A graphene-based multifunctional affinity probe for selective capture and sequential identification of different biomarkers from biosamples†

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Received 29th July 2012, Accepted 31st August 2012
DOI: 10.1039/c2cc35483g

A novel multifunctional graphene-based affinity probe has been explored for selective capture of two different types of peptides from the biosamples for sequential detection.

Many low-abundance endogenous peptides and phosphopeptides in the body fluids or tissues are biomarkers with higher clinical sensitivity and specificity, which could provide valuable information for the detection of many diseases and elucidation of pathological variations.1 Mass spectrometry (MS) is highly sensitive to trace peptides in proteomics.2 However, it is still a great challenge to detect the low-abundance peptides from complex biological samples directly due to extremely low concentrations of these peptides and interferences of high levels of small molecules such as salts, surfactants and other contaminants in samples.3 In addition, detection of the phosphopeptides always suffers from the ion suppression effect of nonphosphopeptides as well as the low ionization efficiency of phosphopeptides due to the negatively charged sites.4 Thus, it is essential to purify and separate these low-abundance peptides and phosphopeptides prior to MS analysis.

In recent years, some magnetic composites were explored to enrich and separate low-abundance peptides or phosphopeptides in object samples. For example, the C8-functionalized magnetic particles were applied to enrich low-concentration peptides in biological samples based on the hydrophobic interaction,5 while affinity materials consisting of magnetic core and metal oxide shell have been used for selective enrichment of the phosphopeptides based on the selective affinity of the metal oxide for the phosphate groups in the phosphopeptides.6,7 Although substantial progress has been made in the low-abundance peptide and the phosphopeptide enrichment, these composite materials cannot extract the low-abundance peptides and the phosphopeptides synchronously. Therefore, some useful and informative peptides/phosphopeptides are lost during the enrichment step and no longer contribute to the practical application. In addition, discrimination of phosphopeptides depends strongly on the tandem MS spectra or phosphatase treatment currently which are indirect determination methods.7 Thus, multifunctional materials for selective capture, fast isolation, and sequential determination of the low-abundance peptides and the phosphopeptides from complex biosamples are of great demand.

Recently, graphene has been successfully utilized as an adsorbent for MS detection of the low-mass molecules (e.g. amino acids, polypeptides, peptides, steroids, nucleosides and nucleotides)8 based on its ultrahigh specific surface area, high loading capacity, unique large delocalized π–π-electron system and hydrophobic interaction.9 Nevertheless, its poor hydrophilicity and difficult surface functionalization often restrict its more extensive bioapplication,10 while the hydrophilic oxidizing graphite (GO) with its functional groups located at both sides of the graphene sheet can be utilized as the supporter for integration with many nanostructures rooted in easy surface modification.11 It is easy to reduce the GO to graphene (rGO) for the preparation of graphene-based multifunctional materials. Compared with the carbon nanotubes used as adsorbents for enrichment of the biomolecules (e.g. proteins and long-chain peptides) by the interaction between carbon atoms and biomolecules, the unique double-sided aromatic chemical structure and high flexibility of graphene may produce a more powerful capture of long carbon chains. These characteristics also can be free from the hindrance of the targeted analytes accessing the carbon nanotubes, and contribute to more efficient adsorption.8 Most recently, our research results indicate that the rare-earth affinity materials can selectively capture and identify the phosphopeptides.12 Herein, a graphene-based multifunctional composite material which combines the hydrophobic interaction of the graphene for enrichment of the low-abundance peptides, the lanthanum phosphate affinity for detection and identification of phosphopeptides and the Fe₃O₄ nanoparticles for fast magnetic isolation has been designed and fabricated. Such multifunctional composite material can realize selective capture, fast magnetic separation and sequential determination of the low-abundance peptides and the phosphopeptides from the complex biosamples.

The synthesis strategy of the graphene-based multifunctional affinity probe is shown in Scheme 1. The LaPO₄-graphene oxide composites (LaG) were first prepared by the electrostatic interaction between the negatively charged GO and the positively
charged lanthanum ion and then the in situ growth of lanthanum phosphate nanorods (Fig. S1, ESI†). After covalent grafting of (3-mercaptopropyl)trimethoxysilane onto the GO (as verified by FTIR in Fig. S2, ESI†), the Fe3O4 nanoparticles were readily grafted to the GO via covalent Fe–S bonds. After reduction of the GO, the multifunctional affinity probe (LaGM) with affinity, hydrolytic catalysis, and strong magnetism as well as hydrophobicity rooted in the rGO (or graphene) was obtained. Notably, the LaGM affinity probe not only can enrich the low-abundance peptides from complex biosamples based on the hydrophobicity from the unique structure of graphene used as the supporter, but also can selectively capture and easily label the phosphopeptides in virtue of affinity and hydrolytic catalysis of the LaPO4 nanorods attached to the graphene. Furthermore, the magnetic nanoparticles covalently grafted on the graphene facilitate the separation and purification procedure. More importantly, the two types of peptides can be selectively captured and then eluted one by one for the sequential MS detection.

SEM and TEM images (Fig. 1) show the triple hierarchical structure of the multifunctional LaGM composites. Obviously, the LaPO4 nanorods (ca. 300 nm in length and 10 nm in average diameter) are evenly distributed on graphene, surrounding the Fe3O4 nanoparticles (ca. 60 nm). Furthermore, no free Fe3O4 nanoparticles or LaPO4 nanorods are observed, indicating that the Fe3O4 nanoparticles, the LaPO4 nanorods, and the graphene have been integrated into an entity. According to high-resolution TEM (HRTEM) images, the lattice fringe images of the LaPO4 nanorods (Fig. 1e) and the Fe3O4 nanoparticles (Fig. 1f) exhibit the crystalline nature that can be indexed to the hexagonal LaPO4 and cubic magnetite, respectively. The X-ray energy-dispersive spectrum (EDS) (Fig. 1g) of the obtained LaGM composites reveals the existence of C, O, La, P and Fe elements (Cu arising from the sample carrier), further confirming the triple hierarchical composites.

The formation of the LaGM triple hierarchical composites can be further confirmed by powder X-ray diffraction (XRD) analysis and Fourier-transform infrared (FTIR) spectroscopy. Compared with the GO, new diffraction peaks in the XRD of LaG (Fig. S3a, ESI†) that match well with those of the hexagonal LaPO4 (JCPDS No. 04-0635) can be clearly observed, and the LaG shows a new absorption peak in FTIR at around 1050 cm\(^{-1}\) (O–P–O of LaPO4) besides the characteristic absorption peaks at 1735 cm\(^{-1}\) (C=O) and 1621 cm\(^{-1}\) (C=C) of graphene oxides (Fig. S3b, ESI†). After attachment of the Fe3O4 and reduction of the GO, the diffraction peaks of the magnetite phase (JCPDS No. 65-3107) can be observed in the XRD patterns of LaGM, indicating the existence of magnetite. In addition, the appearance of the new absorption peak at 565 cm\(^{-1}\) (Fe–O–S) and the disappearance of the peak at 1735 cm\(^{-1}\) (C=O) indicate covalent binding of the Fe3O4 and reduction of the hydrophilic functional groups (e.g., –COOH) on the GO. The magnetic properties of the LaGM and Fe3O4 were examined via a superconducting quantum interface device (SQUID) magnetometer (Fig. S4, ESI†), indicating a strong superparamagnetism of LaGM at room temperature. Although the saturation magnetization value of LaGM (25.1 emu g\(^{-1}\)) is lower than that of Fe3O4 (77.6 emu g\(^{-1}\)), the LaGM can be rapidly separated from the mixture within 1 min with the help of a magnet (Fig. S4 inset, ESI†).

To examine the possibility of the LaGM probe for enrichment of low-abundance peptides, the diluted BSA tryptic digest (10 nM) was employed as a model sample. As shown in the MALDI-TOF mass spectra (Fig. S5a, ESI†), before treatment only five peptides (marked with ‘\(^*\)’) with the low signal-to-noise (S/N) ratio can be detected. However, after enrichment with the LaGM probe, sixteen peptides with the increased MS S/N ratio and intensity can be assigned to the peptides from the BSA tryptic digest (sequence coverage of 27%, Table S1, ESI†), indicating the effectiveness of the LaGM probe for enrichment of the low-abundance peptides (Fig. S5b, ESI†). Furthermore, to inspect the selectivity of the LaGM probe for peptides, human urine containing a high level of salts and contaminants was used as a complex real biosample.

No peptides could be identified for direct detection of human urine due to the interference of the high content salts and contaminants (Fig. S6a, ESI†). However, five peptides with high S/N ratio and intensity were observed after treatment with the LaGM probe (Fig. S6b, ESI†), indicating that the LaGM probe can selectively enrich the target peptides from complex biosamples.

The LaGM probe can also be used for capture and labelling of phosphopeptides based on the affinity and hydrolytic catalysis of the LaPO4 nanorods, as shown in Fig. S7 (ESI†). To investigate the phosphopeptide capturing and labeling efficiency, the β-casein tryptic digest containing the phosphopeptides (Table S2, ESI†) was first used as a model sample. Only one phosphopeptide with weak intensity (marked with ‘\(^\dagger\)’) can be detected in the direct
different concentrations. When the concentration is as low as 10^{-9} M, phosphopeptides can still be detected (Fig. S8d, ESI†). A complex peptide mixture consisting of β-casein and BSA (1 : 50 molar ratio) was further used to evaluate the selectivity of the LaGM probe for capture of phosphopeptides. Apparently, no phosphopeptides can be detected from the peptide mixture due to the suppression effect and interference from the abundant nonphosphopeptides (Fig. S9a, ESI†). However, after selective capture using the affinity probe, three labeled phosphopeptides can be clearly identified in the mass spectrum and free of the interference of nonphosphopeptide peaks (Fig. S9b, ESI†). These results confirm that the LaGM probe can also be used for selective capture of phosphopeptides.

Human serum containing various informative endogenous peptides including the phosphopeptides released by diseased tissue and these peptides have gained considerable interest for the disease biomarker discovery. To prove the practical effect of the LaGM probe for enriching low-abundance peptides from real samples. More importantly, two labeled phosphopeptides are successfully detected with a clean background in the second elution step (Fig. 2b). The MALDI tandem mass spectrometry (MS/MS) further confirms the effectiveness of the LaGM probe for capturing and labeling the phosphopeptides (Fig. S12, ESI†). These results demonstrate that the LaGM probe can be used for selective capture, sequential MS detection and easy discrimination of low-abundance peptides and phosphopeptides. For comparison, two types of commercial affinity products (ZipTipC18 pipette tip and TiO2 nanoparticles) were also used to capture target peptides from diluted human serum under the same experimental conditions (Fig. S10 and S11, ESI†). Obviously, the monofunctional commercial products cannot be used for selective capture and sequential determination of the corresponding biomolecules. While the selectivity and the efficiency of commercial TiO2 nanoparticles for enrichment of phosphopeptides are inferior to those of the LaGM probe.

In summary, a new graphene-based affinity probe consisting of the graphene scaffold, affinity nanorods and magnetic nanoparticles has been synthesized. The multifunctional probe can be used for selective capture, sequential determination and direct identification of two types of peptides. This affinity probe has better sensitivity and selectivity than the commercial ZipTipC18 pipette tip and TiO2 affinity product. Therefore, this work could provide new insights for the design of the multifunctional graphene-based affinity probes for extracting low-abundance biomarkers from complex biosamples in biomedical application.

The work was supported by the Natural Science Foundation of China (NSFC) (Grant No. 20871083 and 21171161).

Notes and references