Analytica Chimica Acta 1245 (2023) 340862

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

New hydrophilic material based on hydrogel polymer for the selective enrichment of intact glycopeptides from serum protein digests

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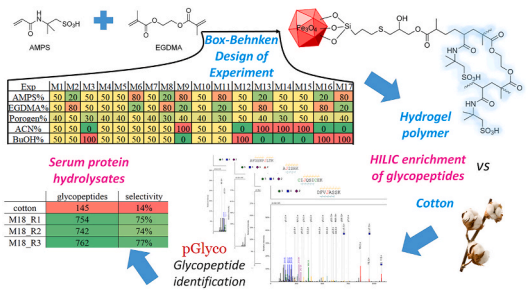
HIGHLIGHTS

- Optimized development of poly (AMPS-*co*-EGDMA) polymer by experimental design.
- Hydrogel features were tuned by experimental design to maximize the enrichment.
- AMPS percent is significant for glycopeptide enrichment and selectivity.
- Preliminary glycopeptide enrichment on fetuin digests shows great potential.
- Comparative study of HILIC material and cotton on serum shows improved performance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Prof. J. Pawliszyn

Keywords:
Glycopeptides
Enrichment
HILIC
Hydrogel
Magnetic solid-phase extraction

ABSTRACT

The paper describes the preparation and characterization of a new HILIC material for the enrichment of N-linked glycopeptides. The material was prepared using 2-acrylamido-2-methyl-1-propanesulfonic acid as the monomer and ethylene glycol dimethacrylate as the cross-linker. The material was developed by a Box-Behnken experimental design, taking into consideration the amount of monomer-to-crosslinker ratio, the composition, and the amount of porogen mixture. By this approach, the property of the resulting polymer could be fine-tuned to modulate the hydrophilicity and porosity. As HILIC enrichment is mostly dependent on hydrophilic interactions, including H-bonding, the amount of swelling was expected to have an important function, therefore the optimization considered a monomer percent in the range of 20–80%, which implied very different water swelling capacities. After assessing the potential of this new polymer family on fetuin digests, the 17 materials resulting from the Box-Behnken experimental design were used for the enrichment of glycopeptides from serum protein digests. The materials displayed a superior performance over cotton HILIC enrichment, both in terms of the number of enriched N-linked glycopeptides and selectivity, providing up to 762 N-linked glycopeptides with 77% selectivity. The optimization indicated that a high amount of monomer significantly affected the number of enriched glycopeptides, which is also closely connected with the hydrogel nature of the resulting polymers. The results not only provide one additional HILIC material for the enrichment of glycopeptides but also pave the way for the use and development of hydrogel materials for the enrichment of N-linked glycopeptides.

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https://doi.org/10.1016/j.aca.2023.340862
Received 12 July 2022; Received in revised form 17 January 2023; Accepted 18 January 2023
Available online 20 January 2023

1. Introduction

Protein glycosylation is an important post-translational modification (PTM), for which increasing evidence has proved that altered or abnormal expression of glycans on glycoproteins is closely related to many diseases, especially cancer. Indeed, glycoproteins provide more than 50% of cancer biomarkers used in clinical diagnosis [1]. Protein glycosylation is challenging from an analytical perspective. Firstly, glycosylation can be very heterogeneous because it results in a great protein diversity, which in turn greatly affects the biological outcome. Therefore, the identification of glycosylated proteins, including site and glycan structure, is of fundamental importance to understand the role of protein glycosylation in an organism [2]. Glycoproteomics is an important tool to provide structural information on protein glycosylation [3]. For many years, deglycosylation-centric glycoproteomics has been the main identification strategy for enriched glycopeptides, which were identified indirectly, by high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) and bioinformatics, thanks to the introduction of a mass tag on the glycosylation site upon deglycosylation [2]. By this approach, the connection between linked glycans and peptides is lost, but the modification site can confidently be identified along the peptide sequence. In the most recent years, developments in analytical methods, MS instrumentation, and bioinformatics software enabled the analysis of intact glycopeptides, which provide information on both the glycosylation site and glycan structure [4].

Glycopeptide analysis is generally challenging due to the low abundance and wide dynamic range of glycoproteins in most biological samples. Selective enrichment of glycopeptides is usually necessary to tackle suppression issues due to more abundant species. Nowadays, various strategies are available for the enrichment of glycopeptides, especially N-linked glycopeptides, including the very common approaches using lectin affinity chromatography, hydrazide chemistry, boronate affinity chromatography, and hydrophilic interaction liquid chromatography (HILIC) [5,6]. Novel HILIC materials, including histidine-, dipeptide-, maltose-, and amide-based ones, have great potential for enrichment of glycopeptides owing to their increased hydrophilic characteristics over non-modified peptides; as such, HILIC can successfully compete with other enrichment methods due to its remarkable advantages of no biases toward specific glycopeptides, satisfactory compatibility with mass spectrometry (MS) analysis, high throughput and low cost [3].

Despite the potential of hydrogels in the development of hydrophilic materials for the enrichment of polar analytes, among which N-linked glycopeptides can be considered, the applications of these materials are limited in the specific field of sample preparation and chromatography. They have been used to develop immunoaffinity nanomaterials [7], metal extraction systems, and sorbents for dye removal from water [8]. The use of hydrogels as stationary phases in chromatography is hindered by their swelling and resulting high osmotic pressure. Nonetheless, core-shell materials, with finely-tuned shells by modulation of the amount of cross-linker, have been recently described for the development of stationary phases for mixed-mode chromatography and HILIC [9–11]. In the specific field of glycopeptide enrichment, only one very recent report exploits this particular feature for the purpose, with good performance in serum samples [12]. In addition, one smart material, with potential hydrogel features, was described previously for enrichment by hydrazide derivatization [13].

2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) is a common monomer for the preparation of polymers and an important ionic monomer in hydrogel production, with pH-independent swelling properties due to the presence of sulfonic acid groups completely dissociated at all pH values. The copolymers of AMPS and methacrylic acid have been demonstrated to possess interesting smart features, with pH- and temperature-dependent responses depending on the relative percent of AMPS and cross-linker in the specific preparation, which in turn greatly affects the swelling of the final material [14]. In particular, when used in low percent to AMPS, ethylene glycol dimethacrylate (EGDMA) as the cross-linker increased the swelling capacity and allowed the preparation of hydrogels while at a higher percent provided strength to the polymer. The results have indicated the possibilities of developing tailor-made hydrogels based on the final application, with tuneable swelling and improved strength of the final polymers [14,15]. In the field of proteomics, AMPS monomer was used for the preparation of monolith columns for strong cation exchange capillary LC of peptides [16]. AMPS provided cation exchange properties, while EGDMA was used as a hydrophilic cross-linker.

Although glycopeptides are not enriched by strong cation exchange separation media, mesoporous silica functionalized with sulfonic acid efficiently enriched glycoproteins and glycopeptides, via hydrogen bonds and hydrophilic interactions, with high selectivity [17]. Much recently, magnetic nanoparticles functionalized with citric acid were also demonstrated suitable for glycopeptide enrichment from saliva [18]. Indeed hydrogen bonding, electrostatic interactions, and material affinity for water are significant for the enrichment and separation of glycopeptides [3].

Based on the above literature, the present work describes the development of a new hydrophilic material for the selective enrichment of glycopeptides from biological media. The material preparation was optimized by fine-tuning the monomer/cross-linker ratio and surface properties with different porogens through a Box–Behnken design (BBD) of experiments. The enrichment efficiency was considered to understand the relative influence of the chosen variables (monomer and cross-linker ratio, porogen mixture amount, and composition) and select the final preparation procedure for application to the enrichment of intact N-linked glycopeptides from serum. The resulting material had hydrogel features and represents a promising application in the development of enrichment materials for N-linked glycopeptides.

2. Materials and methods

2.1. Materials and chemicals

Glycidyl methacrylate, (3-mercaptopropyl)trimethoxysilane (MPTMS), trifluoroacetic acid (TFA), sodium hydroxide, sodium citrate, iron (III) chloride, ethylene glycol, fetuin from fetal bovine serum, cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail, ethylene glycol dimethacrylate 98%, 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), and 2,2′-azobis (2-methylpropionitrile) were provided by Merck (Darmstadt, Germany). Ethanol 96°, butan-1-ol (BuOH), and acetone were purchased from Carlo Erba reagents (Milan, Italy). LC-MS grade acetonitrile (ACN) and water were provided by VWR International (Milan, Italy). LC-MS grade dimethylsulphoxide (DMSO) was bought from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Super purity reagent ammonium hydroxide solution was purchased from Romil (Cambridge, UK). Mass spectrometry grade trypsin was provided by Promega (Madison, WI, USA).

2.2. Preparation of AMPS polymer materials

The glycopeptide enrichment material was prepared by a multistep procedure, as described in Fig. 1.

2.2.1. Synthesis of magnetic nanoparticles

Magnetic nanoparticles were prepared by the solvothermal method, as previously described [19]. Briefly, 1.95 g $FeCl_3$ and 0.60 g sodium citrate were dissolved in 60 mL ethylene glycol. Then, 3.6 g sodium acetate was added and vigorously stirred for 30 min; when the solution was homogeneous, the mixture was transferred to a 100 mL autoclave and reacted for 10 h at 200 °C. When cold, the product was magnetically decanted and washed with ethanol and water. The product was dried at 60 °C.

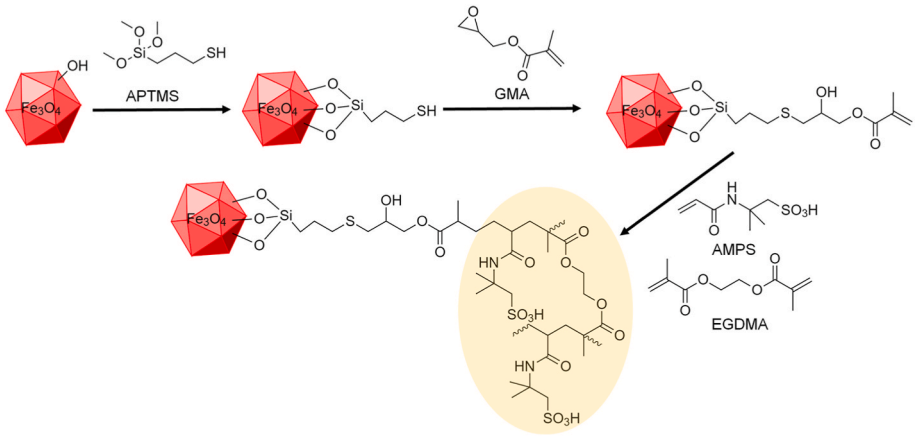

Fig. 1. Schematic depiction of the synthetic procedure for the magnetic HILIC material developed for glycopeptide enrichment.

2.2.2. Silanization of magnetic nanoparticles

The magnetic nanoparticle product was then functionalized to covalently bind the AMPS copolymers, based on a modified procedure devised from the literature that allows direct functionalization of Fe_3O_4 nanoparticles with thiol silanes [20]. (3-mercaptopropyl)trimethoxysilane (MPTMS) was used for the first reaction according to the following procedure: 100 mg of Fe_3O_4 nanoparticles were dispersed for 30 min by sonication (S 60 H Elmasonic) in 45 mL of water at pH 11 (0.001 mol L^{-1} NaOH). MPTMS (300 μL) was dissolved in 2 mL of ethanol and dropwise added to the magnetite nanoparticle suspension. The mixture was left reacting for 19 h under vortex agitation (Vortex Genie 2, Scientific Industries, Bohemia, New York, USA). The product was magnetically decanted, sequentially washed with water, ethanol, and acetone, and finally dried at 60 °C.

2.2.3. Functionalization of magnetic nanoparticles

The product (500 mg) was dispersed in 8 mL of acetone and reacted with glycidyl methacrylate (2.8 mL) under vortex shaking for 1 day [21]. The product was magnetically decanted, washed with acetone, and dried at 60 °C.

2.2.4. Preparation of polymer materials by Box-Behnken design of experiment

A BBD of experiment strategy was used for the optimization of the material preparation. The commercial software Design-Expert 13 (StatEase, Minneapolis, USA) was used to optimize the amount of reagents and solvents in the polymerization. In detail, the monomer-to-crosslinker ratio was optimized by modulating the amount of AMPS in the range of 20–80%. The reaction was carried out in DMSO with a porogen mixture having percent between 30 and 50%. ACN and BuOH were used in the porogen mixture, the composition of which was modulated by changing the percent of ACN in the range 0–100%. The design provided 17 experiments with five replicates at the center point to estimate the pure error, as summarized in Table 1. For each preparation, 30 mg of magnetic nanoparticles were added with the required amount of AMPS dissolved in DMSO; then the suitable amount of EGDMA, ACN, and BuOH was added, according to the amounts reported in Table S1. AIBN was added as 1% (w/w) on total reactants and was used to catalyze the polymerization. The reactions were carried out at 60 °C for 6 h. Then, the materials were ground and washed with water and ACN. Finally, the materials were suspended in 1 mL water and stored at 4 °C until use.

The number of identified glycopeptides and the selectivity for each M1-17 experiment were used in the BBD model to search for the preparation conditions that allowed maximizing the number of glycopeptides and the selectivity. Based on the results of the BBD analysis, the material with the final composition (indicated as M18) was finally prepared, according to the above-described general procedure and using 80% AMPS (24 mg), 50% porogen mixture (150 μL ACN) and 150 μL di DMSO as the solvent. The preparation was done in triplicate. The product was characterized by high-resolution scanning electron microscopy (SEM, Auriga Zeiss) for morphological analysis and microanalysis by Energy Dispersive X-ray Spectrometry (EDS). Further morphological characterization was done by scanning transmission electron microscopy (STEM, Nova NanoSEM 450, FEI, Thermo Fisher Scientific). The water uptake of the material was also evaluated by weight measurements, as previously described [22], according to the following equation:

$$Water\ uptake\ (\%) = \frac{w_{wet} - w_{dry}}{w_{dry}} \times 100$$

W_{wet} is the weight of the wet material, w_{dry} is the weight of the dry material.

The M18 material and related synthetic intermediate materials were analyzed by Fourier transform infrared (FT-IR) analysis using an IR spectrophotometer Nicolet iS50 coupled with a Nicolet Continuum FT-IR microscope (Thermo Scientific). Samples were analyzed using a diamond cell and transmission spectra were acquired in the range 4000–650 cm^{-1}, with a spatial resolution of 8 cm^{-1}. Forty scans were acquired before elaboration by Fourier transform. Background spectra were subtracted for each measure.

2.2.5. Preparation of protein digests

Serum was prepared as previously described from human whole

Table 1
Results of the BBD for the selection of the percent amounts of monomer (AMPS), cross-linker (EGDMA), porogen, and porogen composition (ACN and BuOH percent amounts in the porogen mixture).

Exp	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17
AMPS%	50	20	50	50	50	80	50	20	80	50	80	50	20	50	50	20	80
EGDMA%	50	80	50	50	50	20	50	80	20	50	20	50	80	50	50	80	20
Porogen%	40	50	30	40	40	50	40	30	40	40	30	50	40	30	50	40	40
ACN%	50	50	0	50	50	50	50	50	100	50	50	0	100	100	100	0	0
BuOH%	50	50	100	50	50	50	50	50	0	50	50	100	0	0	0	100	100

blood provided by the UOC Immunohematology and Transfusion Medicine (Sapienza, University of Rome) after informed consent, according to institutional bioethics. The serum was prepared as previously described [23]. Serum was added with cOmplete™ Mini, EDTA-free Protease Inhibitor Cocktail (Merck), and PhosSTOP phosphatase inhibitor cocktail (Merck). Serum protein content was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, manufacturer's protocol), and aliquoted at −80 °C until use. For the preparation of tryptic digests, 1 mg of bovine fetuin or serum proteins were digested using trypsin and purified, as previously described [24].

2.2.6. Glycopeptide enrichment procedure

The glycopeptide enrichment procedure was devised based on previous reports on sulfonic acid-functionalized mesoporous silica [17] and cotton-HILIC enrichment of glycopeptides [25,26]. Digests (100 μg fetuin or 500 μg serum protein digests) were dissolved in 500 μL of ACN/H_2O, 83:17 (*v/v*) with 0.1% TFA. The material (5 mg) was initially washed twice with 500 μL of water for 2 min on a vortex shaker, then twice with 500 μL of loading buffer for 2 min on a vortex shaker. Then, the sample was added and incubated for 30 min under slight agitation, the material was washed with 500 μL of loading buffer (2 min, three times), and the enriched peptides finally eluted with 300 μL of water under agitation for 15 min (three times). All operations were performed by magnetic decantation. Cotton-HILIC enrichments were performed for comparison by adaptation of previous reports [24] to account for the loaded protein amount. One hundred mg of commercial cotton wool was used, packed in a solid-phase extraction cartridge. Cotton was first washed with 500 μL of water (twice) and equilibrated with 500 μL of loading buffer (twice). After loading the sample, cotton wool was washed with 500 μL of loading buffer (three times), then the enriched glycopeptides were eluted with 300 μL of water (three times). All operations were done by gravity. For both procedures, the eluates were pooled, evaporated under low pressure, and finally dissolved in 100 μL of water with 0.1% formic acid for nanoHPLC-MS/MS analysis.

2.2.7. Shotgun proteomic analysis of enriched peptide samples

Peptide mixtures were analyzed by nanoHPLC-MS/MS using an Orbitrap Elite mass spectrometer (Thermo Scientific). Samples (20 μL) were online preconcentrated on a μ-precolumn (Thermo Fisher Scientific, 300 μm i. d. × 5 mm Acclaim PepMap 100C^{18}, 5 μm particle size, 100 Å pore size) at 10 μL min^{-1} flow rate of a premixed mobile phase H_2O/ACN, 98:2 (*v/v*) containing 0.1% (*v/v*) TFA. Peptide samples were separated on an EASY-Spray column (Thermo Fisher Scientific, 15 cm × 75 μm i. d. PepMap C18, 3 μm particles, 100 Å pore size) operated at 300 nL min^{-1} flow rate and 35 °C. A 97 min-long gradient was employed with H_2O and ACN as mobile phases A and B, respectively, both with 0.1% formic acid. The following linear gradient was used: 1% B for 5 min; 1–5% B in 2 min; 5–35% B in 90 min; the column was then washed at 90% B for 20 min, and equilibrated for 35 min at 1% B.

Spectra were acquired to allow the structure elucidation of both peptides and intact N-glycopeptides using Orbitrap Elite mass spectrometer MS/MS acquisition performed as previously described [26]. Each sample was run in triplicate analysis.

2.2.8. Database searching

Intact N-glycopeptides were identified using pGlyco 3.0 [4]. The software used the databases downloaded from Uniprot (Bovin, 6003 sequences, or Human, 20,435 sequences) to substitute N residues in the sequon 'N-X-S/T/C (X is not P)' into 'J'. The resulting database was used for spectra annotation. For both taxonomies, oxidation on methionine and acetyl on protein N-term were set as a variable modification; carbamidomethyl on cysteine was set as a fixed modification. Maximum 2 modifications were considered for each peptide. Digestion was set to tryptic with a maximum of 2 missed cleavages. Spectra were matched considering HCD fragmentation with a 20 ppm error on product ions and a 10 ppm error on precursor ions. The matching of glycopeptides was accepted with a false discovery rate (FDR) of 0.01. The selectivity was evaluated by identification of the co-enriched peptides matched to the same databases used for glycopeptide spectra annotation. The MaxQuant software [27] (version 1.6.3.4) with the Andromeda search engine was used for the purpose as previously described [28].

3. Results and discussion

The use of BBD of experiments has been suggested as an excellent route for the optimized preparation of polymers, with custom features [29]. To disclose the optimized polymerization conditions for preparing a polymer with the best enrichment capacity for glycopeptides, a BBD of experiments was used to evaluate the significance of three independent variables (AMPS monomer percent, porogen percent, porogen composition), on the glycopeptide enrichment performance, measured as the total number of matched glycopeptides and selectivity, for each experimental condition. In a BBD, the number of experiments "N" necessary for the evaluation of three independent variables is calculated according to the following equation:

$$N = k^2 + k + cp$$

where "k" represents the number of factors (variables, 3) involved in the study, and "cp" is the number of replicates of the central point (5). As such, 17 materials (M1-17) were prepared for optimization.

The rationale behind the BBD optimization of the polymer preparation was finding a tailored composition most suited for N-linked glycopeptides enrichment considering several factors, which were expected to possibly affect the enrichment. The amount of AMPS was the most important variable to be optimized. In fact, it was demonstrated in previous studies that AMPS polymers can have very variable water content, depending on the percent of monomer and type of cross-linker. In particular, at 100% AMPS, the swelling was largest, and it dropped to ca. 18% of the maximum value when AMPS was lowered to 20% in the polymerization feed mixture [14]. As HILIC enrichment is mostly dependent on hydrophilic interactions, including H-bonding, the amount of swelling was expected to have an important function. Therefore, the optimization would consider the effect of AMPS percent on the enrichment over a range in which the water swelling varied significantly, in turn affecting the hydrogel features and the binding selectivity for N-linked glycopeptides. As the amount of the cross-linker EGDMA was dependent on that of AMPS, the mechanical strength of the resulting polymers was also optimized in the same study [14,15].

The use of the experimental design approach allowed considering additional factors, which potentially could affect the enrichment performance, in particular the effect of porogen additives on the solvent mixture. The porosity of the materials was expected to change, as demonstrated in previous works on monolith polymers [30].

The polymer optimization was done using a magnetic core as the substrate to develop a versatile enrichment system suitable for miniaturized procedures based on magnetic solid-phase extraction [31]. In this specific case, the core was functionalized to provide a covalent link to the outer polymers while granting protection of the magnetic core to oxidation and corrosion processes, which may decrease the material stability over time and finally decrease the enrichment capability [20,32]. To achieve this goal and reduce the number of synthesis steps, the outer thiol functionalization was achieved without the presence of the intermediate silica coating, as recently described [20].

3.1. Enrichment of glycopeptides from fetuin digests

To assess the potential of the AMPS monomer to prepare a polymer family suitable for the enrichment of glycopeptides, one of the 17 materials was initially tested and compared to cotton-HILIC for enrichment capability and selectivity. Material M14 was randomly chosen from the 17 materials and allowed enrichment of 84 unique glycopeptides

(corresponding to 11 peptide sequences, 62 linked-glycans, and 10 glycosylations sites of which 7 were from minor fetal bovine serum proteins other than fetuin, Table S2). By comparison, the cotton material allowed the identification of 13 unique glycopeptides (corresponding to 3 peptide sequences, 9 linked glycans, and 3 glycosylation sites on fetuin, Table S3). No other glycoproteins were detected in these experiments. From these results, the material provided better performance, with a very high affinity for N-linked glycopeptides, as demonstrated by the enrichment of trace amounts of glycoproteins. The selectivity was evaluated by the identification of the co-enriched peptides. The results indicated that material M14 had a very high selectivity, with only 63 co-enriched peptides matched to 23 proteins (Table S4). They were common with the cotton-HILIC experiments, which in turn provided 380 peptides matched to 57 proteins. Label-free analysis of the matched peptides indicated that the areas associated with the glycopeptides were comparable in the two enrichment systems. The co-enriched peptides were more abundant than the glycopeptides for both materials, with the areas being ca. twice that of the enriched glycopeptides in cotton results and ca. four times that of the enriched glycopeptides in the M14 material (Fig. 2).

The discrepancy between areas and the number of matched co-enriched peptides in the HILIC material can be attributed to the non-optimized sorbent amount, suitable for non-specific adsorption. The hydrophobicity of the co-enriched peptides was evaluated using the grand average of hydropathy (GRAVY) index calculated by the ProPAS freeware [33] and showed that the profiles were similar for both materials and the non-glycopeptides were hydrophilic (GRAVY <0 for 73% and 87% of co-enriched peptides for cotton and M14 materials, respectively).

The intermediate materials without polymer coating were also tested to clearly understand any contribution to the enrichment or selectivity for glycopeptide analysis. The bare magnetic nanoparticles and the sequentially functionalized magnetite with MPTMS and GMA were used in enrichment experiments on fetuin protein digests. These experiments provided a low number of glycopeptide identifications, i.e., 11 matched glycopeptides for magnetic nanoparticles, 4 for MPTMS functionalized material and 7 for the GMA functionalized material (Tables S5–7). The selectivity was low for the bare magnetic nanoparticles, with 289 matched peptides, while the functionalized materials displayed low binding of peptides in general, with only 12 matched peptides for the MPTMS derivative and 48 for the GMA functionalized material (Table S8). The label-free quantitative analysis of these materials further supported the limited binding of peptides, especially for the MPTMS and GMA functionalized preparation steps (Fig. 2). These results support a significant improvement in glycopeptide enrichment associated with the polymer functionalization with little contribution from the other components used for the preparation of the composite material with hydrogel functionalization.

The recovery of glycopeptides from the fetuin digests was estimated by comparing the areas between non-enriched fetuin digests and M14 samples of selected *m/z* values matched to glycopeptides in both M14 and cotton enrichment samples. The selected 8 features (*m/z* at 987.159, 1019.18, 1358.58, 1110.47, 1151.22, 1183.24, 1256.01, 1176.10) were detected in the non-enriched samples, and only three of them were fragmented (*m/z* at 987.159, 1019.18, 1358.56). The recovery for the latter was 100–175%. In all other cases, an excessive matrix effect was observed, due to suppression in the non-enriched samples, where 637 non-glycopeptides were matched. The observed suppression confirms the existent literature [34]. The reproducibility of the enrichment was estimated as relative standard deviation in triple experimental replicate and was 1–24%. The results further support the potential of the AMPS hydrogel family to enrich glycopeptides.

3.2. Enrichment of glycopeptides from serum digests

The 17 materials and cotton were used to enrich glycopeptides from serum protein digests in a comparative study where the number of glycopeptides, co-enriched peptides, and selectivity were evaluated to assess the performance of the prepared polymers and identify the most suitable preparation for the goal of glycopeptide enrichment. The identification results are collected in Table 2 and the complete lists of matched glycopeptides and co-enriched peptides are provided in the Supplementary Information (Table S9 displays the results of glycopeptide identification from cotton experiments, Tables S10–S26 display the results of glycopeptide identifications from M1-M17 experiments). The chosen monomer was effective in enriching glycopeptides in most preparations, providing 103–643 matched glycopeptides vs 145 of the cotton material. The selectivity was evaluated by the identification of the co-enriched peptides (Table S27). The materials provided a variable number of co-enriched peptides, in the range 87–457 vs 928 of the cotton material. The related selectivity was in the range of 32–78% for the 17 materials vs 14% for the cotton material. From these results, a superior affinity for N-liked glycopeptides was assessed in the screening experiments.

A label-free relative quantitative analysis, based on peak areas, was also done to assess the difference in selectivity among the cotton HILIC procedure and the 17 materials. The label-free quantitative analysis also indicated that the cotton material had a larger recovery of co-enriched peptides, whose average intensity was significantly larger than that for the 17 materials. Peak intensities were ca. 10-fold more intense in the

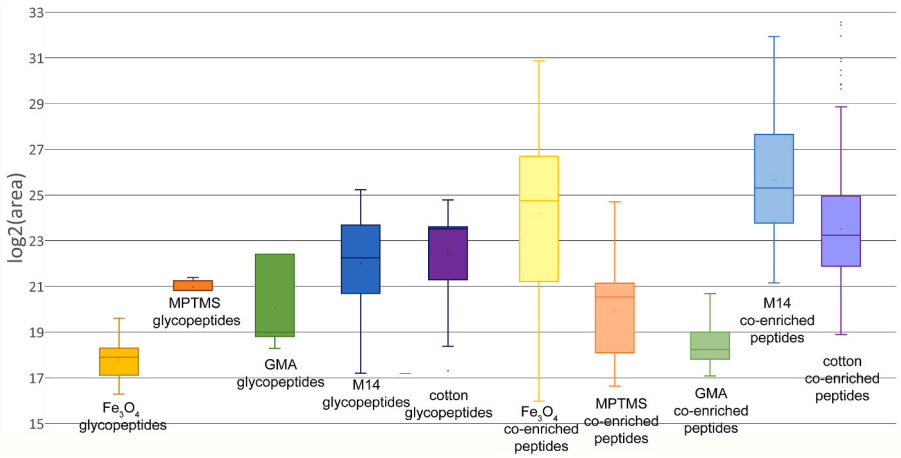

Fig. 2. Label-free relative quantification of glycopeptides and co-enriched peptides in fetuin digests using material M14, its three preparation steps (i.e., bare Fe_3O_4 nanoparticles, MTPTMS functionalized material, and GMA functionalized material), and cotton for comparison.

Table 2
Results of serum glycopeptide enrichment experiments for the M1-17 materials of the BBD and the reference cotton material.

	glycopeptides	co-enriched peptides	selectivity	Unique peptide sequences	Unique linked-glycans	Unique glycoproteins	Unique glycosylation sites
cotton	145	928	14%	22	23	16	55
M1	516	243	68%	71	48	51	182
M2	391	208	65%	136	47	97	331
M3	214	457	32%	80	27	67	160
M4	603	411	59%	66	32	53	191
M5	508	336	60%	66	33	52	140
M6	637	372	63%	69	37	46	168
M7	454	235	66%	59	27	44	120
M8	251	394	39%	44	22	32	93
M9	516	145	78%	126	62	90	361
M10	643	218	75%	76	30	54	166
M11	612	408	60%	56	30	44	138
M12	365	451	45%	46	18	42	88
M13	311	130	71%	53	21	40	93
M14	532	348	61%	56	15	47	105
M15	602	177	77%	63	27	49	136
M16	103	87	54%	18	7	17	25
M17	580	225	72%	60	33	45	133

cotton-HILIC enrichment than in the 17 materials (Fig. 3).

The use of a much more complex sample than fetuin indicated that the co-enrichment of non-glycosylated peptides occurred when there was material still available for interaction.

Despite very different numbers of co-enriched peptides being identified under the tested conditions, the physicochemical features of co-enriched peptides were shared among the 17 materials and cotton as well. The GRAVY index value, isoelectric point, and molecular weight (MW) associated with the co-enriched were calculated using the ProPAS freeware [33]. The GRAVY index value profile was similar in both all materials and cotton enrichment experiments (Fig. S1), with ca. 70% of the co-enriched peptides being hydrophilic (ca. 70%). This result could be justified by the enrichment conditions, where water is used in a low amount in the loading buffer, therefore the partitioning of polar peptides favors the water-rich materials rather than the ACN-rich loading buffer. The MW was found also similar in all materials, and ca. 70% of the co-enriched peptides were <2000 (Fig. S2). No significant differences were observed for the pI as well, as ca. 80% had a pI $\leq$ 7 for all the tested experimental conditions (Fig. S3).

3.3. Application of the Box-Behnken design of experiment and selection of the final polymer composition

For the BBD of experiments, three factors were considered as significantly affecting the enrichment process, i.e. the number of identified glycopeptides and the selectivity (calculated as the total number of glycopeptides divided by the total number of peptides); the variables selected for optimization were the percent of monomer in the polymerization mixture (AMPS in the range 20–80%), porogen amount in the solvent mixture (30–50%), and the percent of ACN in the porogen mixture (0–100%). The BBD resulted in 17 experiments, with the central point repeated 5 times. The ANOVA test results showed the best fit with the linear models for both the glycopeptide and selectivity values. From the fitting, the linear model indicated that the AMPS percent was a significant variable to maximize the enrichment of glycopeptides (Table S28), but it did not affect the selectivity (Table S29). The result possibly indicated that the hydrophilic interaction is significant in the binding of glycopeptides but does not affect the binding of the co-enriched peptides under the tested experimental conditions. ACN percent was also found significant for both the enrichment of glycopeptides and selectivity. The porogen percent was found to be significant for the selectivity, which could hint at the fact that it can affect the pore structure of hydrogels. Butanol as the porogen did not provide better results than ACN, therefore a size exclusion mechanism can be excluded as significant in the enrichment, and the surface area can be considered much more important.

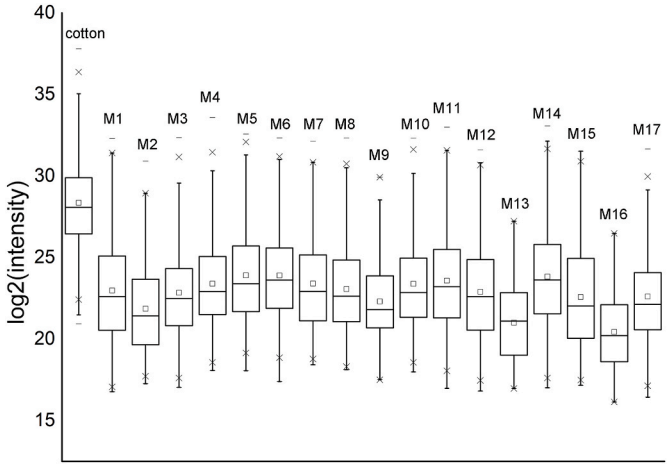

Fig. 3. Box-plot of the log2 (intensity) values for the co-enriched peptides identified in M1-M17 and cotton HILIC enrichment experiments of serum protein digests.

The result of the BBD analysis is graphically displayed in Fig. 4. The suggested results were 76.599% AMPS, 49.293% porogen, 52.045% ACN for the maximization of the glycopeptide identifications, and 80.000% AMPS, 50.000% porogen, 99.999% ACN for maximization of the selectivity. The two solutions are very similar in terms of material preparation, and the final conditions with 80% AMPS, 50% porogen, and 100% ACN was chosen for further tests.

Composite materials with the final polymer preparation composition (M18) were finally tested for comparison (Table 3). The results, from three experimental replicates, provided 742–762 glycopeptides with a selectivity of 74–77%, which demonstrates that the material can be prepared with good reproducibility (Tables S30–33).

In addition, the performance was superior than that of cotton enrichment (Table 2), not only for the number of matched glycopeptides and selectivity but also for the number of unique linked glycans, glycosylation sites, and glycoproteins identified by the final polymer composition.

The use of hydrogels for the enrichment of intact glycopeptides is, surprisingly, not common despite the potential. To the best of our knowledge, only one hydrogel material with this feature was recently described during the writing of this work and applied to serum [12]. The material had a very good performance, identifying up to 283 intact glycopeptides from 95 glycoproteins in serum samples, which was however lower than the results from our optimized material. The results

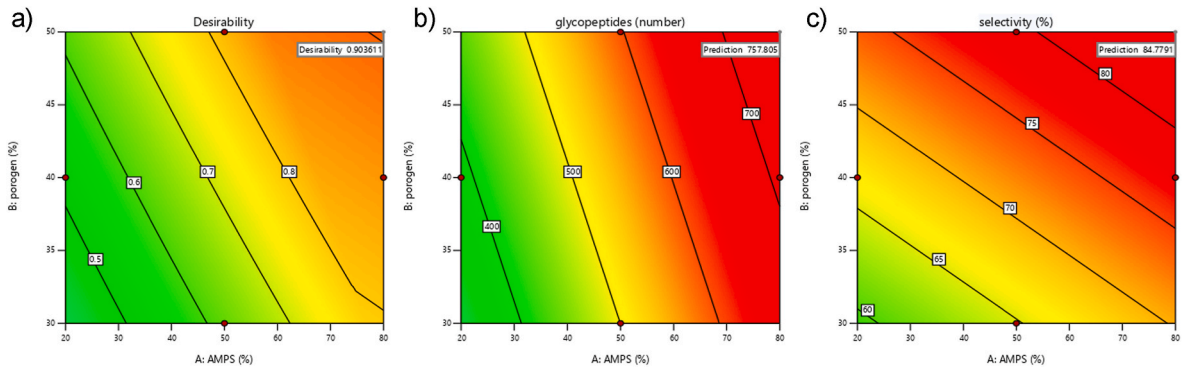

Fig. 4. Contour plots resulting from the BBD of experiments and describing the trends of the desirability (a), the number of glycopeptides (b), and the selectivity (c) based on the percentages of porogen (30–50%) and AMPS (20–80%).

Table 3
Results of serum glycopeptide enrichment experiments for the final material (M18).

	glycopeptides	co-enriched peptides	selectivity	Unique peptide sequences	Unique linked-glycans	Unique glycoproteins	Unique glycosylation sites
M18_R1	754	252	75%	137	39	96	342
M18_R2	742	255	74%	146	69	103	354
M18_R3	762	228	77%	151	51	104	337

clearly demonstrate the potential of hydrogels for the enrichment of N-linked intact glycopeptides and their potential for the development of new HILIC materials.

3.4. Characterization of M18 and stability test

The best-performing material, M18, was further characterized to establish the morphology and stability over time. SEM analysis was carried out to assess the morphology at the different preparation steps, while EDS was used to shed light on the surface composition. Magnetic nanoparticles were spherical aggregates of smaller particles (ca. 8 nm) with diameters within 100–400 nm (Fig. S4a). The elemental composition showed ca. 55% Fe and 37% O (Fig. S5). In the next preparation step, the dimension was not much affected due to silanization, but the presence of individual magnetite nanoparticles was less visible (Fig. S4b). The EDS analysis showed a composition of 59% O, 32% Fe, 7% C, 0.6% Si, 0.4% S (Fig. S6). The surface functionalization with GMA also did not significantly affect the morphology of the nanoparticle material (Fig. S4c). The composition was 46% O, 28% C, 18% Fe, 5% Si, 2% S (Fig. S7). The final modification with the polymer material induced an increase to ca. 200–600 nm of the spherical material, with additional lamellar amorphous material (Fig. S4d). The composition was 48% O, 29% C, 22% Fe, 1% N, 0.6% S, 0.3% Si (Fig. S8).

Further characterization was done by STEM analysis and comparison between transmission images and scanning transmission electron microscopy high-angle annular dark-field imaging, by means of which the magnetic core is clearly visible inside the composite materials (Fig. 5).

The successful preparation of the final M18 material and the related intermediate preparation steps was further studied by FT-IR analysis. Magnetic nanoparticles (6, red line) displayed the expected profile [19]. The bands at 1640 and 1370 cm^{-1} were associated with the carboxylate group. The bands at 1060 and 3400 cm^{-1} were ascribed to the *C*–H vibrating and *O*–H vibrating, respectively. Functionalized magnetic nanoparticles with MPTMS (Fig. 6, green line) FT-IR spectra also agreed with previous data [20]. The peaks at ca. 3000 cm^{-1} were related to *C*–H stretching. The strong peaks at 1100 and 800 cm^{-1} were associated with

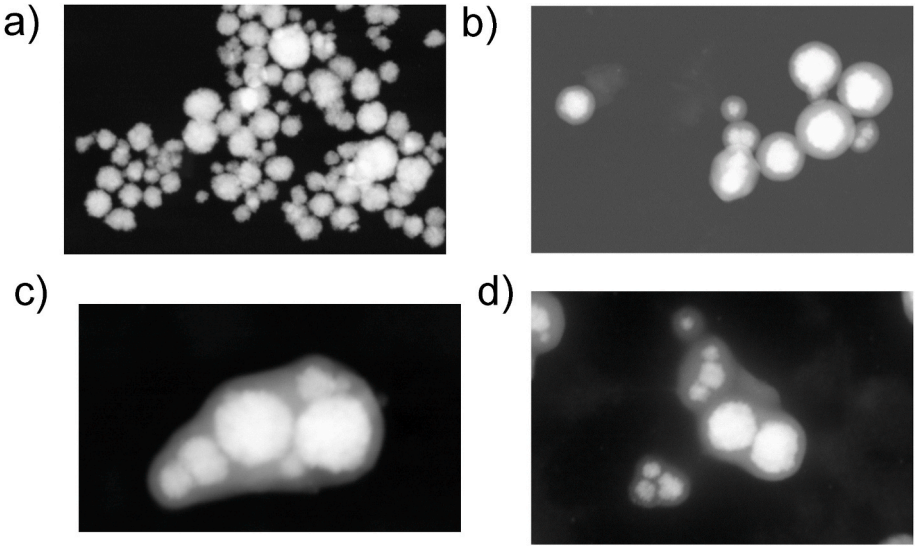

Fig. 5. STEM analysis of the material at different preparation steps: a) magnetic nanoparticles; b) silanization; c) GMA; d) final product.

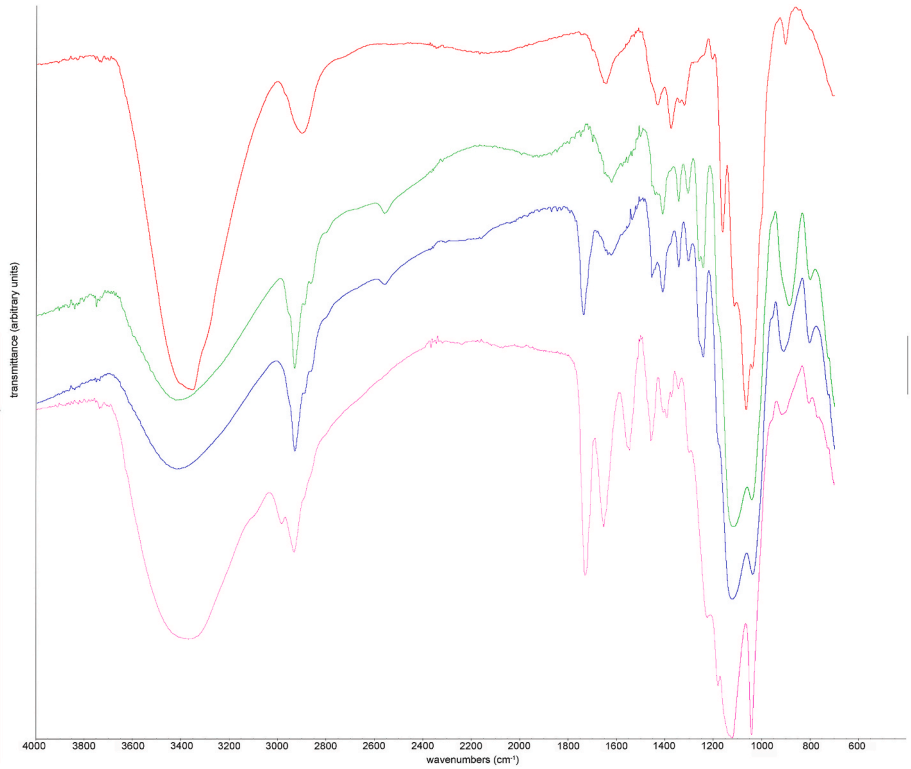

Fig. 6. FT-IR transmission spectra of M18 (pink line) and previous synthesis steps, i.e. bare magnetic nanoparticles (red), magnetic nanoparticles functionalized with MPTMS (green), and further derivatized with GMA (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the *Si*–*O*–Si and *Si*–O stretching vibrations, respectively, which confirmed the covalent bond to the bare magnetic nanoparticles. The typical peak of *S*–H was weak but visible at ca. 2550 cm^{-1}. The next functionalization step with GMA (Fig. 6, blue line) introduced a strong signal at 1700 cm^{-1}, associated with the carbonyl ester of GMA. Finally, M18 final material displayed peaks associated with the hydrogel copolymer, i.e., the bands at 1649 cm^{-1}, 1543 cm^{-1}, 1451 cm^{-1}, 1212 cm^{-1}, 1039 cm^{-1}, which were assigned to carbonyl stretching vibration of the amide group, bending of the N–H group, *C*–N stretching, $–SO_2$ asymmetric, symmetric stretching of the $–SO_2$ group of AMPS, respectively (Fig. 6, pink line). The signal at 1723 cm^{-1} was attributed to the carbonyl group of EGDMA and was merged with the one of GMA. The FT-IR spectra of the different materials confirmed the expected functionalization.

The magnetic responsiveness was assessed by dispersing the material in water and applying an external magnet for 1 min (Fig. S9), which demonstrated an excellent magnetic response.

The water uptake (%) was finally calculated, by weight measurement of the dry and wet material and resulted in 668%, indicating large hydrophilicity of the material, in turn, capable of forming hydrogels. The result agreed with the literature on similar hydrogel materials [14].

To assess the stability of the material in terms of glycopeptide enrichment performance, the enrichment was repeated one month after the preparation of the material on fetuin digests, as discussed in the preliminary experiments. The number was significantly lower, with only 6 glycopeptide identifications (Table S34), which indicates that the material must be freshly prepared for an efficient result.

4. Conclusions

The paper describes the preparation of a composite magnetic material with an outer hydrophilic shell, suitable for the enrichment of N-linked intact glycopeptides. The material preparation was optimized by BBD, to modulate the hydrophilic characteristics of the material and pore structure. Different monomer-to-cross-linker ratios and porogenic solvent mixtures were tested and applied to the enrichment of intact glycopeptides from tryptic digests of serum proteins. The final polymer composition contained 80% of the monomer, which was essential because it results in a very hydrophilic polymer with hydrogel features. The high water content in the polymer shell due to swelling was assessed by the water uptake of the final material composition and provided an excellent enrichment capability for intact glycopeptides, superior to traditional cotton-HILIC material enrichment. The result laid a promising ground for the development of cation exchange HILIC materials and hydrogels for the enrichment of glycopeptides. The water swelling feature is particularly interesting because the water-rich nature of hydrogels is well suited for interaction with very polar analytes, in the specific case of N-linked glycopeptides. The developed material represents one of the first applications of hydrogels for the enrichment of glycopeptides and provides results useful for the development of new HILIC materials with hydrogel features. The use of functionalized magnetic materials allows bypassing the limits of packed chromatographic columns and fully exploiting the hydrogel affinity for water in the extraction process.

CRediT authorship contribution statement

Andrea Cerrato: Formal analysis, Investigation. **Chiara Cavaliere:** Supervision, Writing – review & editing. **Carmela Maria Montone:** Conceptualization, Methodology, Writing – original draft. **Susy Piovesana:** Conceptualization, Supervision, Project administration, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

Acknowledgments

This work has been supported by the University of Rome "La Sapienza" with the starting grant number AR22117A868F8CAD.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.340862.

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