ChemComm

COMMUNICATION

View Article Online
View Journal

Cite this: DOI: 10.1039/c5cc03756e

Received 6th May 2015,
Accepted 3rd June 2015

DOI: 10.1039/c5cc03756e

www.rsc.org/chemcomm

Preparation of mesoporous SiO_2@azobenzene–COOH chemoselective nanoprobes for comprehensive mapping of amino metabolites in human serum†

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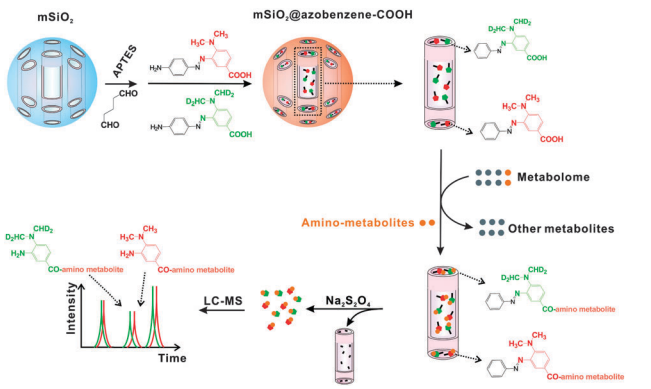
A novel type of mesoporous SiO_2@H_4/D_4 tagged azobenzene–COOH chemoselective nanoprobe was developed for comprehensive mapping of amino metabolites in complex biological samples with high specificity and sensitivity.

Amino metabolites such as amino acids, catecholamines, dipeptides, and polyamines play vital roles in biosynthesis, cellular growth, immunomodulatory and signal transduction.[1a,b] However, their determination is a challenging task due to low abundance, poor retention and ionization when using reversed-phase liquid chromatography coupled with mass spectrometry. The traditional derivatization reagents such as dansyl chloride,[2a] 7-fluoro-4-nitrobenzoxadiazole,[2b] 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate,[2c] isobaric Tag[2d] and AccQ Tag[2e] are used to improve the detection sensitivity, ameliorate separation and reduce ion suppression. However, lack of specificity, using excess derivatization reagents, and considering special reaction conditions during the derivatization process are unfavorable for targets' stability and can produce additional matrix effects. Recently, some chemoselective probes have been designed for the analysis of amino metabolites, carboxylic acids, ketone-aldehydes, and thiols. These probes are constructed with the resin supported selective reaction groups and the cleavable linkers for cleavage with the existence of acids,[3a] enzymes,[3b] or optical irradiation.[3c,d] Although these chemoselective probes present excellent specificity towards target compounds, the recovery of target molecules and the improvement of MS sensitivity are not satisfactory because of small specific surface area of the resins and low ionization efficiency of derivatization groups.[3b] Therefore, a new method with high recovery, MS sensitivity, specificity and low matrix effects is necessary by introducing high ionization efficiency of the cleavable linker and large surface area of supports into the chemoselective probes.

In this work, a novel chemoselective probe called $mSiO_2$@azobenzene–COOH for amino metabolites was constructed using mesoporous SiO_2 ($mSiO_2$) nanoparticles as the solid supports and azobenzoic acid as the cleavable linker. On one hand, azobenzoic acid has a special reactivity towards amino groups under the selected mild conditions which can realize selective harvest of the amino metabolites; on the other hand, azobenzoic acid is easy to be cleaved into two parts by sodium dithionite and the part with the benzene ring and the tertiary amine structure append to the target amino metabolites can also serve as a derivatization reagent and could enhance their retention and MS sensitivity.[4a,b] Moreover, $mSiO_2$ nanoparticles with a large surface area can supply a large amount of reaction sites for binding azobenzoic acid, and their mesoporous structures are conducive to diffusion and enrichment of amino metabolites.[5a,b]

The procedure for the construction of $mSiO_2$@azobenzene–COOH nanoprobes is shown in Scheme 1. Briefly, $mSiO_2$ nanoparticles were synthesized through the reported approach;[6] then amino and aldehyde groups were sequentially grafted onto the surface of $mSiO_2$; finally, the nanoparticles were further functionalized by H_4/D_4 tagged NH_2–azobenzene–COOH (ESI,† Fig. S1). As shown in Fig. 1a, $mSiO_2$ nanoparticles were about 70 nm in



Scheme 1 Synthetic strategy of $mSiO_2$@azobenzene–COOH and its workflow for the amino metabolite enrichment.

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† Electronic supplementary information (ESI) available: Experimental protocol as well as figures and tables displaying results. See DOI: 10.1039/c5cc03756e

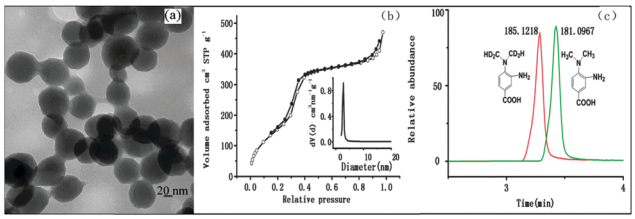

Fig. 1 (a) TEM image of $mSiO_2$ nanoparticles, (b) N_2 adsorption–desorption isotherms and pore size distribution of $mSiO_2$ nanoparticles, (c) extracted ion chromatogram of the derivatization reagent cleaved from H_4/D_4 tagged $mSiO_2$@azobenzene–COOH with $Na_2S_2O_4$.

average diameter. The nitrogen adsorption–desorption isotherms demonstrated that the $mSiO_2$ nanoparticles had ordered mesoporous with an average pore size of 2.7 nm (Fig. 1b). Moreover, the $mSiO_2$ with large pore volume (0.726 $cm^3\ g^{-1}$) and surface areas (647.8 $m^2\ g^{-1}$) were favorable for the diffusion and enrichment of small molecules. The zeta potential of the $mSiO_2$ nanoparticles changed from −11.7 mV to 7.59 mV after the amino groups were grafted, and rose to 32.46 mV after being functionalized with NH_2–azobenzene–COOH, which could reflect that these groups with positive charge sequentially bonded on the surface of $mSiO_2$. The stretching vibration at 1500 cm^{-1}, 1721 cm^{-1} and 1462 cm^{-1} analysed using FT-IR spectroscopy further demonstrated the involvement of aromatic C=C and C=O bonds of carboxyl and N=N bond (ESI,† Fig. S2). In addition, the conjugated system of C=C, C=O and N=N made IR absorption wave numbers lower in FT-IR spectroscopy. The extracted ion chromatogram of the derivatization reagent cleaved from H_4/D_4 tagged $mSiO_2$@azobenzene–COOH with $Na_2S_2O_4$ (Fig. 1c) could confirm the azobenzene–COOH group modified on the surface of $mSiO_2$.

The selective enrichment of amino metabolites by $mSiO_2$@azobenzene–COOH nanoprobes was based on the condensation reaction between amino and carboxyl groups with the assistance of 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HATU) and *N*-hydroxysuccinimide (NHS), which show better selectivity towards amino groups than phenolic hydroxyl groups.[7] Then the enriched amino metabolites were derivatized and released through the cleavage of the azo bonds by sodium dithionite (Scheme 1). Firstly, the amounts, activating time of the activators including HATU, *N*,*N*-diisopropylethylamine (DIPEA) and NHS for the condensation reaction, as well as the coupling time of amino metabolites with the activated carboxyl on the surface of the nanoprobes were optimized, respectively. The results exhibited that the activation could be rapidly accomplished within 2 min when 40 mg $mSiO_2$@azobenzene–COOH nanoprobes were treated with 200 μL of 10 mM HATU, DIPEA and NHS, and the coupling time of the activated nanoprobes with the amino metabolites can reach equilibrium in 5 min (ESI,† Fig. S3–S6, ESI†). Since azobenzene can be efficiently cleaved by $Na_2S_2O_4$ to produce two aniline parts,[8a,b] the concentration of $Na_2S_2O_4$ was optimized to realize complete cleavage. Treatment with 0.2 mM $Na_2S_2O_4$ for 60 min could fulfill the release of the captured metabolites.

To evaluate the derivatization efficiency and the selectivity of $mSiO_2$@azobenzene–COOH for amino metabolites, 100 μL mixtures of amino acids, aliphatic amines, aromatic amines, amides or phenolic hydroxyl standards were treated with $mSiO_2$@azobenzene–COOH, respectively. The conventional derivatization strategy with dansyl chloride for the same amount of amino metabolites, amides and phenolic hydroxyl mixture was used as a comparison. As shown in Fig. 2, nearly all of the amino metabolites had very weak MS signals before derivatization, except aromatic amines (Fig. 2a-i, b-i and c-i). After being derivatized with $mSiO_2$@azobenzene–COOH and dansyl chloride, the MS signal intensity of amino acids and aliphatic amines was greatly enhanced (Fig. 2a-ii, a-iii, b-ii and b-iii). Especially, the signal intensity of aliphatic amines could be further magnified a few dozen times by $mSiO_2$@azobenzene–COOH compared with dansyl chloride derivatization. The derivatization efficiency of aromatic amines was not satisfactory with either derivatization method, which might be ascribed to their inherent high MS response. However, a more acceptable signal-to-noise ratio could still be maintained after derivatization with $mSiO_2$@azobenzene–COOH nanoprobes than dansyl chloride (Fig. 2c-ii and c-iii). Theoretically, the derivatization products of dansyl chloride

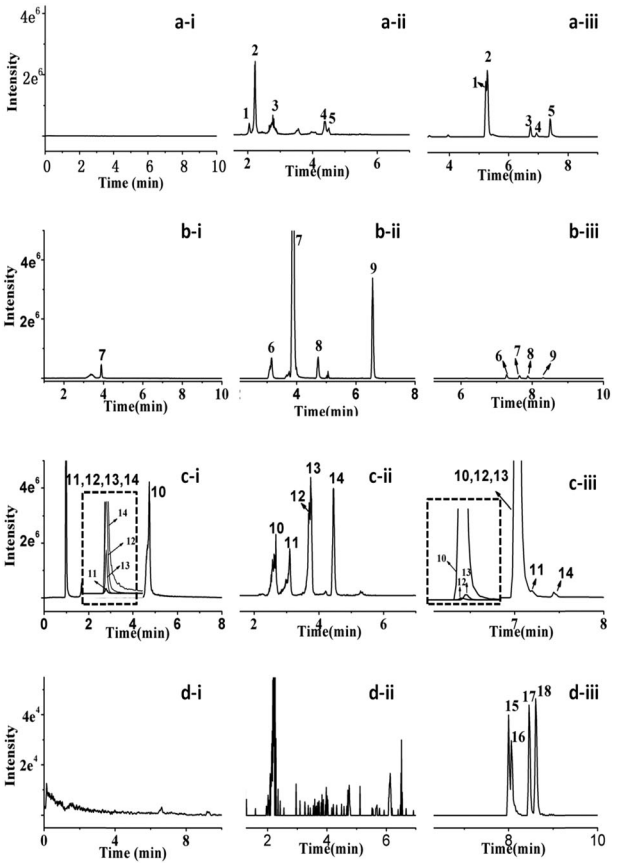

Fig. 2 Extracted ion chromatograms of (a) 500 ng mL^{-1} amino acids, (b) 500 ng mL^{-1} aliphatic amines, (c) 5 μg mL^{-1} aromatic amines, (d) 5 μg mL^{-1} phenol mixture before (i) and after $mSiO_2$@azobenzene–COOH nanoprobes (ii) and dansyl chloride derivatization (iii) ((1) glycine, (2) L-alanyl-L-alanine, (3) L-methionine, (4) L-leucine, (5) L-tryptophan, (6). propylamine, (7) isobutylamine, (8) cyclohexylamine, (9) heptylamine, (10) *N*-methylanthranilic acid, (11) dopamine, (12) *N*-methyltyramine, (13) 1-phenylethanolamine, (14) *p*-anisidine, (15) guaiacol, (16) 4-nitrophenol, (17) 3-ethyl-5-methylphenol, (18) 2-phenylphenol).

should have better MS sensitivity because they are eluted in a higher organic phase ratio.[4a] However, the MS sensitivity of most amino metabolites after being derivatized with $mSiO_2$@azobenzene–COOH was much higher than that with dansyl chloride due to the additional enrichment of $mSiO_2$@azobenzene–COOH. Noticeably, dansyl chloride could react with both amino and phenolic hydroxyl groups under basic conditions.[2a,c] As shown in Fig. 2d-iii, phenolic hydroxyl compounds had strong MS sensitivity after being derivatized with dansyl chloride, meanwhile, no derivatization products of phenolic hydroxyl compounds after being treated with $mSiO_2$@azobenzene–COOH could be detected (Fig. 2d-ii). All the results indicated that high selectivity derivatization for amino metabolites was achieved by $mSiO_2$@azobenzene–COOH nanoprobes.

The LODs for the mixed amino standards were further investigated as listed in Table S1 (ESI†). Most of the amino metabolites had a detection limit above 10 ng mL^{-1} when they were directly analyzed. Propylamine and isobutylamine are difficult to be detected in untargeted LC-MS profiling because their molecular weight are less than 100.[9] The MS sensitivity of amino metabolites was enhanced up to 10 000 fold after being treated with $mSiO_2$@azobenzene–COOH nanoprobes, and the LODs of most amino metabolites were greatly lower down to the 20–500 pg mL^{-1} level, which was 1–2 orders of magnitude lower than those with the dansyl chloride derivatization. The results prove that the nanoprobes are more powerful in the derivatization of low abundant amino metabolites. For amino metabolites such as amino acids and aliphatic amines, which had a weak retention on a reversed-phase column or a low ionization efficacy, the derivatization method could reduce their limit of detection greatly. Similar to the signal intensity, the derivatization made the LOD of only a few aromatic amines better, either with nanoprobes or dansyl chloride. The LOD of secondary amines such as Pro, *N*-methylanthranilic acid and *N*-methyltyramine did not decrease after nanoprobe derivatization probably due to weak reactivity caused by steric hindrance. Actually, 4 out of 15 amino metabolites have lower LOD with dansyl chloride derivatization than nanoprobes. Therefore, $mSiO_2$@azobenzene–COOH nanoprobes are an alternative tool for the detection of amino metabolites.

A mixture containing three deuterated amino acids (Leu-d_3, Trp-d_5 and Met-d_3) was utilized to investigate the linearity, recovery and precision of the developed LC-MS method based on the chemoselective nanoprobes. As shown in Table S2 (ESI†), all of the three deuterated amino acids have good linearity in the concentration range of 1 ng mL^{-1} to 1000 ng mL^{-1} with the regression coefficients of above 0.99. The recoveries of three deuterated amino acids are around 87.0% to 97.3% with excellent precision (RSD $<$ 5.7%).

The stable isotope tagged derivatization is an approach through introducing a stable isotope-tagged moiety to the endogenous metabolites in order to overcome matrix effects and satisfy accurate quantification/qualification.[10] The H_4/D_4 tagged $mSiO_2$@azobenzene–COOH nanoprobes were used to extract and assist in exploring the low abundant amino metabolites from human serum by making use of the MS features of their H_4/D_4 tagged derivatization products. As shown in Fig. S7 (ESI†), the *m*/*z* difference between H_4 and D_4 tagged derivatization products is a constant value of 4.0252, and the retention times of D_4 tagged derivatization products are a little shorter than those of the corresponding H_4 tagged derivatization products, in the range of 0 to 15 s. Moreover, the MS intensities of H_4 and D_4 tagged derivatization products were close to each other. According to the above patterns, the amino metabolites extracted from human serum with the nanoprobes could be defined by the in-house developed software.[11] Since the proteins and peptides in human serum containing abundant amino groups could occupy the reactive sites for derivatization and hinder the diffusion of amino metabolites in mesoporous,[12] the serum was firstly ultrafiltrated to remove these interferences. After being pretreated with the H_4/D_4 tagged nanoprobes, 94 derivatizated amino metabolites were extracted from 20 μL serum based on the features of H_4 and D_4 tagged derivatization products by LC-MS analysis. Among them, 56 metabolites were identified through Metlin and HMDB databases and 21 amino metabolites were verified with standards as shown in Table S3 (ESI†). Besides amino acids, modified metabolites (such as trimethyllysine), low-abundant amino metabolites (such as ethanolamine, aminoacetone and butylamine), and some dipeptides or tripeptides were obtained after being derivatized with $mSiO_2$@azobenzene–COOH. Extracted ion chromatograms of a part of the extracted amino metabolites after being derivatized with the H_4/D_4 tagged nanoprobes or directly analyzed are shown in Fig. 3. Several common amino acids at a high concentration could be detected without derivatization, such as leucine and valine (Fig. 3a), however, they were retained weakly on a reversed-phase column and coeluted with other compounds at 1.18 min (nearly at dead time). After being derivatized with the nanoprobes, these amino metabolites were separated effectively with increased sensitivity and retention time (valine at 5.01 min and leucine at 6.44 min), what is more, a lot of new amino metabolites at a low concentration were found with the help of MS features of H_4/D_4 tagged derivatization products, such as imidazole lactate at 4.05 min, trimethyllysine at 7.12 min and pipecolinic acid at 4.64 min (Fig. 3b). In addition, among the 94 amino metabolites obtained by the chemoselective probes,

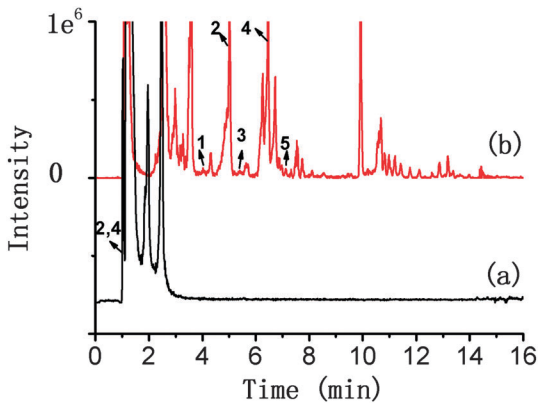

Fig. 3 Extracted ion chromatograms of part of amino metabolites defined from 20 μL human serum analyzed directly (a) and derivatized with $mSiO_2$@azobenzene–COOH nanoprobes (b) ((1) imidazole lactate, (2) valine, (3) pipecolinic acid, (4) leucine, (5) trimethyllysine).

only 40 amino metabolites were detected with dansyl chloride derivatization and most of them were amino acids. Many amino metabolites could be found with the chemoselective nanoprobes, but were not observed with the dansyl chloride derivatization such as methylamine, cyclohexylammonium and dihydroxyindole. These amino metabolites usually participate in important physiological processes and their abnormal levels might imply different physiological and pathological states.

In conclusion, the synthetic $mSiO_2$@azobenzene–COOH chemoselective nanoprobes show several advantages including (i) metabolites enrichment (captured and released by $mSiO_2$@azobenzene–COOH nanoprobes), (ii) chemoselectivity (coupling reagent-catalyzed reaction between metabolites and solid supports), (iii) derivatization on solid supports (specific structures with high ionization efficiency tagged to metabolites). Compared with the conventional dansyl chloride derivatization, $mSiO_2$@azobenzene–COOH nanoprobes presented high selectivity towards amino groups and could further enhance the MS sensitivity of amino metabolites by 1–2 orders of magnitude. Finally, the H_4/D_4 tagged $mSiO_2$@azobenzene–COOH was successfully applied to extract and explore amino metabolites from a small amount of serum and 94 amino metabolites were found including many low abundant amino metabolites. This newly developed method based on the $mSiO_2$@azobenzene–COOH nanoprobes was feasible and practical in comprehensive qualitative/quantitative analysis of amino metabolites, and it would be of great promise in the exploration of unknown amino metabolites from very tiny and precious samples. In the future, this derivatization strategy can further be utilized for other metabolites such as carboxylic acids, aldehyde-ketones, and phenols by changing the reactive groups on azobenzene linkers.

This study was supported by the National Basic Research Program (No. 2012CB720801) from the State Ministry of Science and Technology of China, and the foundations (No. 21275141 and 21435006) and the creative research group project (No. 21321064) from the National Natural Science Foundation of China.

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