APPLICATION NOTE

Carbon Nanotubes (2,5-Dihydroxybenzoyl Hydrazine) Derivative as pH Adjustable Enriching Reagent and Matrix for MALDI Analysis of Trace Peptides

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A functionalized carbon nanotube (CNT), CNT 2,5-dihydroxybenzoyl hydrazine derivative, was synthesized and used as both pH adjustable enriching reagent and matrix in matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis of trace peptides. The derivative reagent, 2,5-dihydroxybenzoyl hydrazine, introduced phenolic hydroxyl and phenyl groups to the surface of the CNT. The former group can provide adjustable surface charge and a source of protons for chemical ionization, and the latter helps to keep strong ultraviolet absorption for enhancing pulsed laser desorption and ionization. It was found that the functionalized CNT was less twisted in a basic condition (pH 10.5), which afforded an increased surface area to volume ratio for adsorption towards trace peptides. However, functionalized CNT becomes deposited in an acidic condition (pH 5) and can be isolated readily from the sample solutions once the nanoparticles have trapped the target analytes, thus providing a novel and convenient alternative method for quick isolation. Compared with the previously reported method on enriching analytes using the pristine CNT, it is observed that the detection limit for analytes can be greatly improved due to enhancing adsorption capacity of the functionalized CNT. Moreover, peptide mixture at concentration as low as 0.01 pg/μL still can be detected after enrichment mediated by the functionalized CNT, while it is difficult to be detected without enrichment at concentration 0.1 pg/μL using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. Therefore, high efficiency of adsorption and enrichment towards trace peptides can be achieved by adjusting pH value of the functionalized CNT dispersion. (J Am Soc Mass Spectrom 2006, 17, 1023–1027) © 2006 American Society for Mass Spectrometry

MALDI-TOF MS is now a routine analysis tool for biomolecules [1]. However, many proteins are quite scarce owing to the difficulties of sample isolation. This is especially true for proteins that are expressed in low abundance [2]. Moreover, a further decrease in sample yield may occur during the preparation of a peptide digest before MALDI-TOF MS analysis, which therefore requires that peptide preparations undergo an enrichment step beforehand.

Recently, several methods of sample preparation have been developed for the MALDI-MS analysis of biomolecules using nanomaterials such as gold nanoparticles [3], magnetic nanoparticles [4], fullerene [5–7], and carbon nanotubes (CNTs) [8–10]. The ability of CNTs as a sort of unique enriching reagents has been demonstrated by some studies [8–10]. In addition,

CNTs have been used as the matrix in MALDI-TOF-MS for analysis of small molecules without the matrix ion interference [10–12]. However, pristine CNTs typically exist as ropes or bundles because of strong van der Waals interactions, resulting in stacking and entangling and, thus, decreasing the surface area to volume ratio, finally limiting the adsorption capacity of the CNT. One method to combat particle aggregation and allow efficient analyte binding is surface modification. Shiea et al. and Woods et al. have reported successful applications of covalently functionalized nanocarbon as matrices for MALDI-TOF analysis of bioanalyte [5–7]. Herein, we emphasized on examining functionalized CNTs as an enriching reagent as well as matrix for MALDI analysis of trace peptides and expected to gain more efficient results.

CNTs surface can be modified by introducing various organic functional groups, thus providing a strongly physisorbing surface area, adjustable surface charge, and a source of protons for chemical ionization. It has been demonstrated that the surface of CNTs can

be easily modified in numerous ways, either by covalent or noncovalent functionalization [13, 14].

In the current work, we derivatize multiwalled carbon nanotube (10–30 nm in diameter) with 2,5-dihydroxybenzoyl hydrazine, and carefully evaluated the performance of the functionalized CNT functioning both as pH adjustable enriching reagent and the matrix for MALDI MS to analyze trace peptides. The properties of the nanoparticles can be adjusted after the introduction of organic groups such as phenolic hydroxyl to the surface of the CNT. We found that the functionalized CNT can be well dispersed in a basic condition (pH 10.5) while become deposited in an acidic condition (pH 5). Therefore, the adsorption capacity is good when pH of the dispersion was adjusted to basic state. However, the functionalized CNT particles will quickly deposit to the bottom of the vial when pH is adjusted to acidic value, and in this way these particles can be isolated readily from the sample solutions once the nanoparticles have trapped the target analytes. Therefore, we can achieve high efficiency of adsorption and enrichment of trace peptides by adjusting pH value of the functionalized CNT dispersion.

Experimental

Chemicals and Materials

The peptides (angiotensin I and angiotensin II), chicken egg white lysozyme, trypsin, and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Sigma (St. Louis, MO). Dithiothreitol (DTT) was obtained from Aldrich (Milwaukee, WI). The multiwalled CNT particles used in the study were obtained from Sun Nanotech (Shanghai, China). Methanol used in our experiments was HPLC grade, and MilliQ water (Millipore, El Paso, TX) was used. Filter membranes (type VCTP, 100-nm pore) were obtained from Millipore. The other chemical reagents were of analytical grade and were used without further purification.

Synthesis Procedure for Functionalized CNTs

To prepare CNT 2,5-dihydroxybenzoyl hydrazine derivative, the following procedures were developed, including oxidation of the CNT, activation of the carboxyl moieties with thionyl chloride, and subsequent reaction with hydrazine. The details of the experiment processes can be seen in the Supplementary Material section S1 which can be found in the online version of this article.

Preparation of Analyte Solutions

Angiotensin I and Angiotensin II were dissolved in water at the concentration of 1 mg/mL as storage solution, and other different concentrations were prepared by dilution step by step. Different concentrations of lysozyme digestion were also prepared by dilution step by step. All storage solutions were refrigerated at around 4 °C for usage. The tryptic digest reaction proceeded as following: 1 mg of lysozyme was dissolved in 990 μl of 0.03 M ammonium bicarbonate. A 1 μmol amount of DTT was added to the soluble lysozyme mixture and the resulting solution was incubated at 50 °C for 15 min. Then iodoacetic acid (20 μL, 1 mM) was added to the cooling solution. The resulting solution was diluted 1:1 with water. The lysozyme was digested with trypsin (2%, wt/wt) at 37 °C overnight.

Extraction Procedure for Functionalized CNTs and CNTs

Ten μL functionalized CNTs suspensions (10 mg/mL) were added to standard peptides or peptide digest solutions (1 mL) at varied concentrations. Then ammonia was added to the mixture (pH 10.5). With agitation less than 5 s, functionalized CNTs were homogeneously spread in the solution and the analytes were extracted from the liquid phase to the surface of functionalized CNTs in 30 min. Then acetic acid was added to the solution (pH 5). After centrifugation at 10,000 for 5 min, functionalized CNTs adsorbed with analytes were deposited on the bottom of the centrifuge tube. Then the supernatant was removed, and 5 μL dispersant solution of 50% acetonitrile (vol/vol) was added into centrifuged tube to resuspend the functionalized CNT. Finally, about 1 μL suspension of the functionalized CNTs was pipetted onto the sample target for further analysis by MALDI-TOF-MS. When using pristine CNTs to adsorb the analytes, the CNT was suspended in the solution of analytes. After the analytes were extracted from the liquid phase to the surface of CNTs, the solution was centrifuged and the deposited CNTs adsorbed with analytes were suspended by the 50% acetonitrile (vol/vol) solution for directly MALDI-TOF MS analysis.

Instrument Setup

The MALDI-TOF-MS analyses were performed by employing delayed extraction in positive ion mode on a time-of-flight mass spectrometer (Voyager-DE STR, Applied Biosystems, Framingham, MA) with a 2.0-m flight tube. Desorption/ionization was obtained by using a 337-nm nitrogen laser with a 3-ns pulse width. Available accelerating potential is in the range of ±20 kV. Laser power was adjusted to slightly above the threshold to obtain good resolution and signal-to-noise ratios, and mass calibration was achieved using "calibration mixture one" provided by Applied Biosystems (Darmstadt, Germany) as an external standard. The functionalized CNT was suspended in water with ultrasonically dispersion for 5 min. The suspension was deposited

onto lacey carbon-coated copper grid and air-dried for transmission electronic microscopy (TEM, Philips CM-120, Eindhoven, The Netherlands) analysis.

Results and Discussion

Design, Synthesis, and Characterization of the Functionalized CNT

The adsorption of CNTs can be credited to physical interaction, so the capacity of adsorption is correlated with the surface area to volume ratio of the CNT. Surface modification is reported to be a feasible method to separate discrete CN molecules from the tight bundles [15]. We synthesized the CNT derivative by amidation of oxidized CNTs (see Supporting Information S1, which can be found in the electronic version of this article.), which is one of the general approaches for chemical modification of CNTs reported so far [16, 17]. We chose 2,5-dihydroxybenzoyl hydrazine as derivative reagent because of the acidic phenolic hydroxyl and aromatic phenyl groups it holds. Phenolic hydroxyl not only can serve as proton source for ionization but also can make surface charge adjustable. Meanwhile, the phenyl group may help to conserve or even enhance the efficiency of the CNT as matrix for it has strong ultraviolet (UV) adsorption. Moreover, this CNT derivative can be synthesized according to the method reported previously [16].

To confirm that we had successfully generated the CNT 2,5-dihydroxybenzoyl hydrazine derivative, ^{1}H NMR and thermal gravimetric analysis (TGA) were employed to characterize it. Weak signals for the newly formed acylhydrazine protons present in the ^{1}H NMR spectrum for the CNT derivative. [^{1}H NMR (DMSO-d_6): δ = 10.947 Hz (Ar-OH), 10.365 (Ar-OH), 9.152 (NH), 7.219-6.849 (Ar-H)]. In addition, TGA (see Supporting Information S2) analysis results show that the onset temperature of the weight loss for the functionalized CNTs is about at 500 °C, while that of pristine CNT is higher than 700 °C. The weight-loss on the TGA curve for the CNT derivative can be assigned to the decomposition of the functionalized moieties of the CNT derivative. The results, therefore, confirmed the formation of the CNT 2,5-dihydroxybenzoyl hydrazine.

The introduction of phenolic hydroxyl group to the surface of CNTs 2,5-dihydroxybenzoyl hydrazine derivative can provide adjustable surface charge. Hence, by changing the pH value, the dispersion ability of the functionalized CNT can be adjusted. Figure 1a and b show TEM images of the functionalized CNT in neutral and basic conditions, respectively. By comparing Figure 1a and 1b,we can see that the CNT were less twist and its dispersion ability was increased after treatment with ammonia. The phenomenon is attributed to the strong electrostatic repulsion of the negative charge, which amassed on the surface of the functionalized CNT when the solution was adjusted to basic state (pH 10.5) and prevented the formation of large congeries. Therefore,
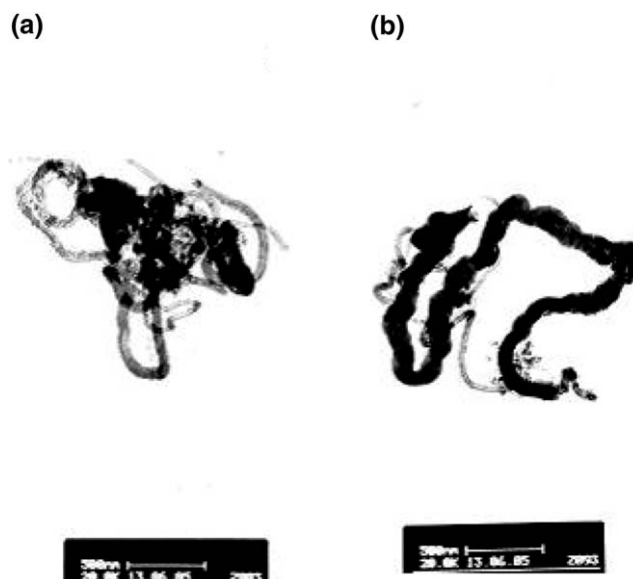
Figure 1. TEM micrograph of the functionalized CNT (**a**) in neutral condition, (**b**) in basic condition. The functionalized CNT were less twist in basic condition.

the TEM data demonstrated the feasibility of using the CNT 2,5-dihydroxybenzoyl hydrazine derivative as pH adjustable enriching reagent.

MALDI Analysis of Trace Peptides After Extraction Procedure Using the Functionalized CNT as Enriching Reagent and Matrix

In this work, we principally invested the performance of the CNT 2,5-dihydroxybenzoyl hydrazine derivative as a pH adjustable enriching reagent rather than as a matrix for MALDI MS analysis of peptides.

Figure 2a and b show mass spectra of the peptide (Angiotensin II, MW = 1296 Da) extracted from 1 mL solutions at 100 pg/μL with (Figure 2a) pristine CNTs and (Figure 2b) the functionalized CNTs as pH adjustable enriching reagent and matrix for MALDI, respectively. It can be found that there is an intense ion signal for angiotensin II at m/z 1297 in Figure 2b, while only a weak ion signal at m/z 1297 in Figure 2a, which demonstrated that all parameters for mass spectrum on functionalized CNTs are better than those on pristine CNTs.

The improvement of intensity and signal-to-noise (S/N) of analyte might result from the fact that the capacity of the adsorption of the functionalized CNTs was enhanced after the addition of ammonia solution, which has been demonstrated by TEM image as shown in Figure 1. Moreover, when the solution was adjusted to acid state (pH 5) using acetic acid, the dispersion ability of the functionalized CNT decreased quickly. Therefore, these particles can be isolated readily from the sample solutions once the nanoparticles have trapped the target analytes. It is reminiscent of the magnetic-nanoparticles previously reported, which
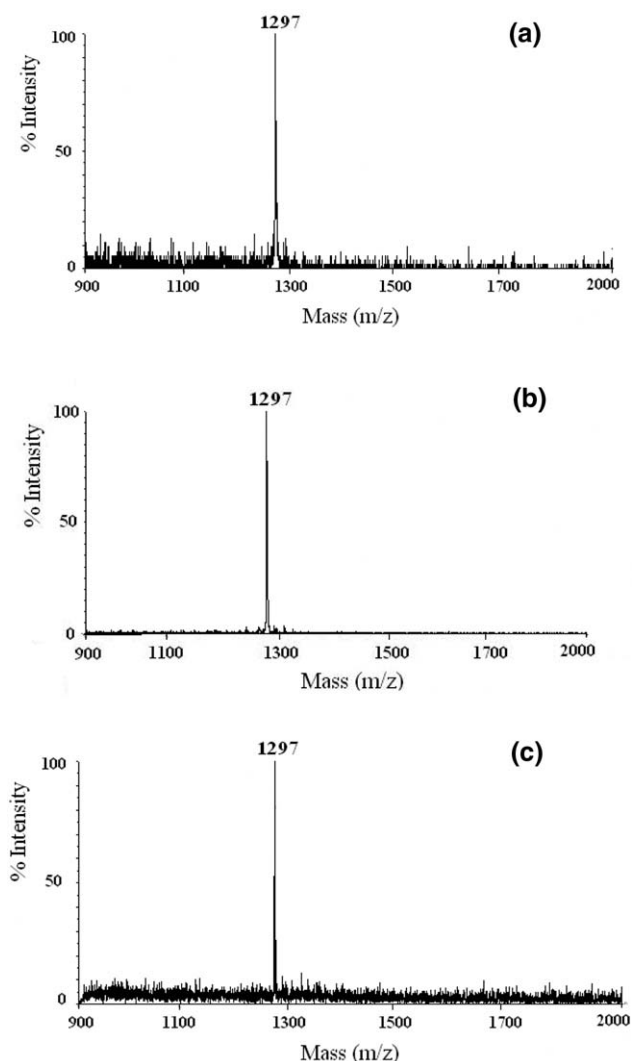
Figure 2. MALDI-TOF mass spectra for peptide (angiotension II, 1296 Da) at a concentration of 100 pg μL^{-1} (**a**) enriched with the pristine CNT, (**b**) enriched with the functionalized CNT treated by ammonia and acetic acid, and (**c**) DHB as a matrix without enrichment.

droxyl and phenyl groups to the surface of the CNT, which provides a source of protons for chemical ionization [12] and keeps strong ultraviolet absorption for enhancing pulsed laser desorption and ionization, respectively and, thus, assuring that the functionalized CNT conserve the properties as efficient matrix (as shown in Figure 2b). Note that DHB is a choice of matrix for peptide analysis, and that enhanced performance of DHB derivative as matrix will also most likely be due to the fact DHB is such a good matrix.

Then we further investigated the trap capacity of our functionalized CNTs toward trace peptides by analyzing the tryptic digest product of lysozyme. Figure 3a and b present the MALDI mass spectra of the peptides from the tryptic digest product of lysozyme enriched using the pristine CNT and the functionalized CNT particles, respectively. Figure 3b displays the MS result of a lysozyme (500 pg/μL) digest enriched by the functionalized CNT. A database search [18] identified about nine peptides that represent 50–65% coverage of the lysozyme sequence. We can see that signals are intense and the spectrum is clean in Figure 3b. In comparison with the MS data from the high-lysozyme concentration (5000 pg/μL) digest enriched by the pristine CNT (Figure 3a), where only three weak signals for peptides of the digest product can be seen. The advantage of the functionalized CNT-mediated enrichment is obvious: a clear tryptic fragment profile is present, and the S/N is much higher even for a preparation containing tenfold less protein. The result also
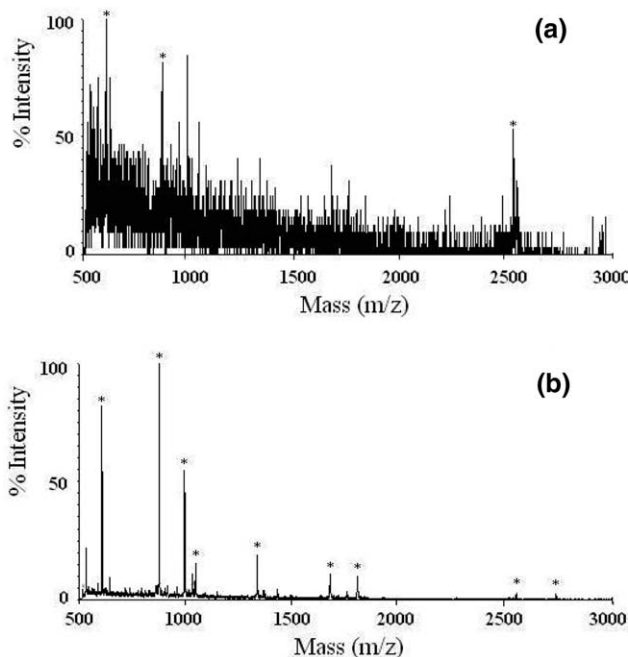
magnetize the target analytes and isolate them readily from the sample solution by employing an external magnetic field [4]; both of these methods can afford similar results. Distinctly, our method involves a simple procedure of pH value changing and, thus, providing a novel and convenient alternative method for quick isolation.

In addition, Figure 2c shows mass spectrum of angiotensin II at 100 pg/μL using DHB as matrix without enrichment. By comparing Figure 2b and 2c, we can see that the functionalized CNT is working better than DHB. Thus the enhancement with the CNT derivative can be attributed to its ability of concentrating the analyte.

In a previous study, CNTs were demonstrated as a kind of suitable matrix for the analysis of small biomolecules [11]. In this work, the modified reagent, 2,5-dihydroxybenzoyl hydrazine introduced phenolic hy-

Figure 3. MALDI-TOF mass spectra for tryptic digest product of lysozyme enriched using (**a**) the pristine CNT with sample at 5000 pg/μL, (**b**) the functionalized CNT treated by ammonia and acetic acid with sample at 500 pg/μL. The peaks marked with an asterisk represent the lysozyme fragments.

indicates that the functionalized CNT appears not to discriminate against the various peptides. Hence, it demonstrates that the functionalized CNT can provide better enriching results than the pristine CNT do.

To further study the enrichment effect of the functionalized CNT on trace peptide analysis, the functionalized CNT was dispersed in a dilute aqueous solution containing two peptides (0.01–1 pg/μL for each peptide) (Peptide I: Angiotensin I, Mw 1046 Da; and peptide II: Angiotensin II, Mw 1296 Da) After centrifugation and redispersion, the suspension of peptide-adsorbed functionalized CNTs were mixed with a normal MALDI matrix (CHCA). We compare the results of analysis peptides with or without the functionalized CNT-mediate enrichment (mass spectra can be seen in Supplementary Material S3). We found that the peptides at concentration lower than 1 pg/μL were scarcely detected by MS using CHCA as matrix without the functionalized CNT-enrichment step. However, after enrichment, peaks of all peptides became clearly apparent, even at a peptide concentration as low as 0.1 pg/μL. Notably, when the peptide solution was diluted to 0.01 pg/μL, the two peptides could still be detected after enrichment. The results demonstrate the remarkable ability of functionalized CNT for enrichment of trace peptides.

Conclusions

In summary, we synthesized a kind of functionalized carbon nanotube (CNT), CNT 2,5-dihydroxybenzoyl hydrazine derivative. And the functionalized CNT was successfully applied in the enrichment and identification of trace peptides by functioning both as pH adjustable enriching reagent and a matrix used in MALDI-MS. Using the functionalized CNT as pH adjustable enriching reagent, we have demonstrated that it provided some advantages over the pristine CNT. First, the efficiency of enrichment was greatly enhanced as a result of increased surface area to volume ratio for adsorption in basic conditions. Second, it provided a novel and convenient alternative method for quick isolation since the functionalized CNT adsorbed with analytes can be isolated readily from the sample solutions in acidic condition for the decreased dispersion ability. In addition, compared with the method using CHCA as matrix without enrichment, the detection limit for analytes can be enhanced about 10 to 100 times after enrichment by the functionalized CNT. Therefore, this technique is expected to open up new horizons for the preconcentration and identification of trace peptides, as well as a new application of CNTs.

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