Facilitating Serum Determination of Neuron Specific Enolase at Clinically Relevant Levels by Coupling On-line Molecularly Imprinted Solid-Phase Extraction to LC-MS/MS

Nicholas McKitterick[1], Tugrul Cem Bicak[2], Peter A. G. Cormack[2,*], Léon Reubsaet[1] and Trine Grønhaug Halvorsen[1,*]

[1] Section for Pharmaceutical Chemistry, Department of Pharmacy, University of Oslo, PO Box 1068 Blindern, 0316 Oslo, Norway

[2] WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, Thomas Graham Building, 295 Cathedral Street, Glasgow G1 1XL, Scotland, UK

Corresponding authors:

Trine Grønhaug Halvorsen
Section for Pharmaceutical Chemistry
Department of Pharmacy
University of Oslo
P.O. Box 1068 Blindern
0316 Oslo
Norway
E-mail: trinegha@farmasi.uio.no
Phone: +47 22855735

Peter A. G. Cormack
WestCHEM, Department of Pure and Applied Chemistry
University of Strathclyde
Thomas Graham Building
295 Cathedral Street
Glasgow G1 1XL
Scotland
UK
E-mail: peter.cormack@strath.ac.uk
Phone: +44-(0)141-548 4951

Abstract

The identification and quantification of biomarkers is essential for the diagnosis, treatment, and long-term monitoring of many human diseases. In the present work, macromolecular synthetic receptors with pre-determined affinity and selectivity for the signature peptide of a prognostically significant small cell lung cancer (SCLC) biomarker - neuron-specific enolase (NSE) – were prepared in a porous polymer microsphere format using a template-directed synthesis strategy performed under precipitation polymerization conditions. The polymer microspheres were packed into short trap columns and then exploited as molecularly selective sorbents in a fully automated, on-line molecularly imprinted solid-phase extraction (MISPE) protocol. The on-line MISPE protocol was optimised with respect to the composition of the loading mobile phase, the flow rate, and the extraction time. The molecularly imprinted polymers (MIPs) showed high affinity and useful selectivity for the peptide target - the hexapeptide ELPLYR - compared to non-imprinted control polymers. The MIPs were able to retain the biomarker on-column for extraction times of up to 20 minutes, and the on-line MISPE method enabled complete recovery of the biomarker over the linear range 10-100 ng mL^{-1} when the biomarker was present in spiked buffer (R^2=0.994). For extractions of ELPLYR from very complex biological matrices, the recoveries of ELPLYR from reversed-phase SPE (RP-SPE)-treated and untreated digested human serum were 100.8 ± 6.2% and 61.6 ± 1.9%, respectively. Extractions of ELPLYR from spiked untreated digested serum were linear in the range of 7.5-375 ng mL^{-1} (R^2 = 0.99). The limit of detection (LOD) and limit of quantification (LOQ) for the biomarker in digested serum were estimated to be 1.8 ng mL^{-1} and 6.0 ng mL^{-1}, respectively, which is below the median reference level of NSE in humans (8.6 ng mL^{-1}). This work sets in place the basis for a new diagnostic tool for SCLC that is sensitive, robust, automated, and antibody-free, and which works very well with complex human plasma samples.

Keywords: on-line solid-phase extraction; molecularly imprinted polymers; liquid chromatography tandem mass spectrometry; low-abundant biomarkers; bottom-up proteomics

1.0 Introduction

The identification and quantification of biomarkers of disease is essential for the diagnosis, treatment, and long-term monitoring of many common health conditions, including cancers. In this regard, several thousand putative biomarkers have been reported in the literature to date, and many new biomarkers are discovered every single year [1]. The classical methods used for biomarker analysis are normally antibody-based methods, such as enzyme-linked immunosorbent assays (ELISA) [2]. Whilst such antibody-based methods are usually sensitive and rapid, the raising of antibodies against biomarkers can be an expensive undertaking. Furthermore, the lead-in times for the development and production of new antibodies tend to be lengthy, which means that new antibody production struggles to keep pace with the rapid rate of discovery of new biomarkers. Furthermore, antibody-based assays can give rise to false positive and false negative results, although this particular limitation can be addressed to some extent by combining antibody clean-up with mass spectrometric determination. Nevertheless, the development and exploitation of antibody-free strategies in combination with mass spectrometry for targeted, rapid, sensitive, and accurate biomarker detection is very timely and of critical importance to human health.

Molecularly imprinted polymers (MIPs) are synthetic polymers with unique physicochemical properties that allow the targeted capture of neutral or charged molecules [3]. When used as antibody binding mimics, MIPs can bind strongly and selectively to a range of targets, from small molecule analytes through to macromolecules such as proteins, and even to whole cells [4]. Most commonly, the affinity of a MIP for its target is based upon non-covalent intermolecular interactions, such as hydrogen bonding, however the size and shape of the binding sites within the MIP play a role too [5].

MIPs have been shown to be robust, reusable materials [6], and, compared to the production of typical single-use antibodies, they can be produced efficiently using streamlined synthetic protocols. This is a major reason why MIPs are attracting interest as cost-effective alternatives to antibodies for the

82 analysis of some biomarkers. These recent developments are in addition to the use of MIPs in a range
83 of other fields, including electrochemical sensors [7], drug delivery [8], protein crystallization [9],
84 catalysis [10], and in separation science (*e.g.*, solid-phase extraction, SPE) [11]. MIPs have been used
85 extensively for numerous applications in separation science for over 20 years [11]. Once optimised for
86 loading and elution solvents, the versatility of MIPs allows for successful off-line and on-line SPE for a
87 range of targets, including biomolecules [6]. On-line molecularly imprinted solid-phase extraction
88 (MISPE) includes the field of imprinted materials incorporated into appropriate physical formats for
89 packing into columns for on-line chromatographic analysis [12]. On-line MISPE in combination with
90 liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers a robust automated platform
91 for the analysis of biomarkers, and MISPE columns can be interfaced readily with both micro- and
92 nano-LC systems [13-15]. A key advantage of moving to an automated, on-line mode of operation is
93 reduced manual handling, which gives higher throughput and better reproducibility and repeatability
94 than the analogous off-line methods.

95 A key development of MIPs is the extraction of biomolecules from biological fluids (*e.g.*, human
96 serum). MIPs targeting many proteins and peptides have been developed. For proteins, the imprinting
97 strategy involves either whole protein imprinting or imprinting of a smaller fraction of the protein such
98 as the epitope or tryptic signature peptide. [16] Whole protein imprinting has been utilised for some
99 high abundant proteins, while imprinting of a smaller fraction of the protein is used more commonly
100 for low-abundant proteins [16]. Typically, the MIPs are exploited as microparticles and can be packed
101 into SPE columns used in combination with LC-MS/MS for the selective extraction and quantification
102 of low abundant protein and peptide biomarkers in complex matrices (*e.g.*, human serum) [17]. MIPs
103 targeting the signature peptide of the small cell lung cancer (SCLC) biomarker progastrin-releasing
104 peptide (ProGRP) were developed for use in on-line analysis [12] and used to extract the nonapeptide
105 NLLGLIEAK from human serum, including from serum samples from patients diagnosed with SCLC.
106 ProGRP is the most selective biomarker known for the detection and follow-up of SCLC (*i.e.*, most
107 patients testing negative are not in a diseased state); however, in the clinic, ProGRP is usually

determined in parallel with a second SCLC biomarker, neuron-specific enolase (NSE). NSE is present in elevated levels in a range of cancers (*e.g.*, neuroblastoma, melanomas, and breast cancers) [18], however the combined determination of NSE and ProGRP improves the accuracy of the SCLC diagnosis. MIPs targeting NSE have been developed for use in electrochemical sensors [19], but not for off-line or on-line MISPE work. A method determining NSE from serum using LC-MS/MS and the bottom-up approach after immunocapture of the intact protein has been developed previously [18]. In this work, the peptide ELPLYR was identified as a suitable signature peptide owing to its specificity combined with strong ionisation in electrospray ionisation. Therefore, ELPLYR presents an attractive template for MIPs for use in NSE determination as well.

The objective of the present work was to develop MIPs in an appropriate physical format for use as molecularly selective sorbents in on-line MISPE, for the capture of the signature peptide of NSE (the hexapeptide ELPLYR) from biofluids. To this end, precipitation polymerisation was used to deliver high quality, molecularly imprinted polymer microspheres that could be packed directly into trap columns. Subsequently, an on-line MISPE methodology was devised and optimised with a view to extracting ELPLYR from complex matrices, thereby validating the potential of MIPs for the analysis of NSE in human serum. Success will represent a significant advancement to the state-of-the-art and diagnostic power since it will now be possible to determine two diagnostically important SCLC biomarkers in human serum by MIP-based enrichment of signature peptides followed by LC-MS/MS.

2.0 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

The peptide template (Z-ELPLY[Nle]F, 98.51%) was purchased from LifeTein (Somerset, NJ, USA). 1,2,2,6,6-Pentamethylpiperidine (PMP, 99%), tetrabutylammonium hydroxide solution (TBA.OH, 1.0 M in methanol, 25%), acetonitrile (ACN, 99%), divinylbenzene-80 (DVB-80, 80% DVB isomers and 20%

ethylvinylbenzene isomers), tetrahydrofuran (THF, 99%), methanol (MeOH, 99.8%), dimethyl sulfoxide (DMSO, 99%), hydrochloric acid (HCl, 37%), formic acid (FA, MS grade, ≥98), dithiothreitol ((≥98%, DTT) and iodoacetic acid (≥98%, IAA) were purchased from Sigma Aldrich, St Louis, MO, USA. *N*-3,5-*bis*(Trifluoromethyl)-phenyl-*N*'-4-vinylphenylurea (TPVU, purity >95%) was kindly donated by Malmö University. 2,2'-Azo*bis*isobutyronitrile (AIBN, ≥98%) was purchased from BDH (Dubai, UAE). Acetonitrile LC-MS grade (MeCN, 99.9%), methanol LC-MS grade (MeOH, 99.9%), and acetic acid (AcOH, 100%) were purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (BioUltra, ≥99.5%) was purchased from Fluka (Milwaukee, WI, USA). Purified NSE (γγ-dimer, Protein ID: P09104) was obtained from Scripps Laboratories (San Diego, CA, USA). Stable isotope labelled internal standard (IS) peptide ELPLY[R_$^{13}C_6{}^{15}N_2$] (>95%) was purchased from Innovagen (Lund, Sweden). Bovine serum albumin (BSA) and trypsin (TPCK-treated) from bovine pancreas (sequencing grade) were purchased from Sigma Aldrich. Water was filtered through a Merck Millipore Milli-Q Integral 3 water dispenser (resistivity: 18.2 MΩ cm). Human serum from consenting healthy individuals was obtained from Oslo University Hospital, Ullevål (Oslo, Norway). All serum samples were stored at -32 °C.

2.2 PREPARATION OF PROTEINS, STANDARD SOLUTIONS, AND PURIFICATION OF REAGENTS

NSE concentration was determined *via* UV absorbance (280 nm, A280) and the NSE stock diluted to the desired concentrations with 50 mM ammonium bicarbonate (ABC) and stored at -20 °C.

A stock solution of the IS peptide was prepared in water at a concentration of 10 mM. The standards were diluted in 50 mM ABC for further use.

DVB-80 was passed through a short column of neutral alumina prior to use, and AIBN was recrystallized from acetone at low temperature.

2.3 MIP SYNTHESES

See *Supplementary Information* for the MIP characterization data.

MIP 1 was prepared *via* precipitation polymerization using an adaptation of literature methods: To a borosilicate Kimax tube was added Z-ELPLY[Nle] (6.6 mg, 7.5 µmol), PMP (1.8 µL, 10 µmol), DVB-80 (1.01 g, 1.1 mL, 7.74 mmol), MeCN (20 mL) and THF (2.5 mL), and the mixture ultrasonicated for two minutes until a clear solution was obtained. TPVU (26.2 mg, 70 µmol) and TBA.OH (70 µL, 70 µmol) were then added, and the mixture ultrasonicated for a further two minutes to dissolve all components. Then, AIBN (19.7 mg, 120 µmol) was added and the mixture ultrasonicated for an additional five minutes and purged with oxygen-free nitrogen gas for 10 minutes at ambient temperature prior to sealing of the reaction vessel under nitrogen. The sealed tube was then placed into a pre-heated incubator (UVP hybridizer oven, Analytik Jena, Jena, Germany) at 60 °C, and left for 48 h at a rotation speed of ~ 8 rpm, during which time a milky suspension of polymer microspheres formed. After cooling to room temperature, the polymer microspheres were isolated from the reaction medium by vacuum filtration on a 0.45 µm nylon membrane filter and washed sequentially with MeCN (50 mL), MeOH/0.1 M aq. HCl (90/10, v/v, 50 mL) and MeOH (50 mL). Finally, the product was dried overnight in a vacuum oven (60 °C, 50 mbar) to constant mass (yield: 49%). The corresponding non-imprinted polymer (NIP), NIP 1, was synthesized and isolated using the same procedure, except that the template and PMP were omitted from the synthetic protocol (Yield: 45%).

MIP 2 was prepared similarly to MIP 1, however higher amounts of TPVU (13.1 mg, 35 µmol) and TBA.OH (35 µL, 35 µmol) were used and the yield of product was 51%. The corresponding NIP, NIP 2, was synthesized and isolated using the same procedure as MIP 2, except that the template and PMP were omitted from the synthetic protocol (Yield: 48%).

2.4 COLUMN PACKING

Each polymer (MIP 1 and MIP 2 and their corresponding NIPs) was wet packed into 1.5 x 5 mm PEEK cartridges. For this, each polymer (6 mg) was suspended in 380 µL MeCN prior to ultrasonication for

five minutes. The polymers were vacuum-packed using heptane as the packing solvent at 100 bar. Each packed column was inspected under an optical microscope and asymmetry analysis performed to evaluate the packing quality (*see Supporting Information*).

2.5 ON-LINE MISPE-LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

The chromatographic system consisted of an ISO-3100 A loading pump, an LPG-3400 M pump with a degasser, a WPS-3000TRS autosampler, and an FLM3000 flow-manager (all Dionex, Sunnyvale, CA, USA). Extraction was performed by a MIP trap column. The chromatographic separation was carried out using an Aquasil C_{18} analytical column (Thermo Scientific, 100 Å, 3 µm, 50 mm × 1 mm). The extraction was performed by injection of 2 µL of sample onto the MIP trap column. The MIP was then washed using an isocratic flow of 97:3 20 mM FA:MeCN at 20 µL min^{-1} for 10 min (see set-up in Fig. 1A). Following extraction, the system was switched to forward-flush the sample through the MIP column, the analytical column, and finally MS analysis. The microflow pump was directed to waste and kept at an isocratic flow of 50 µL min^{-1} at 100% mobile phase A (20 mM FA and MeCN 95:5, v/v) during extraction (*i.e.*, for the first 10 min). After 10 min of loading, the chromatographic separation was performed using a 20 min linear-gradient from 0 to 85% mobile phase B (20 mM FA and MeCN 5:95, v/v) after 3 min isocratic elution by mobile phase A. After the gradient was run, the column was washed for 3 min with 90% mobile phase B and re-equilibrated with mobile phase A for 20 min. The column temperature was set and kept constant at 25 °C.

A triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo Scientific, Waltham, MA, USA) was used to determine the signature peptide and its corresponding IS by selected reaction monitoring (SRM) in positive mode. The following transitions pairs were monitored: for NSE- γ signature peptide ELPLYR, 395.7 → 274.7 and 395.7 → 548 with collision energy (CE) of 14 V; for the ELPLYR IS, 401.0 → 279.7 and 401.0 → 558.3 with a CE of 14 V. The heated capillary temperature was set at 265 °C, and

the spray voltage was 4.0 kV. Auxiliary gas (N_2): 10 arb., sheath gas (N_2): 5 arb. 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.

2.6 METHOD OPTIMISATION

Retention factor (k) calculation

k was calculated using the following equation:

$$\mathrm{k} = (t_R - t_0)/t_0$$

Where t_R = Retention time, t_0 = hold-up time

2.6.1 Optimization of loading mobile phase composition and flow rate

MIP 1 and MIP 2 (and their corresponding NIPs) were coupled directly to the MS (see Fig. 1B) and 2 µL of ELPLYR IS was injected directly onto the MIP/NIP columns. This LC system consisted of the ISO-3100 A loading pump, the WPS-3000TRS autosampler, and the FLM3000 flow-manager. The loading mobile phases consisted of three different ratios of 20 mM FA and MeCN (100:0, 95:5, and 90:10 v/v). Three different flow rates were used: 20, 30, and 40 µL min^{-1}.

2.6.2 Fine-tuning of the loading mobile phase composition

The loading mobile phase was optimized further using MIP 1 and NIP 1. As above, 2 µL of ELPLYR IS was injected directly onto the columns. The loading mobile phase consisted of 100:0, 99:1, 98:2, 97:3, 96:4 and 95:5 v/v 20 mM FA and MeCN. The flow rate was set to 20 µL min^{-1}.

2.6.3 Optimisation of loading time (extraction time)

The loading time was evaluated using MIP 1 as a trap column (Fig. 1B). ELPLYR IS (2 µL) was injected onto MIP 1 using the loading mobile phase consisting of 97:3 20 mM FA:MeCN at 20 µL min^{-1}. The following loading (extraction) times were evaluated: 5, 7.5, 10, 12.5 and 20 min (n=3). Digested BSA peptides (50 nM, 2 µL) were analysed at 5, 10, and 20 min to evaluate the selectivity and optimal extraction time for sample clean-up. (For BSA MS transitions see *Supporting Information* Table S1)

2.6.4 Imprinting factor (IF) calculation

IFs were calculated for each set of conditions using the k values for each MIP and its respective NIP using the following equation:

$$IF = \mathrm{k}\,(MIP)/\mathrm{k}\,(NIP)$$

2.7 METHOD EVALUATION

2.7.1 Protein digestion

NSE-γ standard solutions were diluted by ABC to a volume and concentration of 470 µL and 53.2 µg mL^{-1}, respectively. 10 µL of 2.5 mM DTT (freshly prepared in ABC buffer) was added to the protein mixture and incubated at 60 °C (800 rpm) for 20 min. Afterward, the solution was cooled, and 10 µL of 10 mM IAA (freshly prepared in ABC buffer) was added. Incubation was carried out for 15 min at room temperature (800 rpm) in the dark. Digestion was initiated by the addition of trypsin (10 µL, 125 µg mL^{-1} in 50 mM ABC) for an enzyme to substrate ratio of 1:20 (w/w) overnight at 37 °C. The resulting digest had a final concentration and volume of 50 µg mL^{-1} and 500 µL, respectively. The digest was divided into 10 µL aliquots and stored at -32 °C until further use.

BSA standards were diluted with ABC (50 mM) to a final volume and concentration of 500 µL and 50 µg mL^{-1}, respectively. 2.5 µL of 50 mM DTT (freshly prepared in ABC buffer) was added to the protein mixture and incubated at 60 °C (800 rpm) 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 (800 rpm) in the dark. Digestion was then accomplished by adding trypsin at an enzyme to substrate ratio of 1:20 (w/w) overnight at 37 °C.

2.7.2 NSE digest extractions

The on-line MISPE method was evaluated by determining the recovery and linearity of an NSE digest. For recovery determination, NSE digest 100 ng mL^{-1} (10 µL) was diluted to a final concentration of 10 ng mL^{-1} by 50 mM ABC containing 25 nM of digested BSA (90 µL) (n=3). For linearity determination, NSE digest (50 µg mL^{-1}) was diluted to 10, 25, 50 and 100 ng mL^{-1} (n=3) with 50 mM ABC containing 100 nM digested BSA. The samples were injected (2 µL) onto MIP 1. The loading/extraction time was set to 10 min followed by gradient elution and analysis. To determine the extraction recovery, a control sample of 25 ng mL^{-1} of NSE digest in 50 mM ABC was analysed without the presence of the MIP column to determine the signal w/o MIP (=100% recovery).

2.8 COMPLEX MATRIX EXTRACTIONS

2.8.1 RP-SPE treatment of digested serum

For analyses of more complex matrices, RP-SPE treated serum was selected as a sample with complexity intermediate between a simple NSE digest and untreated digested serum. An in-house RP-SPE treatment was performed as described previously [20]. Briefly, SPE tips were made in-house by punching out six discs of C8 3M Empore material (Teknolab AS, Kolbotn, Norway) and packing them into the lower part of a 300 µL bevel point pipette tip (VWR, Hanover, Germany) using a metal wire. The SPE material was activated with 100 µL of MeCN followed by 100 µL of 20 mM FA. Digested serum (50 µL) was transferred to the SPE tip. The tips were then washed with 100 µL of 20 mM FA before being eluted with 100 µL of MeCN/0.01% (v/v) TFA in the ratio 80:20. The eluent was evaporated to dryness at 60 °C under N_2 gas and reconstituted in 50 µL of 50 mM ABC.

2.8.2 Human serum digestion

Human serum was defrosted at 4 °C for 1 h. 200 µL of serum was diluted 1:1 with 100 mM ABC before the addition of 200 mM freshly prepared DTT (10 µL) in 50 mM ABC. The samples were incubated at 60 °C for 1 h. and allowed to cool to room temperature before the addition of 10 µL of freshly prepared 500 mM IAA in 50 mM ABC. The samples were incubated at room temperature (800 rpm) for 30 min in the dark prior to the addition of 70 µL of 10 mg mL^{-1} trypsin freshly prepared in 50 mM ABC. The samples were digested overnight at 37 °C. Digested serum was frozen at -20 °C until required.

2.8.3 Determination of recoveries in complex matrices

Recoveries were evaluated in two complex matrices: NSE digest spiked into RP-SPE-treated digested human serum, and NSE digest spiked into digested serum. NSE digest (1875 ng mL^{-1}; 10 µL) was spiked into 90 µL of RP-SPE-treated digested human serum or 1:1 diluted (in 50 mM ABC) digested human serum for a final NSE digest concentration of 375 ng mL^{-1}. To determine the extraction recoveries, a control sample of 187.5 ng mL^{-1} of NSE digest in 50 mM ABC was analysed without the presence of the MIP column to determine the signal w/o MIP (=100% recovery). Following this, each sample type (n=3) was injected onto the standard trap system and the relative signal intensity used to determine recoveries.

2.8.4 Linearity, LOD and LOQ

Digested serum samples (40 µL) were spiked with 5 µL of various concentrations of digested NSE and 5 µL of ELPLYR IS to final concentrations of 7.5, 37.5, 75, 187.5, and 375 ng mL^{-1} NSE and 2 ng mL^{-1} of IS and weighted 1/x (n=3). The signal (S) of ELPLYR in the 7.5 ng/mL digested NSE in digested serum sample and the noise (N) in the digested blank serum sample were used to calculate the LOQ (S/N = 10) and LOD (S/N = 3). The respective chromatograms are seen in Fig. S2.

3.0 Results and Discussion

3.1 TEMPLATE DESIGN AND OPTIMIZATION OF MIP SYNTHESES

Precipitation polymerization (PP) is a surfactant/stabilizer-free method for synthesising high quality crosslinked polymer microspheres in good yields, with good control over microsphere diameter, chemical functionality, and porous morphology. Typically, monomers are polymerised under dilute conditions in near-theta solvents, and products with narrow particle size distributions and bead diameters in the low-micron size range are formed. Such products are very well-suited for high-performance chemical separation work, including as stationary phases in LC and as SPE sorbents. It was for this reason that PP was the polymer synthesis method of choice in the present work.

ELPLYR (*i.e.*, H-Glu-Leu-Pro-Leu-Tyr-Arg-OH) (Fig. 2A) was selected as the signature peptide for NSE based on a previous study which used this peptide as a surrogate for the determination of NSE from serum [18]. The peptide is specific for the γ-subunit of NSE which is present in both the heterodimeric (αγ) and homodimeric (γγ) form of NSE. The sequence ELPLYR (AA 127-132) is found on the outside of each the γ-monomer. Therefore, it may be more readily accessible to trypsin during digestion, improving cleavage efficiency, and generating a more intense signal during analysis. ELPLYR was not used as the template in the production of MIPs because bleeding/leaching of residual template from ELPLYR imprinted polymers would be expected to occur during exploitation of such MIPs for ELPLYR capture from tryptic digests, and bleeding/leaching of the template, where the template is structurally identical to the analyte, is unacceptable in ultra-trace analyses where the analyte concentration is very low (N.B. the reference levels of NSE in humans in 8.6 ng/mL). A template analogue approach must, therefore, be used. In the design of a suitable template analogue for ELPLYR, some of the key considerations for the template analogue were: 1) Structural similarity to ELPLYR; 2) Cost and availability; 3) Solubility (in the porogens preferred for MIP production by PP); 4) Conformation and conformational rigidity; 5) Stability; 6) Functional groups available for templating. In a tryptic digest, any peptides present which contain C-terminal arginine are expected to bind appreciably and in a non-selective manner to a MIP prepared using a peptide template where the C-terminus of the template is arginine and this residue was targeted *via* a non-covalent molecular imprinting protocol. Thus it was

317 decided to mutate the C-terminal arginine and build selectivity into the MIPs through amino acid
318 residues other than the C-terminal arginine.

319 Based on the above considerations, the peptide used as a template analogue for the NSE signature
320 peptide was Z-ELPLYR, where Z = Cbz = Carboxybenzyl (a protecting group for the peptide N-terminus),
321 and Nle = norleucine (Fig. 2B). Points to note: 1) The introduction of the Z group, and the change of
322 Arg to Nle, is expected to increase the solubility of the template in the porogens preferred for non-
323 covalent molecular imprinting and PP; 2) The ionisable groups in the template analogue are all
324 Brønsted acidic (the Brønsted basic groups present in H-ELPLYR-OH have been either protected or
325 replaced); 3) The Glu side-chain and the C-terminal carboxylic acid are useful functional group handles
326 for templating. Urea-based monomers for oxyanion binding are obvious functional monomer (FM)
327 candidates; 4) The Tyr side-chain is also a potentially useful functional group handle for templating.

328 Establishing an appropriate ratio of FM to crosslinker is an important part of MIP design for SPE
329 applications. If too little crosslinker is used then the polymers may not be porous in the wet state and
330 access to binding sites may be impeded or prevented. On the other hand, whilst higher crosslinker
331 levels are more likely to yield permanently porous materials, this may be at the expense of lower
332 nominal binding capacity. Appropriate ratios of FM to crosslinker are normally arrived at through a
333 process of trial-and-error. Given this background, the approach was to increase the relative amount
334 of FM in the polymer network in a stepwise fashion in order to identify the best performing MIP,
335 however when the FM content in the monomer feed was at a FM to crosslinker mol ratio of 1:50, the
336 polymer particles formed through polymerisation started to aggregate and high quality particles could
337 no longer be obtained (probably due to the less effective self-stabilisation of DVB particles bearing
338 urea moieties derived from the FM). Therefore, we decided to synthesise and evaluate two different
339 sets of MIPs (and their corresponding NIPs) where the ratios of FM to crosslinker in the monomer feed
340 were around 1:200 and 1:100.

3.2 METHOD OPTIMISATION

3.2.1 Initial testing of MIP/NIP pairs #1 and #2

The initial round of testing of the MIP columns focused on optimising the composition of the loading mobile phase and the flow rate. For this, each column was coupled directly to the MS using a single pump (Fig. 1A), and ELPLYR IS (5 nM) in 50 mM ABC injected (2 µL) onto each column (n=3). For each column, the k values of ELPLYR IS were determined under a range of conditions (mobile phases of different elution strength were used, and the flow rates were varied) to establish the chromatographic conditions under which the MIP could bind the target strongly and selectively. To establish binding selectivity imparted into the MIPs through templating, retention factors were measured in parallel for non-imprinted control polymers, and then an imprinting factor (IF) calculated for each set of conditions. The initial optimization of the composition of the loading mobile phase was performed using three different ratios of 20 mM FA and MeCN (v/v) since mixtures of 20 mM FA and MeCN were shown to be effective mobile phases in earlier on-line MISPE work targeting tryptic peptides [12]. Similarly, the flow rates investigated (20, 30, and 40 µL min^{-1}) were based on earlier studies, with faster flow rates being preferred provided that shorter analysis times are not at the expense of analyte recovery.

The first mobile phase evaluated was 100% 20 mM FA (data not shown). It was found that there was no observable MS signal at any flow rate on either of the MIPs or NIPs, even after one hour of isocratic flow. This showed that both the MIPs and the NIPs retained the peptide strongly (no selectivity), which is unsurprising since non-selective binding is expected to dominate for divinylbenzene-based polymers when the mobile phase is fully aqueous. However, the peptide could be eluted off the polymers in the subsequent washing step, so the peptide is not bound irreversibly.

Subsequently, mixtures of 20 mM FA and MeCN (at ratios 95:5 and 90:10 (v/v)) were evaluated as the loading mobile phase. The k values are high right across the board, and range from 14.4 to 21.7, which

shows high affinity of the polymers for the target. When comparing the two MIPs (MIP 1 and MIP 2), MIP 1 has a consistently higher affinity for the target, and the difference between the polymers is greatest for the mobile phase with the highest organic content (90:10 20 mM FA:MeCN (v/v)). It was also found that the k values were influenced very little by the flow rate, which suggested that the contact time between polymers and peptide was sufficiently long for all flow rates investigated. The highest k value was obtained for MIP 1 at a flow rate of 20 µL min^{-1} when using 95:5 20 mM FA:MeCN (v/v) as the mobile phase (21.7±1.3). Under the same mobile phase and flow rate conditions, the corresponding NIP 1 had a k value of 19.8±0.3, which gives an imprinting factor of 1.1. Whilst this is a modest imprinting factor, the affinity of MIP 1 for the target is high, and high levels of non-specific binding to the non-imprinted sorbents arise due to a combination of hydrophobic effects and hydrogen bonding interactions between carboxylate groups in the target and the pendent urea moieties that are distributed randomly throughout the polymers.

For MIP 2 and NIP 2, the k values were also found to be highest when using 95:5 20 mM FA:MeCN (v/v) as the loading mobile phase at a flow rate of 20 µL min^{-1} (20.1±0.7 and 19.40±0.3, respectively), however, the IF value was lower (IF=1.04.). Thus, whilst affinity remains high there is a fall in selectivity. When the amount of MeCN in the mobile phase was increased from 5% to 10% (*i.e.*, to 90:10 20 mM FA:MeCN v/v) the k values decreased somewhat for all MIPs and NIPs which is consistent with a disruption of non-selective binding *via* hydrophobic effects. For all the mobile phases and flow rates evaluated, there is no significant difference (two-sided T-test, $P \geq 0.05$) in k values between any MIP and the corresponding NIP except for MIP 1 and NIP 1 at a flow rate of 40 µL min^{-1} when using 90:10 20 mM FA:MeCN (v/v) as the mobile phase ($P = 0.0009$). However, with this mobile phase, the k values for MIP 1 (16.5±0.3) is up to 25% lower than that obtained when using the 95:5 20 mM FA:MeCN (v/v) mobile phase, and higher retention factors are normally preferable when working with complex samples where there will be more competition for binding sites.

In this initial round of testing, MIP 1 turned out to be the most promising polymer; it had a high affinity for the target together with a degree of selectivity. To improve the selectivity it was decided to fine-tune the mobile phase composition in the range 100:0 to 95:5 20 mM FA:MeCN (v/v). For this part of the MISPE optimisation, the flow rate was fixed at 20 µL min^{-1}.

3.2.2 Fine-tuning of the loading of MIP 1

The fine-tuning of the mobile phase composition for the loading of MIP 1 was performed using mobile phases containing 95:5, 96:4, 97:3, 98:2, and 99:1 ratios of 20 mM FA:MeCN (v/v). Similarly to the results obtained when the mobile phase was 100% 20 mM FA (*i.e.*, under fully aqueous loading conditions), ELPLYR was not eluted within one hour when using 98:2 and 99:1 20 mM FA:MeCN (v/v) as the mobile phase (data not shown). However, for the three other mobile phase compositions investigated, there was a clear trend of increasing k values with decreasing MeCN. At 95:5, 96:4 and 97:3 20 mM FA:MeCN (v/v), the k values recorded for MIP 1 increased steadily from 19.5±0.1 to 20.3±0.4 to 23.8±0.6, respectively (Fig. 3). NIP 1 showed a similar, but weaker, trend, with k values of 18.6±0.1, 19.1±0.3, and 20.6±0.4, respectively. For all three of these mobile phases a significant difference between the MIP 1 and NIP 1 k values was seen (P = 0.014-0.0005). When loading with 97:3 20 mM FA:MeCN (v/v), the highest k value on MIP 1 was obtained (23.8), giving an IF of 1.2. This is the highest IF obtained across all the conditions tested, and is slightly higher than the IF reported for earlier work concerning on-line-MISPE of tryptic peptides [12]. Accordingly, for the further optimisation work the mobile phase was fixed as 97:3 20 mM FA:MeCN (v/v), since strong affinity and useful selectivity were apparent under these conditions.

3.2.3 Loading time (extraction time)

The loading (extraction) time during on-line analysis is critical for ensuring maximal retention of the analyte of interest while simultaneously reducing the sample complexity as much as possible before analysis. This process ensures maximum sensitivity due to reduced matrix effects in the MS. A pragmatic balance must be struck between the duration of the extraction step, the recovery of the

target analyte, and the overall analysis time. To evaluate the loading (extraction) time, MIP 1 was used as a trap column and coupled to a triple quadrupole MS, as described in the Experimental (Fig 1A).

The recovery of ELPLYR when using a 5 min loading time was 95.3±1.2% (Table 1). The recovery increased to 100 % when loading for 7.5 min or longer. Thus MIP 1 can retain ELPLYR exceptionally well (there was no loss of peptide after 20 min, which equates to approximately 40 column volumes). However, to investigate in more detail the selectivity of the MIP for ELPLYR over other peptides, digested BSA was loaded onto the MIP.

The recovery of seven tryptic BSA peptides was determined using three different loading times (5, 10, and 20 min; Table 2). With a loading time of 5 min, the recoveries ranged from 9.5-107.5%, with an average recovery of 66.5±12.4%. When the loading time was doubled to 10 min, the average recovery was 59.3±6.3%. Increasing the loading time yet further to 20 min resulted in an average recovery of 55.0±5.4%. An average recovery of 55-67 % for the seven tryptic BSA peptides, compared to essentially quantitative recovery of the target, implies moderate selectivity for ELPLYR, however only one peptide was retained as strongly as ELPLYR, LGEYGFQNALIVR, and this is most likely due to its isoelectric point (pI = 6.1) being comparable to ELPLYR (6.0). Since there were no significant differences in non-specific binding as a function of extraction time, 10 min was selected as the optimum extraction time since the recovery of ELPLYR was 100% even after washing with over 20 column volumes of mobile phase.

3.3 INITIAL EVALUATION OF ANALYTICAL PERFORMANCE

The initial evaluation of the method involved determining the recovery and linearity to establish the binding capacity of the MIP in simple matrices. This gives an insight into the potential downstream compatibility of the MIP with extractions of complex matrices. The extraction recovery of ELPLYR was determined to 100.4±5.0% in a sample of digested NSE in 50 mM ABC containing digested BSA (10 times molar amount). Linearity was determined by injecting digested NSE in the range 10-100 ng mL^{-1} in 50 mM ABC containing a 100 nM digested BSA (n=3) onto the MIP column. Linear regression was

obtained by plotting the intensity of ELPLYR *vs*. the added concentration. The correlation value (R^2=0.99) was within acceptable limits and is higher than for previously reported correlation columns [12].

3.4 EXTRACTIONS FROM COMPLEX MATRICES

Extractions from matrices of increasing complexity were evaluated to determine the compatibility of the on-line MISPE method with real biological samples and to verify the need to use a MIP for extraction when analysing complex samples. The latter was investigated as the MIP and NIP had shown similar retention factors of ELPLYR in extractions from simple matrices. Digested NSE was spiked into digested human serum pre-treated by off-line RP-SPE and also into untreated digested human serum; the recovery of ELPLYR was then determined for the two sample types (Fig. 4) using the MIP as a trap column; the latter sample was also analysed using the NIP as a trap column. When using the MIP as a trap column, the extraction recovery from RP-SPE treated serum was 100.8±6.2%. Therefore, the extraction efficiency was comparable to those in a simple matrix. MIP extractions from the most complex matrix (digested serum), on the other hand, showed a lower but still acceptable recovery of ELPLYR (61.6±1.9%). In comparison, when the NIP was used to extract digested NSE from serum, the recovery dropped to 25.2±1.7%, demonstrating the effect of the imprinting in extraction from complex matrices.

The high recovery of ELPLYR from RP-SPE treated serum demonstrates that the MIP can enrich low abundant ELPLYR even when it is in the presence of high numbers of non-specific peptides. The recovery of ELPLYR from untreated, 1:1 diluted serum is lower than for the less complex matrices. The recovery is, however, within the ranges reported for other MIPs targeting proteins: A MISPE method targeting the signature peptide NLLGLIEAK (from the SCLC biomarker ProGRP) gave a recovery of <25% in precipitated serum [17], and an epitope imprinted on-line MISPE method targeting the sequence MIQRTPKIQ from beta2-microglobulin showed recoveries of ≥83% in spiked serum samples (μg mL^{-1} range). An NSE-imprinted electrochemical sensor based assay reported recoveries between

approximately 96-100% in 1:100 diluted serum [19]. Most likely, the dilution factor of the latter method reduces the complexity sufficiently to ensure complete recoveries. Nevertheless, MIP 1 has excellent compatibility with complex matrices, such as minimally diluted serum, and is therefore likely to be able to analyse NSE in serum at levels below the clinical reference range. In addition, since the recovery drops when the MIP is replaced by the NIP when extracting complex samples, then imprinting is likely to play an essential role in the extraction. The lack of selective binding sites in the NIP leads to the drop in recovery as ELPLYR must compete with the myriad tryptic peptides in the digested serum for interaction with the solid-phase.

The efficient extraction of ELPLYR for complex matrices using MIP demonstrates the value of MIPs as potential tools in diagnostics as well as aiding in biomarker validation.

3.5 ANALYTICAL EVALUATION IN COMPLEX MATRICES

The analytical performance was evaluated by determining the linearity, LOD, and LOQ of NSE in digested human serum (n=3). The method is linear in the disease range (3.4–344.2 ng mL^{-1}) [21], and the linear regression was within acceptable limits (R^2>0.99, slope 0.0026, intercept -0.0019). The LOD and LOQ were estimated to be 1.8 (S/N=3) and 6.0 ng mL^{-1} (S/N=10), respectively, which is a very significant finding because the LOQ is below the median reference levels in humans (8.6 ng mL^{-1}) [22]. Therefore, the on-line MISPE method has great potential in NSE analysis and quantification, subject to efficient digestion of the complex samples. Presently, the digestion efficiency of NSE is approximately 50%, therefore further improvements in the digestion could yield up to two-fold reductions in the LOD and LOQ. Furthermore, the use of more contemporary MS systems in combination with nanoflow LC are expected to reduce the LOD and LOQ yet further. Irrespective of the further refinements that are possible, the on-line MISPE protocol developed and presented here can quantify NSE below the reference level in human serum. The good performance of MIPs in contact with complex matrices demonstrates the potential value MIPs have in diagnostics.

4.0 Conclusions

The present work demonstrates the successful design, synthesis, and exploitation of robust synthetic receptors targeting the signature peptide of the biomarker NSE for the diagnosis of SCLC. Two MIPs were synthesized in a convenient beaded format using precipitation polymerisation and packed into columns for on-line MISPE-LC-MS/MS. Initial optimisation work revealed that the polymers had high affinity for the target, and further optimisation of the MISPE protocol enabled conditions to be established where the target could be extracted efficiently from water-rich samples. Thereafter, the analytical potential of the most promising synthetic receptor (MIP 1) was evaluated with complex matrices. The on-line extraction method had acceptable recoveries, excellent linearity, and LODs and LOQs for NSE which were in the low ng mL^{-1} range and, very significantly, below the human reference level in serum (which is 8.6 ng mL^{-1}). The low cost and stability of the MIP, combined with the automated on-line MISPE enrichment and LC-MS/MS of the target molecule, is very promising for SCLC diagnosis in particular, and the low-cost analysis of analytes in complex matrices in general, as well as for aiding biomarker validation.

5.0 Acknowledgements

This work was supported by the Horizon 2020 Research Framework Programme of the European Commission BioCapture project (722171). We thank Dr. R. Trones from G&T Septech AS, Norway, for assisting with the packing of the MIPs and NIPs into columns.

References

[1] G. Poste, Bring on the biomarkers, Nature 469(7329) (2011) 156-157.

[2] R.M. Lequin, Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA), Clin. Chem. 51(12) (2005) 2415-2418.

[3] K. Haupt, K. Mosbach, Molecularly imprinted polymers and their use in biomimetic sensors, Chem. Rev. 100(7) (2000) 2495-2504.

[4] A. Mujahid, N. Iqbal, A. Afzal, Bioimprinting strategies: From soft lithography to biomimetic sensors and beyond, Biotechnol. Adv. 31(8) (2013) 1435-1447.

[5] A. Poma, A.P. Turner, S.A. Piletsky, Advances in the manufacture of MIP nanoparticles, Trends Biotechnol. 28(12) (2010) 629-637.

[6] G. Vasapollo, R.D. Sole, L. Mergola, M.R. Lazzoi, A. Scardino, S. Scorrano, G. Mele, Molecularly imprinted polymers: present and future prospective, Int. J. Mol. Sci. 12(9) (2011) 5908-5945.

[7] M.X. Li, X.H. Wang, L.M. Zhang, X.P. Wei, A high sensitive epitope imprinted electrochemical sensor for bovine serum albumin based on enzyme amplifying, Anal. Biochem. 530 (2017) 68-74.

[8] J.Z. Hilt, M.E. Byrne, Configurational biomimesis in drug delivery: molecular imprinting of biologically significant molecules, Adv. Drug Delivery Rev. 56(11) (2004) 1599-620.

[9] E. Saridakis, S. Khurshid, L. Govada, Q. Phan, D. Hawkins, G.V. Crichlow, E. Lolis, S.M. Reddy, N.E. Chayen, Protein crystallization facilitated by molecularly imprinted polymers, Proc. Natl. Acad. Sci. U. S. A. 108(27) (2011) 11081-6.

[10] L. Chen, X. Wang, W. Lu, X. Wu, J. Li, Molecular imprinting: perspectives and applications, Chem. Soc. Rev. 45(8) (2016) 2137-211.

[11] W.J. Cheong, S.H. Yang, F. Ali, Molecular imprinted polymers for separation science: a review of reviews, J. Sep. Sci. 36(3) (2013) 609-628.

[12] C. Rossetti, M.A. Świtnicka-Plak, T.G. Halvorsen, P.A. Cormack, B. Sellergren, L. Reubsaet, Automated Protein Biomarker Analysis: on-line extraction of clinical samples by Molecularly Imprinted Polymers, Sci. Rep. 7 (2017) 44298.

[13] A. Rachkov, M. Hu, E. Bulgarevich, T. Matsumoto, N. Minoura, Molecularly imprinted polymers prepared in aqueous solution selective for [Sar1, Ala8] angiotensin II, Anal. Chim. Acta 504(1) (2004) 191-197.

[14] F. Yang, D. Deng, X. Dong, S. Lin, Preparation of an epitope-imprinted polymer with antibody-like selectivity for beta2-microglobulin and application in serum sample analysis with a facile method of on-line solid-phase extraction coupling with high performance liquid chromatography, J. Chromatogr. A 1494 (2017) 18-26.

[15] M. Kempe, K. Mosbach, Separation of amino acids, peptides and proteins on molecularly imprinted stationary phases, J. Chromatogr. A 691(1-2) (1995) 317-323.

[16] R. Boysen, Advances in the development of molecularly imprinted polymers for the separation and analysis of proteins with liquid chromatography, J. Sep. Sci. 42(1) (2019) 51-71.

[17] C. Rossetti, A. Abdel Qader, T.G. Halvorsen, B.r. Sellergren, L. Reubsaet, Antibody-free biomarker determination: Exploring molecularly imprinted polymers for pro-gastrin releasing peptide, Anal. Chem. 86(24) (2014) 12291-12298.

[18] S.B. Torsetnes, S.G. Løvbak, C. Claus, H. Lund, M.S. Nordlund, E. Paus, T.G. Halvorsen, L. Reubsaet, Immunocapture and LC–MS/MS for selective quantification and differentiation of the isozymes of the biomarker neuron-specific enolase in serum, J. Chromatogr. B 929 (2013) 125-132.

[19] X. Wang, Y. Wang, X. Ye, T. Wu, H. Deng, P. Wu, C. Li, Sensing platform for neuron specific enolase based on molecularly imprinted polymerized ionic liquids in between gold nanoarrays, Biosens. Bioelectron. 99 (2018) 34-39.

[20] C. Rosting, A. Gjelstad, T.G. Halvorsen, Water-soluble dried blood spot in protein analysis: a proof-of-concept study, Anal. Chem. 87(15) (2015) 7918-7924.

[21] D. Yu, K. Du, T. Liu, G. Chen, Prognostic value of tumor markers, NSE, CA125 and SCC, in operable NSCLC Patients, Int. J. Mol. Sci. 14(6) (2013) 11145-11156.
[22] T. Esscher, L. Steinholtz, J. Bergh, E. Nöu, K. Nilsson, S. Påhlman, Neurone specific enolase: a useful diagnostic serum marker for small cell carcinoma of the lung, Thorax 40(2) (1985) 85-90.

Figure captions

Figure 1. (A**)** The on-line MISPE system. Firstly, the analyte is injected onto the MIP (left). Following sample loading, the system switches and the analyte is gradient eluted onto a C_{18} analytical column before determination by a triple quadrupole MS (right). (**B**) Set-up for initial optimization. The MIP (or NIP) is coupled directly to the MS.

Figure 2. Chemical structures of the NSE signature peptide (A) and the structural analogue of the NSE signature peptide used as template during MIP syntheses (B)**.**

Figure 3. Retention factors (k) of MIP 1 and NIP 1 at 20 µL min^{-1} isocratic flow with mobile phases 95:5, 96:4 and 97:3 mM FA:MeCN (v/v). *P≤0.05

Figure 4. Recoveries of ELPLYR after spiking 5 ng mL^{-1} digested NSE to matrices of increasing complexity (50 mM ABC containing 50 nM digested BSA, SPE-treated digested human serum, and untreated digested human serum, n=3).

581 Table 1. Recoveries of ELPLYR tryptic peptide using loading (extraction) times of 5, 7.5, 10, 12.5, and

582 20 min.

Extraction time (min)	ELPLYR Recovery (%)	RSD (%)
5	95.3	1.4
7.5	103	2.9
10	100	5.0
12.5	105	9.5
20	102	4.4

583

584

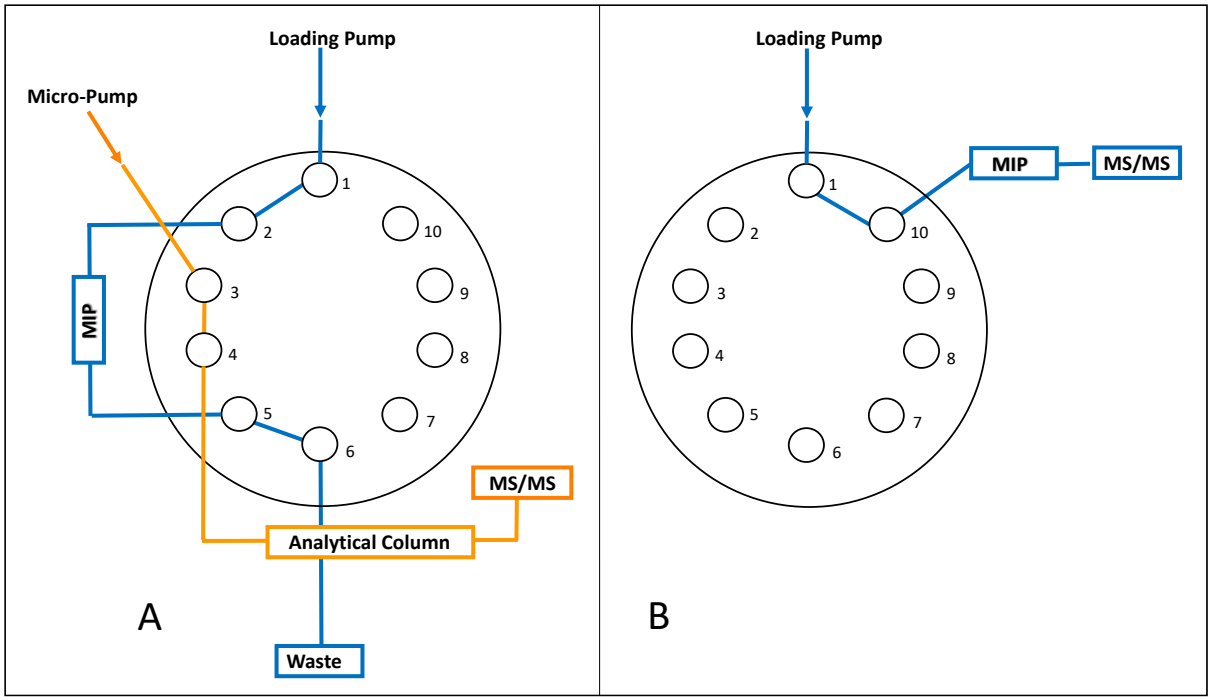
585

586 Table 2. Recoveries of seven BSA tryptic peptides loaded for 5, 10, and 20 min (n=3).

	5 min		10 min		20 min	
Peptide	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
DAELGSFLYEYSR	78.9	37.4	80.3	1.1	73.4	12.4
YICDNQDTISSK	83.9	6.5	88.9	3.8	82.2	9.1
HLVDEPQNLIL	94.7	0.8	86.9	12.3	79.5	3.0
AEFVEVTK	45.0	54.0	19.8	11.6	11.9	114.6
LVTDLTK	9.5	173.2	0	0	0	0
HPEYAVSVLLR	46.1	19.0	41.9	9.2	42.9	11.9
LGEYGFQNALIVR	107.5	14.2	97.0	5.8	95.1	0.1
Average	**66.5**	**39.5**	**59.3**	**6.3**	**51.9**	**21.6**

587

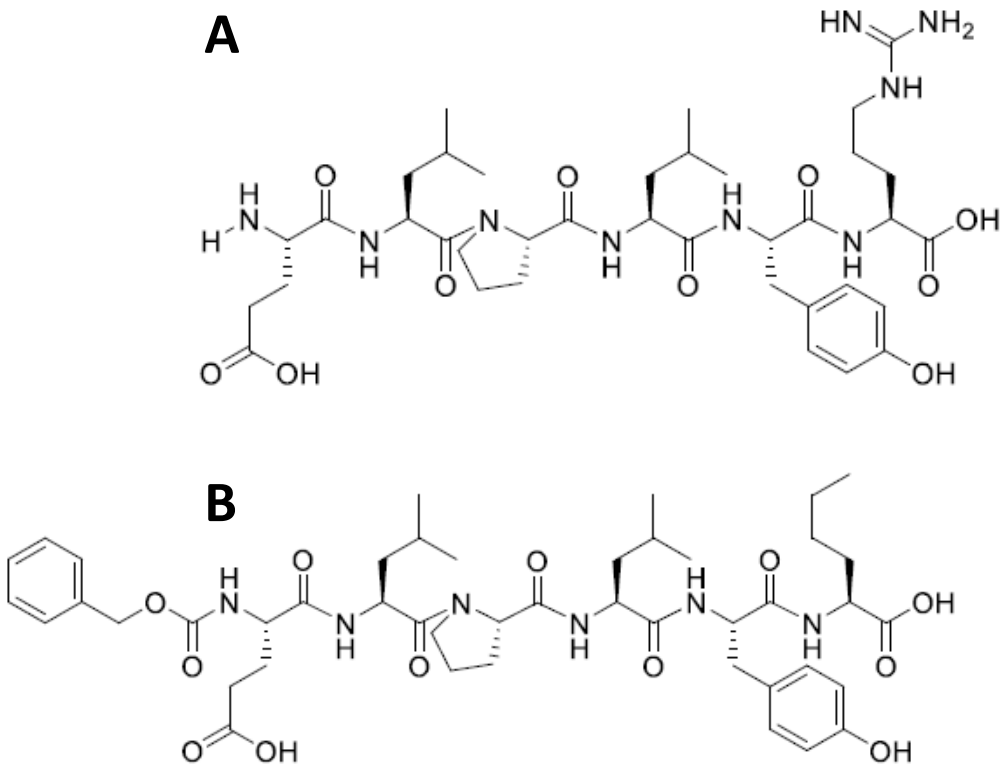
588



589

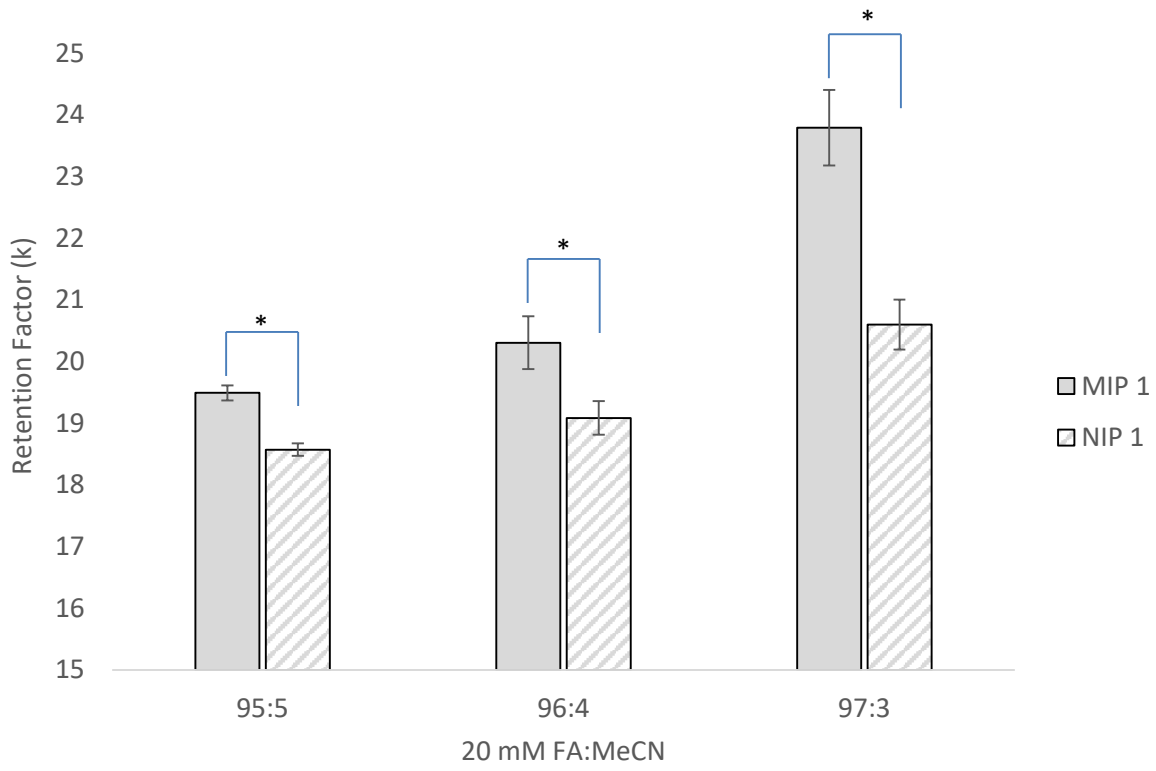
590 **Figure 1**

591


A
HN
NH2
NH
H
N
O
N
H
O
N
H
O
N
H
O
N
H
O
OH
O
OH
OH

B
O
H
N
O
N
H
O
N
O
N
H
O
H
N
O
N
H
O
OH
O
OH
OH

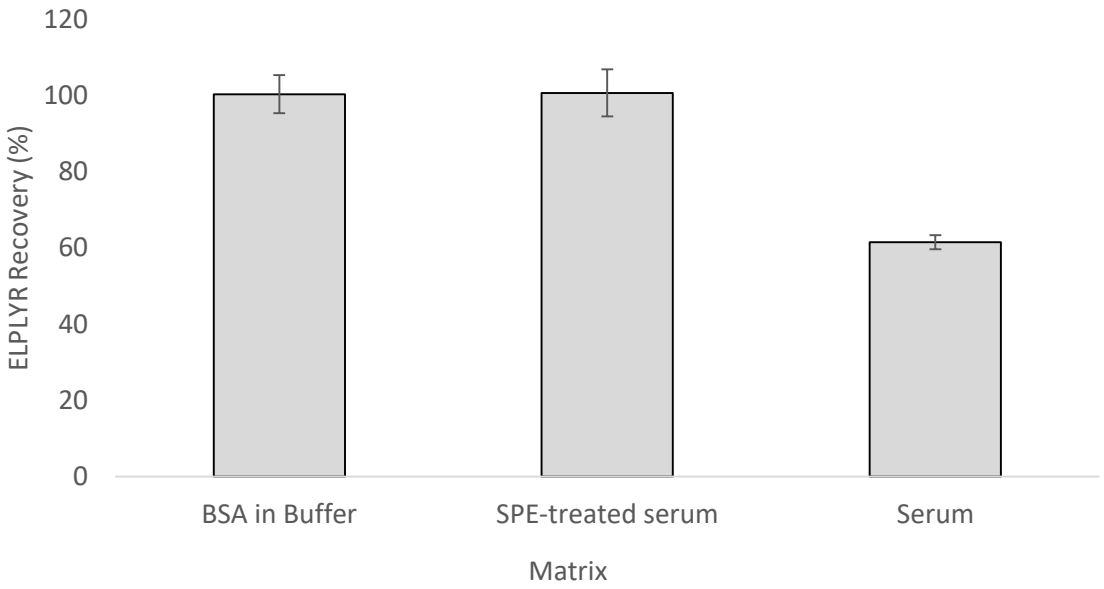
Figure 2



599

600 **Figure 3**

601



602

603 **Figure 4**

604

605