



Preparation of a hydrophilic interaction liquid chromatography material by sequential electrostatic deposition of layers of polyethyleneimine and hyaluronic acid for enrichment of glycopeptides

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Received: 17 April 2019 / Accepted: 22 July 2019 / Published online: 3 August 2019
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Abstract

A hydrophilic interaction liquid chromatography (HILIC) material with application in glycoproteomics was obtained by sequential deposition of polyethyleneimine (PEI) and hyaluronic acid (HA) on a negatively charged substrate by means of electrostatic self-assembly. This kind of surface modification endows the material with excellent hydrophilicity and warrants efficient glycopeptides enrichment. The feasibility of this enrichment was verified by using dendritic mesoporous silica nanoparticles (DMSNs) and magnetic graphene oxide (MagG) as negatively charged substrates for PEI and HA adhesion. The two final products (DMSNs@PEI@HA and MagG@PEI@HA) exhibit high enrichment selectivity (molar ratios of IgG and BSA digests = 1:500 and 1:1000), sensitivity (detection limit, 2 fmol/μL), recovery (>90%) and enrichment capacity (300 mg/g). When using DMSNs@PEI@HA, 419 N-glycopeptides derived from 105 glycoproteins were identified. When using MagG@PEI@HA, 376 N-glycopeptides derived from 102 glycoproteins were identified, both from a 2 μL serum sample. This is better than by methods described in previous reports.

Keywords Post-translational modification · Mass spectrometry · Functional nanomaterial · Electrostatic self-assembly · Glycopeptides enrichment

Introduction

Protein glycosylation, one of the most important post-translational modifications, regulates the localization and folding of proteins. It also plays an important role in cell biology processes, such as signal transmission, immune

response, metabolism [1–3]. Numerous diseases are associated with the abnormal glycosylation sites and levels [4]. Studying protein glycosylation under different physiological conditions is important to understand the mechanism of disease generation and related signaling pathways, thus providing useful information for disease diagnosis [5–8]. Although mass spectrometry (MS) is currently the most effective tool for identifying protein glycosylation, the MS responses of low-abundant glycopeptides are always severely suppressed by the abundant non-glycopeptides in complex biological samples [9, 10]. Therefore, highly efficient enrichment of low-abundant glycopeptides from complex biological samples prior to MS analysis is needed [11, 12].

Hydrophilic interaction liquid chromatography (HILIC) is a commonly used technique for glycopeptides enrichment based on the difference in hydrophilicity between glycopeptides and non-glycopeptides. Due to its ease of operation and outstanding repeatability, HILIC has received increasing attention in separation of biomolecular. [13, 14]. To date, various hydrophilic molecules, such as maltose [15], zwitterion

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00604-019-3712-2>) contains supplementary material, which is available to authorized users.

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[16], hydrophilic dendrimer [17], have been used for surface modification to improve the hydrophilicity of HILIC materials. Although these HILIC materials can be used for selective enrichment of glycopeptides, the cumbersome and demanding modification processes of materials result in time-consuming and low-efficient functionalization. For example, maltose is typically modified on the surface of the substrates by click chemistry [18]. In this reaction, it is necessary to introduce N_3 groups onto the substrate using an azide and to modify the maltose with an alkyne groups. Subsequently, the N_3 groups on the substrate are reacted with the alkyne-terminated maltose by click chemistry to complete the modification of maltose. The tedious manipulations and harsh conditions make this modification method unsuitable for universal application. In order to improve the modification efficiency and application range of HILIC materials, it is a trend to develop a facile and universal modification strategy.

The electrostatic self-assembly technique has been widely used to modify the spherical and planar substrates due to its simplicity, time-saving and high modification efficiency. Actually, the surface modification method of HILIC materials based on electrostatic self-assembly technology is a facile and universal modification strategy, which will promote the development of HILIC materials in glycopeptides enrichment. Polyethyleneimine (PEI), a positively charged polymer, can be easily modified on negatively charged substrates by electrostatic interaction. Moreover, owing to its intrinsic hydrophilicity, PEI not only improves the hydrophilicity of the substrates, but also acts as a connector to further connect with other hydrophilic molecules. And hyaluronic acid (HA), a common anionic polysaccharide with abundant carboxyl and hydroxyl [19, 20], can also be combined with PEI by electrostatic interaction to further improve the hydrophilicity of HILIC materials. Therefore, it can be considered that the use of PEI and HA for surface modification of HILIC materials by electrostatic self-assembly technique is an ideal modification method used for glycopeptides enrichment.

Herein, a universal modification strategy was developed to synthesize HILIC materials for glycopeptides enrichment, in which PEI and HA were sequentially assembled on negatively charged substrates by means of electrostatic self-assembly technique. PEI with a high density of amino groups can be easily modified on negatively charged substrates by electrostatic interaction, and then HA with numerous hydroxyl groups can be combined with PEI in the same manner. Dendritic mesoporous silica nanoparticles (DMSNs) and magnetic graphene oxide (MagG) [21] with large surface area were respectively selected as negatively charged substrates for PEI and HA adhesion. The products (DMSNs@PEI@HA and MagG@PEI@HA) exhibited excellent hydrophilicity and high selectivity, sensitivity, recovery and enrichment capacity for glycopeptides enrichment, as well as high enrichment efficiency in identifying low-abundance N-glycopeptides from 2 μ L of human serum.

The enrichment results demonstrate the feasibility of this facile and universal modification strategy for the synthesis of HILIC materials, and it shows obvious application potential in glycoproteomics analysis.

Experimental

Materials and chemicals

Triethanolamine (TEA), cetyltrimethylammonium bromide (CTAB), sodium salicylate (NaSal), tetraethyl orthosilicate (TEOS), polyethyleneimine (PEI, 50%, Mw = 70000), ferric chloride ($FeCl_3$), sodium citrate, sodium acetate (NaAc), ethylene glycol were purchased from Aladdin (Shanghai, China, <https://www.aladdin-e.com/>). Hyaluronic acid (HA) was provided by Nine Ding chemistry (Shanghai, China, <http://www.9dingchem.com/>) Ammonium bicarbonate (NH_4HCO_3), urea, dithiothreitol (DTT), iodoacetamide (IAA), trypsin, immunoglobulin G from human serum (IgG), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (USA, <https://www.sigmaaldrich.com>). PNGase F was acquired from New England Biolabs (UK, <https://www.neb.uk.com/>). Acetonitrile (ACN), trifluoroacetic acid (TFA), and formic acid (FA) were chromatographic grade. Ultra-pure water was used after processing by Milli-Q system (Millipore, Bedford, MA). Human serum was extracted from healthy people in Sixth People's Hospital of Shanghai. All of the above reagents were used directly without further purification.

Modification of two different substrates

The synthesis of DMSNs and MagG was according to our previous work (ESM for details) [22, 23]. Then DMSNs and MagG were modified with PEI and HA by electrostatic self-assembly technology. Briefly, 0.8 g of PEI was dissolved in 80 mL of deionized water. Then 0.08 g of DMSNs or MagG was added and dispersed by ultrasound. The mixture was stirred for 12 h. Subsequently, the products were washed with water for three times and then dispersed in 80 mL of HA (50 mg) aqueous solution with stirring for 24 h. Finally the products DMSNs@PEI@HA and MagG@PEI@HA were washed with water and lyophilized to dryness.

Sample preparation

Standard protein (IgG, 1 mg) or BSA (1 mg) was dissolved in 100 μ L of denaturing buffer solution (8 M urea, 50 mM NH_4HCO_3 , pH 8.2). Subsequently, 5 μ L of DTT (200 mM) was added to the solution under vibrating at 56 $^{\circ}C$ for 45 min and then 20 μ L of IAA (200 mM) was added and incubated for another 30 min in the dark. Finally, NH_4HCO_3 (50 mM) was supplemented to the solution to a total volume of 1 mL

and trypsin (trypsin/protein = 1/25, w/w) was added with additional incubation at 37 °C for 17 h. The tryptic digest was stored at -80 °C for further use.

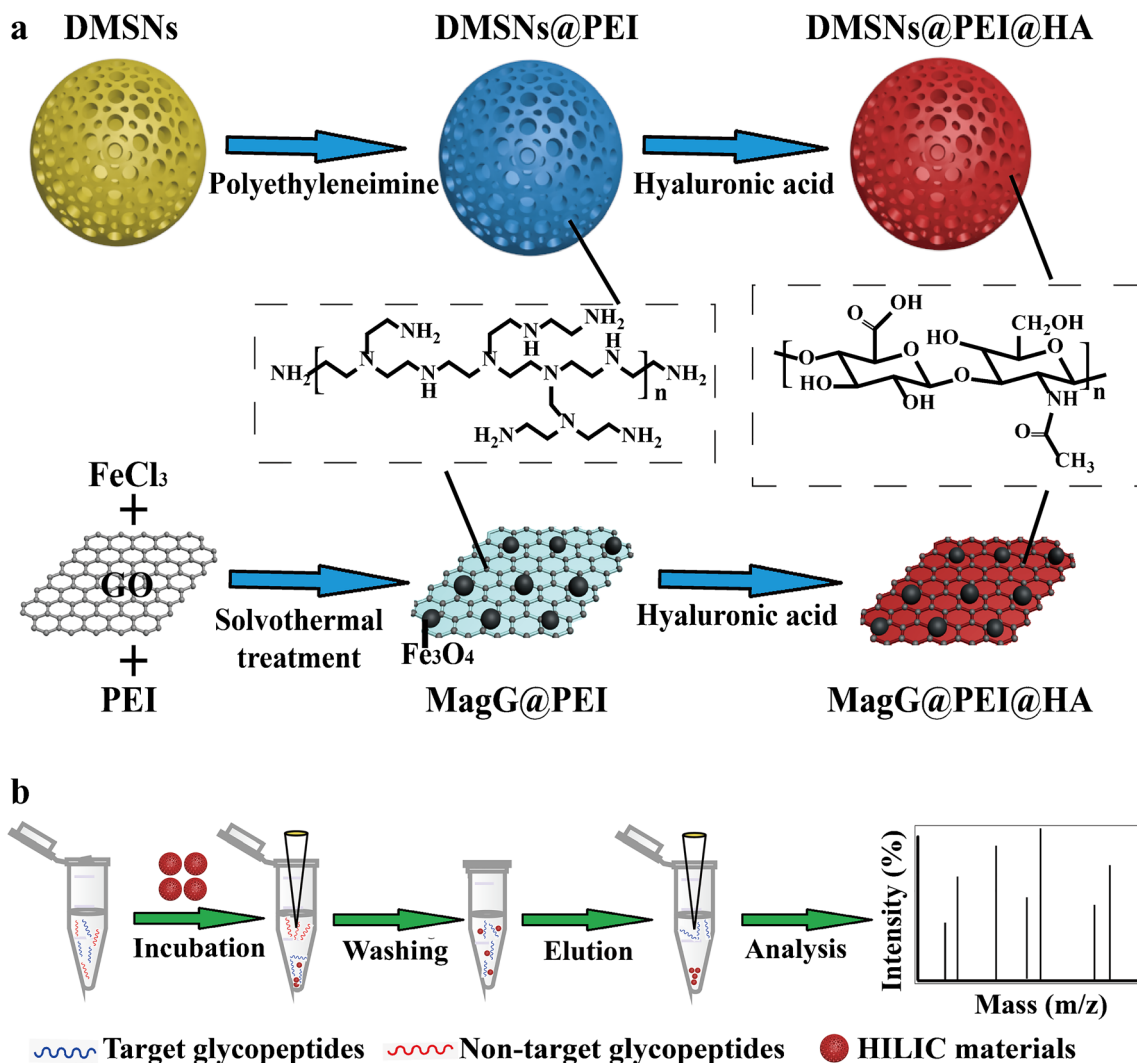
The preparation of human serum digestion was similar to our previous work [24]. Typically, 2 μL of human serum and 18 μL of NH₄HCO₃ (50 mM, 8 M urea) were mixed together. Subsequently, 5 μL of DTT (40 mM) was added and incubated at 56 °C for 45 min. Then 4 μL of IAA (200 mM) was added and incubated for another 30 min in the dark. Finally, 16 μL of NH₄HCO₃ and 5 μL of trypsin (trypsin/protein = 1/25, w/w) were added to the mixture with additional incubation at 37 °C for 17 h. The tryptic digest was lyophilized and stored at -80 °C for further use.

Selective enrichment of glycopeptides

The illustration of the enrichment process is shown in Scheme 1. 6 μL of IgG tryptic digests was dissolved in 200 μL of loading buffer (ACN/H₂O/TFA = 90/8/2, v/v/v),

and then 20 μg of nanocomposites was added. The mixture was incubated at room temperature for 30 min. After removing the supernatant, the nanocomposites were washed with 100 μL of washing buffer (ACN/H₂O/TFA = 90/9.9/0.1, v/v/v) for three times. The captured glycopeptides were eluted from the nanocomposites with 10 μL elution buffer (H₂O/TFA = 97/3, v/v) and then analyzed by MALDI-TOF MS. It is worth noting that it is a magnetic separation for MagG@PEI@HA and centrifugation for DMSNs@PEI@HA.

For human serum tryptic digests enrichment, 2 μL of digests was mixed with 500 μL of loading buffer (ACN/H₂O/TFA = 90/8/2, v/v/v) and 400 μg of nanocomposites, then the mixture was incubated for 30 min. The nanocomposites were washed with washing buffer (500 μL, ACN/H₂O/TFA = 90/9.9/0.1, v/v/v) for three times. The elution step was repeated three times with 100 μL of elution buffer (H₂O/TFA = 97/3, v/v) and then the eluent was combined and lyophilized. The lyophilized glycopeptides were redissolved in a mixture containing 17 μL of H₂O, 2 μL of 10X Glycobuffer 2 and 1 μL (500 unites) of



Scheme 1 Preparation process of DMSNs@PEI@HA and MagG@PEI@HA and application flowchart of glycopeptides enrichment

PNGase F with incubation at 37 °C for 17 h. Finally, the mixture was lyophilized and analyzed by nano LC-MS/MS.

Characterization and MS analysis

The detailed instruments and characterization are displayed in the Electronic supplementary material.

Result and discussion

Synthesis and characterization of DMSNs@PEI@HA and MagG@PEI@HA HILIC materials

The synthesis process of DMSNs@PEI@HA and MagG@PEI@HA is presented in Scheme 1a. The DMSNs

were obtained by using CTAB as a template with NaSal assisted [25]. And the MagG was synthesized by a solvothermal method. Both DMSNs and MagG have a large number of negatively charged groups on the surface [26, 27]. Therefore, PEI can be easily modified on the surface of DMSNs and MagG by electrostatic interaction due to its abundant amino groups. Subsequently, HA with numerous hydroxyl groups can be combined with PEI in the same manner. This modification strategy is applicable to other negatively charged substrates for the synthesis of HILIC materials.

The microscopic morphology of DMSNs@PEI@HA was carried out by TEM and SEM. Figure 1a shows that the DMSNs exhibit a spherical structure with obvious central-radial dendritic mesoporous and a diameter of ~150 nm. After PEI and HA coating, the film is visible on the surface

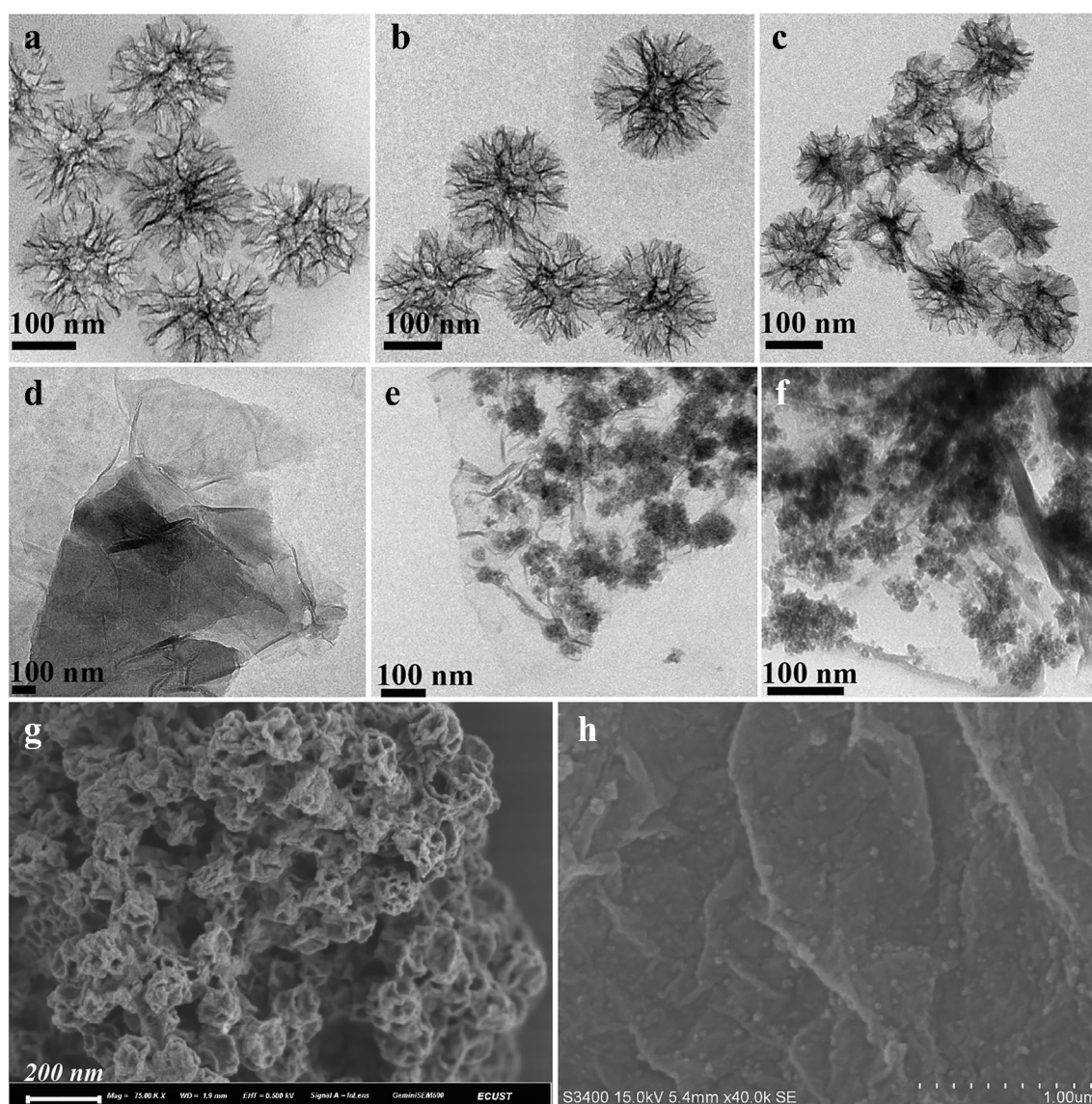


Fig. 1 TEM images of DMSNs (a), DMSNs@PEI (b), DMSNs@PEI@HA (c), graphene oxide (d), MagG@PEI (e) and MagG@PEI@HA (f). SEM images of DMSNs@PEI@HA (g) and MagG@PEI@HA (h)

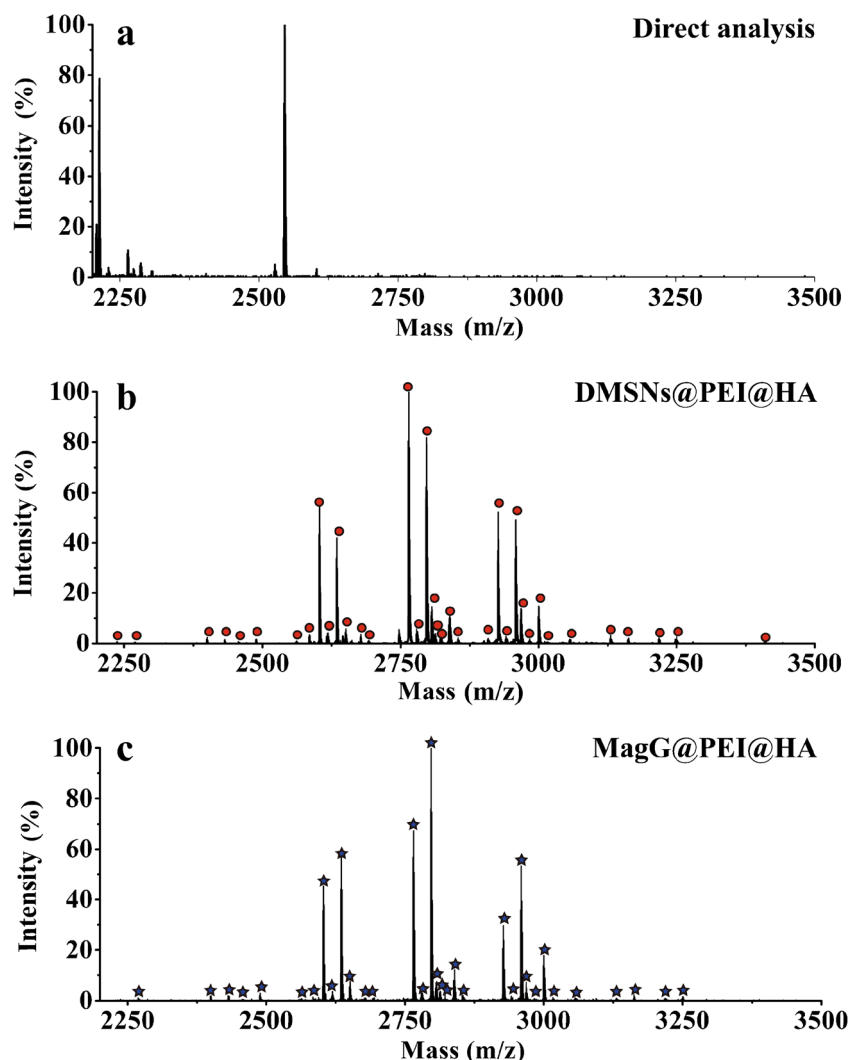
of the DMSNs, while the size and pore structure of DMSNs@PEI and DMSNs@PEI@HA have no obvious change (Fig. 1b, c). The FE-SEM image of DMSNs@PEI@HA (Fig. 1g) also reveals that the DMSNs@PEI@HA still have abundant pores after modification. And for MagG@PEI@HA, lamellar graphene oxide and Fe₃O₄ nanoparticles can be clearly observed from both TEM (Fig. 1f) and SEM (Fig. 1h) images.

In addition, the successful modification of PEI and HA step by step was confirmed by various characterizations. The zeta potential changes of the materials are shown in Fig. S1 (A) and (B). While after PEI coating, the zeta potential changes from negative to positive due to the presence of abundant amino groups in PEI. Subsequently, the zeta potential of material is significantly decreased after treatment with HA. In addition, TGA and FT-IR results also demonstrate that PEI and HA are successfully modified on two negatively charged substrates. The detailed information and corresponding instructions are display in the ESM (Fig. S1).

Fig. 2 MALDI-TOF mass spectra of the IgG tryptic digest before (a) and after DMSNs@PEI@HA (b) and MagG@PEI@HA (c) enrichment. Glycopeptides enriched by DMSNs@PEI@HA and MagG@PEI@HA are labeled with “●” and “★”, respectively

Selective enrichment of glycopeptides from standard protein

In order to verify that this modification strategy has great application value in glycopeptides enrichment, standard glycoprotein (IgG) tryptic digest was selected as model sample to evaluate the glycopeptides enrichment efficiency of DMSNs@PEI@HA and MagG@PEI@HA. The enrichment procedure is shown in Scheme 1b. Firstly, a certain concentration of IgG trypsin digest was fully incubated with the nanomaterials to ensure maximum adsorption of glycopeptides. And then the non-specifically adsorbed peptides were removed by washing with washing buffer. Finally, the captured glycopeptides were eluted with elution buffer and analyzed by MS. For glycopeptides enrichment, the ratio of ACN to H₂O in loading buffer is the key to determining the enrichment efficiency [28]. Therefore, different concentrations of ACN (95%, 92%, 90%, 88%, 85% and 80%) with 2% TFA were used to determine the optimal percentage of ACN in the loading buffer. As shown in Fig. S2 and S3, both DMSNs@PEI@HA and



MagG@PEI@HA shows optimal enrichment efficiency when the concentration of ACN was 90% according to the number and intensity of glycopeptides signal specifically. Thus, ACN/H₂O/TFA (90/8/2, v/v/v) was adopted as loading buffer in the experiment. In addition, we also optimized the acidity of the elution buffer by using different concentrations of TFA aqueous solution (6%, 3%, 2%, 1%, 0.5% and 0.1%). The results shows that 3% TFA aqueous solution as the elution buffer possessed optimal enrichment effect for both DMSNs@PEI@HA and MagG@PEI@HA (Fig. S4 and S5).

The enrichment selectivity, sensitivity, recovery and capacity of the two nanocomposites were measured under the optimal enrichment conditions. The MALDI-TOF mass spectra of IgG digest before and after enrichment are shown in Fig. 2. It can be seen that no signal of glycopeptides is identified before enrichment (Fig. 2a) due to the severe inhibition of abundant non-glycopeptides. However, after enrichment with DMSNs@PEI@HA and MagG@PEI@HA (Fig. 2b, c), the number of glycopeptides increases significantly and the signal of non-glycopeptides almost disappears. 36 and 33 glycopeptides are identified by DMSNs@PEI@HA and MagG@PEI@HA, respectively. The results are better than

many previous hydrophilic nanomaterials. The detailed information of the identified glycopeptides are listed in Table S1. The excellent enrichment performance is mainly attributed to the excellent hydrophilicity endowed by PEI and HA. The result verifies the feasibility of this modification strategy in glycopeptides enrichment.

Subsequently, mixtures of IgG and BSA digests at different molar ratios were used to simulate complex systems for investigation of the enrichment selectivity of DMSNs@PEI@HA and MagG@PEI@HA. As shown in Fig. 3a, direct analysis of the mixture of IgG and BSA digests at a molar ratio of 1:100, only non-glycopeptides signals can be observed in the spectrum. However, after treatment with DMSNs@PEI@HA (Fig. 3b), most of the signals belonged to glycopeptides and 20 glycopeptides are detected. Even when the molar ratio of IgG and BSA increased to 1:500, DMSNs@PEI@HA still captured 14 glycopeptides from such a complex system (Fig. 3c). And for MagG@PEI@HA, the enrichment results also indicate an excellent enrichment selectivity (Fig. 3d, e and f). These results further demonstrate that this modification strategy is suitable for glycopeptides enrichment and exhibits good enrichment selectivity even in the presence of plenty non-glycopeptides.

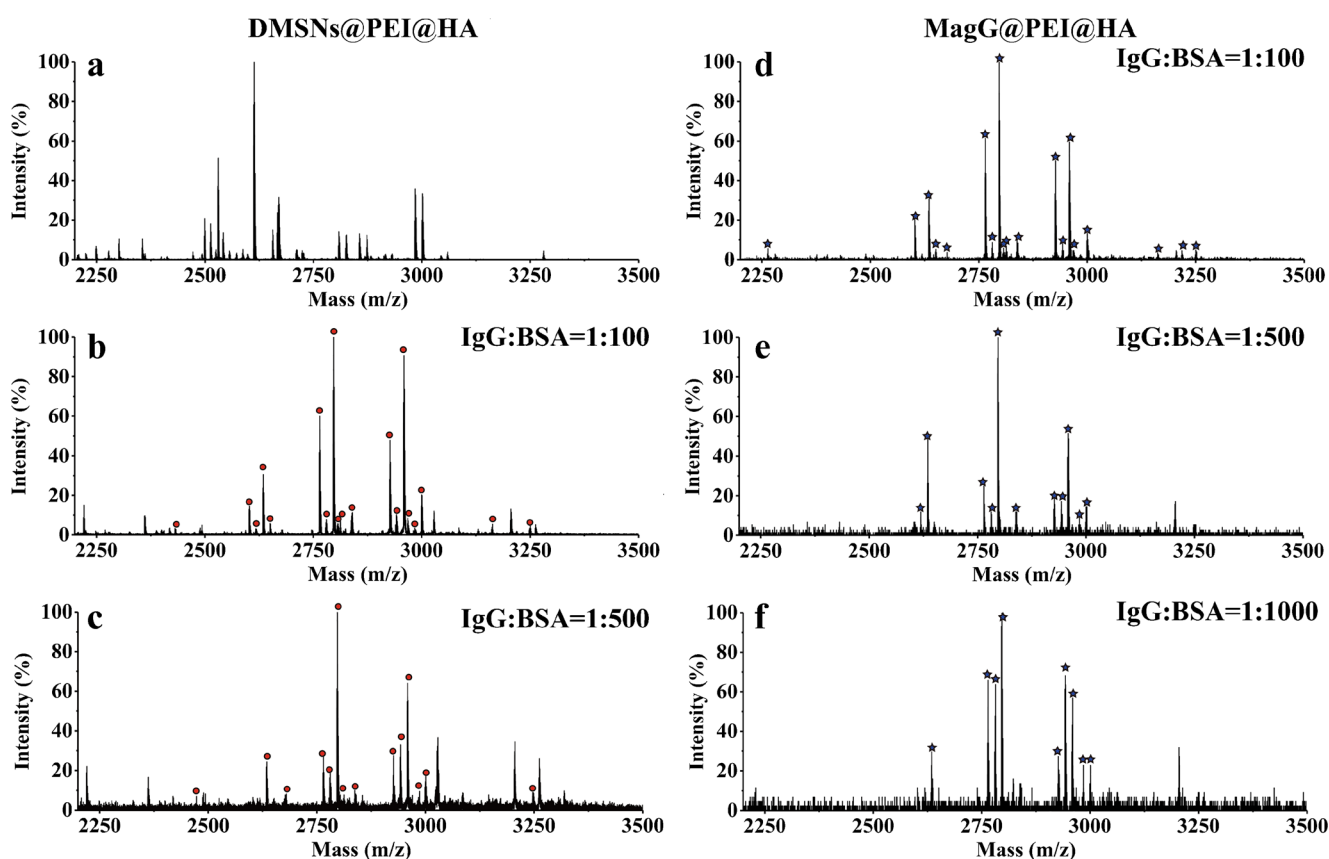


Fig. 3 MALDI-TOF mass spectra of IgG and BSA mixed digests at different molar ratios. **a** Direct analysis of the mixture at a molar ratio of 1:100; after enrichment by DMSNs@PEI@HA at molar ratios of **(b)** 1:100 and **(c)** 1:500; after enrichment by MagG@PEI@HA at molar

ratios of **(d)** 1:100, **(e)** 1:500 and **(f)** 1:1000. Glycopeptides enriched by DMSNs@PEI@HA and MagG@PEI@HA are labeled with “●” and “★”, respectively

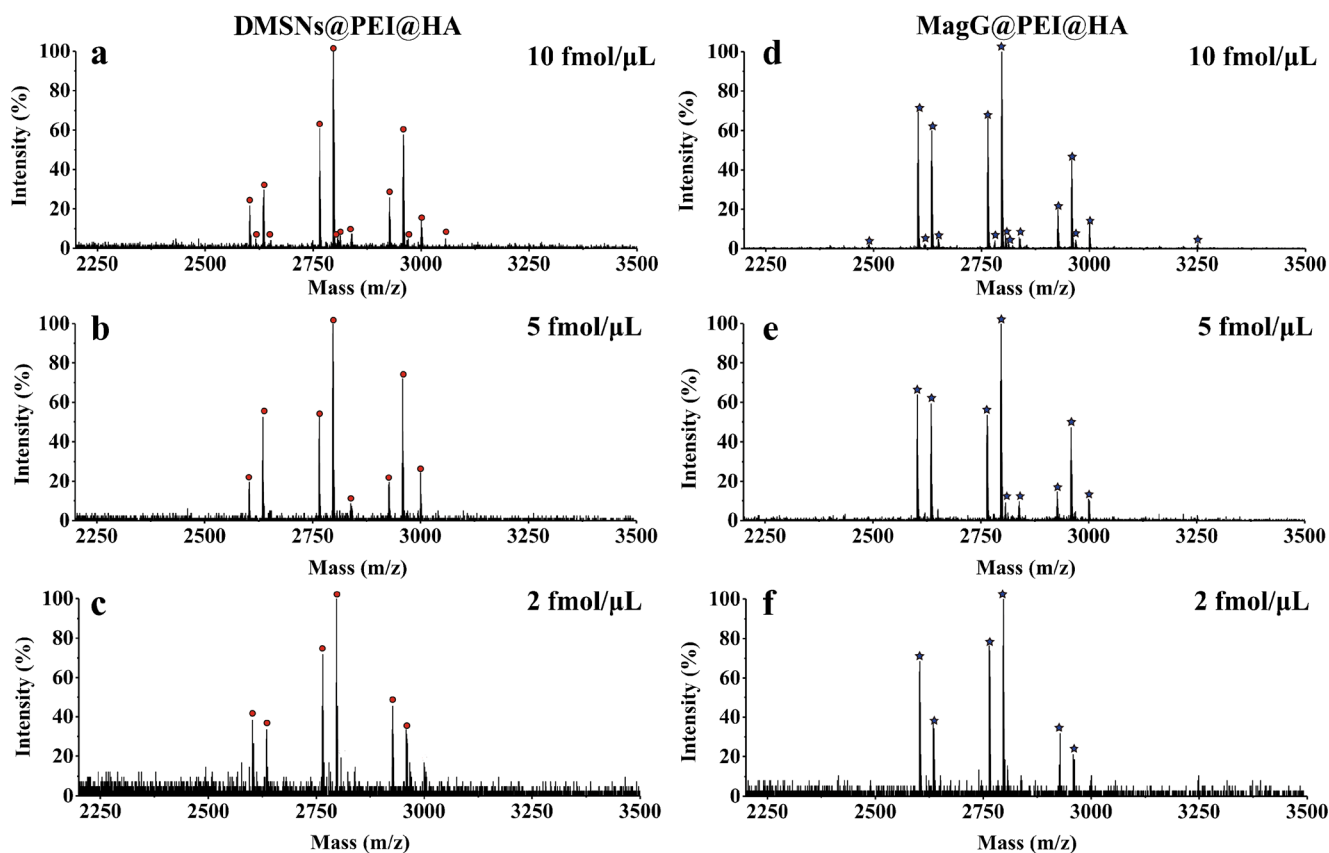


Fig. 4 MALDI-TOF mass spectra of glycopeptides enriched from IgG tryptic digest with different concentrations using DMSNs@PEI@HA and MagG@PEI@HA. Glycopeptides enriched by DMSNs@PEI@HA and MagG@PEI@HA are labeled with “●” and “★”, respectively

In addition, different concentrations of IgG tryptic digest (10 fmol/μL, 5 fmol/μL and 2 fmol/μL) were used to investigate the detection limits of DMSNs@PEI@HA and MagG@PEI@HA for glycopeptides enrichment. As shown in Fig. 4a, 14 glycopeptides is detected from 10 fmol/μL IgG tryptic digest after enrichment by DMSNs@PEI@HA. Even when the concentration of IgG tryptic digest is reduced to 2 fmol/μL (Fig. 4c), 6 glycopeptides are still observed despite significantly enhanced background noise. And the enrichment situation of MagG@PEI@HA is similar to

DMSNs@PEI@HA. When the concentration of IgG tryptic digest is as low as 2 fmol/μL, 6 glycopeptides are also shown in Fig. 4f after treatment with MagG@PEI@HA. Therefore, the detection limits of DMSNs@PEI@HA and MagG@PEI@HA are as low as 2 fmol/μL. The result means that HILIC materials prepared by the above modification strategy has good sensitivity to glycopeptides enrichment.

The enrichment capacity of the two nanocomposites was also measured by enriching the glycopeptides from a fixed

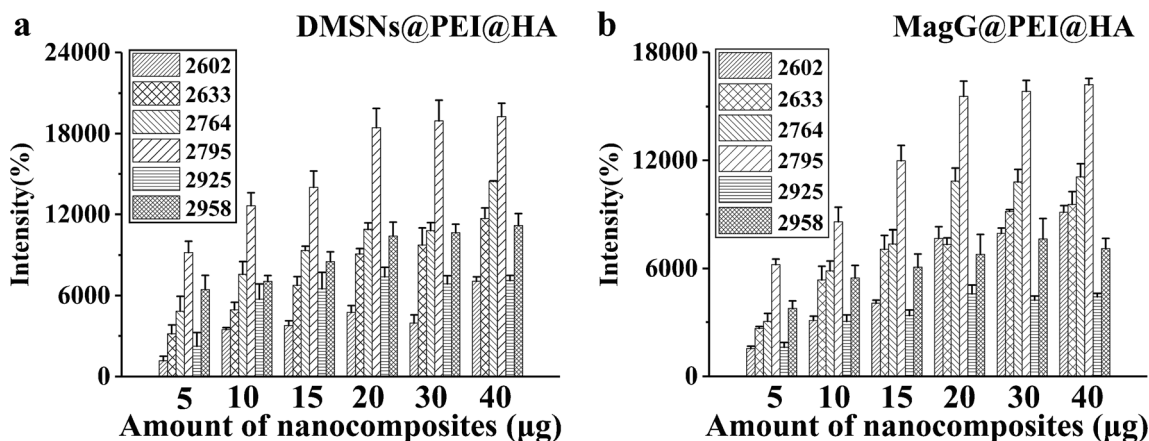


Fig. 5 Evaluation of the loading capacity of DMSNs@PEI@HA (a) and MagG@PEI@HA (b)

Table 1 An overview on recently reported nanomaterial-based glycopeptide enrichment procedure

Materials	Modification Method	Sensitivity	LODs	Recovery	capacity	NO. of identified N-glycopeptides	Ref.
Fe ₃ O ₄ – PEI – pMaltose	Click chemistry	1:100	10 fmol	84.5%	200 mg/g	17	[29]
MagHN/Au-GSH	Affinity interactions	1:100	2 fmol	89.65%	100 mg/g	21	[30]
Fe ₃ O ₄ @SiO ₂ @PMSA	Reflux-precipitation polymerization	/	0.1 fmol	73.6%	100 mg/g	27	[31]
MMP	Cross-linking polymerization	1:50	1 fmol	70.18%	400 mg/g	28	[32]
DMSNs@PEI@HA	Electrostatic interaction	1:500	2 fmol	90.87%	300 mg/g	36	Our work
MagG@PEI@HA	Electrostatic interaction	1:1000	2 fmol	98.25%	300 mg/g	33	Our work

amount of IgG trypsin digest (6 µg) using different amounts of materials (5 µg, 10 µg, 15 µg, 20 µg, 30 µg and 40 µg). As shown in Fig. 5, when the amount of materials is gradually increased from 5 µg to 20 µg, the signal intensity of the 6 selected glycopeptides is also increased. The signal intensity reaches a maximum at 20 µg and then remains constant. This indicates that 20 µg of material can capture all glycopeptides in 6 µg of IgG trypsin digest. Therefore, the enrichment capacity of both DMSNs@PEI@HA and MagG@PEI@HA is calculated to be about 300 mg g⁻¹. Furthermore, IgG tryptic digest (3 µg) is used to measure the enrichment recovery of DMSNs@PEI@HA and MagG@PEI@HA toward glycopeptides by using stable isotopic dimethyl labeling technology (Fig. S6). The enrichment recovery is reflected by the MS intensity ratio of heavy (deuterium, D) to light (hydrogen, H) labelled peptides. Table S2 lists the calculation results of enrichment recovery of DMSNs@PEI@HA and MagG@PEI@HA, showing that the recovery for glycopeptides enrichment exceeds 90% (Table 1).

Identification of N-glycopeptides from human serum

Encouraged by the excellent enrichment performance of the two nanocomposites, Peptides in human serum containing abundant glycoprotein biomarkers is used as real sample to demonstrate the utility of the two materials. 2 µL of Healthy human serum was digested with trypsin and then enriched by 400 µg of material. The eluted N-glycopeptides were deglycosylated by using PNGase F and then analyzed by nano LC-MS/MS. The identified deamidation occurs in asparagines residues (mass increment, 0.9858 Da) that conforms to the N-glycosylation consensus sequence (n-! Proline-[S/T/C]) is considered as glycosylation sites. A total of 419 N-glycopeptides derived from 105 glycoproteins and 376 N-glycopeptides derived from 102 glycoproteins are identified from only 2 µL of human serum after enrichment with DMSNs@PEI@HA and MagG@PEI@HA, respectively (Table S3 and S4 for detailed information). The enrichment efficiency of the two nanocomposites is higher than those of previous publications, such as

MIL-101(Cr)-NH₂ (10 µL human serum: 116 N-glycopeptides, 42 glycoproteins) [33], Fe₃O₄@G₆P (2 µL human serum: 243 N-glycopeptides, 92 glycoproteins) [34] and UiO-66-COOH (2 µL human serum: 255 N-glycopeptides, 93 glycoproteins) [35]. These enrichment results indicate that the DMSNs@PEI@HA and MagG@PEI@HA can be used to capture N-glycopeptides from real biological samples with high efficiency.

Conclusions

A universal modification strategy was developed for the synthesis of HILIC materials used for highly efficient enrichment of glycopeptides. Based on the electrostatic self-assembly technique, PEI with positive charge and HA with negative charge were sequentially modified on the negatively charged substrates (DMSNs and MagG). This modification strategy was carried out at room temperature and was suitable for surface functionalization of most negatively charged substrates. PEI with a high density of amino groups and HA with numerous hydroxyl groups endowed the materials with excellent hydrophilicity. Therefore, both DMSNs@PEI@HA and MagG@PEI@HA as HILIC materials possessed outstanding performances for glycopeptides enrichment, such as high enrichment selectivity, sensitivity, capacity and recovery. In addition, DMSNs@PEI@HA and MagG@PEI@HA also exhibited high efficiency in the enrichment of glycopeptides from human serum. All enrichment results indicate the feasibility of this facile and universal modification strategy in glycopeptides enrichment, which have high application potential in surface modification of materials in N-glycoproteome research.

Acknowledgements This work was supported by Natural Science Foundation of Shanghai (No. 19ZR1412000) and the Fundamental Research Funds for the Central Universities (No. 50321101917022).

Compliance with ethical standards The author(s) declare that they have no competing interests.

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