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 abundance 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 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 clean-up 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* non-covalent 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) is 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 pre-formed 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. mMIPshave 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: a mMIP

targeting lysozyme demonstrated clear enrichment in egg white.[22] Whilst a 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 clean-up 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, non-magnetic MIPs have been developed to extract NLLGLIEAK from serum using off-line MISPE[25] and on-line 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 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 aluminium 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 labelled 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. Afterwards, 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, 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_2O for 5 min. The bound peptides were eluted with 100 µL 80:15:5 H_2O:MeCN:FA for 5

191 min. The eluent was dried under N_2 and reconstituted in 100 µL ABC containing 0.1% FA.

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BINDING ISOTHERMS

mMIP C and its corresponding non-imprinted 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:

$$\%\boldsymbol{Bound} = \mathbf{100} - \left(\frac{\mathbf{SI_{EX}}}{\mathbf{SI_{QC}}}\right) \cdot \mathbf{100}\%$$

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:

$$\boldsymbol{IF} = \frac{\textbf{Bmax mMIP (nmol/mg)}}{\textbf{Bmax mNIP (nmol/mg)}}$$

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 (10,000 x *g*). 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_2O: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.

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 non-imprinted magnetic core-shell polymer microspheres (*Synthesis Method 1*) and imprinted and non-imprinted magnetic polymer microspheres (*Synthesis Method 2*). This outcome was achieved by adapting a literature protocol for microgel magnetisation, and by drawing upon our extensive in-house knowledge on polymer synthesis using precipitation polymerisation (PP) and molecular imprinting. A non-covalent 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 polymerisation 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, synthesised and then screened for their ability to recognise and bind to the target peptide in aqueous media followed by a magnetic capture; a list of the template, functional monomers and crosslinker 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 non-imprinted 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 magnetisation of these microspheres (*see Supporting Information.*), they were used as seed particles in a subsequent PP. Accordingly, non-magnetic 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 non-imprinted 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 crosslinker, the porogen was acetonitrile, the initiator concentration was 2 mol% (w.r.t. the total number of moles of polymerisable 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-vinylphenylurea 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 non-covalent molecular imprinting approach.

Overall, the polymer synthesis programme delivered good yields of micron-sized imprinted and non-imprinted magnetic core-shell polymer microspheres (*Synthesis Method 1*) and imprinted and non-imprinted 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.

Table 1. Structural information[1] of the mMIPs and NIPs

	Template	Functional Monomers	Crosslinker	SIZE (µm)
mMIP A	Z-NLLGLIEA[Nle]	EAMA.HCI, BTPV	DVB-80	4-5
mNIP A	-	EAMA.HCI, BTPV	DVB-80	4-5
mMIP B	Z-NLLGLIEA[Nle]	EAMA.HCI	DVB-80	4-5
mNIP B	-	EAMA.HCI	DVB-80	4-5
mMIP C	Z-NLLGLIEA[Nle]	EAMA.HCI	DVB-80	4-5
mNIP C	-	EAMA.HCI	DVB-80	1-5
mMIP D	Z-NLLGLIEA[Nle]	EAMA.HCI, BTPV	DVB-80	approx. 1
mNIP D	-	EAMA.HCI, BTPV	DVB-80	approx. 1

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

SELECTION OF STANDARD SOLUTIONS

Optimisation 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 optimisation 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 optimisation experiments allowing IS correction. Furthermore, ProGRP was used for the evaluation of the final optimised aqueous extraction method. 50 mM ABC buffer

was used to ensure compatibility with the increasing sample complexity in further optimisation, such as tryptic digests, addition of digested BSA and finally digested ProGRP in serum.

Digested BSA was selected as the source of non-selectively bound competing peptides in the optimisation 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, however, since the mMIPs were synthesized in the presence of MeCN and were 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 (Fig. 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 maximise the binding efficiency (Fig. S2). This was accomplished by loading 5 nM NLLGLIEAK IS (100 µL) onto increasing amounts of mMIP (200 to 600 µg) and extracting for between 10 and 120 min.

Supernatants were collected and analysed 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 to 30%). Maximum binding efficiency of 91.0 ± 4.6% was reached after 60 min. Similarly, 400 µg mMIP had moderate recoveries between 10-20 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 50-120 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 non-specific 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 two-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 (Fig. 1), which suggested that mMIP C was an 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 synthesised using a functional monomer (EAMA.HCl) which gave rise to high fidelity binding sites for NLLGLIEAK in our earlier published work on on-line MISPE; this is why mMIP C outperforms the other MIPs.

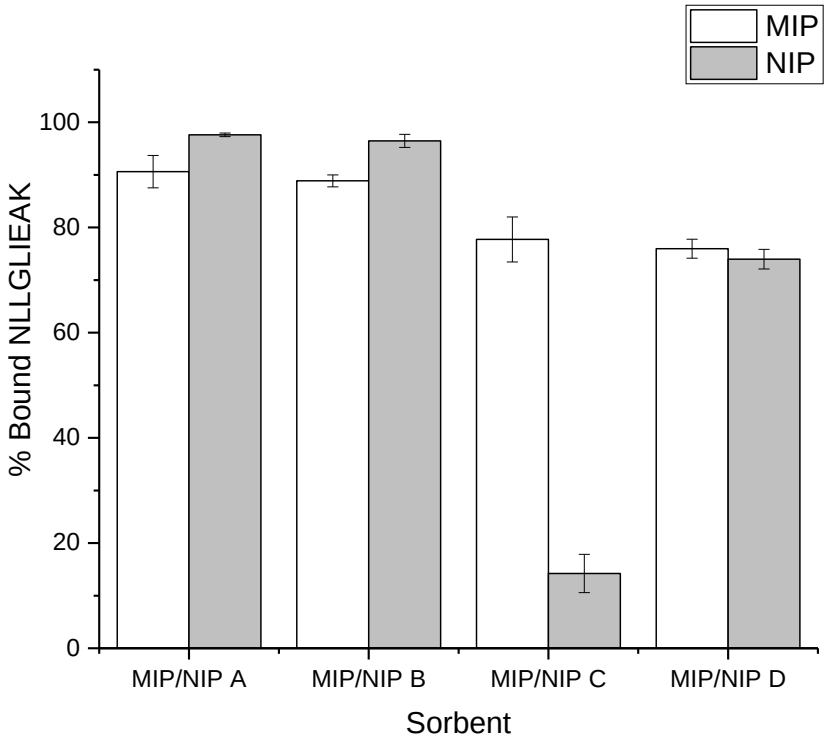


Figure 1. Selectivity of the mMIP/mNIP pairs towards target peptide determined as binding efficiency (% bound NLLGLIEAK IS ± standard deviation of NLLGLIEAK IS). Samples consisted of NLLGLIEAK IS (5 nM) in ABC (50 mM) containing 10 nM digested BSA (n=3).

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 non-linear shape of the mMIP curve (Fig. 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 non-selective in nature. Saturation was reached for the mMIP after 13 extractions, with a B_{max} of 7.4 pmol NLLGLIEAK/mg mMIP (Fig. 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.

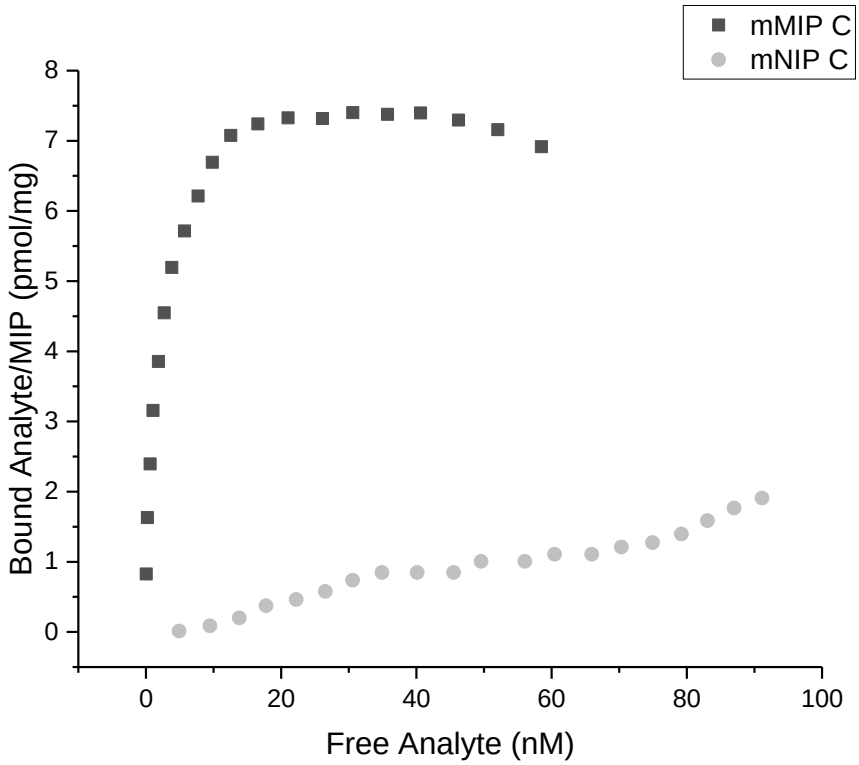


Figure 2. Binding isotherms for mMIP C and mNIP C, expressed as bound analyte/mg mMIP or mNIP *vs.* concentration of free analyte. Samples consisted of NLLGLIEAK IS (5 nM) in ABC (50 mM) containing 10 nM digested BSA (n=2).

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. Whilst 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 non-selective 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 realised 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 non-target 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 optimised 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.

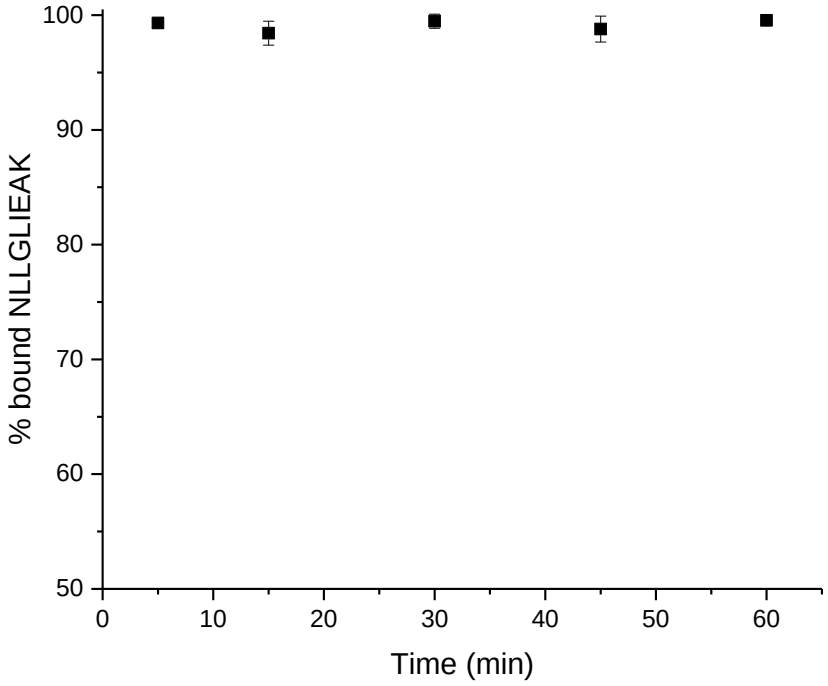
Sample Loading

The sample loading procedure was fine-tuned for mMIP C. NLLGLIEAK (5 nM), NLLGLIEAK IS (5 nM) and digested BSA (10 nM) was 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 analysed 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 non-target 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 whilst 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 analysed 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. (Fig. 3). The results show that 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.



411

412 **Figure 3. Effect of increasing the extraction time on the binding efficiency (% bound**
413 **NLLGLIEAK ± standard deviation) of NLLGLIEAK using mMIP C. Samples consisted**
414 **of NLLGLIEAK IS (5 nM), NLLGLIEAK (5 nM) and 10 nM digested BSA in 50 mM**
415 **ABC. Samples were extracted for 5, 15, 30, 45 and 60 min (n=3).**

416

417 **Washing Step**

418 Next, the washing step was optimised. Washing of the mMIP is essential to remove non-
419 specifically bound peptides, and other adsorbed components, from the polymer prior to elution
420 to ensure a clean extract for analysis. Care must be taken to avoid loss of the target peptide
421 during washing, and a compromise may have to be struck between the loss of target peptide
422 and efficient removal of adsorbed compounds. To identify an optimal wash buffer,
423 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 minimise any loss of the target peptide. As can be seen in Fig. 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 0-5 % were evaluated as well; the results are shown in Fig. 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 non-specific peptides. Given all of these results, a fully aqueous washing buffer was selected for use in the subsequent experiments.

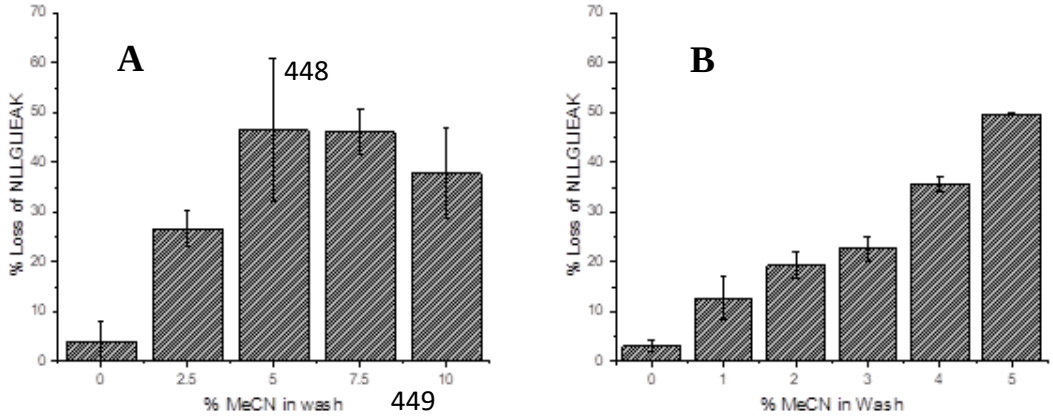


Figure 4. Effect of increasing MeCN in the wash buffer on the loss of NLLGLIEAK (% loss NLLGLIEAK ± standard deviation) of NLLGLIEAK using mMIP C. Samples consisted of NLLGLIEAK IS (5 nM), NLLGLIEAK (5 nM) and 10 nM digested BSA in 50 mM ABC, and were extracted for 5 min. (A) Samples were washed with 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).

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. Firstly, two eluents were evaluated based on the outcomes of the earlier wash experiments: one eluent was 7.5:92.5 MeCN:H_2O and the other was 7.5:92.5 MeCN:0.1% FA in H_2O. FA was included as a component in one of the eluents since acidic conditions were expected to disrupt the non-covalent 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 only (6.2 ± 10.7 %). A more potent eluent (80:15:5 MeCN:H_2O:FA) was therefore evaluated, an eluent which had a high organic content (to promote efficient wetting of the polymer and solubilisation 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_2O:FA was hence selected as the preferred eluent for the remainder of the experiments.

Table 2. Recoveries of NLLGLIEAK after elution with a range of eluents, as represented by %recovery NLLGLIEAK ± standard dev.[1]

Eluent	Recovery (%)	RSD (%)
7.5:92.5 MeCN:H_2O	2.5	173
7.5:92.5 MeCN:H_2O (0.1% FA)	6.2	173
80:15:5 MeCN:H_2O:FA	84.8	14.1

[1]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).

AFFINITY OF mMIPs TOWARDS OTHER PEPTIDES

To evaluate the effectiveness and selectivity of the optimised 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 (Fig. 5).

The wash fraction had normalised 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 normalised 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 towards 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: ALGNQQPSWDSEDSSNFK 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. ALGNQQPSWDSEDSSNFK, 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 ALGNQQPSWDSEDSSNFK it will have a higher affinity for an eluent with a high MeCN content. A consequence of ALGNQQPSWDSEDSSNFK 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.

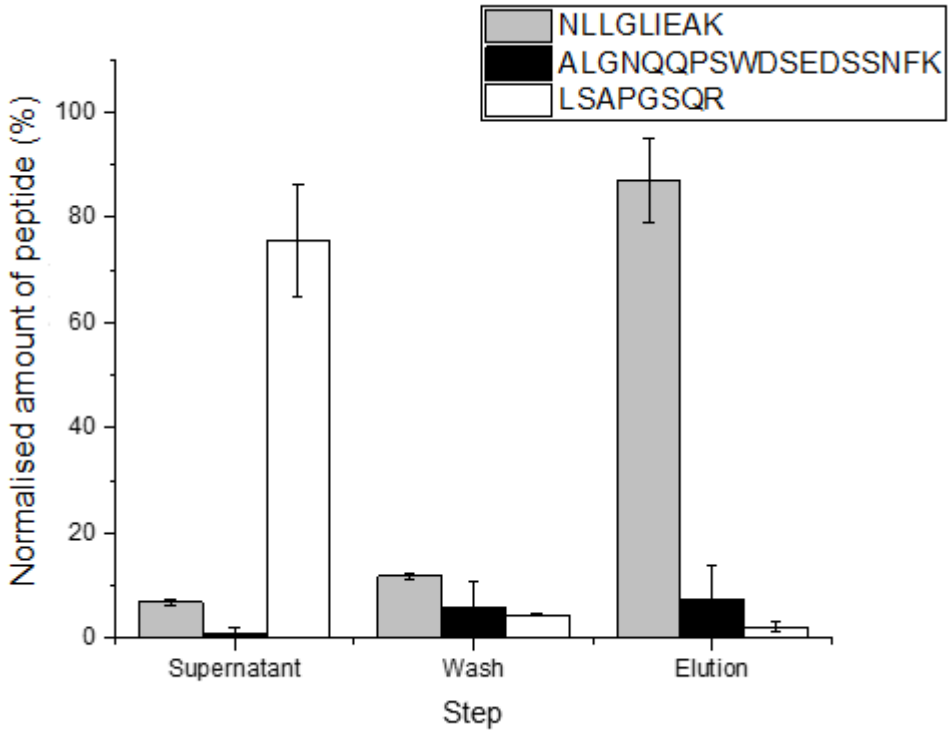


Figure 5. Evaluation of the selectivity of each step in the mSPE method using digested ProGRP, as represented by normalised amount of peptide (%) ± standard deviation of three ProGRP peptides for the three steps. Samples consisted of ProGRP (182 nM) and NLLGLIEAK IS (5 nM) in ABC (50 mM) containing 10 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).

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 optimised method. Initially, the recovery of the target for this extraction of a complex matrix was low (5.6 ± 0.5%; Fig. 6). This is most likely due to the high abundant, non-target peptides binding non-selectively 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 3x600 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 non-magnetic MIPs[25] as well as antibody-based clean-up 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.

554 An estimate of the detection and quantification limits (LOD and LOQ, respectively) was
555 carried out based on the signal intensity of NLLGLIEAK after analysis of the spiked serum
556 sample. LOD (S/N=3) and LOQ (S/N=10) were estimated to be 39 pM and 129 pM,
557 respectively. This is significantly lower than the LOD reported for crushed and ground MIP
558 particles packed into SPE-cartridges (LOD 625 pM)[25] and of the same order of magnitude as
559 reported for MIP microparticles applied in on-line SPE (LOD 11 pM).[7] The observed LOD is
560 5.6 times higher than the upper reference level for humans in humans[24], but this should be
561 within reach after further optimisation of the mSPE method and/or use of a more sensitive LC-
562 MS/MS system. The recovery is most likely affected by two factors: limited binding capacity
563 and interference from matrix components. In respect of interference from matrix components,
564 the mMIP is likely to interact with many abundant tryptic peptides in the matrix, as has been
565 observed previously for MIPs with similar compositions targeting NLLGLIEAK.[7,32] It is
566 expected that use of mMIPs with higher binding capacities will yield higher recoveries despite
567 non-specific interactions of the matrix components (N.B. mMIP C was synthesised using a
568 template to crosslinker mole ratio in the feed of 1:533, thus there is significant scope for
569 preparing mMIPs with significantly higher binding capacities, if desired, by increasing the
570 template to crosslinker ratio during the polymer synthesis stage).

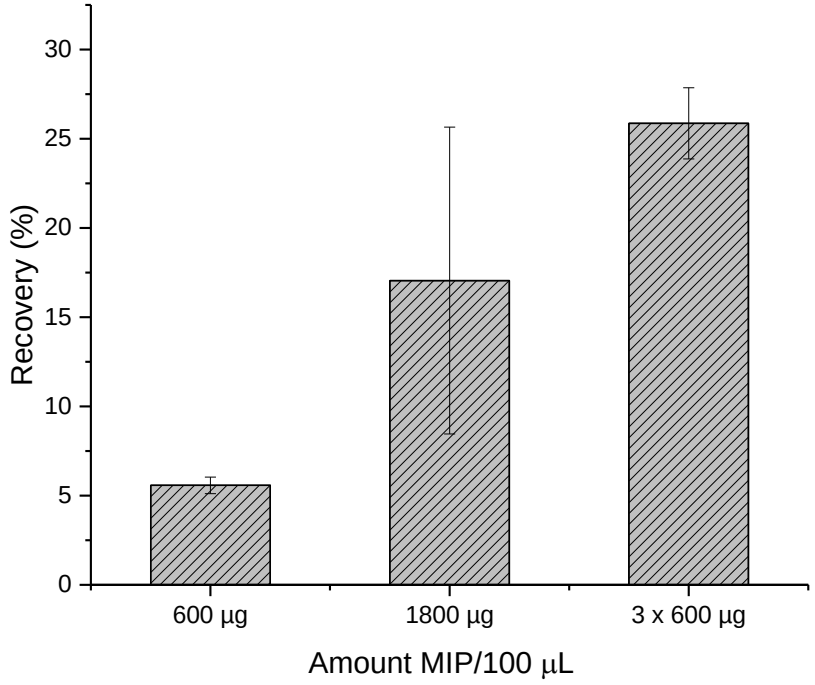


Figure 6. Recoveries of NLLGLIEAK from human serum using digested ProGRP, as represented by %recovery NLLGLIEAK ± standard deviation. Samples consisted of ProGRP (10 nM) and NLLGLIEAK IS (10 nM) in 50 µL serum diluted 1:1 in ABC (50 mM). Serum was digested with trypsin and samples were extracted for 5 min. with 600 µg, 1800 µg and 3x600 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).

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 magnetisation procedure that can be applied to a range of porous media, non-restricted to beaded materials. The magnetisation 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 optimisation 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 clean-up NLLLGIEAK 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 optimisation 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.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

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.

RAW DATA FILES

Raw data files have been uploaded to Peptide Atlas:

http://www.peptideatlas.org/PASS/PASS01592

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