CHEMISTRY
A EUROPEAN JOURNAL

ChemPubSoc
Europe

DOI: 10.1002/chem.201202838

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Surface-Assisted Laser Desorption/Ionization Mass Spectrometric Detection of Biomolecules by Using Functional Single-Walled Carbon Nanohorns as the Matrix

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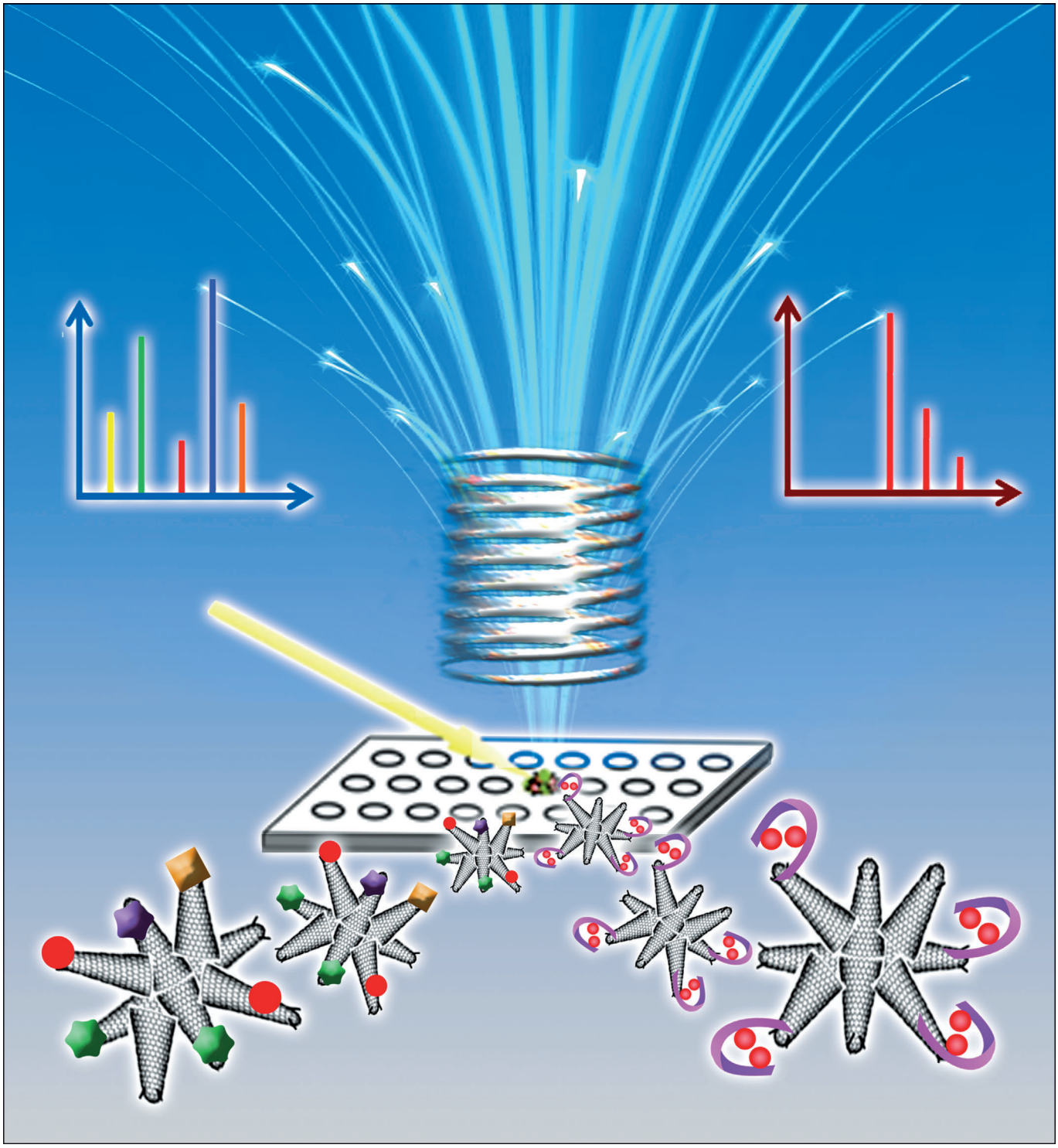

Abstract: A surface-assisted laser desorption/ionization time-of-flight mass spectrometric (SALDI-TOF MS) method was developed for the analysis of small biomolecules by using functional single-walled carbon nanohorns (SWNHs) as matrix. The functional SWNHs could transfer energy to the analyte under laser irradiation for accelerating its desorption and ionization, which led to low matrix effect, avoided fragmentation of the analyte, and provided high salt tolerance. Biomolecules including amino acids, peptides, and fatty acids could successfully be analyzed with about 3- and 5-fold higher signals than those obtained using conventional matrix. By integrating the advantages of SWNHs and the recognition ability of aptamers, a selective approach was proposed for simultaneous capture, enrichment, ionization, and MS detection of adenosine triphosphate (ATP). This method showed a greatly improved detection limit (1.0 μM) for the analysis of ATP in complex biological samples. This newly designed protocol not only opened a new application of SWNHs, but also offered a new technique for selective MS analysis of biomolecules based on aptamer recognition systems.

Keywords: adenosine triphosphate · aptamers · carbon nanohorns · mass spectrometry · SALDI-TOF

Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a powerful tool in the analysis of species with a high molecular mass such as proteins, DNA/RNA, polysaccharides, and synthetic polymers.[1–6] Owing to the high background signal in the low-mass region (<700 Da) produced from conventional matrix systems, this technique is difficult to use for the analysis of low molecular weight compounds.[7] The inhomogeneous crystallization of analytes in traditional organic matrices such as α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) also produces "sweet spots" on the sample probe, which results in poor shot-to-shot and sample-to-sample reproducibility. These disadvantages hinder its application in some important fields such as drug discovery and biotechnology, where small molecule detection and identification are significant.[8] To overcome these disadvantages, surface-assisted laser desorption/ionization time-of-flight mass spectrometry (SALDI-TOF MS) has been developed to eliminate the interference of matrix ions and improve sample homogeneity[9,10] by using titania particles,[11–13] porous silicon,[14–16] and ionic liquids[17] as the matrix. Similar to the role fulfilled by organic matrices, these materials can induce desorption and ionization of the analyte by efficiently transferring the energy from laser irradiation. Thus, probing the new materials as efficient matrices is becoming an urgent topic for extending the application of MALDI-TOF MS.

Carbon-based materials, especially carbon nanotubes[18–20] and graphene,[21,22] have attracted particular attention as potential matrices in SALDI-TOF MS owing to their good flexibility for different samples, high surface area, and energy transfer efficiency. Carbon nanotubes were first used as matrix to eliminate the conventional matrix interference for desorption/ionization of peptides, organic compounds, and β-cyclodextrin, which showed high detection sensitivity and excellent reproducibility.[18] These matrix systems have been used for monitoring enzyme reactions and for the screening of enzyme inhibitors.[19] The monolayer structure and unique electronic properties of graphene can greatly improve the efficiency of desorption/ionization, thus leading to low matrix background ions and improved shot-to-shot reproducibility.[21] By using negative ion mode on graphene a method for the SALDI-TOF MS analysis of small molecules has been developed, which shows very "clean" mass spectra.[22] To improve the specificity of SALDI assay techniques, some affinity reagents such as antibodies[23,24] and aptamers[25] have been conjugated to porous silicon and graphene for affinity capture of the target from complex samples. The aptamer-conjugated graphene oxides have been used for direct MALDI readouts of adenosine and cocaine in the positive ion mode.[25] This work further explored the application of single-walled carbon nanohorns (SWNHs), a new carbon nanomaterial, as an efficient MALDI matrix. By combining the advantages of SWNHs with the specificity of aptamers and the good analytical performance obtained upon negative ion mode, a new method for SALDI-TOF determination of small biomolecules such as adenosine triphosphate (ATP) was proposed.

SWNHs, which are composed of thousands of graphitic tubule closed ends with cone-shaped horns,[26,27] possess large surface area, plentiful inner nanospaces, highly defective horns, and high energy transfer efficiency.[28,29] The oxidation treatment of SWNHs can produce extensive oxygen-functionalized sites at the horns[30] for the convenient immo-

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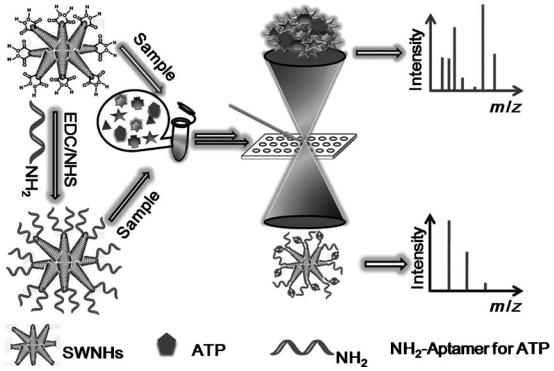
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201202838.

bilization of biorecognition molecules.[31] The large surface area makes SWNHs more tightly attached to the target plate to prevent their detachment under vacuum, which avoids the contamination of the ion source. The plentiful nanospaces and high energy transfer efficiency result in high desorption and ionization efficiency for analytes. Thus, the designed method showed excellent performance for SALDI analysis of amino acids, peptides, and fatty acids without any background problems. After functionalization with ATP aptamers, the functional SWNHs could act as an affinity extraction and detection platform for simultaneous capture, enrichment, and ionization of ATP from complex media (Scheme 1). This method greatly improved the detection limit, and could be highly beneficial to sample preparation and selective MS analysis and high-throughput detection of biomolecules based on affinity recognition.



Scheme 1. Schematic diagram of aptamer modification of SWNHs and SALDI-TOF MS analysis using functional SWNHs as matrix.

Results and Discussion

Characterization of functional SWNHs: To realize the selective capture and detection of ATP by SALDI-TOF MS, an amino-functionalized aptamer (Apt) for ATP was introduced to SWNHs, which could intercalate two ATP molecules by forming noncanonical G:A base pairs.[32] The shape and size of the Apt–SWNHs were characterized by TEM, which showed the typical morphology of dahlia-like SWNH bundles with a diameter of 80–100 nm (Figure 1 A), indicating that the original dahlia flower-like morphology of the SWNH aggregates was sustained to provide the advantages of SWNHs for MALDI-TOF MS analysis. The covalent modification of ATP aptamers on oxidized SWNHs could be confirmed by XPS and FT-IR spectra. The survey XPS spectrum of oxidized SWNHs exhibited C1s and O1s signals with binding energies of 285 and 531 eV, respectively (Figure 1 B, curve a), and their atomic concentrations were 83.76 and 16.24 %. After binding with ATP aptamers, the survey XPS spectrum of Apt–SWNHs resulted in two new signals of N1s and P2p with binding energies of 398 and 131 eV, respectively (Figure 1 B, curve b). These results demonstrated that ATP aptamers are anchored to SWNHs.

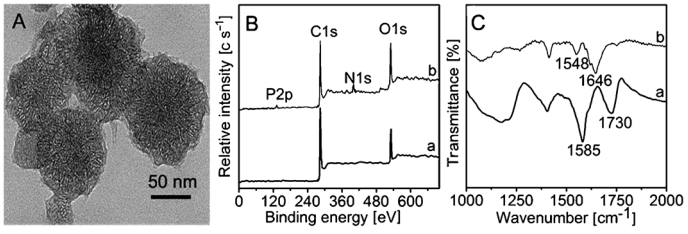


Figure 1. A) TEM image of Apt–SWNHs, B) FT-IR spectra of a) SWNHs and b) Apt–SWNHs, and C) XPS analysis of a) SWNHs and b) Apt–SWNHs.

The FT-IR spectrum of oxidized SWNHs displayed two signals at 1730 and 1585 cm^{-1} (Figure 1 C, curve a), which were assigned to the carbonyl stretch mode of −COOH and −COO$^-$.[31] After covalent conjugation of the amino-modified ATP aptamer to the SWNHs, the vibration of amide I and amide II could be observed at 1646 and 1548 cm^{-1} (Figure 1 C, curve b), while the signal assigned to the carbonyl stretch mode decreased greatly, indicating the effective reaction of the carboxylic group of SWNHs with the amino group of the ATP aptamer-NH_2. These results further confirmed the attachment of ATP aptamer to SWNHs upon covalent binding.

Performance of SWNH-assisted SALDI MS for analysis of amino acids: Amino acids were firstly chosen as representative small molecules to examine the application of SWNHs as the matrix of MALDI. The MALDI-TOF mass spectra of a standard solution containing five 0.5 mM amino acids of Val (M_r 117.15), Gln (M_r 146.14), Phe (M_r 165.19), Arg (M_r 174.20) and Trp (M_r 204.23) with CHCA or SWNHs as matrix are shown in Figure 2. Not surprisingly, CHCA as a conventional matrix showed strong background signals corresponding to the matrix-related ions, including a protonated matrix molecule $[M+H]^+$ at 190 Da, a fragment ion at 172 Da, a sodiated matrix molecule $[M+Na]^+$ at 212 Da, and a $[2M+H]^+$ ion at m/z 379 (Figure 2 A and Table S1 in the Supporting Information). Some background signals would interfere the detection of amino acids with $[M+H]^+$ ions at m/z 118.13, 147.12, 166.13, 175.17, and 205.14, respectively. Thus, CHCA is not a useful matrix for amino acid analysis.

In the positive ion mode the mass spectrum using functional SWNHs as assisted matrix displayed only the signals corresponding to potassium or/and sodium adduct ions of amino acids such as $[M+K]^+$, $[M+Na]^+$, $[M+2K-H]^+$, $[M+2Na-H]^+$, or $[M+Na+K-H]^+$ and the $[M+H]^+$ signal for Arg (Figure 2 B and Table S2 in the Supporting Information). No fragment ion signals of the analytes were observed. Furthermore, the matrix ion interference was completely eliminated, suggesting that SWNHs were the novel "soft" matrix for MALDI-TOF MS. Compared with CHCA matrix, the lack of the $[M+H]^+$ signal for other amino acids was possibly due to the fact that CHCA is a stronger proton donor than SWNHs, which is also one of the reasons that CHCA is generally used in the positive ion mode.

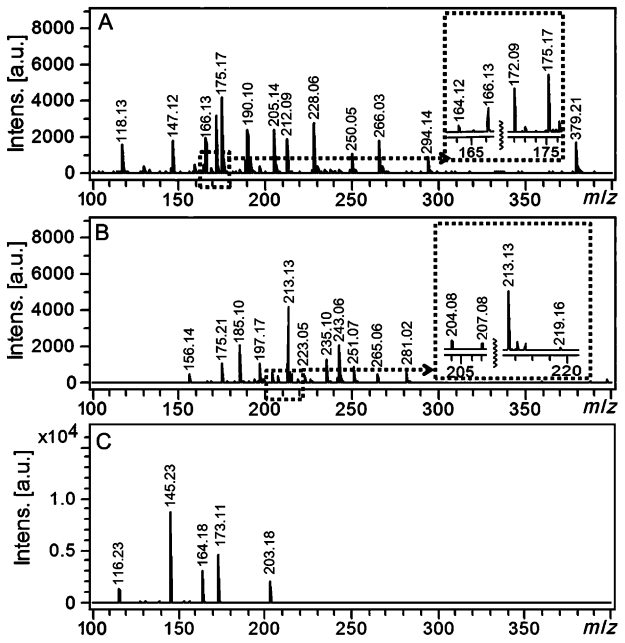


Figure 2. MALDI-TOF MS spectra of 1 μL solution containing 0.5 mM Val, Gln, Phe, Arg, and Trp using A) CHCA and B) SWNHs as matrix in the positive ion mode, and C) SWNHs as matrix in the negative ion mode.

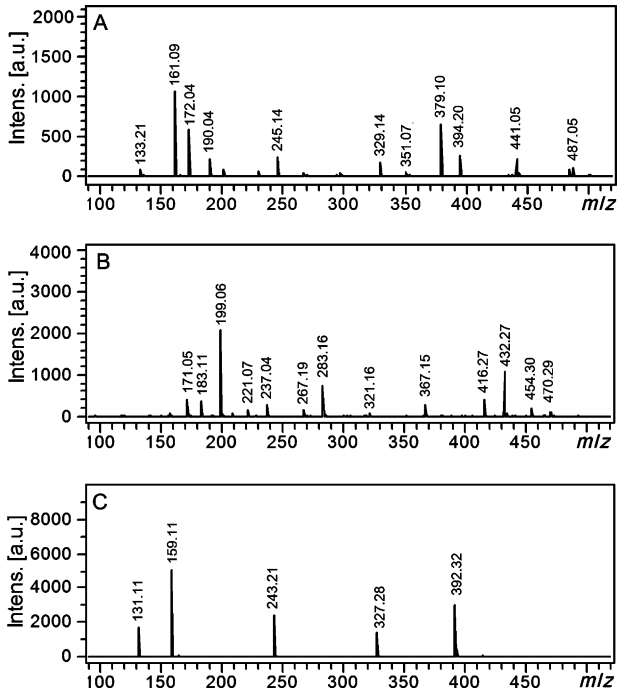


Figure 3. MALDI-TOF MS spectra of 1 μL solution containing five kinds of 0.5 mM peptides using A) CHCA and B) SWNHs as matrix in the positive ion mode, and C) SWNHs as matrix in the negative ion mode.

To improve the performance of functional SWNHs, the SALDI TOF mass spectrum in the negative ion mode was examined under the same experimental conditions. Figure 2C indicates that both the fragmentation of analytes and the signals corresponding to the metal adduct ions of amino acids did not occur, and the matrix ion interference was also eliminated. The very clean $[M-H]^-$ ion signals at m/z 116.23, 145.23, 164.18, 173.11, and 203.18 could be used for detection of Val, Gln, Phe, Arg, and Trp, respectively. In comparison with CHCA matrix, the functional SWNH-assisted SALDI mass spectrum showed about 3-fold stronger signal intensity, which resulted from the high energy transfer efficiency of the SWNHs, thus indicating higher sensitivity.

Due to the "hot spots" produced from the co-crystallization of analyte and matrix, the conventional MALDI method often suffers from poor shot-to-shot and spot-to-spot reproducibility. Here, the analyte was adsorbed on SWNHs, which avoided the co-crystallization. Thus the SWNH-assisted SALDI mass spectra showed acceptable shot-to-shot reproducibility (see Figure S1 in the Supporting Information).

Analysis of peptides: Similarly, peptides such as Gly–Gly (M_r 132.12), Ala–Ala (M_r 160.17), Leu–Leu (M_r 244.33), Tyr–Phe (M_r 328.36) and Glu–Val–Phe (M_r 393.43) were also used to examine the performance of SWNHs. The traditional matrix CHCA could be used for detection of these peptides with the signals corresponding to $[M+H]^+$, but it brought strong background interference signals corresponding to the CHCA-related ions (Figure 3A). The mass spectrum of peptides in the positive ion mode obtained using functional SWNHs as assisted matrix also showed the sodium or potassium adducts (Figure 3B and Table S3 in the Supporting Information). A clean mass spectrum with high signal intensity and free matrix background interference could be obtained in the negative ion mode (Figure 3C), which displayed the $[M-H]^-$ ion signals corresponding to these peptides at m/z 131.11, 159.11, 243.21, 327.28, and 392.32, respectively. Compared with the CHCA matrix, the functional SWNHs led to 5-fold stronger signal intensity. Thus, a sensitive SWNH-assisted SALDI-TOF MS method could be applied to the analysis of peptides.

Effect of salt tolerance: Salt tolerance is an extensively concerned problem in MS analysis. So it was examined in the functional SWNH-assisted SALDI-TOF MS analysis using fatty acids as model analytes. The mass spectra of 0.5 mM C12 (M_r 200.32), C14 (M_r 228.37), C16 (M_r 256.42), C18 (M_r 284.48), and C20 (M_r 312.53) recorded in the negative ion mode are shown in Figure 4. They display free matrix background interference and clean signals corresponding to $[M-H]^-$ ions at m/z 199.19, 227.20, 255.20, 283.22, and 311.24. Interestingly, the signal intensity increased with the increasing number of carbon atoms, which was possibly due to the stronger interaction between SWNHs and longer fatty acid, which resulted in a more efficient energy transfer. In the absence and presence of 10, 100, and 1000 mM NaCl, the mass spectra showed the same responses, and no obvious change could be observed, thus proving that this system possessed high salt tolerance. Similar results were also observed in the detection of amino acids (Figure S2) and peptides

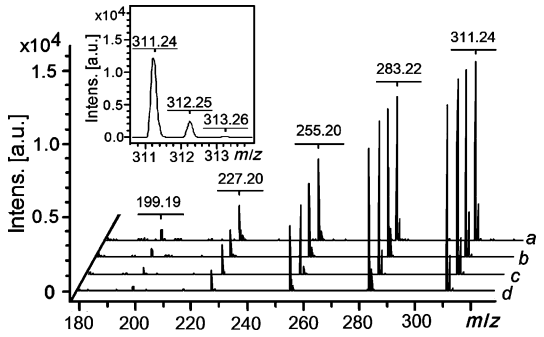


Figure 4. SALDI-TOF MS spectra using SWNHs as matrix in the negative ion mode for 0.5 mM C12, C14, C16, C18, and C20 fatty acids in a) absence of salt and presence of b) 10, c) 100, and d) 1000 mM NaCl.

(Figure S3). This should be due to the homogeneous distribution of analyte molecules on the SWNHs, which contrasted with the heterogeneous crystals formed from analyte molecules in conventional organic matrices.

SWNHs as assisted matrix for determination of ATP: To confirm the performance of functional SWNHs in affinity capture and ionization, ATP aptamers were conjugated to SWNHs. As a control experiment, SWNH-assisted SALDI-TOF MS was first used for the analysis of ATP. The SALDI-TOF MS displayed a clean spectrum with three signals at m/z 346.11, 426.22, and 506.18 that were attributed to $[ATP-2Pi]^-$, $[ATP-Pi]^-$, and $[ATP-H]^-$, respectively (Figure 5A). Although both signals at m/z 346.11 and 426.22 showed good sensitivity and could be used for the analysis of ATP, the response attributed to $[ATP-H]^-$ at 0.5 mM was very weak. Considering the fact that oppositely charged surfactant additives could affect the ionization efficiency of charged analytes, a surfactant CTAB was added in the functional SWNHs solution to improve their surface activity and MS signal intensities.[33,34] Upon addition of CTAB, all three signals heightened, and the optimal concentration of CTAB

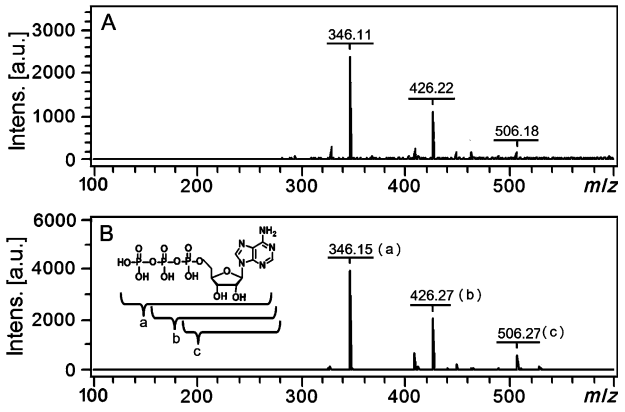


Figure 5. MALDI-TOF MS spectra of 0.5 mM ATP using A) SWNHs and B) SWNHs +10 μM CTAB as matrix. The signals at a) m/z 346.15, b) 426.27, and c) 506.27 are assigned to $[ATP-2Pi]^-$, $[ATP-Pi]^-$, and $[ATP-H]^-$ ions, respectively.

was found at 10 μM (see Figure S4 in the Supporting Information), which was optimized with their total signal intensity. Figure 5B shows the significantly improved mass spectrum. In the presence of CTAB (10 μM) the signal attributed to $[ATP-H]^-$ increased by 3.8 times.

Apt–SWNH-assisted SALDI MS analysis of ATP: After ATP was spiked in urine sample, the MS spectrum obtained after extraction with functional SWNHs solution containing CTAB (10 μM) showed the three characteristic ATP signals, and some new background signals assigned to the species in urine sample were also observed (Figure 6A). However,

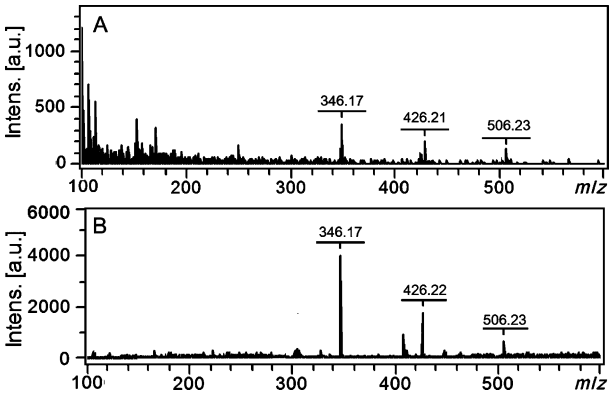


Figure 6. SALDI-TOF MS analysis in presence of CTAB (10 μM) for ATP (20 μM) spiked in urine sample after extraction with A) SWNHs and B) Apt–SWNHs.

when the sample was extracted with Apt–SWNHs, the background signals could be eliminated from the MS spectrum, and much higher responses were observed for the three characteristic signals (Figure 6B), indicating the efficient capture and extraction of ATP from the urine sample by the Apt–SWNHs. Therefore, the Apt–SWNHs could serve as a dual platform for both selective capture and ionization, which provided a selective and sensitive method for SALDI MS analysis of ATP.

With the decreasing amount of ATP spiked in urine sample, the MS intensity of all the three characteristic signals decreased, and the spectrum clearly showed these signals at ATP concentration as low as 1.0 μM (Figure 7), which could be considered as the detection limit for ATP (10 pmol). The detection limit was comparable with our previously reported results obtained using ion-pairing LC-ESI MS (2.5 μM),[35] HPLC-ESI MS (0.49 μM),[36] and capillary LC-high resolution MS (0.2 μM),[37] and that of about 1 ng mL^{-1} (1.97 μM) obtained using HPLC-ESI MS/MS after extraction with methanol/water (1:1).[38] Evidently, the newly developed method has some intrinsic merits, which include simple sample preparation, tolerance to high salt tolerance, as well as high-throughput and fast analysis.

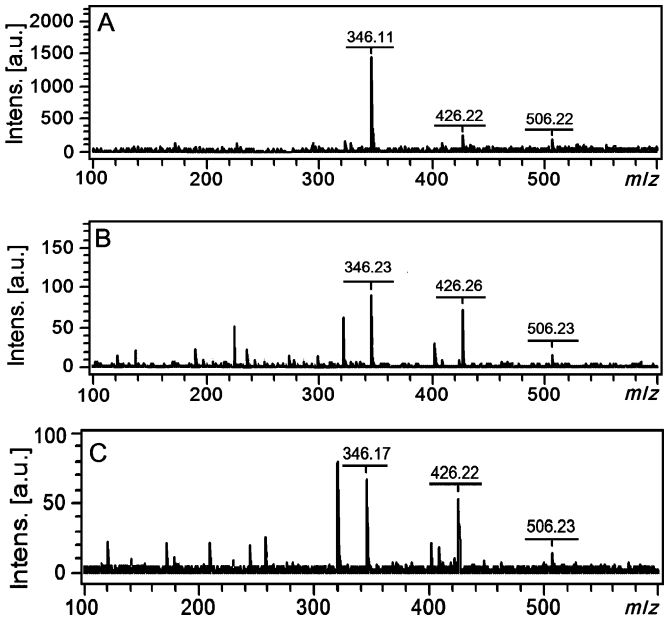


Figure 7. SALDI-TOF MS analysis in presence of CTAB (10 μM) for A) 10, B) 2.0, and C) 1.0 μM ATP spiked in urine samples after extraction with Apt–SWNHs.

Conclusion

This work presented a novel matrix system, that is, functional SWNHs, for MALDI-TOF MS analysis of small biomolecules including peptides, amino acids, fatty acids, and ATP. Compared with conventional matrices, functional SWNHs exhibited several advantages, including no fragmentation of the analyte, free matrix background interference, high salt tolerance, and excellent shot-to-shot reproducibility. By combining the advantages of SWNHs with the specificity of aptamers and the good analytical performance of negative ion mode, a selective and sensitive method was developed for Apt–SWNH-assisted SALDI MS analysis of ATP in complex biological samples. The Apt–SWNHs showed the excellent capture ability to eliminate the background signals from the species co-existed in urine sample. Owing to the controllable modification of SWNHs with different kinds of recognition elements, this strategy possessed good extensibility. This work not only expanded the application of SWNHs but also provided a novel method for bioanalysis.

Experimental Section

Materials and reagents: L-Glutamine (Gln), L-tryptophan (Trp), L-valine (Val), L-phenylalanine (Phe), L-arginine (Arg), peptides including Gly–Gly, Ala–Ala, Leu–Leu, Tyr–Phe, and Glu–Val–Phe, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), ATP, CHCA, hexadecyltrimethyl-ammonium bromide (CTAB), sinapinic acid (SA) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO). Saturated fatty acids including *n*-dodecanoic acid (C12), *n*-tetradecanoic acid (C14), *n*-hexadecanoic acid (C16), *n*-octadecanoic acid (C18), and *n*-eicosanoic acid (C20) were obtained from Acros Organics (New Jersey). Acetonitrile was of HPLC grade and supplied by TEDIA (Fairfield, OH). Anhydrous ethanol was provided by Acros Organics (New Jersey). The 5′-amino-modified ATP aptamers with the sequence of 5′-NH_2-$(CH_2)_6$-ACC TGG GGG AGT ATT GCG GAG GAA GGT-3′ were purchased from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China) and purified using HPLC. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore, Billerica, MA) was used in all assays. All other reagents were of analytical grade and used without further purification. SWNHs were kindly provided from Professor Sumio Iijima, who leads the carbon nanotube project in the Japan Science and Technology Agency.

Apparatus: X-ray photoelectron spectroscopic (XPS) measurements were performed with an ESCALAB 250 spectrometer (Thermo-VG Scientific, U.S.A.) with an ultrahigh vacuum generator. Infrared spectra were obtained on a Nicolet Magna 550 Series II Fourier transform infrared spectrometer (Madison, WI). The transmission electron micrograph (TEM) was gained on a JEM-2100 TEM (JEOL, Japan). MALDI-TOF MS experiments were performed on a Bruker Autoflex II mass spectrometer (Bruker Daltonics, Germany) equipped with a pulsed nitrogen laser (337 nm wavelength). The instrument was operated with accelerating voltage, grid voltage and delayed extraction time set to 19 kV, 90%, and 60 ns, respectively. A MTP 384 massive target from Bruker Daltonics was used for sample plate. All mass spectra reported were obtained in the reflector mode and calibrated using an external standard of SA for the analysis of small molecule compounds with molecule weight below 700 Da in this research. Each mass spectrum was acquired as an average of 100 laser shots.

Preparation of functional SWNHs: First, SWNHs (40 mg) were dispersed in 60 mL of 30% HNO_3 and then refluxed for 24 h at 140 °C to obtain carboxylic group functionalized SWNHs. The resulting suspension was centrifuged, and the precipitate was washed thoroughly with water until the pH value was about 7.0. The black solid was collected and dried in vacuum at 80 °C. The functional SWNHs were homogeneously dispersed in deionized water to a concentration of 1 mg mL^{-1}.

The SWNHs functionalized with carboxylic groups were further conjugated with ATP aptamers by forming the amide between the -COOH group on the SWNHs and the -NH_2 moiety of the aptamer via EDC-NHS coupling (Scheme 1). Before modification, 50 μL of the oxidized SWNHs dispersion was mixed with EDC (50 μL, 6 mM) and NHS (50 μL, 15 mM), which were prepared in 2-morpholinoethanesulfonic buffer (pH 5.5), and reacted at room temperature for 15 min. After centrifugation the resulting SWNHs were washed thrice with PBS (0.01 M) and resuspended in PBS (50 μL, 0.01 M), which was mixed with 5′-amino-modified ATP aptamers (50 μL, 100 μM), at ambient temperature for 2 h, followed by centrifugation four times at 14000 rpm for ten min each and washing to remove excess unbound aptamer. Finally, the bioconjugate precipitate, Apt–SWNHs, was dispersed in PBS and stored in refrigerator at 4 °C.

Preparation of samples: 5 mM fatty acids including C12, C14, C16, C18, and C20 were separately dissolved in anhydrous ethanol as the storage solutions. Amino acids and peptides (5 mM) were prepared in a 1:1 (v/v) mixture of methanol and water. These stock solutions were kept at −20 °C prior to use. Saturated CHCA and SA solutions were prepared in 2:1 (v/v) mixture of acetonitrile and water containing TFA (0.1%). The analyte solution (0.5 μL) was mixed with the matrix (0.5 μL) in the well of the target plate and dried at room temperature for MALDI-TOF MS analysis. To improve the efficiency of LDI, CTAB was added to the functional SWNHs solution (1 mg mL^{-1}), which was then mixed with analyte solution at 1:1 (v/v). Afterwards, 1 μL of the mixture was dropped onto the target plate and dried at room temperature for SALDI detection. In salt tolerance experiments, NaCl (3 M) was used to adjust the salt to the corresponding concentration.

Aptamer-assisted ATP extraction and SALDI MS analysis: The known amounts of ATP were spiked in urine samples of a healthy volunteer and firstly boiled for 5 min and centrifuged at 16000 rpm for 20 min to remove the precipitated proteins and other solid particles. 2 μL of SWNHs modified covalently with aptamers was centrifuged and then redispersed in a pH 7.5 binding buffer containing Tris-HCl (20 mM), NaCl

(140 mM), and $MgCl_2$ (2 mM) to mix with 10 μL ATP-spiked urine sample (or 20 μL for 1.0 μM ATP-spiked urine sample). After 30 min, the resulting mixture was centrifuged, and the precipitates were washed with a binding buffer (10 μL) and then water (10 μL), and redispersed in 2 μL of a solution containing TFA (0.1%) and CTAB (10 μM). As a control experiment, ATP-spiked urine sample was mixed with functional SWNHs solution containing CTAB (10 μM) at 1:1 (v/v). Finally, 1 μL of the suspension was pipetted onto the target plate and dried at room temperature for 20 min to perform the SALDI MS analysis.

Acknowledgements

This work was funded by the National Basic Research Program of China (2010CB732400), the National Natural Science Foundation of China (21121091, 21135002, and 21175025), and the Natural Science Foundation of Jiangsu (BK2010193).

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Received: August 8, 2012
Published online: December 12, 2012

