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ORIGINAL PAPER

Iminodiacetic acid (IDA)-generated mesoporous nanopolymer: a template to relate surface area, hydrophilicity, and glycopeptides enrichment

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Abstract

A three-step strategy is introduced to develop inherent iminodiacetic (IDA)-functionalized nanopolymer. SEM micrographs show homogenous spherical beads with a particle size of 500 nm. Further modification to COOH-functionalized 1,2-epoxy-5-hexene/DVB mesoporous nanopolymer enriches glycopeptides via hydrophilic interactions followed by their MS determination. Significantly high BET surface area 433.4336 $m^2\ g^{-1}$ contributes to the improved surface hydrophilicity which is also shown by high concentration of ionizable carboxylic acids, 14.59 ± 0.25 mmol g^{-1}. Measured surface area is the highest among DVB-based polymers and in general much higher in comparison to the previously reported BET surface areas of co-polymers, terpolymers, MOFs, and graphene-based composites. Thirty-one, 19, and 16 N-glycopeptides are enriched/identified by nanopolymer beads from tryptic digests of immunoglobulin G, horseradish peroxidase, and chicken avidin, respectively, without additional desalting steps. Material exhibits high selectivity (1:400 IgG:BSA), sensitivity (down to 0.1 fmol), regeneration ability up to three cycles, and batch-to-batch reproducibility (RSD > 1%). Furthermore, from 1 μL of digested human serum, 343 N-glycopeptide characteristics of 134 glycoproteins including 30 FDA-approved serum biomarkers are identified via nano-LC–MS/MS. The developed strategy to self-generate IDA on polymeric surface with improved surface area, porosity, and ordered morphology is insignia of its potential as chromatographic tool contributing to future developments in large-scale biomedical glycoproteomics studies.

Keywords Mesoporous polymer · Zwitterion · HILIC · Glycopeptides · MALDI-MS

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Introduction

Mass spectrometry (MS) is the primary tool for glycoproteomics because of its capacity to analyze isoforms related to disease, high sensitivity, and throughput [1]. However, high complexity of real samples (tissue and body fluids), heterogeneity of glycoforms, and ion suppression because of non-glycosylated peptides [2] limit the application of MS to endorse research at clinical levels. Therefore, enrichment of glycopeptides is formidable prior to MS analysis.

Popular enrichment methodologies developed in glycoproteomics include lectin affinity chromatography (LAC) [3], hydrazide chemistry (HC) [4], boronic acid-based affinity chromatography [5], and hydrophilic interaction liquid chromatography (HILIC) [6]. HILIC is simple, reproducible, selective [7], and compatible with MS [8]. HILIC-based strategies remove non-glycosylated peptides and salts in a single step. In HILIC, glycopeptides bind with the stable

hydrophilic layer created on the surface of material, while non-glycosylated peptides and salts are retained in acetonitrile-based loading buffer and washed off during the washing step [9]. The HILIC retention mechanism is mainly based on the hydrophilic partitioning of analyte to the enriched superficial water layer surrounding the surface of polar phase [11]. Ionic interaction and hydrogen bonding may also be involved in the separation depending on the sample properties and the character of HILIC phase [10]. Various HILIC phases have been fabricated for glycopeptides enrichment which include polymeric magnetic nanoparticles [11], silica [12], metal organic frameworks [13], graphene oxide [14], soluble polymers [15], and sepharose [16] with terminal hydrophilic groups such as amino acids [17], carbohydrates [18], amides [19], hydroxyls [20], amines [14], dipeptides [21], and sulphonic acid [7].

Polymeric microspheres are being used in gas storage, chemical separations, sorptions, solid supports as catalysts, and chromatography [22] because of their resistance to conformational changes by pH, organic solvents, and heat [23]. Polymers containing oxirane group provide ease of modification with reagents to introduce amine, thiol, amide, and hydroxyl groups in HILIC media. Currently reported HILIC sorbents have limited number of surface functionalities, and the development of versatile synthetic routes to generate hydrophilicity on specific support material is in demand.

In the present study, co-polymer (1,2-epoxy-5-hexene and divinylbenzene) with terminal oxirane group is synthesized for the first time by soap-free emulsion polymerization. High density of hydrophilic groups (-COOH, -OH) enhances hydrophilicity through facile functionalization with diethylenetriamine (DETA) and chloroacetic acid. COOH-functionalized mesoporous polymeric beads provide high surface area and porosity, superior hydrophilicity, solvent resistance, and good bio-capability for enriching N-Linked glycopeptides. Sensitivity, selectivity, reusability, and batch-to-batch reproducibility are tested using model glycoproteins (HRP, IgG, and avidin) and analyzed by MALDI-TOF–MS. Furthermore, N-glycosylation sites in N-glycopeptides of characteristic glycoproteins are identified from digested human serum.

Experimental

Reagents and chemicals

The detail of chemicals and reagents is provided in the Supporting information. Moreover, the serum samples were pooled from human donors according to standard protocols. The utilization of human serum was complied with guidelines of the ethics committee of the institute, and all participants (age above 18) gave their informed consent. Furthermore, it is confirmed that the experimental protocols to execute the described experiments were approved by the ethics committee of the institute. It is however clarified that the reported study in this work does not directly involve any in vivo animal studies. The samples were stored prior to further use.

Synthesis of co-polymer

1,2-Epoxy-5-hexene/DVB co-polymer was synthesized according to a reported method with modifications [24]. Briefly, 1,2-epoxy-5-hexene (1.5 mL) and DVB (1.0 mL) in deionized water (50 mL) were mechanically stirred for 25 min with nitrogen purging. Potassium periodate (35 mg) in deionized water (1 mL) was added drop-wise to the reaction mixture, and temperature was raised to 80 °C for 8 h. Finally, the content was cooled to room temperature; co-polymer was collected by centrifugation and washed with excess water.

Modification with diethylenetriamine (DETA)

A total of 0.1 g 1,2-epoxy-5-hexene/DVB co-polymer beads were added to DETA (10 mL). The mixture was stirred for 16 h at room temperature and 3 h at 105 °C. The reaction mixture was added to chilled cold water (100 mL) to quench the reaction. After filtration, the residue was washed with excess water followed by overnight drying in oven.

Carboxymethylation of co-polymer beads

A solution of chloroacetic acid (4.75 g) in water (20 mL) was prepared and made ice cold followed by the drop-wise addition of potassium carbonate (0.1 M, 6.25 mL). DETA-treated co-polymer beads were stirred in that solution for 24 h at room temperature and at 70 °C for 3 h. Finally, the product was washed with excess water and dried overnight at 65 °C.

Digestion of model glycoproteins and serum

The details can be found out in the Supporting information.

Preparation of sample for selectivity and sensitivity studies

For the selectivity study, range of peptide mixtures was produced using BSA digest keeping the IgG digest amount constant and applied to HILIC sorbent for enrichment. The applied ratios (w/w) were 1:1, 1:100, 1:200, 1:300, and 1:400.

To test the sensitivity of HILIC sorbent, dilutions of IgG tryptic digest were prepared using stock solution (1 nmol). All calculations were performed using solution dilution calculator provided by Sigma-Aldrich (https://www.sigmaaldrich.com). The concentrations (1000 fmol to 0.1 fmol) were used for the sensitivity study. All dilutions were prepared in loading buffer.

Enrichment of glycopeptides

Carboxylated co-polymer beads were equilibrated with loading buffer (92% v/v ACN, 1% v/v TFA). Tryptic digest (5.0 µL) was diluted with 95 µL loading buffer and incubated at room temperature with carboxylated co-polymer beads on a platform shaker at 450 rpm for 20 min. After centrifugation for 2 min at 10,000 g, supernatant was removed and washed three times with washing buffer (100 µL of 92% v/v ACN, 1% v/v TFA). Finally, glycopeptides were eluted with eluting buffer (20 µL of 30% v/v ACN, 0.1% v/v TFA). Eluted peptides with matrix were spotted onto MALDI plate for MS analysis.

Regeneration of IDA-functionalized polymeric beads

After the enrichment process, COOH-functionalized mesoporous polymeric beads were washed thrice by sonication for 1 min with TA-30 (250 µL of 30% v/v ACN and 0.1% v/v TFA), followed by once with water and dried. To start another batch of enrichment, COOH-functionalized mesoporous polymeric beads were equilibrated with loading buffer. This cycle of regeneration and re-use for enrichment was repeated 3 times.

Glycopeptides enrichment from human serum

Lyophilized tryptic digest of human serum proteins was diluted with loading buffer (500 µL of 92% v/v ACN, 1% v/v TFA), followed by the addition of 1 mg carboxylated co-polymer beads. The mixture was incubated for 20 min with shaking at room temperature. After centrifugation, supernatant was removed, and loaded beads were washed twice with washing buffer I (100 µL of 92% v/v ACN, 1% v/v TFA). Washing was performed once with washing buffer II (100 µL of 85% v/v ACN, 0.1% v/v H_3PO_4). Glycopeptides were eluted with eluting buffer (20 µL of 30% v/v ACN, 0.1% v/v TFA) at room temperature. Finally, the eluted glycopeptides were lyophilized for de-glycosylation.

De-glycosylation of serum-enriched glycopeptides

Eluted serum glycopeptides were diluted with ammonium bicarbonate (ABC; 50 µL, 50 mM). 1000 U of PNGase-F was added and incubated overnight at 37 °C to release N-glycans. De-glycosylated peptides were subjected to nano-LC–MS/MS analysis.

MS analysis

Eluted fractions of model proteins are analyzed by MALDI-MS. The detailed procedure is provided in the Supporting information. Enriched glycopeptides from serum are subjected to nano-LC–MS/MS. The applied parameters and adopted method is provided in the Supporting information.

Results and discussion

Characterization of COOH-functionalized nanopolymer beads

In HILIC, glycopeptides retain predominantly by hydrogen bonding and electrostatic interaction through dipole–dipole, ion–dipole, dipole-induced dipole, and even hydrophobic interactions between HILIC sorbent and the mobile phase [25]. Glycoproteins have sugar moieties with –OH groups which impart more hydrophilic character than simple proteins, resulting in improved HILIC enrichments. In common practice, IDA is chemically introduced on the affinity material particularly in the case of IMAC and modified further. This renders the possibility of enhanced hydrophilicity as functionalization with hydrophilic species via IDA crosslinking become dependent on the number of attached IDA groups. COOH-functionalized 1,2-epoxy-5-hexene/DVB mesoporous nanopolymer offers higher hydrophilicity due to inherent IDA groups during synthesis. Synthetic scheme of HILIC sorbent is depicted in Fig. S1.

Firstly, 1,2-epoxy-5-hexene/DVB co-polymer beads are prepared using soap-free emulsion polymerization (Fig. S1a). The oxirane groups on co-polymer are opened with diethylenetriamine (Fig. S1b) which result in the development of IDA on nanopolymer. Finally, carboxymethylation of DETA-treated polymeric beads is performed (Fig. S1c) to get COOH functionality. The numbers of COOH groups indirectly show the self-IDA generation during nanopolymer synthesis. The extent of immobilized carboxylic groups is potentiometrically determined, and ionizable carboxylic acids are 14.59 ± 0.25 mmol g^{-1}, which are much higher than previously reported.

Further characteristics of nanopolymer like morphology, porosity, and composition support the performance of affinity material in separation. SEM of 1,2-epoxy-5-hexene/DVB mesoporous nanopolymer reveals homogenous spherical particles of size around 500 nm (Fig. 1a–c).

The ordered morphology improves the chromatographic operations in terms of separation of rare target molecules,

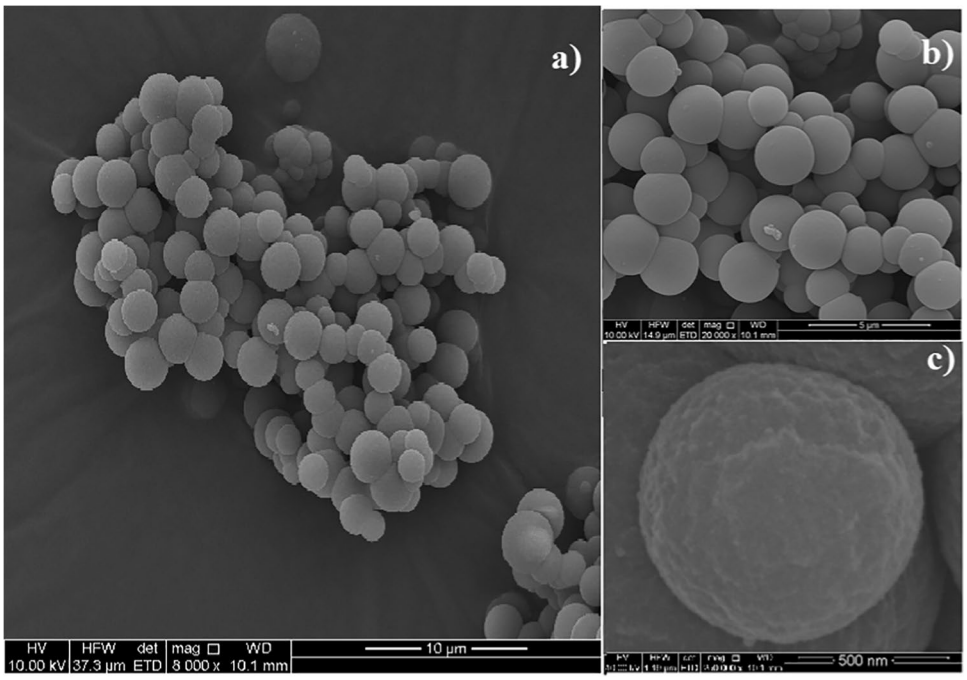

Fig. 1 SEM micrographs of COOH-functionalized 1,2-epoxy-5-hexene/DVB co-polymer at magnifications of **a** 10 µm, **b** 5 µm, and **c** 500 nm

speed of analysis, material flexibility to pH control, and ease of operation. There is no agglomeration seen which facilitates better dispersion in SPE-based enrichment. EDX elemental analysis (Fig. S2) shows carbon C as 96.8% and oxygen O as 1.9% with no impurity from reactants or by-products. Impurities and solvent residuals from polymeric beads are degassed under vacuum at 160 °C for 16 h. Surface area and hydrophilicity being contributors in N-glycopeptides enrichment are explored. Surface area is determined by nitrogen adsorption analysis at 77.33 K. BET surface area is calculated as 433.43 $m^2\ g^{-1}$ with pore size 20.27 Å and pore volume 0.22 $cm^3\ g^{-1}$. Pore size suggests mesoporous nanopolymer beads. The measured surface area is the highest in comparison to the previously reported BET surface areas of co-polymers, terpolymers, MOFs, and graphene-based composites (Table S4). Higher surface area may be attributed to the low mass density along with the adoption of soap-free polymerization which decreases co-polymer size and increases surface area, improving dispersion as well.

Co-polymer is further characterized by ^{1}H-NMR (Fig. S3). δ 7.2–7.7 ppm corresponds to phenyl double bond, while strong peak at δ 1.25 ppm relates to aliphatic hydrogen. Peak at δ 3.1–3.4 ppm indicates protons of epoxy ring that confirm oxirane groups being intact after polymerization for further immobilization.

The synthesis of co-polymer and further derivatizations are confirmed by Fourier-transform infrared (FT-IR) spectroscopy. FT-IR spectrum of 1,2-epoxy-5-hexen/DVB polymer is compared with IDA-generated 1,2-epoxy-5-hexene/DVB polymeric HILIC sorbent. The band at 2903 cm^{-1} corresponds to C-H stretching, while bands at 1254 cm^{-1} and 902 cm^{-1} belong to stretching vibrations of oxirane groups (Fig. S4a). Bands at 3442 cm^{-1} and 1742 cm^{-1} represent stretching of −OH and C=O, respectively (Fig. S4b), which indicate the generation of IDA group onto the polymer.

Enrichment of N-linked glycopeptides from model protein digests

An enrichment workflow illustrates the steps involved and complete view of developed approach (Fig. 2).

MALDI-MS spectrum is recorded for the digested IgG protein (Fig. 3a) prior to enrichment where a number of non-glycopeptides are detected. Using the Net Prediction Utility software, hydrophobicity calculations show majority of non-glycopeptides as hydrophobic (Table S1). IgG contains both hydrophilic and hydrophobic peptides and is thus a good model glycoprotein for testing the selective hydrophilic nature of synthesized polymeric beads. Also, the presence of one glycosylation site at 180, with amino acid sequence EEQF*N*STFR, makes difficult to enrich maximum isoforms of glycopeptide with structurally different glycans attached. A total of 31 glycopeptides are detected in MS spectrum recorded after enrichment (Fig. 3b).

All identified glycopeptides have hydrophobicity values less than 0.2 indicating hydrophilic nature. The zoomed mass ranges for IgG digest after enrichment are shown as in-set of Fig. S5. MS/MS of selected masses at 2603 ([Hex]3[HexNAc]4[Fuc]1, EEQF**N**STFR, 0.2155072), 2765 ([Hex]4[HexNAc]4[Fuc]1, EEQF**N**STFR, 0.2155072), 2797 ([Hex]4[HexNAc]4[Fuc]1, EEQY**N**STYR, 0.08546869), 2959 ([Hex]5[HexNAc]4[Fuc]1, EEQY**N**STYR,

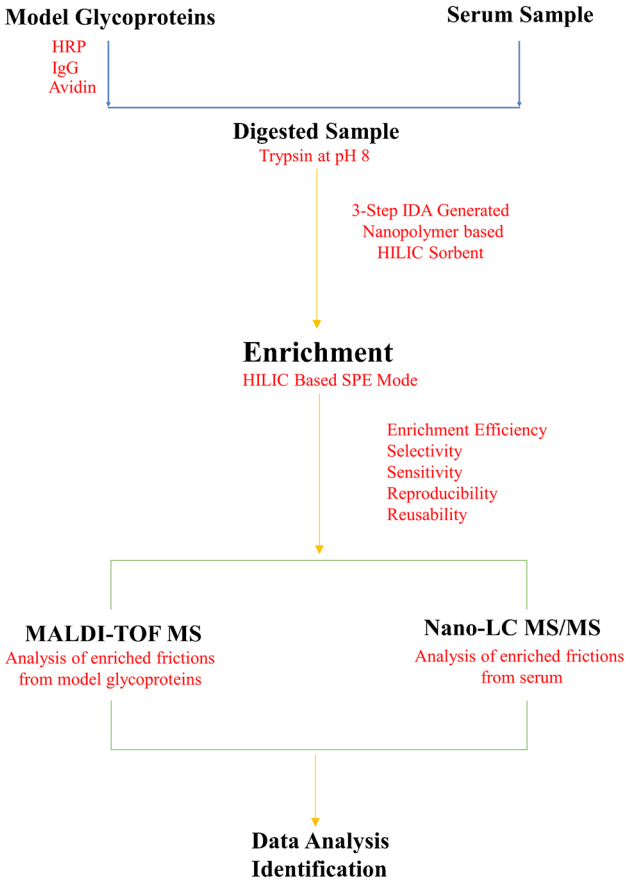

Fig. 2 Glycopeptides analysis workflow using IDA-generated mesoporous HILIC sorbent

0.08546869), and 2927 ([Hex]5[HexNAc]4[Fuc]1, EEQFNSTFR, 0.2155072) are also recorded (ESI, Fig. S6 to S10)).

MS/MS spectra of representative intact glycopeptides show dominant peak at m/z 1242 for (peptide + CHCHNHAc)$^+$ ion that corresponds to $^{0,2}X_O$ cross-ring fragmentation of ClcNAc. MS/MS spectra thus confirm the enriched peptides belonging to IgG [26].

Horseradish peroxidase (HRP, accession number P00433) glycoprotein contains eight glycosylation sites at amino acid positions 43, 87, 188, 216, 228, 244, 285, and 298. Tryptic digestion of protein constitutes a complex sample of varying hydrophobic non-glycopeptides to hydrophilic glycopeptides. Before enrichment, the detected non-glycopeptides (Fig. 4a) have hydrophobicity values in the range of 0.3 to 0.7 (Table S2) as compared to the hydrophobicity below 0.5 for enriched glycopeptides by COOH-functionalized polymeric beads (Fig. 4b). The hydrophobicity of HRP glycopeptides is higher than that of IgG glycopeptides due to presence of uncharged hydrophobic amino acids like F (phenylalanine), I (isoleucine), L (leucine), M (methionine), V (valine), W (tryptophan), A (alanine), and P (proline) in the peptide sequence.

Chicken avidin has single glycosylation at Asn-41 (WTNDLGS*N*MTIGAVNSR, 34–50). MS spectra recorded before and after enrichment show differences in detected entities (Fig. S11a and b). A total of 16 glycopeptides are detected after enrichment with COOH-functionalized polymeric beads. The detail of glycopeptides is given in Table S3.

The distribution of identified glycopeptides according to the number of missed cleavages and correct parameters increase the number of successful hits. Traditionally 0 or 1 missed cleavage is set as search parameter in data analysis where 65% of the tryptic peptides are not identified. The histogram is shown for the number of glycopeptides

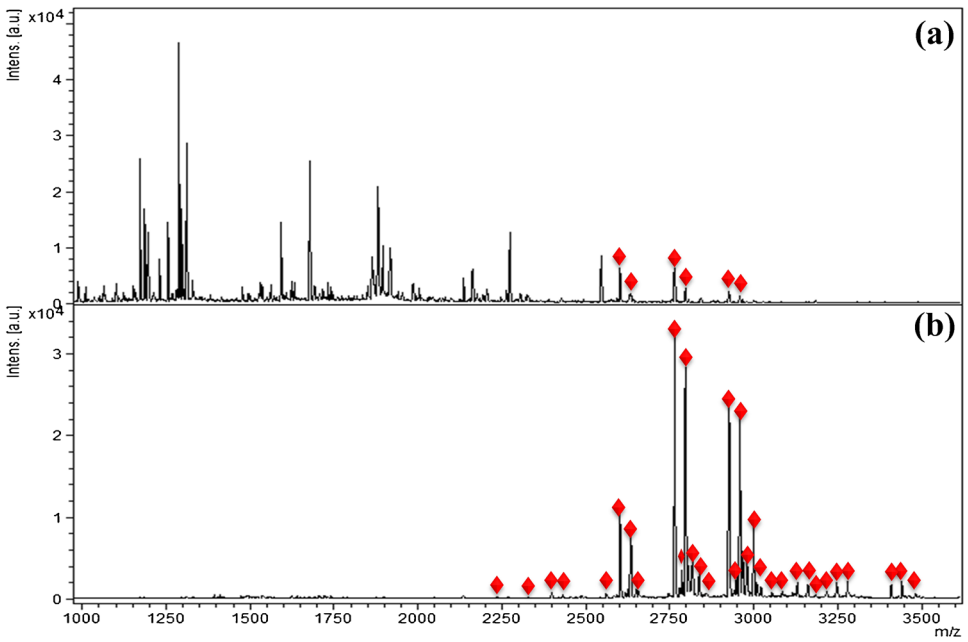

Fig. 3 MALDI-MS spectra of IgG tryptic digest (**a**) prior to enrichment and (**b**) after enrichment using COOH-functionalized 1,2-epoxy-5-hexene/DVB co-polymer. Detected glycopeptides are highlighted as red diamond. Detail of detected glycopeptides and non-glycopeptides is given in Table S1

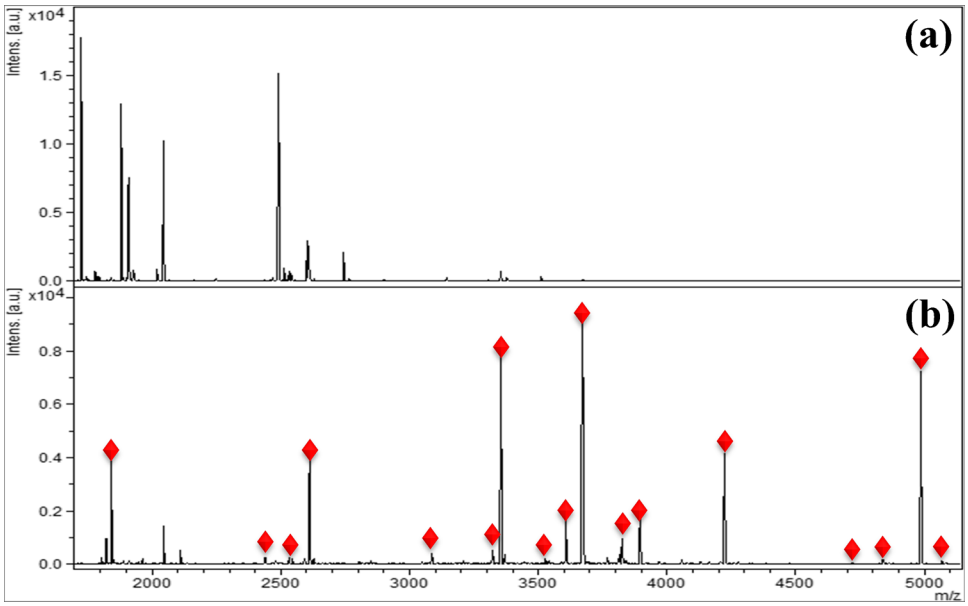

Fig. 4 MALDI-MS spectra of HRP tryptic digest (**a**) prior to enrichment and (**b**) after enrichment by COOH-functionalized 1,2-epoxy-5-hexene/DVB co-polymer. Detected glycopeptides are highlighted as red diamond. Detail of HRP-derived glycopeptides and non-glycopeptides is given in Table S2

to missed cleavage for HRP, IgG, and avidin samples (Fig. S12). The average number of glycopeptides increases with the number of enzymatic cleaving sites missed. This is advantageous for yielding longer peptides in the middle range sequencing.

Material validation

Validation parameters like selectivity, sensitivity, and intra-laboratory reproducibility (precision) are tested through enrichments by COOH-functionalized polymeric beads from IgG tryptic digest. Selectivity is termed as the ability of affinity material to differentiate analyte(s) of interest from components in the matrix or in the sample. IgG contains one glycosylation site, and most of the enriched glycopeptides are having repeated amino acid sequence but varying sugar moieties attached to asparagine which structurally differentiate each glycopeptide. IgG tryptic digest has number of non-glycopeptides present as endogenous components which can non-specifically interact with affinity material. Spiking IgG in BSA further increases background complexity. Spiked samples are prepared as 1:1, 1:100, 1:200, 1:300, and 1:400. The number of enriched IgG glycopeptides is high with relative signal intensity of $10^{\times 4}$ when BSA content is low. There is no difference observed as the ratio of BSA is increased to 1: 200 (Fig. S13a–c). In 1:300 ratio, 4 glycopeptides in the mass range of 2250–2500 Da are detected with lower intensity as shown in the in-set (Fig. S14a). Further increase in BSA reduces signal intensity to 1500, but still, the maximum number of abundant glycopeptides is detected above signal to noise ratio (Fig. S14b). The highest selectivity achieved by employing COOH-polymeric beads is 1:400.

Sensitivity is assessed based on instrument response to analyte concentration. The sensitivity of the analyte in biological sample may vary from the sensitivity assessed in standard solution. Matrix causes interference and affects the sensitivity of the developed method. MALDI-MS spectra are recorded for different concentrations of IgG digest. Ten pmol, 1 pmol, and 100 fmol concentrations show no decrease in the number of glycopeptides; however, relative signal intensity has decreased (Fig. S15a–c). Ten 10 fmol and 1 fmol concentrations show similar pattern of MS spectra (Fig. S16a and b). The lowest concentration of 0.1 fmol (100 attomole) is close to the detection limit of instrument, and thus less abundant glycopeptides below 2400 Da and above 3000 Da are not observed above the S/N ratio (Fig. S16c).

The enrichment reproducibility of COOH-functionalized polymeric beads is determined by using IgG tryptic digest (Fig. S17). Three runs of batch-to-batch reproducibility are carried out where 31 N-linked glycopeptides are observed in the first, second, and third runs. Data is subjected to statistical analysis, and relative standard deviations (RSDs) are calculated (Table S5). RSDs of all peptides are < 1 (0.000827–0.001847) showing high reproducibility of enrichment. Comparison with the reported materials regarding sensitivity, selectivity, number of glycopeptides enriched from the model, and serum digests is given in Table S6. IDA-generated mesoporous nanopolymer HILIC sorbent shows better performance in terms of all mentioned parameters.

Regeneration of COOH-polymeric beads

SPE-based affinity materials are generally difficult to re-use because of loss of affinity sites in the enrichment process of multiple buffer treatments with loading, washing, and elution pH. It is however important to re-use affinity material due to laborious SPE chemistry. COOH- functionalized polymeric beads are tested with IgG digest for their re-use in three regeneration cycles (Fig. S18a–c). The beads are regenerated using TA-30 (30% v/v ACN, 0.1% v/v TFA) followed by washing with deionized water.

Identification of serum glycoproteins

Serum glycoproteins can highlight diseases particularly cancers as the link of glycosylation has been reported with pancreatic, colon, breast, colorectal, cervical, renal, hepatocellular, prostate, gall bladder, and ovarian carcinomas (Fig. S19).

COOH-polymeric beads enrich glycopeptides from digested serum and subjected to nano-LC–MS/MS after deglycosylation with PNGase-F. The total of 332 N-glycopeptides with 347 glycosylation sites are detected (Table S7). SwissProt annotation is used with bottom-up approach to identify the glycoproteins of human serum. The identified glycoproteins are linked to cancers where 21% correspond to lung cancer and 3% to pancreatic carcinoma. About 30 of identified glycoproteins are the FDA-approved human serum biomarkers [27]. These thirty identified serum protein biomarkers are listed with their accession number in Table S8. The above results demonstrate that the new affinity sorbent can be applied to serum/plasma at the clinical level for diagnosis and treatment of diseases along with the discovery of new biomarkers for providing valuable information regarding the human glycobiology.

Conclusion

HILIC affinity based on a new polymeric material modified with carboxylic acid identifies glycoproteins/glycopeptides. High surface area promotes the attachment of carboxylic groups in high concentrations increasing the hydrophilicity of polymeric beads. Surface-generated IDA promotes the induced hydrophilicity of polymeric support making it more pertinent for hydrophilic biomolecules as glycoproteins/glycopeptides. Nanosize, homogenous spherical structure, and no agglomeration (better dispersibility) result in efficient SPE enrichments. The choice of digested standard samples, altered dilutions, and inclusion of non-specific background tests the ability of designed material for its HILIC-based enrichment. Enrichment of serum glycoproteins benefits its use for real sample application. Owing to its enhanced hydrophilicity, its further applications can be broadened to other hydrophilic-based biomolecules. In combination with the optimized enrichment protocols, COOH-functionalized polymeric beads help in the site-specific and in-depth glycoproteomics research.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00604-021-05074-8.

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Data availability Data is available via the ProteomeXchange with identifier PXD027783.

Declarations

Conflict of interests The authors declare no competing interests.

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