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Technical Note

Magnetic Synthetic Receptors for Selective Clean-Up in Protein Biomarker Quantification

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ABSTRACT: Biomarker analysis by mass spectrometry (MS) can allow for the rapid quantification of low abundant biomarkers. However, the complexity of human serum is a limiting factor in MS-based bioanalysis; therefore, novel biomarker enrichment strategies are of interest, particularly if the enrichment strategies are of low cost and are easy to use. One such strategy involves the use of molecularly imprinted polymers (MIPs) as synthetic receptors for biomarker enrichment. In the present study, a magnetic solid-phase extraction (mSPE) platform, based on magnetic MIP (mMIP) sorbents, is disclosed, for use in the MS-based quantification of proteins by the bottom-up approach. Progastrin releasing peptide (ProGRP), a low abundant and clinically sensitive biomarker for small cell lung cancer (SCLC), was used to exemplify the mSPE platform. Four different mMIPs were synthesized, and an mSPE method was developed and optimized for the extraction of low concentrations of tryptic peptides from human serum. The mSPE method enabled the selective extraction of the ProGRP signature peptide, the nonapeptide NLLGLIEAK, prior to quantification of the target via LC-MS/MS. Overall, the mSPE method demonstrated its potential as a low cost, rapid, and straightforward sample preparation method, with demonstrably strong binding, acceptable recoveries, and good compatibility with MS. mMIPs are a potential low-cost alternative to current clinical methods for biomarker analysis.
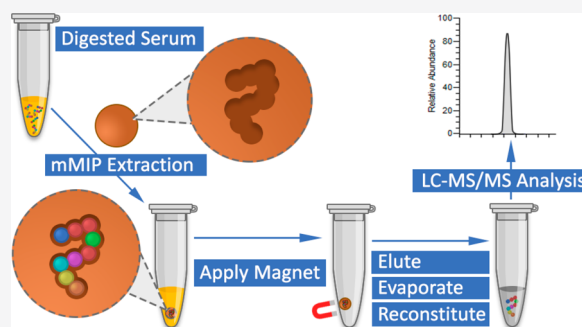
KEYWORDS: LC-MS/MS, low-abundant biomarkers, bottom-up protein analysis, magnetic capture, molecularly imprinted polymers

INTRODUCTION

The role of biomarkers in the diagnosis and management of disease is an increasingly critical aspect of clinical pathology. Therefore, it is of utmost importance that there is robust, accurate, and rapid quantitation of biomarkers, and especially for biomarkers of aggressive diseases (e.g., cancers). Many serum biomarkers can be used to diagnose malignancies without the need for invasive procedures such as biopsies of internal organs.[1]

Low abundant protein biomarkers present analytical challenges in MS-based proteomics, namely difficulty in selective enrichment and quantification due to interference from high abundant proteins and other serum components.[2] MS analysis of proteins is typically achieved using one of two approaches: top-down and bottom-up proteomics. Top-down approaches involve the analysis of whole proteins by MS, whereas bottom-up analysis involves enzymatic digestion of proteins and analysis of proteolytic peptides. The use of bottom-up workflows in tandem with enrichment methods has gained interest in recent years. LC-MS/MS analysis of signature peptides has the potential to yield high accuracy and precision, with low limits of detection (LOD) compared to top-down analysis, metrics that are essential when quantifying

low abundant biomarkers.[3] To utilize fully the quantitative potential of bottom-up LC-MS/MS, selective enrichment of the target biomarker marker is critical. Typically, antibody-based selective enrichment has been used in sample cleanup owing to the high selectivity of antibodies for targets. However, producing highly selective antibodies is expensive, laborious, complex, and time-consuming. Therefore, alternative materials with molecularly selective binding properties are desirable, and MIPs are one such alternative in this regard.

MIPs are robust, synthetic polymers designed to have unique chemical and structural properties that allow selective recognition of a desired target.[4] These properties have been exploited to allow MIPs to bind strongly and selectively to a variety of targets, from small molecules to large macromolecular targets such as proteins, and even to cells.[5] Usually, MIPs bind to targets via noncovalent forces (including

hydrogen bonding, electrostatic interactions, hydrophobic interactions, and van der Waals forces), although binding can also be through the formation of covalent bonds.[6] Thus, MIPs can be considered to be antibody-binding mimics, and are sometimes even referred to as plastic antibodies. Compared to antibody production and use, MIPs are more cost-effective, reusable, and require less complex and time-consuming synthesis, and this has led to many applications for MIPs: they have been utilized successfully as solid-phase extraction (SPE) sorbents,[7] electrochemical sensors,[8] in drug delivery,[9] for protein crystallization,[10] and for catalysis.[11] To date, MIP-based assays typically have higher detection limits compared to antibody-based immunoassay methods. However, magnetic MIPs (mMIPs) are a promising emerging format that has shown some promise for the extraction of peptides present at low levels in serum.[12]

mMIPs are MIPs with magnetic properties, and some of these materials have been developed to target and quantify peptides and proteins.[13] Typically, mMIPs can be produced in one of two distinct ways: either by encapsulation of a preformed magnetic component during a template-directed synthesis[14] or by magnetization of a MIP.[15] mMIPs allow for the simplification of off-line SPE, with the use of a magnet allowing for the circumvention of several centrifugation steps to remove the sample matrix,[16] greatly speeding up work-flow. mMIPs have been used for the extraction and top-down quantification of proteins such as bovine serum albumin (BSA),[17] lysozyme,[18] hemoglobin,[13] and RNase A.[19] However, the analysis of whole proteins (i.e., top-down proteomics) typically gives higher LODs because the MS analysis of whole proteins is less sensitive than (bottom-up) peptide analysis. MIPs targeting peptides have been shown to function well in complex matrices: An epitope imprinted MIP targeting the low abundant biomarker protein cardiac troponin I allowed enrichment of the target protein in a matrix designed to mimic human serum,[20] an epitope imprinted MIP targeting the high abundant protein HTR was found to enrich the target protein qualitatively.[21] Similarity, mMIPs have been shown to function in complex matrices: an mMIP targeting lysozyme demonstrated clear enrichment in egg white.[22] While an mMIP targeting the peptide hormones angiotensin I and II demonstrated the value of mMIPs for the enrichment and quantification of peptides using LC-MS/MS,[12] the use of mMIPs for target enrichment and cleanup in bottom-up proteomics has not yet been reported.

To demonstrate the ability of the mMIP platform to enable the determination of tryptic peptides, the small-cell lung cancer biomarker ProGRP is an appealing model because a fully validated LC-MS method has been developed for its tryptic peptides.[23] Furthermore, ProGRP is a low abundant biomarker that is known to be clinically sensitive (most patients testing positive for ProGRP are in a diseased state) and selective (most patients testing negative are not in the diseased state).[24] The signature peptide of ProGRP, NLLGLIEAK, is a very reproducibly produced tryptic peptide and has high MS sensitivity. Previously, nonmagnetic MIPs have been developed to extract NLLGLIEAK from serum using off-line MISPE[25] and online MISPE (MISPE is molecularly imprinted SPE).[26]

The aim of the current work was to develop mMIPs targeting NLLGLIEAK and to explore the potential for the selective and rapid extraction of tryptic peptides in serum. Four mMIPs were designed and synthesized, and an mSPE method was developed and optimized using increasingly complex matrices to demonstrate the clinical viability of mMIPs for the extraction of NLLGLIEAK from human serum.

■ MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile LC-MS grade (MeCN, 99.9%), methanol LC-MS grade (MeOH, 99.9%), acetic acid (AcOH, 100%), ethanol (EtOH, ≥99.5%), and dimethyl sulfoxide (DMSO, ≥98%) were purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (BioUltra, ≥99.5%) was purchased from Fluka (Milwaukee, WI, USA). Formic acid (FA, MS grade, ≥98%), divinylbenzene-80 (DVB-80, 80%), methacrylic acid (MAA, purity ≥98.0%), 1,2,2,6,6-pentamethylpiperidine (PMP, purity >99%), tetrabutylammonium hydroxide solution (TBA·OH, 1.0 M in methanol, ≤50%), hydrochloric acid (37% (w/w) in H_2O), Tween 20, sodium hydroxide (NaOH, purity ≥97%), iron(III) chloride ($FeCl_3$, purity 97%), iron(II) chloride ($FeCl_2$, purity 98%) DL-dithiothreitol (≥99.5%, DTT), iodoacetic acid (≥98%, IAA), and 28−30% ammonium hydroxide solution (NH_4OH) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-Aminoethyl methacrylamide hydrochloride (EAMA·HCl, purity ≥98%) was purchased from Polysciences Inc. (Niles, IL, USA). N-3,5-bis(Trifluoromethyl)-phenyl-N′-4-vinylphenylurea (BTPV, purity >95%) is not commercially available and was kindly donated by Dortmund University. Z-NLLGLIEA[Nle] (purity 96.58%) was purchased from LifeTein. 2,2′-Azo-bis-isobutyronitrile (AIBN, purity 98%) was purchased from BDH Lab. Supplies (Dubai, UAE). Water was filtered through a Merck Millipore Milli-Q Integral 3 water dispenser (resistivity: 18.2 MΩ cm^{-1}).

Preparation of Reagents, Proteins, and Peptides. DVB-80 was purified by filtration through a short plug of neutral aluminum oxide prior to use. AIBN was recrystallized from acetone at low temperature.

Recombinant ProGRP was obtained from Radiumhospitalet, Oslo University Hospital, Oslo, Norway. ProGRP isoform 1 was cloned from human cDNA (Origene technologies) and expressed in *Escherichia coli* (Promega) via pGEX-6P-3 constructs (GE Healthcare) and purified as described previously.[25] ProGRP concentrations were determined via UV absorbance (280 nm), diluted to the desired concentration with 50 mM ammonium bicarbonate (ABC) and stored at −20 °C.

Synthetic NLLGLIEAK (>95%) and the stable isotope labeled internal standard (IS) peptide NLLGLIEA-[K_$^{13}C_6^{15}N_2$] (>95%) were purchased from Innovagen (Lund, Sweden). Stock solutions of each peptide were prepared in water at a concentration of 10 mM. The standards were diluted in 50 mM ABC for further use.

Bovine serum albumin (BSA) and trypsin (TPCK-treated) from bovine pancreas (sequencing grade) were purchased from Sigma-Aldrich

Human Serum. Human serum from healthy individuals was obtained from Oslo University Hospital, Ullevål (Oslo, Norway). All serum samples were stored at −32 °C.

mMIP Synthesis. Two mMIP formats were synthesized: magnetic core−shell MIPs and magnetized MIP microspheres.

Magnetic core−shell MIPs were synthesized by a two-step precipitation polymerization (PP). For this, poly(MAA-co-DVB-80) microspheres were synthesized and then magnetized in a first step, with these magnetic core particles then being used as seeds for the production of imprinted shells in a second

precipitation polymerization. The magnetized MIP microspheres were prepared by the partial in-filling of the pores in MIP microspheres using a magnetic component. For the detailed synthesis of the polymers, see Supporting Information.

Liquid Chromatography−Tandem Mass Spectrometry

LC-MS/MS analysis was performed using a triple quadrupole mass spectrometer according to established methods for ProGRP.[27] The chromatographic system consisted of an LPG-3400 M pump with a degasser, a WPS-3000TRS autosampler, and an FLM3000 flow-manager (all Dionex, Sunnyvale, CA, USA). The LC system was controlled by Chromeleon v. 6.80 SR6 (Dionex). The chromatographic separation was carried out using an Aquasil C18 analytical column (Thermo Scientific) (100 Å, 3 μm, 50 mm × 1 mm). The chromatographic separation was performed by loading 10 μL of sample with mobile phase A (20 mM formic acid (FA) and acetonitrile (MeCN) 99:1, v/v) and eluting with a 30 min linear gradient from 0 to 85% mobile phase B (20 mM FA and MeCN 1:99, v/v). After the gradient was run, the column was washed for 3 min with 90% mobile phase B and re-equilibrated with mobile phase A. The column temperature was set and kept constant at 25 °C. A triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo Scientific) was used to determine signature peptides by selected reaction monitoring (SRM). The following transition pairs were monitored: for the ProGRP signature peptide NLLGLIEAK, 485.8 → 630.3 and 485.8 → 743.4; for the NLLGLIEAK IS, 489.9 → 638.3 and 489.9 → 751.4; for the ProGRP signature peptide LSAPGSQR, 408.2 → 272.6 and 408.2 → 544.4; for the ProGRP signature peptide ALGNQQPSWDSEDSSNFK, 1005.450 → 595.300, 1005.450 → 913.300, 1005.450 → 1028.300 and 1005.450 → 1398.500. TSQ data were processed by Xcalibur's QualBrowser (version 2.2 SP 1.48, Thermo Scientific), and MS responses based on the peak intensity, automatically processed by genesis peak detection algorithm, were used. Among them, only peaks with a signal-to-noise (S/N)-ratio above 10 and with retention time and ion ratios corresponding to those of reference samples at high concentration were considered.

Protein Digestion

ProGRP standard solutions were diluted with ABC (50 mM) to a final concentration of 50 nM. Digestion was carried out with trypsin with an enzyme to substrate ratio of 1:40 at 37 °C, overnight.

BSA standards were diluted to a volume and concentration of 500 μL and 100 nM, respectively, with ABC (50 mM). 2.5 μL of 50 mM DTT (freshly prepared in ABC buffer) was added to the protein mixture in 50 mM freshly prepared ABC buffer and incubated at 800 rpm at 60 °C for 20 min. Afterward, the solution was cooled, and 2.5 μL of 200 mM IAA (freshly prepared in ABC buffer) was added. Incubation was carried out for 15 min at room temperature in the dark. Digestion was then accomplished by adding trypsin as described above.

mMIP Preconditioning

Prior to use, the mMIP was washed by gentle inversion overnight in 9:1 MeOH:HCl to remove any bound template. MeOH:HCl was removed by washing twice with MeCN for 5 min.

Initial Testing of mMIPs

The initial tests were performed on one batch of core−shell mMIP (mMIP A) to determine the requirements for conditioning, mass mMIP, extraction time and loading buffer (see Supporting Information for more details).

Final Aqueous mSPE Protocol

The mMIP was conditioned in 50 mM ABC (100 μL) before the addition of 100 μL of loading buffer spiked with 5 nM digested ProGRP, 5 nM IS, and 10 nM digested BSA and extracted for 5 min. The supernatant was collected and the mMIP particles washed with 100 μL Milli-Q H$_2$O for 5 min. The bound peptides were eluted with 100 μL 80:15:5 H$_2$O:MeCN:FA for 5 min. The eluent was dried under N$_2$ and reconstituted in 100 μL ABC containing 0.1% FA. The eluent was analyzed by LC-MS/MS.

Binding Isotherms

mMIP C and its corresponding nonimprinted polymer (mNIP C, i.e., a polymer synthesized under identical conditions to mMIP C except for the omission of template) were conditioned (as described in mMIP Preconditioning) before the addition of 100 μL of loading buffer spiked with 5 nM IS and 10 nM digested BSA. After 5 min, the supernatant was collected. This procedure was repeated for a total of $n = 20$ with the same mMIP/mNIP pair. The supernatants were analyzed to determine the binding profiles using the formula:

$$\%\text{Bound} = 100 - \left(\frac{\text{SI}_{\text{EX}}}{\text{SI}_{\text{QC}}}\right) \cdot 100\%$$

where SI_{EX} is the signal intensity from the supernatants after extraction, and SI_{QC} is the mean of signal intensities from the QC-samples.

Imprinting Factor (IF)

Imprinting factors were determined using the ratio of the relative B_{max} (maximum specific binding) of the binding isotherms for the mMIP and mNIP, using the formula:

$$\text{IF} = \frac{B_{\text{max}}\ \text{mMIP (nmol/mg)}}{B_{\text{max}}\ \text{mNIP (nmol/mg)}}$$

Enrichment of NLLGLIEAK from Spiked Human Serum

Human serum samples (500 μL) were spiked to 10 nM NLLGLIEAK IS and 10 nM ProGRP, diluted 1:1 in 50 mM ABC and vortexed for 30 s. High molecular weight proteins were precipitated with MeCN at −30 °C using a sample:MeCN ratio of 1:0.7.[28] The precipitated proteins were removed by centrifugation (10 000g). Digestion was carried out with trypsin at a substrate to enzyme ratio of 1:20 (of calculated remaining protein concentration) at 37 °C, overnight. The mMIP (600 μg) was conditioned as described in mMIP Preconditioning and loaded with 100 μL of digested sample. Extraction was performed for 5 min. The mMIP was washed twice with 100 μL of water. Peptides were eluted with 100 μL 80:15:5 H$_2$O:MeCN:FA for 5 min. The supernatant was then extracted 2 more times with fresh mMIP (600 μg) to ensure maximum recovery. The eluents were pooled and dried under N$_2$ and reconstituted in 50 mM ABC (100 μL) containing 0.1% FA and analyzed LC-MS/MS.

Table 1. Structural Information[a] of the mMIPs and mNIPs

	template	functional monomers	cross-linker	size (μm)
mMIP A	Z-NLLGLIEA[Nle]	EAMA·HCl, BTPV	DVB-80	4−5
mNIP A	−	EAMA·HCl, BTPV	DVB-80	4−5
mMIP B	Z-NLLGLIEA[Nle]	EAMA·HCl	DVB-80	4−5
mNIP B	−	EAMA·HCl	DVB-80	4−5
mMIP C	Z-NLLGLIEA[Nle]	EAMA·HCl	DVB-80	4−5
mNIP C	−	EAMA·HCl	DVB-80	1−5
mMIP D	Z-NLLGLIEA[Nle]	EAMA·HCl, BTPV	DVB-80	approximately 1
mNIP D	−	EAMA·HCl, BTPV	DVB-80	approximately 1

[a]For detailed information regarding concentrations and ratios of the synthetic components see Supporting Information: Tables S3 and S6.

■ RESULTS AND DISCUSSION

Polymer Synthesis

New approaches for the synthesis of magnetic MIPs and NIPs were developed, which allowed for the synthesis of imprinted and nonimprinted magnetic core−shell polymer microspheres (Synthesis Method 1) and imprinted and nonimprinted magnetic polymer microspheres (Synthesis Method 2). This outcome was achieved by adapting a literature protocol for microgel magnetization, and by drawing upon our extensive in-house knowledge on polymer synthesis using precipitation polymerization (PP) and molecular imprinting. A noncovalent molecular imprinting strategy was adopted to impart affinity into selected polymers for the signature peptide of ProGRP, thereby building upon recent disclosures in this area. Precipitation polymerization was used as the polymer synthesis method of choice since it can deliver high quality polymer microspheres in the low-micron size range. A range of polymers was designed, synthesized, and then screened for their ability to recognize and bind to the target peptide in aqueous media followed by a magnetic capture; a list of the template, functional monomers and cross-linker used to prepare mMIPs and mNIPs is presented in Table 1, together with a statement of the microsphere diameters. For full details about polymer synthesis and properties, see Supporting Information; however, the most salient points are outlined here.

Magnetic Core−Shell Polymer Microspheres (mMIP A, mNIP A, mMIP B, and mNIP B). The synthesis of mMIP A and mMIP B, and their corresponding NIPs, necessitated the synthesis of nonimprinted porous polymer microspheres bearing carboxylic acid groups (to enable the in-filling of pores with a magnetic component), thus poly(DVB-80-co-MAA) microspheres with diameters ∼5 μm were targeted. For this, PP conditions reported previously were applied. A monomer concentration of 3.28% w/v (with respect to the solvent) and an initiator concentration of 3.35 mol % (with respect to the total number of moles of polymerizable double bonds), together with a mixture of acetonitrile and toluene as porogens (75:25 (v/v)), allowed for the synthesis of porous polymer microspheres of an appropriate size. Following the magnetization of these microspheres (see Supporting Information), they were used as seed particles in a subsequent PP. Accordingly, nonmagnetic shells were formed around the magnetic cores, taking advantage of the fact that the PP mechanism is one of nucleation and growth. A 2:1 w/w ratio of magnetic cores to monomer was used for the synthesis of the core−shell particles. Such a ratio allowed for the synthesis of core−shell polymer microspheres with shell thicknesses of ∼0.1 μm. MIPs (mMIP A and mMIP B) and the corresponding NIPs (mNIP A and mNIP B) were prepared by the delayed addition of template (for the MIP syntheses) and functional monomer(s), timed 1.5 h after the start of the PP.

Magnetic Polymer Microspheres (mMIP C, mNIP C, mMIP D, and mNIP D). mMIP C and mMIP D, and their corresponding NIPs, were prepared by magnetization of imprinted and nonimprinted porous polymer microspheres which had been produced via a PP protocol. Therefore, the first step was the synthesis of porous MIP microspheres (and their corresponding NIPs) with Z-NLLGLIEA[Nle] as template, which was followed by the magnetization procedure. For success, PP must involve the polymerization of monomers in dilute solution (typically <5% w/v monomer in solvent) in a near-Θ solvent; therefore, DVB-80 was selected as cross-linker, the porogen was acetonitrile, the initiator concentration was 2 mol % (w.r.t. the total number of moles of polymerizable double bonds), and the monomer concentration was 2% w/v (w.r.t. to the solvent). A small volume of DMSO was required to promote solubility of template and keep all components in solution prior to polymerization, but the use of DMSO was kept to a minimum. N-(2-Aminoethyl)methacrylamide hydrochloride and N-3,5-bis(aminoethylmethyl)-phenyl-N′-4-vinyl-phenylurea were selected as functional monomers since the carboxylic acid groups in the glutamic acid (E) residue and C-terminus of the template were targeted via a noncovalent molecular imprinting approach.

Overall, the polymer synthesis program delivered good yields of micron-sized imprinted and nonimprinted magnetic core−shell polymer microspheres (Synthesis Method 1) and imprinted and nonimprinted magnetic polymer microspheres (Synthesis Method 2), in a convenient beaded format. The magnetic susceptibility of the polymers meant that they could be used for the capture and quantification of an SCLC biomarker in a magnetic SPE platform.

Selection of Standard Solutions

Optimization of the mSPE method required an understanding of the optimal conditions for binding of the target by the mMIPs. For this, NLLGLIEAK IS was utilized in the initial optimization experiments as it circumvents the digestion step and simplifies sample preparation. The IS has chemical and chromatographic properties indistinguishable from native NLLGLIEAK but is distinct in m/z (Δm = +8 Da). Synthetic NLLGLIEAK was incorporated in optimization experiments allowing IS correction. Furthermore, ProGRP was used for the evaluation of the final optimized aqueous extraction method. 50 mM ABC buffer was used to ensure compatibility with the increasing sample complexity in further optimization, such as

tryptic digests, addition of digested BSA and finally digested ProGRP in serum.

Digested BSA was selected as the source of nonselectively bound competing peptides in the optimization of the mSPE protocol.

Initial Testing

Initial tests were carried out on mMIP A to determine the mSPE conditions (conditioning, loading matrix, extraction time, and mass of mMIP). Conditioning of the sorbent is essential for ensuring optimal interactions between the analyte and solid phase during extraction. Since the mMIPs are designed to enrich NLLGLIEAK from serum, the loading matrix should be aqueous to ensure downstream compatibility with tryptic digests. As such, the mMIP was loaded with the NLLGLIEAK IS (5 ng/mL) in ABC (50 mM). Extractions of the target from an organic matrix (100% MeCN) were also performed, since the mMIPs were synthesized in the presence of MeCN and therefore expected to show affinity for the target in this solvent. The binding efficiency (% bound analyte) was found to be 99.9 ± 0.0% and 99.9 ± 0.3% in the aqueous and organic matrices, respectively (Figure S1). Therefore, the mMIPs were expected to have excellent compatibility with aqueous matrices and the potential to extract NLLGLIEAK directly from aqueous matrices such as serum.

Two essential aspects of mSPE optimization are the determination of an appropriate sorbent concentration and extraction time. Short extraction times are critical for low stability analytes, but also allow for a higher throughput of samples. The determination of optimal sorbent concentration is essential to ensure binding capacity is balanced against cost-effectiveness. A range of mMIP concentrations and extraction times were explored to maximize the binding efficiency (Figure S2). This was accomplished by loading 5 nM NLLGLIEAK IS (100 μL) onto increasing amounts of mMIP (200−600 μg) and extracting for between 10 and 120 min. Supernatants were collected and analyzed directly to determine binding efficiency. The binding efficiency with 200 μg mMIP was moderate between 10 and 40 min (25.4−38.4%), with high standard deviations for the shortest extraction times (10−30%). Maximum binding efficiency of 91.0 ± 4.6% was reached after 60 min. Similarly, 400 μg mMIP had moderate recoveries between 10 and 20 min with standard deviations from 7 to 23%; however, 92.8 ± 2.2% of NLLGLIEAK IS was bound after 50 min. With 600 μg mMIP, there was consistent, high binding efficiency from the earliest time point (10 min; 92.3 ± 2.8%), with up to 99.5% of the peptide being bound from 50 to 120 min. Accordingly, all further experiments were performed using 600 μg of mMIP and 100 μL of sample (i.e., 6 mg mMIP per mL sample) since this gave high binding of the target within short incubation times.

mMIP Evaluation

The molecular recognition properties of the mMIPs were evaluated by investigating their binding strength and selectivity compared to their mNIP counterparts. The performance of all mMIP/mNIP pairs (mMIP/mNIP A-D) was assessed by determining their binding efficiencies via extraction of the NLLGLIEAK IS (5 nM) from ABC (50 mM) containing 10 nM digested BSA. BSA (10 nM) was included to model a potential source of nonspecific binding from endogenous proteins, to illustrate selectivity while maintaining a simple matrix. To evaluate binding, the supernatant was measured directly; therefore, serum equivalent levels of BSA are

impractical. While considerably lower than serum levels of albumin were used, a 2-fold concentration of BSA compared to NLLGLIEAK ought to allow influence on binding selectivity to be determined. Under the conditions of the extraction, mMIP C was found to have particularly high affinity and selectivity for the target (Figure 1), which suggested that mMIP C was an
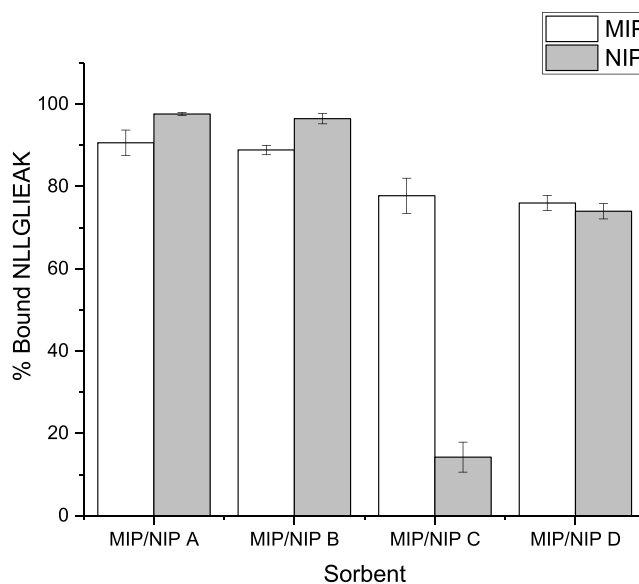

Figure 1. Selectivity of the mMIP/mNIP pairs toward target peptide determined as binding efficiency (% bound NLLGLIEAK IS ± standard deviation of NLLGLIEAK IS). Samples consisted of NLLGLIEAK IS (5 nM) in ABC (50 mM) containing 10 nM digested BSA (n = 3).

excellent candidate for use with complex matrices where both affinity and selectivity are important criteria.[29] The other mMIP/mNIP pairs showed high affinity for the target as well, but poor selectivity under the conditions of the test, therefore mMIP C was selected as the mMIP to be used in the subsequent experiments. It is noteworthy that mMIP C was expected to have higher selectivity than any of the core−shell materials, and was synthesized using a functional monomer (EAMA·HCl) which gave rise to high fidelity binding sites for NLLGLIEAK in our earlier published work on online MISPE; this is why mMIP C outperforms the other MIPs.

Binding Isotherms

Binding isotherms give a broader picture with respect to single concentration extractions of the molecular recognition capabilities of MIPs across a range of concentrations, and were constructed for the mMIP/mNIP C pair for binding to NLLGLIEAK. The nonlinear shape of the mMIP curve (Figure 2) is indicative of selective binding of the target molecule to the molecularly imprinted binding sites in the mMIP, whereas the plot for mNIP C is typical of a situation where binding of the target to the polymer is nonselective in nature. Saturation was reached for the mMIP after 13 extractions, with a B_{max} of 7.4 pmol NLLGLIEAK/mg mMIP (Figure 2). The dissociation constant (K_d) for mMIP C was calculated to be 2.18 × 10^{-9} M. Values of K_d in the low nanomolar range (as are observed here) indicates high affinity between mMIP C and NLLGLIEAK, and is in line with the K_d ranges observed for antigen−antibody binding.
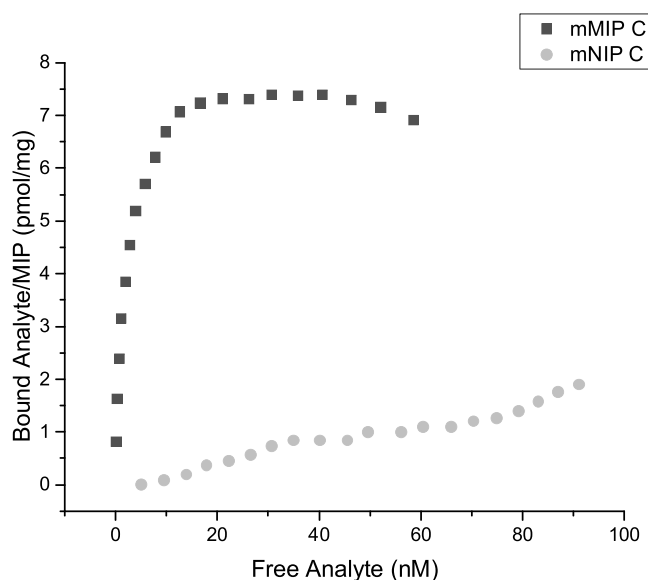
Figure 2. Binding isotherms for mMIP C and mNIP C, expressed as bound analyte/mg mMIP or mNIP vs concentration of free analyte. Samples consisted of NLLGLIEAK IS (5 nM) in ABC (50 mM) containing 10 nM digested BSA ($n = 2$).

Imprinting Factor

A measure of the efficiency of a molecular imprinting process can be gained by determination of the imprinting factor (IF), wherein the binding of an analyte to a MIP is compared to the binding of the same analyte to a polymeric control under nominally identical conditions. While the IF for a MIP does not have a fixed value—since the value measured depends on a number of factors, including the balance of selective and nonselective binding to the MIP under the conditions of the measurement—higher values indicate that there are conditions under which selective binding of an analyte to a MIP can be realized and potentially exploited. In the present case, the IF of mMIP C was calculated to be 6.1, which gave us confidence that molecular imprinting was successful and that binding conditions had been identified under which NLLGLIEAK could be extracted selectively from aqueous media. By comparison, other magnetic MIPs targeting the peptides angiotensin I and angiotensin II were reported to have IFs of 4.9 and 5.2, respectively.[12] Furthermore, an epitope imprinted nanogel for human serum transferrin (HTR) had a similar IF (5.49).[21] Since IF is an indicator of imprinting efficiency,[29] the higher the IF the more likely it is that the MIP will be able to discriminate between the target peptide and nontarget peptides during extractions involving complex matrices such as serum.

Optimization of the mSPE Method

With mMIP C having been identified as the most promising polymer, the mSPE protocol was optimized further with mMIP C to ensure that a robust protocol was in place for the extraction of target peptide from serum. This involved optimization of the loading, washing, and elution steps using synthetic NLLGLIEAK (and NLLGLIEAK IS) in 50 mM ABC containing digested BSA.

Sample Loading. The sample loading procedure was fine-tuned for mMIP C. NLLGLIEAK (5 nM), NLLGLIEAK IS (5 nM), and digested BSA (10 nM) were spiked in 50 mM ABC with increasing MeCN (0−10%). mMIP C (6 mg/mL) was added, and the samples agitated for an hour. Following

magnetic capture of mMIP C, the supernatants were analyzed to determine the binding efficiency. The binding efficiency was highest under fully aqueous conditions (50 mM ABC), with 98.9 ± 0.2% NLLGLIEAK bound. The introduction of small amounts of MeCN reduced the binding efficiency; for 2.5% MeCN, the binding efficiency dropped to 91.6 ± 7.3%, whereas further increases in MeCN levels resulted in large variations in binding efficiency (RSD > 100%). This data shows that mMIP C functioned very well in aqueous media, even when in the presence of nontarget peptides (digested BSA), and is well-suited for compatibility with complex matrix mSPE because the conditions in digested serum are aqueous. All subsequent extractions were performed in 100% aqueous media to ensure downstream compatibility with serum extractions and ensure good repeatability.

Extraction Time. The extraction time was evaluated to determine the shortest extraction time possible while still retaining a high level of binding of NLLGLIEAK. NLLGLIEAK (5 nM), NLLGLIEAK IS (5 nM), and digested BSA (10 nM) were spiked in 50 mM ABC, and a 100 μL sample extracted for 5−60 min; following magnetic separation, the supernatant was analyzed to determine the dependence of the extraction time on the binding efficiency. It was found that mMIP C was able to bind NLLGLIEAK efficiently (98.2 ± 0.2%; $n = 3$) in just 5 min (Figure 3). The results show that
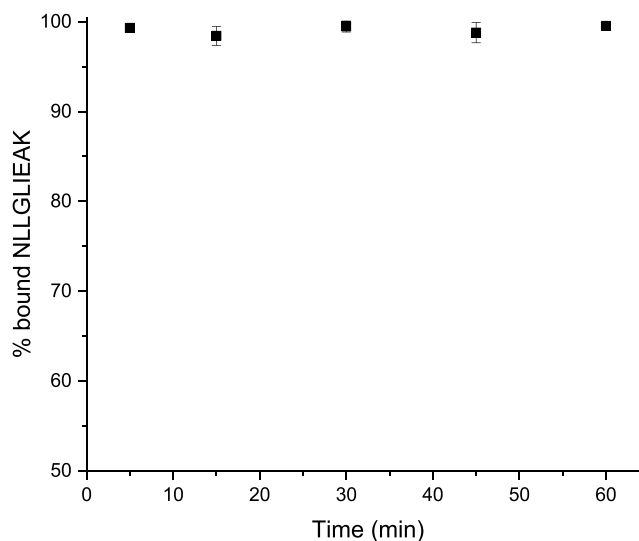
Figure 3. Effect of increasing the extraction time on the binding efficiency (% bound NLLGLIEAK ± standard deviation) of NLLGLIEAK using mMIP C. Samples consisted of NLLGLIEAK IS (5 nM), NLLGLIEAK (5 nM), and 10 nM digested BSA in 50 mM ABC. Samples were extracted for 5, 15, 30, 45, and 60 min ($n = 3$).

mMIP C can extract NLLGLIEAK with high recovery using short extraction times (5 min). Short extraction times are particularly advantageous if the targets have low stability at room temperature, but they also facilitate high sample throughput.

Washing Step. Next, the washing step was optimized. Washing of the mMIP is essential to remove nonspecifically bound peptides, and other adsorbed components, from the polymer prior to elution to ensure a clean extract for analysis. Care must be taken to avoid loss of the target peptide during washing, and a compromise may have to be struck between the loss of target peptide and efficient removal of adsorbed
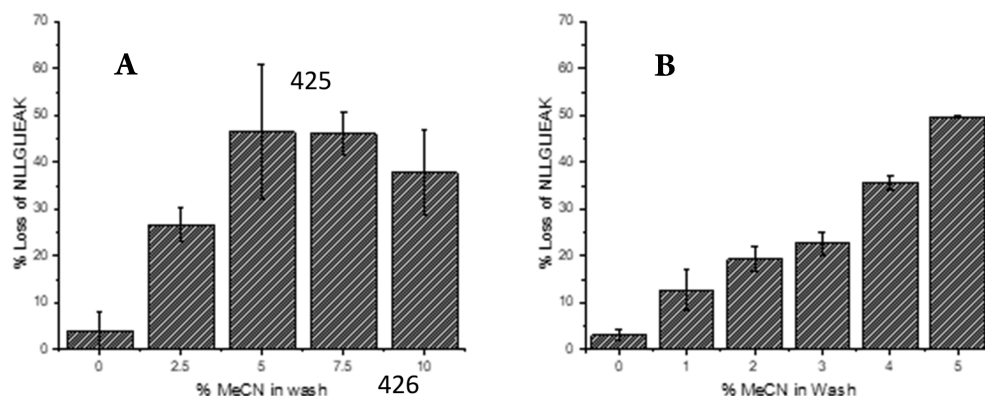
Figure 4. Effect of increasing MeCN in the wash buffer on the loss of NLLGLIEAK (% loss NLLGLIEAK ± standard deviation) of NLLGLIEAK using mMIP C. Samples consisted of NLLGLIEAK IS (5 nM), NLLGLIEAK (5 nM), and 10 nM digested BSA in 50 mM ABC, and were extracted for 5 min. (A) Samples were washed with buffers containing 0, 2.5, 5, 7.5, and 10% MeCN ($n = 3$). (B) Fine-tune washing using 0, 1, 2, 3, 4, and 5% MeCN ($n = 3$).

compounds. To identify an optimal wash buffer, NLLGLIEAK (5 nM), NLLGLIEAK IS (5 nM), and digested BSA (10 nM) were spiked in 50 mM ABC and 100 μL samples extracted for 5 min. The mMIPs were then washed in buffers containing increasing concentrations of MeCN (0, 2.5, 5, 7.5, and 10%). The wash time was set to 5 min to ensure a short sample preparation time and to minimize any loss of the target peptide. As can be seen in Figure 4a, the general trend is that more NLLGLIEAK is lost as the MeCN content of the washing solution rises (this is in agreement with the sample loading findings). Considerable losses (>35%) were observed using 5, 7.5 and 10% MeCN in the wash solution, together with high standard deviations (RSD ≥ 24%) for 5 and 10% MeCN. However, there was minimal loss of target peptide (2.2 ± 1.6%) using a 100% aqueous wash solution. Since the differences in loss of target were so large between 0 and 5% MeCN, MeCN contents ranging from 0 to 5% were evaluated as well; the results are shown in Figure 4b. A similar trend was observed, in that the amount of target lost was directly proportional to the amount of the MeCN in the wash buffer. As there were significant losses at even minor increments of MeCN, it was decided that no consideration would be made with regards to removal of nonspecific peptides. Given all of these results, a fully aqueous washing buffer was selected for use in the subsequent experiments.

Elution of Target Peptide. The final stage of the mSPE procedure is the elution of the target peptide from the polymer using an elution buffer. Elution efficiency (determined as the % recovery) was evaluated using mMIP C with NLLGLIEAK (5 nM), NLLGLIEAK IS (5 nM), and digested BSA (10 nM) spiked in 50 mM ABC. The sample (100 μL) was extracted for 5 min with mMIP C and was then washed with water (100 μL) for 5 min. First, two eluents were evaluated based on the outcomes of the earlier wash experiments: one eluent was 7.5:92.5 MeCN:H$_2$O and the other was 7.5:92.5 MeCN:0.1% FA in H$_2$O. FA was included as a component in one of the eluents since acidic conditions were expected to disrupt the noncovalent interactions between the functional monomers EAMA·HCl and BTPV of mMIP C and NLLGLIEAK. In the washing experiments, 7.5% MeCN in ABC led to approximately 50% loss of NLLGLIEAK; however, when used with water as an eluent it gave rise to low and variable recoveries (2.5 ± 4.3%; Table 2). Furthermore, acidifying the eluent with a low level of FA gave a marginal improvement in recovery

Table 2. Recoveries of NLLGLIEAK after Elution with a Range of Eluents, as Represented by % Recovery NLLGLIEAK ± Standard Deviation[a]

| eluent	recovery (%)	RSD (%)
7.5:92.5 MeCN:H$_2$O	2.5	173
7.5:92.5 MeCN:H$_2$O (0.1% FA)	6.2	173
80:15:5 MeCN:H$_2$O:FA	84.8	14.1

[a]Samples consisted of NLLGLIEAK (5 nM) and NLLGLIEAK IS (5 nM) in ABC (50 mM) containing 10 nM digested BSA. Samples were extracted for 5 min, washed in 50 mM ABC (100 μL) for 5 min and eluted for 5 min ($n = 3$).

only (6.2 ± 10.7%). A more potent eluent (80:15:5 MeCN:H$_2$O:FA) was therefore evaluated, an eluent which had a high organic content (to promote efficient wetting of the polymer and solubilization of the bound target) and a higher FA content (to break selective interactions); in earlier work, this eluent had been used successfully to elute NLLGLIEAK from imprinted polymers.[30] With this eluent, the recovery was markedly increased to 84.8%, and with a satisfactory RSD (<15%) (Table 2). 80:15:5 MeCN:H$_2$O:FA was hence selected as the preferred eluent for the remainder of the experiments.

Affinity of mMIPs toward Other Peptides

To evaluate the effectiveness and selectivity of the optimized mSPE method, the whole procedure was performed using digested ProGRP (250 ng/mL) in ABC (50 mM). Each step in the procedure was evaluated: binding efficiency, loss in washing, and elution recovery. Three peptides were monitored: the target peptide, NLLGLIEAK, and two other ProGRP isoform 1 peptides, ALGNQQPSWDSEDSSNFK and LSAPGSQR. In these experiments, binding efficiency was determined as the normalized amount of peptide in the supernatant recovery (i.e., ratio of the amount of peptide measured in the supernatant and amount of peptide measured in the control, where a low supernatant recovery suggests efficient binding to the mMIP). LSAPGSQR bound poorly to mMIP C, with 75.6 ± 10.6% unbound after incubation with the sample, however ALGNQQPSWDSEDSSNFK bound strongly to mMIP C. The latter observation can be explained on the basis that ALGNQQPSWDSEDSSNFK contains carboxylate side-chains that can bind strongly but non-selectively to amine moieties throughout the polymer.

Unsurprisingly, the target peptide, NLLGLIEAK, also binds strongly to mMIP C when extracting from a digested ProGRP sample (Figure 5).
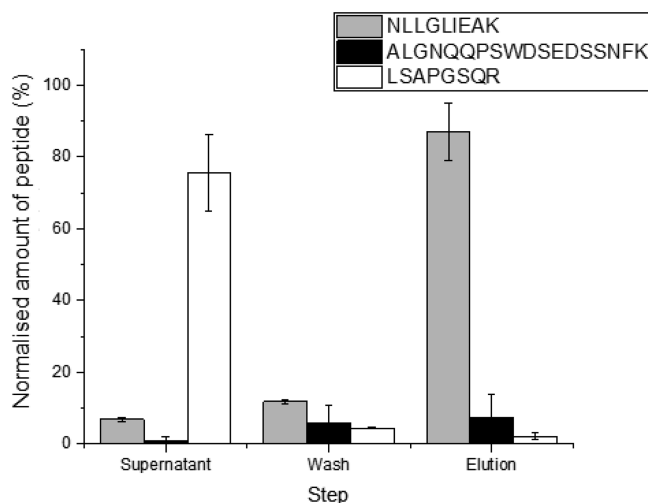
Figure 5. Evaluation of the selectivity of each step in the mSPE method using digested ProGRP, as represented by normalized amount of peptide (%) ± standard deviation of three ProGRP peptides for the three steps. Samples consisted of ProGRP (182 nM) and NLLGLIEAK IS (5 nM) in ABC (50 mM) containing 10 nM digested BSA. Samples were extracted for 5 min, washed in 50 mM ABC (100 μL) for 5 min and eluted with 80:15:5 MeCN:H_2O:FA for 5 min (n = 3).

The wash fraction had normalized amounts of LSAPGSQR and ALGNQQPSWDSEDSSNFK of 4.5 ± 0.7% and 5.9 ± 4.4% respectively. For NLLGLIEAK in the wash this was 11.8 ± 0.6%.

The normalized amounts of LSAPGSQR and ALGNQQPSWDSEDSSNFK in the elution step (i.e., elution recovery) were poor, with an elution recovery of 7.5 ± 6.5% and 2.2 ± 1.0% respectively. However, NLLGLIEAK had an elution recovery of 87 ± 8.1%, showing, under these conditions, mMIP C's selectivity toward NLLGLIEAK compared to LSAPGSQR and ALGNQQPSWDSEDSSNFK as NLLGLIEAK is eluted almost quantitatively off mMIP C. The differences in elution between the peptides are likely to be due to differences in their physicochemical properties. The size (i.e., molecular weight), hydrophobicity (i.e., grand average of hydrophobicity, GRAVY) and isoelectric points (pI) of the tightly bound peptides are quite different: ALGNQQPSWD-SEDSSNFK has a M_w of 2010.06 Da, GRAVY of −1.450 and a pI of 3.68, while NLLGLIEAK has a M_w of 970.18 Da, GRAVY of 0.711 and a pI of 6.00. Therefore, under the elution conditions (approximately pH 2), the acidic groups of NLLGLIEAK will be protonated, disrupting the interactions with the functional groups in the polymer. ALGNQQPSWD-SEDSSNFK, on the other hand, has a pI of 3.68 and is, therefore, more likely to remain bound to EAMA. Furthermore, since NLLGLIEAK is less polar than ALGNQ-QPSWDSEDSSNFK it will have a higher affinity for an eluent with a high MeCN content. A consequence of ALGNQ-QPSWDSEDSSNFK remaining bound to mMIP C after the elution step there may be interferences with the binding of NLLGLIEAK to mMIP C in subsequent extractions. To mitigate this, it would be advisable to perform a thorough wash step before reuse. This wash step should be similar to the initial particle wash protocol, as described in mMIP Preconditioning. This would limit the reuse time to once every day; however, the reusability of the mMIPs ensures low-cost analysis.

Applicability to Complex Matrices

To round-off the study, mMIP C was applied to the mSPE of a real biological sample, specifically a human serum sample containing the biomarker ProGRP. For this, serum was spiked with ProGRP and NLLGLIEAK IS before precipitation of the high molecular weight proteins, as described previously.[7,26] After protein precipitation, evaporation, and reconstitution, the serum was digested and mSPE performed using the optimized method. Initially, the recovery of the target for this extraction of a complex matrix was low (5.6 ± 0.5%; Figure 6). This is
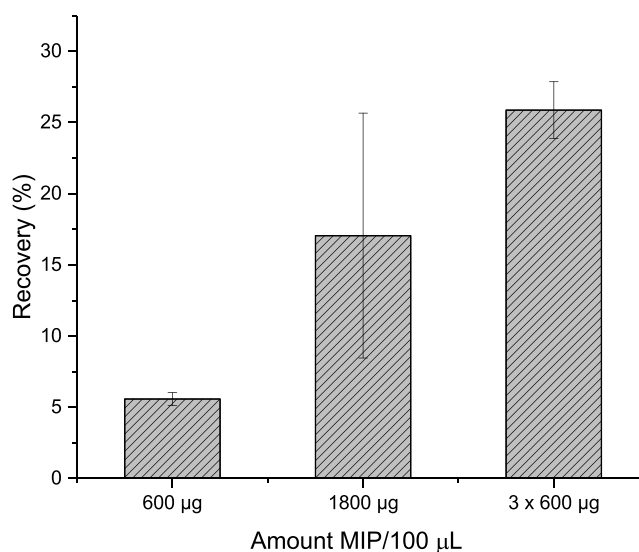
Figure 6. Recoveries of NLLGLIEAK from human serum using digested ProGRP, as represented by % recovery NLLGLIEAK ± standard deviation. Samples consisted of ProGRP (10 nM) and NLLGLIEAK IS (10 nM) in 50 μL serum diluted 1:1 in ABC (50 mM). Serum was digested with trypsin and samples were extracted for 5 min with 600 μg, 1800 μg and 3 × 600 μg mMIP C/100 μL sample, samples were washed in 50 mM ABC (100 μL) for 5 min and eluted with 80:15:5 MeCN:H_2O:FA for 5 min (n = 3).

most likely due to the high abundant, nontarget peptides binding nonselectively to the mMIP binding sites and preventing NLLGLIEAK capture, which suggests capacity limitations, i.e., too few binding sites, an effect that has been described previously.[25] Furthermore, the complexity of serum can limit the digestion efficiency, thereby also lowering the recovery of target. The volume of extracted serum was 50 μL, diluted 1:1 in 50 mM ABC, and low sample volumes can present challenges with recoveries and LODs. To improve the recovery of the process, an increase in the mass of mMIP C used (1800 μg mMIP C/100 μL sample) and sequential extractions using 3 × 600 μg mMIP C/100 μL sample were explored. The use of a higher amount of polymer increased the recovery to 17.1 ± 8.6%, and the use of sequential extraction further increased the recovery to 25.9 ± 2.0%. While both methods used a total of 1800 μg of mMIP C, the sequential extractions yielded higher recoveries and lower variation. This increased recovery is in accordance with conventional extraction theory (e.g., for liquid−liquid extractions).

A recovery of 25% is comparable to a recovery reported for nonmagnetic MIPs[25] as well as antibody-based cleanup of low abundant proteins in human serum.[31] This is considered to be satisfactory if the method otherwise provides repeatable and accurate results and at sufficiently low detection and quantification limits.

An estimate of the detection and quantification limits (LOD and LOQ, respectively) was carried out based on the signal intensity of NLLGLIEAK after analysis of the spiked serum sample. LOD (S/N = 3) and LOQ (S/N = 10) were estimated to be 39 pM and 129 pM, respectively. This is significantly lower than the LOD reported for crushed and ground MIP particles packed into SPE-cartridges (LOD 625 pM)[25] and of the same order of magnitude as reported for MIP micro-particles applied in online SPE (LOD 11 pM).[7] The observed LOD is 5.6 times higher than the upper reference level for humans in humans,[24] but this should be within reach after further optimization of the mSPE method and/or use of a more sensitive LC-MS/MS system. The recovery is most likely affected by two factors: limited binding capacity and interference from matrix components. In respect of interference from matrix components, the mMIP is likely to interact with many abundant tryptic peptides in the matrix, as has been observed previously for MIPs with similar compositions targeting NLLGLIEAK.[7,32] It is expected that use of mMIPs with higher binding capacities will yield higher recoveries despite nonspecific interactions of the matrix components (N.B. mMIP C was synthesized using a template to cross-linker mole ratio in the feed of 1:533, thus there is significant scope for preparing mMIPs with significantly higher binding capacities, if desired, by increasing the template to cross-linker ratio during the polymer synthesis stage).

CONCLUSIONS

In the present study, four magnetic synthetic receptors (mMIPs) were synthesized in two distinct beaded formats, magnetic polymer microspheres, and magnetic core–shell polymer microspheres, using a simple and straightforward magnetization procedure that can be applied to a range of porous media, nonrestricted to beaded materials. The magnetization of the synthetic receptors enabled them to be evaluated for the targeting of the signature peptide of the SCLC biomarker, ProGRP, using a magnetic SPE (mSPE) platform coupled with LC-MS/MS for bottom-up proteomics. The binding selectivity of each mMIP was assessed to determine the most promising mMIP for the optimization of the mSPE method, with one imprinted material (mMIP C) displaying particularly high fidelity for the target, even in fully aqueous media. In this regard, a dissociation constant in the low nanomolar range was estimated for mMIP C which, when taken together with its magnetic character, enabled an optimized mSPE protocol to be established to selectively cleanup NLLGLIEAK from a digested ProGRP sample. Extractions of the biomarker from digested serum samples were also possible, with satisfactory repeatability, which demonstrated the applicability of the mMIP platform to real samples. Sample volumes were low, high recoveries were obtained within very short extraction times (5 min), and the LOD was 39 pM (this LOD is significantly lower than the LOD reported for crushed and ground MIP particles packed into SPE-cartridges). With further optimization and testing, these mMIPs may have potential in clinical settings given their high selectivity and good recoveries at a much lower price point than conventional methods.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00258.

Figures showing initial optimization of mMIP extraction; supplementary results and discussion of mMIP and mNIP production including figures and tables showing production conditions and particle characterization; overview of raw data files uploaded to Peptide Atlas (PDF)

Additional data (XLSX)

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Notes

The authors declare no competing financial interest.

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