ways to achieve a high-quality protein digestion for MS peptide mapping.

Immobilized enzyme has been widely utilized owing to their advantage of allowing the usage of higher enzyme concentrations which lead to shorter digestion time.[3] In addition, enzyme autolysis which always occurred in the high enzyme concentrations could be to a large extent reduced compared with the unfixed enzyme.[4,5] In MALDI-MS analysis, on-plate proteolysis combined with the immobilization of enzyme approach were developed in order to simplify the analysis process. By covalent linking, trypsin was immobilized on magnetic nanoparticles which can be spotted on the MALDI plate, protein samples were then deposited on the same spots and allowed to digest in humidified enclosures with the aid of heat.[6] But it was necessary to remove the nanoparticles using a magnet after digestion because the nanoparticles always interfered with the MS analysis. However, this process may inevitably lead to the loss of samples. Others immobilized the trypsin directly on the spots of the MALDI plate, but the trypsin amount is relatively limited.[7]

Herein, we firstly modified carbon nanotubes (CNTs) with dendrimers and then used the dendrimer-modified CNTs (dCNTs) as the probe for immobilization of trypsin. A schematic illustration of trypsin immobilization onto the amine-terminal dCNTs is shown in Scheme 1. A fourth-generation (G4.0) NH_2-terminated dendrimer with a trimesyl core was covalently functionalized on the surface of the CNTs (a structural diagram of the dendrimers (G4.0) is shown in the ESI†, Fig. S1). The grafting of dendrimers (G4.0) on the surface of the CNTs was further confirmed by the FT-IR spectrum (Fig. 1) and TEM observations (ESI†, Fig. S2), respectively. For acid-treated CNTs (Fig. 1c), the peak at 1579 cm^{-1} is due to the C=C stretch mode, the peak appearing around 1715 cm^{-1} arises from the stretching vibration of the C=O group in the carboxyl group (COOH), and the peak at 1631 cm^{-1} arises from the stretching vibration of the C=O group in the carboxylate ion and carbonyl groups. As shown in Fig. 1a, the dendrimers (G4.0) exhibit two strong absorptions centered at 1647 and 1554 cm^{-1}, which are assigned to the C=O stretching (amide I) and N–H bending/C–N stretching (amide II) vibrations of the dendrimers, respectively. For dCNTs (Fig. 1b), new bands appear at 1641 and 1559 cm^{-1} corresponding to amide (–CO–NH–) I and II of dendrimers (G4.0), respectively. The new peaks at 2918 and 2842 cm^{-1} ascribed to C–H stretch modes also confirm the existence of the dendrimers. This indicates the complete reaction of –COOH groups in the carboxy-terminated CNTs with the amino groups of dendrimers (G4.0). Furthermore, one can find that the peak at 1715 cm^{-1} ascribed to C=O of –COOH totally disappears in curve (b). In addition, the content of dendrimers in dCNTs was estimated by TGA analysis (ESI†, Fig. S3).

The resultant trypsin-linked dCNTs possess many advantages. Firstly, the outer dense amine shell of dendrimers allowed the dCNTs to readily conjugate with enzyme through coupling agents like glutaraldehyde, thus cutting short the complicated and time-consuming procedure of surface modification for enzyme immobilization.[8] Remarkably, it was reported that the nanoparticles modified with dendrimers renders higher protein binding capacity on the surface compared to their unmodified counterparts, because the number of terminal

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† Electronic supplementary information (ESI) available: Experimental; structure of the dendrimers; TEM image of dCNTs; TGA analysis; photographs of prepared sample spots; MALDI-TOF mass spectra; Table S1: identified peptides. See DOI: 10.1039/c004911e

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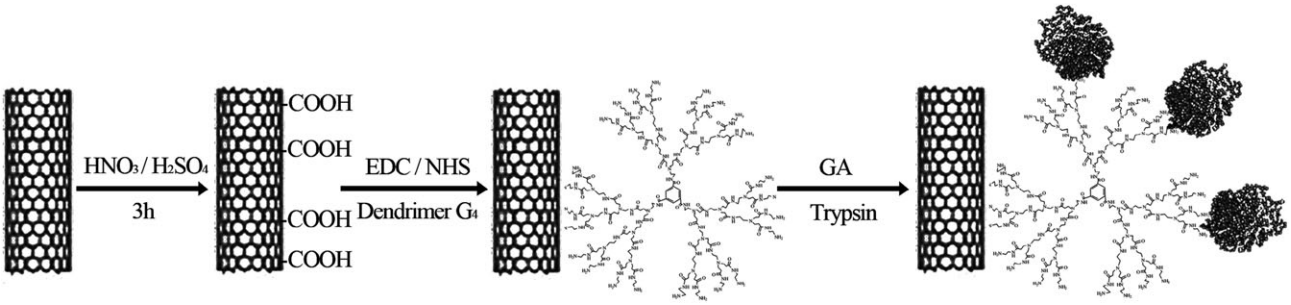

Scheme 1 Schematic illustration of trypsin immobilization onto the NH_2-terminal dCNTs.

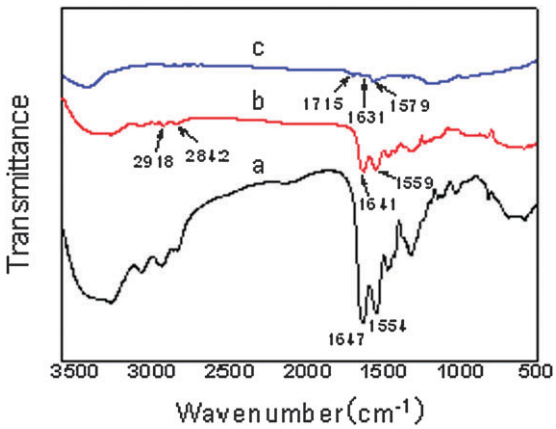

Fig. 1 FT-IR spectra of (a) dendrimer (G4.0), (b) dCNT and (c) acid-treated CNTs.

groups increases after each cycle or "generation" of the synthesis.[9] Therefore, we presumed the relatively high enzyme binding capacity (400 μg mg^{-1}) may be on account of the greater number of amino groups available after four generations. Secondly, with its compact structure, dendrimers may not wrap around enzymes to mask their active sites.[10] Moreover, as aforementioned, the dendrimers introduced a dense outer amine shell on the surface of the CNTs, it helped increase the surface polarity of the CNTs, making it well dispersed in liquid media as a true solution. Further study has indicated that the water-soluble dCNTs do not interfere with the MS analysis at all (as shown in the ESI†, Fig. S4 and S5). Thereby, *in situ* mass spectrometric measurements can be performed after on-plate digestion without any additional procedure to remove the trypsin-linked dCNTs. Therefore, the resultant trypsin-linked dCNTs possess the virtues of soluble enzymes, yet have the added advantages of immobilized ones.

For the purpose of integrating and reducing the sample preparation steps, we utilized the newly developed water-soluble immobilized enzyme to perform on-plate proteolysis followed by MALDI-TOFMS analysis. Standard proteins Cyt-c (MW: 12 384) and Lys (MW: 14 313) were used to test the performance of on-plate digestion using the trypsin-linked dCNTs. Good digestion of Cyt-c and Lys can be achieved using the on-plate proteolysis within 15 min, as demonstrated by the peptide mass fingerprints (PMFs), shown in Fig. 2a and b. Detailed peptide identification results are presented in the ESI†, Table S1. It was found that 88 out of the 104 possible amino acids of Cyt-c and 109 out of the 147 possible amino acids of Lys were matched with the corresponding amino-acid sequence coverage of 84% and 74%, respectively. Protein digestion efficiency also depended highly on the structural properties of the protein to be analyzed because proteins with rigid structures tended to be resistant to digestion. Therefore, Myo (MW: 16 900), a protein known to be rather resistant to proteolysis, was tested. 30% CH_3CN acetonitrile was employed in digestion buffer since it has been reported that mixed aqueous–organic solvents had a positive effect on the digestion efficiency.[11,12] The MALDI-TOF mass spectrum of the digests of Myo (100 ng μL^{-1}) obtained by using 15-min on-plate digestion with trypsin-linked dCNTs is shown in Fig. 2c. 101 out of the 153 possible amino acids of Myo were matched with the corresponding amino-acid sequence coverage of 66%. We compared the on-plate proteolysis method with

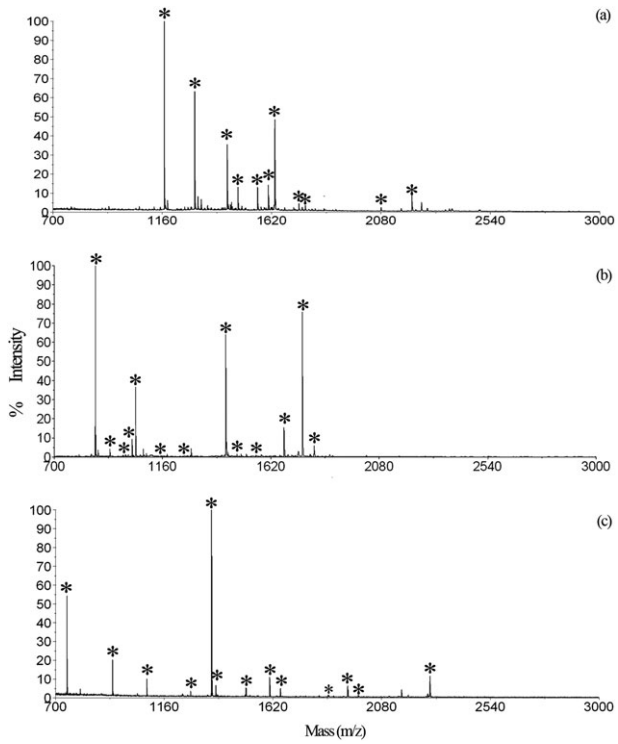

Fig. 2 MALDI-TOF mass spectra of the digests of (a) Cyt-c (100 ng μL^{-1}), (b) Lys (100 ng μL^{-1}), and (c) Myo (100 ng μL^{-1}) in 25 mM NH_4HCO_3 buffer solution (pH 8.1) obtained by using 15-min on-plate digestion with trypsin-linked dCNTs. All matched peptides are marked with "*".

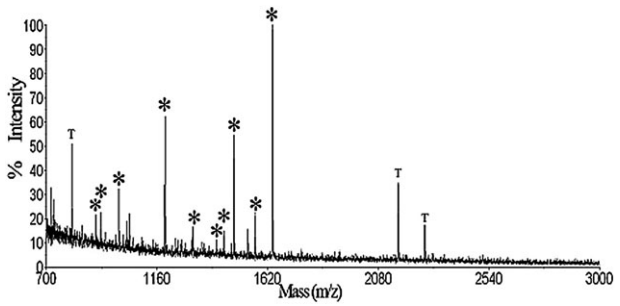

Fig. 3 MALDI-TOF mass spectrum of the digests of BSA (150 fmol μL^{-1}) in 25 mM NH_4HCO_3 buffer solution (pH 8.1) obtained by using 30-min on-plate digestion with trypsin-linked dCNTs. On each spot, 0.5 μL sample solution and 0.5 μL enzyme were spotted and mixed together on the spot with a pipette. All matched peptides are marked with "*", trypsin autolysis peaks are marked with "T".

traditional in-tube digestion. The identification results with the trypsin-linked dCNTs proteolysis are comparable with those by in-tube digestion that required a complicated denaturing procedure and a reaction time of 12 h (see ESI†, Table S1). These results definitely show that the novel on-plate proteolysis method combined with mass spectrometric peptide mass analysis is amenable to explicit identification of protein.

In traditional in-tube digestion, another difficulty frequently arises when the sample volume available is limited, because trace samples tend to be adsorbed by the Eppendorf tube and inevitably lost during the complicated digestion procedure. To check the feasibility of the on-plate digestion procedure for very small amounts of samples (small volume as well as low concentration), standard protein BSA was chosen as a model analyte. The same as before, no reduction and alkylation procedures were performed before the on-plate digestion. Fig. 3 illustrates the MALDI-TOF mass spectrum of 0.5 μL of 150 fmol μL^{-1} BSA digests obtained with on-plate digestion using the trypsin-linked dCNTs. It was found that 10 tryptic peptides were matched with the corresponding amino-acid sequence coverage of 18% for BSA. We also compared the on-plate proteolysis method with the conventional in-tube digestion. For the ease of operation, the total sample volume for in-tube digestion was 50 μL, after digestion, 0.5 μL of the sample was taken out for MS analysis. In-tube digestion of BSA was examined at different trypsin to protein (w/w) ratios. At higher trypsin to protein ratios (10 : 1 or 1 : 1), the mass spectra were dominated by the trypsin autolysis peaks (ESI†, Fig. S6a and b), in that trypsin autolysis becomes a significant competing reaction that reduces substrate conversion in the traditional in-tube digestion methods. While at a lower trypsin to protein ratio (1 : 10), the enzyme was incapable of producing sufficient peptide to allow protein identification (ESI†, Fig. S6c). The result of comparison clearly demonstrated that our novel on-plate digestion can also well accommodate for the digestion of low amount samples.

In summary, novel synthesized trypsin-linked dCNTs were used for on-plate proteolysis for the first time. Excellent digestion performance was demonstrated by the high sequence coverage and higher numbers of identified peptides in a short time without any complicated reduction and alkylation procedures. The high digestion efficiency and minimized sample-handling steps made this on-plate digestion method especially good for samples at extremely low amounts.

Acknowledgements

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