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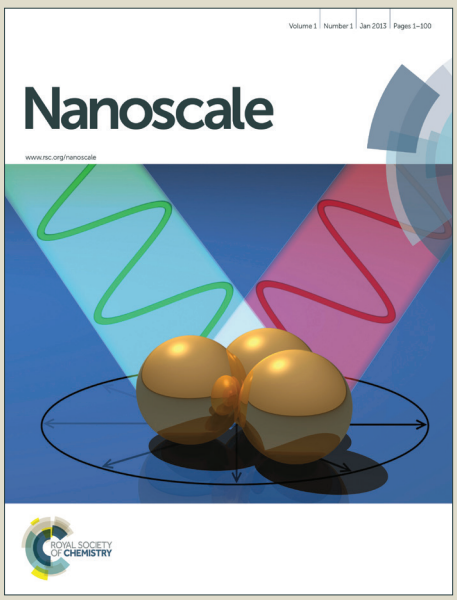

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ARTICLE TYPE

New GO/PEI/Au/L-Cys ZIC-HILIC composites: synthesis and selective enrichment of glycopeptides

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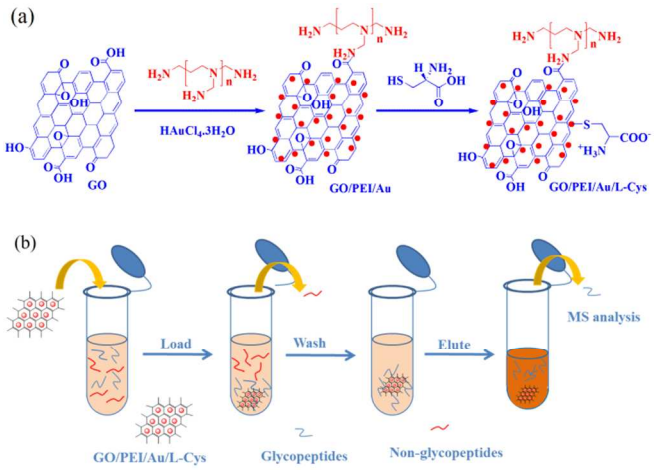
GO/PEI/Au/L-Cys composites were synthesized *via* loading gold nanoparticles on GO surface using polyethylenimine as reducing and stabilizing reagents, followed by L-Cysteine immobilization through Au-S bond. The composites were applied as a kind of novel ZIC-HILIC material to achieve the high selective enrichment of glycopeptides from biological samples.

Protein glycosylation, one of the most important post-translational modifications (PTMs) of proteomes, plays important roles in a number of biological processes, such as intracellular transport, immune response, cell growth, and cell to cell communications.[1-2] To better understand these biological processes, it is necessary to identify glycoproteins and determine their glycosylation sites.[3] Mass spectrometry (MS) coupled with various separation techniques, has become an effective tool for analyzing and characterizing protein glycosylation. However, some challenges still exist. Firstly, glycosylation can be highly labile, which leads to the complexity of the analysis. Secondly, the inherent low abundance of glycoproteins complicates the analysis. Thirdly, MS signals of glycopeptides are strongly suppressed by those of non-glycopeptides, thus making them "lost in the noise".[4] Therefore, the enrichment of glycopeptides from complex biological matrixes is indispensable prior to MS analysis.

Up until now, several methods, including lectin affinity, hydrazine oxidation, boronic acid affinity and hydrophilic interaction chromatography (HILIC), have been developed for the isolation and identification of glycopeptides.[5-8] HILIC displays the advantages of high selectivity, excellent reproducibility and good compatibility with MS analysis, thus making it a promising method for glycoproteome analysis. However, the co-elution of non-glycopeptides with glycopeptides interferes the identification of glycopeptides. Beside to increase the hydrophobicity of non-glycopeptides by adding ion-pair reagents, a more effective solution is to prepare new materials with improved hydrophilicity, and thus to increase the retention of glycopeptides.[9] Zwitterionic (ZIC)-HILIC stationary phase, which contains both positive and negative charges, is proven to have excellent hydrophilicity.[10] Therefore, the preparation of novel ZIC-HILIC materials might be of great potential to improve the selectivity for glycopeptide enrichment.

Graphene oxide (GO) is a novel one-atom-thick carbon nanosheet with various hydroxyl and epoxy groups on the graphitic plane and carboxylic acid group at the sheet edges, which ensures the excellent hydrophilicity.[11] Although GO has recevied increasing attention in biological fields, such as cell imaging, drug/gene delivery and cancer therapy,[12-15] it has not been applied to prepare ZIC-HILIC material. Herein, to the best of our knowledge, for the first time, GO/PEI/Au/L-Cys composites are synthesized *via* loading gold nanoparticles (Au NPs) on GO surface with polyethylenimine (PEI) as reducing and stabilizing reagents, followed by L-Cysteine immobilization through Au-S bond, which shows promising to improve the recognition specificity for glycopeptides.



Scheme 1. (a) Schematics of the synthesis of GO/PEI/Au/L-Cys composites; (b) protocol for glycopeptide enrichment.

The route for preparing GO/PEI/Au/L-Cys composites is shown in Scheme 1a. Firstly, PEI, a cationic polymer, was self-assembled on the surface of anionic GO nanosheets by electrostatic interaction, which could greatly improve the hydrophilicity of GO, owing to the large number of polar groups on the polymer chains. Secondly, Au NPs were introduced by *in situ* growth on the surface of GO/PEI. Finally, L-Cys, a typical zwitterionic, was anchored on GO/PEI/Au by the reaction between Au and the thiol groups of L-Cys. The prepared GO/PEI/Au/L-Cys was further used as a novel kind of ZIC-HILIC material for glycopeptide enrichment.

The morphology of GO, GO/PEI and GO/PEI/Au was investigated by transmission electron microscopy (TEM). As shown in Fig. 1a and b, due to the good hydrophilicity, GO and GO/PEI nanosheets were well dispersed in water. With Au NPs added, as shown in Fig. 1c, such nanoparticles spread homogeneously on the surface of GO/PEI with high density, which could greatly increase the number of reaction sites for further modification. High-resolution TEM revealed the highly crystalline nature of Au NPs with a lattice spacing of 0.238 nm (Fig. 1d).[16] The dynamic light scattering showed that the average hydrated diameter of Au NPs was 15.3±1.3 nm (Fig. S1) deduced from that of residual free AuNPs in solution, which showed the similar size. All these results validated the generation of Au NPs on GO/PEI nanosheets.

The prepared GO/PEI/Au composites could provide large surface area and sufficient anchor sites for the further modification of L-Cys via the interaction of Au and –SH. The morphology of GO/PEI/Au/L-Cys composites was characterized by TEM images (Fig. S2). With excessive L-Cys added, after 4 h reaction, L-Cys began to accumulate on GO surface, which was beneficial to enrichment of glycopeptides. When the time was increased to 24 h, the reaction was completed, with the concentration of residual L-Cys in supernatant unchanged. Therefore, we chose 24 h as the optimized L-Cys immobilization time.

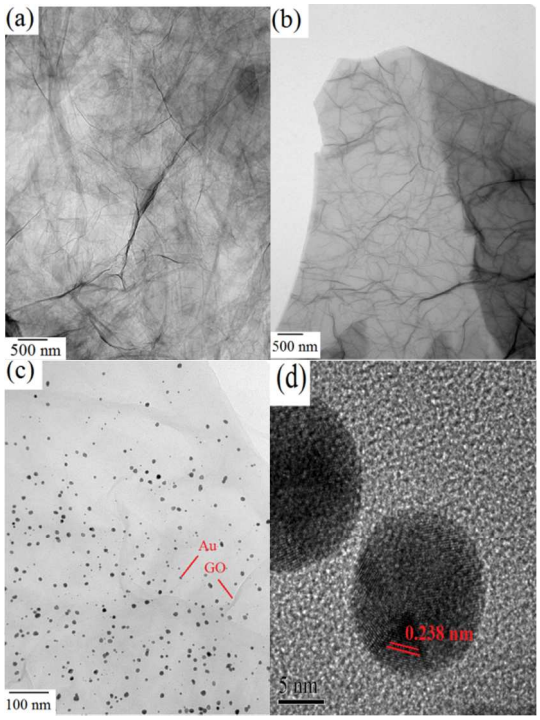


Fig.1 TEM images of (a) GO, (b) GO/PEI, (c) GO/PEI/Au, and (c) HR-TEM image of gold nanoparticles.

The successful synthesis of GO/PEI/Au/L-Cys composites is confirmed by UV-Vis absorption spectra. As shown in Fig. 2a, GO presents a characteristic peak at 230 nm corresponding to π–π* transitions of aromatic C–C bonds. After the *in situ* generation of Au NPs on GO/PEI, a new absorbance band appears at 525 nm, characteristic of the colloidal gold plasmon resonance band.[17] Compared with GO/PEI/Au, the Au NPs absorption of GO/PEI/Au/L-Cys composites blue-shifts to 521 nm due to the formation of Au-S. Fourier-transformed infrared spectroscopy (FT-IR) is further applied to characterize the modification of L-Cys on GO/PEI/Au substrate. As shown in Fig. 2b, for GO, the peak around 1728 cm^{-1} is corresponding to C=O stretching vibration of the carboxylic group. After PEI self-assembled to GO surface, C=O stretching vibration almost disappears, and new peaks at 2928 and 2855 cm^{-1} ascribing to C–H stretch appears, confirming the successful self-assembly.[18] L-Cys fingerprint and C–H stretch of PEI are simultaneously found in GO/PEI/Au/L-Cys, which suggests the successful synthesis of such composites.

X-ray photoelectron spectroscopy (XPS) is further used to evaluate and quantify the elements of GO/PEI/Au/L-Cys. The survey scan of GO/PEI/Au/L-Cys is displayed in Fig. S3, in which C 1s, O 1s, N 1s, S 2p and Au 4f core-levels exist distinctly. The immobilization amount of L-Cys is calculated to 3.86 mmol/g by the content of S element. Such a significantly increased amount of L-Cys should be attributed to the large surface area of GO and the abundance of anchored Au NPs.

GO/PEI/Au/L-Cys composites are further evaluated by BET analysis. The specific surface area and total pore volume of ware 107.8 m^2/g and 0.36 cm^3/g, respectively, indicating a relatively high surface-to-volume ratio. As shown in Fig. S4, the hysteresis loop of adsorption and desorption isotherms at $P/P_0>0.8$ indicates the formation of accumulation pore during the preparation, which is further proven by BJH pore-size distribution curve. It can be expected that the prepared novel ZIC-HILIC material, with excellent hydrophilicity, huge amount of L-Cys and large specific surface area, has great potential for the selective enrichment of glycopeptides from complex samples.

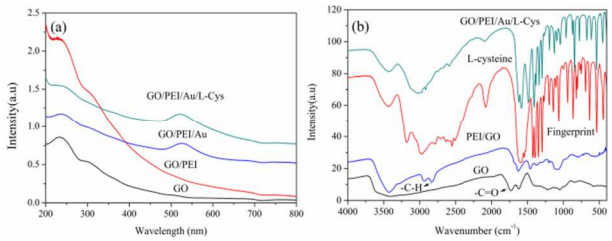


Fig. 2 (a) UV-Vis spectra of GO, GO/PEI, GO/PEI/Au and GO/PEI/Au/L-Cys composites; (b) FT-IR spectra of GO, GO/PEI, L-Cysteine and GO/PEI/Au/L-Cys composites.

The procedure for the enrichment of glycopeptides by GO/PEI/Au/L-Cys composites is illustrated in Scheme. 1b. The performance of ZIC-HILIC material was evaluated by tryptic HRP, with the optimized mass ratio of composites to peptides as at 200:1. ACN/H_2O/FA (80/20/0.1, v/v/v, containing 5 mM NH_4HCO_3) and ACN/H_2O/FA (60/40/0.1, v/v/v, containing 5 mM NH_4HCO_3) were applied as loading and elution buffers, respectively. After incubation with GO/PEI/Au/L-Cys composites for 2.5 min, glycopeptides were disappeared from the supernatant. After 2.5 min elution, glycopeptides were completely desorbed. All these results show that the entire enrichment procedure can be completed within 10 min, much faster than required by PEG brushes hybrid hydrophilic magnetic nanoparticles.[19] The rapid operation should be contributed to the relatively high surface area and structure of GO/PEI/Au/L-Cys composites.

Fig. 3 shows MALDI-TOF MS spectra of tryptic digests of standard glycoproteins before and after enrichment by GO/PEI/Au/L-Cys composites. As shown in Fig. 3a and b, for direct analysis, the signals of glycopeptides in 2.5 pmol of HRP digests (mainly at them/z range of 2500–5000) are seriously suppressed by those of non-glycopeptides (mainly at the m/z range of 1000–2500). However, after enrichment, non-glycopeptides are almost completely removed, and 14

glycopeptides with enhanced intensity dominate the MS spectra (Table S1). Similar results were obtained for the enrichment of glycopeptides in 1 pmol chicken avidin digests (Fig. 3c and d, Table. S2).

To further evaluate the enrichment capacity for sialic acid-containing glycopeptides, the tryptic digests of IgG was taken as the sample. After enrichment, 26 glycopeptides containing 2 sialic acid-containing glycopeptides (m/z 3087 and 3250, Table S3) were identified, confirmed by the further deglycosylation with PNGase F (Fig. S5). All these results demonstrate the superior enrichment ability of GO/PEI/Au/L-Cys composites, attributed to the excellent hydrophilicity.

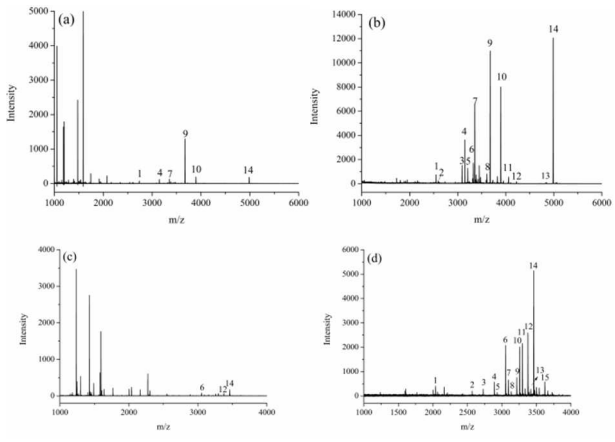


Fig. 3 MALDI-TOF spectra of 2.5 pmol HRP digest (a) before and (b) after enrichment; 1 pmol chicken avidin digest (c) before and (d) after enrichment. Glycopeptide peaks are labeled with number.

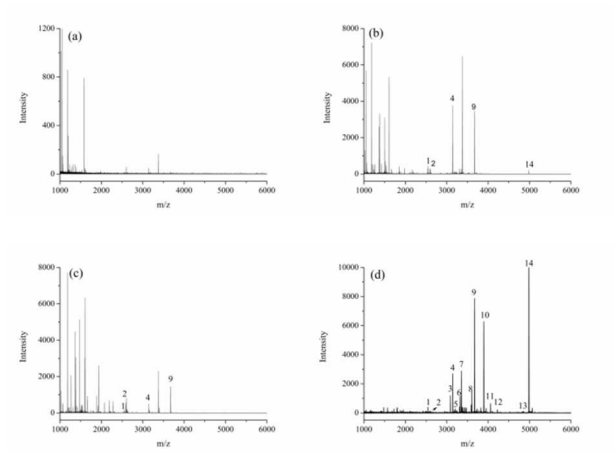


Fig. 4 MALDI-TOF MS spectra of the tryptic digest mixture of Myo and HRP (with the mass ratio of 100:1) after enrichment by (a) GO, (b) GO/PEI, (c) GO/PEI/Au and (d) GO/PEI/Au/L-Cys. Glycopeptide peaks are labeled with number.

The enrichment of glycopeptides in HPR and Myo/HRP systems by GO, GO/PEI and GO/PEI/Au and GO/PEI/Au/L-Cys was further compared. For HRP system, after the enrichment by GO/PEI/Au/L-Cys, 14 glycopeptides were identified, with non-glycopeptides completely removed. However, only 5 glycopeptides were respectively identified after enrichment by GO/PEI and GO/PEI/Au, and no selectivity towards glycopeptides was obtained after enrichment by GO. For HRP/Myo system, as shown in Fig. 4, GO/PEI/Au/L-Cys shows the best selectivity for glycopeptides. Through the measurement of the contact angles of GO, GO/PEI and GO/PEI/Au and GO/PEI/Au/L-Cys (Fig. S6), we proved our deduction that the excellent glycopeptide enrichment capacity of GO/PEI/Au/L-Cys should be contributed to the improved hydrophilicity with PEI and L-Cys introduced. Based on previous study,[20-21] such material might also have the great potential to the sequential enrichment of phosphopeptides and glycopeptides.

Table.1 Recovery of two deglycosylated peptides of human IgG.

Ratio (%)	EEQFN#STYR	EEQYN#STYR
D/H 1	80.1	77.8
D/H 2	77.4	78.8
D/H 3	80.7	83.1
Average recovery	79.4±1.7	79.9±2.8

To evaluate the detection sensitivity of glycopeptides enriched by GO/PEI/Au/L-Cys, the amount of tryptic digests of HRP was further decreased to 25 fmol, near the detection limit in the reflect mode of MALDI-TOF MS. As shown in Fig. S7, after enrichment, 6 glycopeptides are positively identified, indicating GO/PEI/Au/L-Cys shows great potential for trace sample analysis.

IgG (10μg) was further used to evaluate the recovery of glycopeptides after enrichment by GO/PEI/Au/L-Cys, by coupling the dimethyl labeling technique with MS,[8] and the obtained result was up to 79.4% (Table 1), beneficial to improve the coverage of glycoproteome analysis.

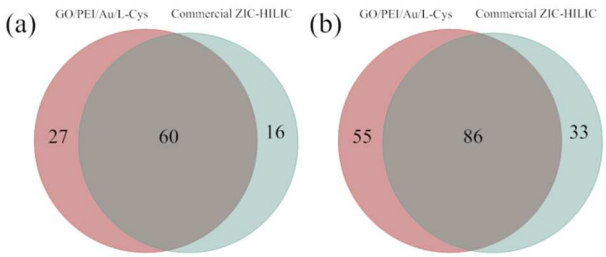


Fig. 5 Overlapping of identified (a) glycoproteins and (b) glycopeptides in low human serum abundance proteins after enrichment by GO/PEI/Au/L-Cys and commercial ZIC-HILIC.

Human plasma possess significant potential for disease diagnosis and rapeutic monitoring.[22] In fact, numerous clinical biomarkers and therapeutic targets are glycoproteins.[23] However, the characterization of human plasma glycoproteins remains a major challenge due to their low concentration and suppression by high abundance proteins.[24] Herein, after the removal of 14 high-abundance proteins in human plasma by a commercial antibody column, GO/PEI/Au/L-cys composites were applied for the enrichment of N-glycopeptides in the digests of low abundance fraction, followed by three independent analysis with capillary reversed-phase liquid chromatography coupled to tandem mass spectrometry (RPLC-MS/MS). A total of 177 proteins and 495 peptides were identified in 5 μg sample, among which 141 glycopeptides, corresponding to 87 glycosylated proteins were identified (Table. S4). For comparison, the commercial ZIC-HILIC material was also used to capture the N-linked glycopeptides under the same conditions. As shown in Fig. 5, GO/PEI/Au/L-Cys exhibits better performance than that of commercial ZIC-HILIC. The results suggest GO/PEI/Au/L-Cys

exhibits excellent performance for enriching glycopeptides from complex biological samples.

In summary, GO/PEI/Au/L-Cys nanocomposites, a novel kind of ZIC-HILIC material, was prepared and successfully applied to the selective enrichment of N-linked glycopeptides from complex biological matrixes. The large surface area, good biocompatibility and excellent hydrophilicity of such composites ensure the advantages of high selectivity and a decrease in time for the enrichment of glycopeptides. This work not only offers a novel material for the high efficient enrichment of glycopeptides, but also opens a new field for the applications of GO.

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