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Immunoaffinity extraction followed by enzymatic digestion for the isolation and identification of proteins employing automated µSPE reactors and mass spectrometry

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Abstract

This work describes a novel automated and rapid method for bottom-up proteomics combining protein isolation with a microimmobilised enzyme reactor (IMER). Crosslinking chemistry based on 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*hydroxysuccinimide (EDC/NHS) coupling was exploited to immobilise trypsin and antibodies onto customisable silica particles coated with carboxymethylated dextran (CMD). This novel silica–CMD solid-phase extraction material was characterised using Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), conductometric titrations and enzymatic colorimetric assays. Micro-solid-phase extraction (μ SPE) cartridges equipped with the modified CMD material were employed and integrated into an automated and repeatable workflow using a sample preparation workstation to achieve rapid and repeatable protein isolation and pre-concentration, followed by tryptic digestion producing peptide fragments that were identified by liquid chromatography mass spectrometry (LC-MS).

Keywords IMER \cdot Immobilised enzyme \cdot Immunosorbent \cdot Protein analysis $\cdot \mu SPE \cdot Mass$ spectrometry

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Introduction

Targeted proteomics and low-level quantification of proteins requires purification and pre-concentration followed by proteolytic digestion to generate peptides that may be easily measured and identified by mass spectrometry. The control of selectivity during sample pre-treatment is often performed using a sorbent containing immobilised antibodies that present a high affinity towards a target analyte. Following selective immunoextraction, an enzymatic digestion is then undertaken in solution. Conventional proteolytic digestions of isolated proteins may be subject to long digestion times, unwanted enzyme autolysis, low activity, poor reproducibility and difficultly of automation [1, 2]. To overcome these drawbacks, the proteolytic enzymes may be immobilised on a solid support and integrated into an analytical workflow, allowing total automation of the analysis [3, 4]. Affinity and enzymatic reactors have been widely used to improve selectivity during sample pre-treatment whilst reducing digestion times, demonstrating promising advances for automation and higher sample throughput. However, only a few studies have presented the combination of both approaches. Hoos et al. reported an online combination of immunoaffinity

chromatography for selective sample pre-treatment followed by solution phase digestion for the detection of covalent adducts of human serum albumin (HSA) [5]. Freije et al. [6] developed an automated and integrated system comprising an immobilised inhibitor cartridge for activity-dependent enrichment followed by an immobilised trypsin reactor for detection of MMP12 spiked into urine samples. Bonichon et al. reported the use of an immunosorbent coupled online to a pepsin IMER (immobilised enzyme reactor) for the total online analysis of HuBuChE-OP adducts from plasma [7]. The same group exploited the online method to investigate the potential of three monoclonal antibodies (mAb2, 3E8 and B2 18-5), grafted on sepharose, to extract HuBuChE from plasma [8]. More recent studies have also shown the development of automated platforms which integrate the multiple steps of the protein sample preparation workflow including protein depletion, fractionation, denaturation, digestion and peptide enrichment into an integrated sample preparation system allowing increased throughput [9, 10].

The choice of an adequate support material is critical for selective immobilisation of a biomolecule. Specifically, the material must be suitable for covalent functionalisation, provide thermal and mechanical resistance and be biocompatible. Ideally, the material should be able to be regenerated for reusability, be stable under reaction conditions and be relatively inexpensive and environmentally friendly [11]. Organic-inorganic hybrids have excellent potential as support materials for enzymes providing enhanced stability, mechanical resistance and compatibility with biological molecules. These support materials comprise inorganic materials, such as silica, inorganic oxides, minerals, carbon materials and magnetic nanoparticles coated with polymers or polysaccharides, such as chitosan [12], poly(ethylene glycol) (PEG) [13], cellulose [14] or dextran [15]. Polysaccharides are effective for the reduction of protein and silica surface interactions and for suppressing unwanted non-specific binding due to increased hydrophilicity of the carrier [16]. It is also possible to chemically modify the support surface to enable numerous immobilisation techniques. Several methods for enzyme immobilisation have been proposed [17], including enzyme entrapment, crosslinking, adsorption, covalent immobilisation and affinity. Among all these methods, covalent immobilisation generally ensures the highest strength of the bonding between support and enzyme, minimising leakage and wash-off issues. Similarly, the immobilisation of antibodies on the bioanalytical platforms is the most critical step as it directly affects analytical performance [18]. This immobilisation may be performed via a wide range of strategies such as physical adsorption, orientated binding by intermediate proteins, covalent binding, biotin-avidin interactions, affinity tags and site-specific binding [19, 20]. It has been recognised that covalent immobilisation is preferred when the coated substrate is subjected to flow or extended times in solution as leaching of the immobilised molecule from the substrate is prevented [21]. The most widely used covalent binding strategy is the heterobifunctional crosslinking of the amino or carboxyl groups on antibodies to the free carboxyl or amino groups on bioanalytical platforms using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) along with N-hydroxysuccinimide (NHS) or sulfo-NHS [22, 23]. The high specificity of antibodies to target analytes renders immunoextraction useful for isolation and enrichment of analytes occurring at very low concentration levels in environmental and biological samples with very complex matrices.

This work details the fabrication of a new inorganic-organic solid-phase support material for the in situ immobilisation of biomolecules. To assess the desired customised support material, several experiments were required including validation of the material and its suitability for trypsin conjugation and antibody immobilisation. Aminopropyl silica (APS) with a particle size of 3 µm was used as an inorganic solid-phase support material. To increase biocompatibility, hydrophilicity and functionality of the surface, silica particles were modified with carboxymethylated dextran (CMD). This customisable material was packed into micro-solid-phase extraction (µSPE) cartridges for accurate and reproducible automated methods of protein isolation and pre-concentration, followed by trypsin digestion to produce peptide fragments that were identified by liquid chromatography mass spectrometry (LC-MS).

Experimental

Chemicals and consumables

Aminopropyl silica (APS) (3 µm, 120 Å) was obtained from Osaka Soda (Amagasaki, Hyogo, Japan). Carboxymethyldextran sodium salt (CMD), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine, sodium hydroxide pellets, potassium hydrogen phthalate (KHP), 2-(N-morpholino)ethanesulfonic acid (MES), phosphate buffered saline (PBS) pH 7.4 solution, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) liquid substrate (ABTS), horseradish peroxidase (HRP), trypsin from bovine pancreas (TPCK treated), bovine serum albumin (BSA), equine cytochrome c (Cyt c), human serum (H4522), Tris base, urea, iodoacetamide (IAA), dithiothreitol (DTT), Nα-benzoyl-l-arginine ethyl ester (BAEE) and formic acid 98% were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Anti-BSA monoclonal antibody (ab9092) was purchased from Abcam (Melbourne, Victoria, Australia). Mouse IgG VisUCyte HRP polymer antibody was obtained from R&D Systems (Minneapolis, MN, USA). Acetonitrile 99.9% purity (ACN) was obtained

from Chem-Supply (Gillman, South Australia, Australia). Ultra-pure water (18.2 M Ω cm) obtained from a Sartorius 611 Arium® pro water generation system was used for all preparation and dilutions unless stated otherwise.

Sample preparation

Protein reduction and alkylation BSA protein solutions were prepared by dissolving 10 mg of protein in 1 mL of 100 mM Tris buffer containing 6 M Urea. 5 μ L of DTT (200 mM in 100 mM Tris) were added to 100 μ L of the protein solution, and samples were reduced at room temperature for 1 h and subsequently alkylated with 20 μ L of IAA (200 mM in 100 mM Tris) for 1 h. Additionally, 20 μ L of DTT were added to consume any unreacted IAA. Finally, 855 μ L of ultra-pure water were added to the mixture to decrease the urea concentration below 1 M. Cyt c protein samples were digested without any further pre-treatment.

In-solution digestion To a 50 μ L of protein solution, 47 μ L of 500 mM Tris buffer pH 8 were added, followed by 2 μ L of 5 ng/ μ L trypsin (1:200 enzyme to protein ratio). Samples were incubated at 37 °C for 18 h, and 1 μ L of concentrated formic acid was added to stop digestion.

Immunoaffinity extraction followed by enzymatic digestion 20 ng/ μ L of BSA were spiked onto a human serum sample. 100 μ L of this solution were then added to a 1.5-mL microcentrifuge tube, and human serum sample was reduced and alkylated followed the same protocol as BSA.

Micro-solid-phase extraction (µSPE)

The µSPE procedure was developed with a digiVOL® Programmable Digital Syringe Driver using ePrep® micro-solid-phase extraction cartridges (µSPEed) obtained from ePrep (Oakleigh, Victoria, Australia) and packed with the novel generic customisable material (see Fig. S1). µSPEed cartridges have two flow paths: the first flow path is designed for the direct aspiration of liquids into the syringe barrel using a one-way check valve, and the second flow path dispenses the liquid through the sorbent bed with a low-dead volume connection. Compared to traditional SPE where 40-50-µm particles are typically used, µSPEed cartridges use small sorbent particle sizes of 3 µm to increase the surface and operation at higher pressures [24], allowing more efficient digestions, faster separation of target analytes and enhanced reproducibility. Once the µSPEed workflows were developed and optimised, they were moved over to the ePrep Sample Preparation Workstation for automation (see Fig. S1).

Development of customisable material

The customisable material was manufactured from aminopropyl silica beads modified with a CMD coating exploiting carbodiimide crosslinking chemistry. In a reaction vessel, 100 mg of CMD, 40 pmol of EDC, 80 pmol of NHS and 10 mL of binding buffer (50 mM MES) were mixed for 2 h at room temperature. The solution was then mixed with 100 mg of APS and allowed to react overnight at room temperature (1:1 ratio of CMD to APS by weight). The resulting material (silica-CMD) was vacuum-filtered, washed with MO water and allowed to dry. A Nicolet 6700 Fourier transform infrared spectrometer (FTIR) (Thermo Fisher, Waltham, MA, USA) using attenuated total reflectance (ATR) was used to characterise the CMD modification. Scanning electron microscopy (SEM) images were taken at 3 kEV using the Zeiss Supra 55 VP (Carl Zeiss AG, Oberkochen, Germany). Conductometric titrations were performed using a Hach HQ14D meter (Hach, Dandenong South, Victoria, Australia) to determine the concentration of carboxylate groups on the novel material. Based on a method previously described by Wu et al. [25], 100 mg of material were suspended into 100 mL of deionised water using ultrasonication; 200 µL of 0.10 M HCl were mixed into the suspension and was titrated under nitrogen against 25 mM of NaOH. To determine the exact NaOH concentration, it was titrated against three aliquots of KHP prepared in ultra-pure water.

To confirm the covalent binding of biomolecules to the novel material, silica-CMD was packed into µSPEed cartridges, and HRP was immobilised as a model enzyme, which catalyses the colorimetric reaction of ABTS in the presence of hydrogen peroxide. The formation of an ABTS cation radical was spectrophotometrically monitored using a Thermo Fisher Multiskan Ascent 96/384 Plate Reader (Thermo Fisher Scientific, North Ryde, NSW, Australia) [26]. For covalent binding of HRP, µSPEed cartridges were conditioned with 500 μ L of 10 mM MES buffer, activated with 0.1 M EDC/NHS in 10 mM MES, loaded with 50 µg of HRP and end-capped with 0.1 M ethanolamine pH 8. The cartridge was then washed with 500 μ L of PBS (1×) buffer prior to loading the 100 µL of ABTS liquid substrate. The loading and colorimetric reaction of ABTS was repeated 10 times within the same cartridge, and fractions were collected in a microwell plate and scanned.

Immobilised enzyme reactor (IMER) through µSPEed cartridges

For the construction of a trypsin IMER, µSPEed cartridges were packed with silica–CMD material, and trypsin was immobilised in-situ. An immobilisation buffer was prepared using MES at a final concentration of 10 mM. A 500 mM tris buffer solution (pH 8) was used to prepare three buffers, a running buffer used as a diluent for protein samples and cartridge conditioning (25 mM Tris with 0.5 mM CaCl₂), a salt wash buffer (25 mM Tris with 10 mM CaCl₂) and an elution buffer (25 mM Tris with 10 mM CaCl₂ and 10% ACN). For end-capping, ethanolamine (0.1 M) was prepared and adjusted to pH 8 with concentrated hydrochloric acid. BSA and Cyt c were chosen as model proteins. BSA was denatured, reduced and alkylated with urea, DTT and IAA, whilst Cyt c was prepared in 500 mM Tris buffer pH 8. Prior to digestion, all protein samples were diluted to working concentrations with the running buffer.

In-situ immobilised trypsin was prepared directly in the µSPEed cartridges using the digiVOL® digital syringe driver. The cartridge was conditioned twice with 500 µL of 10 mM MES buffer and activated with 0.1 M EDC/NHS in 10 mM MES. The cartridge was then loaded with 500 μ L of 50 ng/ μ L trypsin in 10 mM MES buffer and end-capped with 0.1 M ethanolamine at pH 8. Between each step, cartridges were washed with 500 µL of 10 mM MES buffer. Finally, to remove any noncovalently bound trypsin, the cartridge was washed three times with 500 µL salt wash buffer (25 mM Tris with 10 mM CaCl₂).

For protein digestion, the prepared cartridges were conditioned twice with 500 µL of running buffer before loading the protein samples. 100 µL of protein sample (10 ng/µL) were loaded onto the cartridge and cycled through various times for different digest contact times ranging from 5 to 10 min. To recover generated protein fragments, 100 µL of elution buffer were passed through the cartridge, collected and spiked with 1 µL of concentrated formic acid for LC-MS analysis.

To investigate the cartridge's digest performance, in-situ immobilised trypsin cartridges were prepared to perform digests of 1000 µM BAEE prepared in PBS (1×) buffer.

Each cartridge was conditioned with $2 \times 500 \,\mu\text{L}$ salt buffer and $2 \times 500 \ \mu\text{L}$ PBS (1×) buffer. The BAEE solution was then cycled through allowing 5 min of contact time with the trypsin cartridge before elution into an LC vial (15 cycles of 100 µL at 300 µL/min). A further 100 µL of 1% formic acid were passed through the cartridge into the LC vial to dilute the sample 1 in 2 and quench the enzymatic cleavage.

Covalent immobilisation and antibody-antigen (Ab-Ag) complex formation

To ensure covalent immobilisation of the antibody onto silica-CMD, a colorimetric visualisation using HRP was performed. Approximately, 10 mg of loose silica-CMD were added to an Eppendorf tube followed by 200 µL of 10 mM MES buffer, 10 µg of anti-BSA Ab, 1 mg of NHS and 2 mg of EDC and allowed to react for 1 h. The material was washed with 500 µL of PBS buffer by a vortex, spun down with a microcentrifuge and the solution decanted off. This was performed 5 times with PBS (10x) followed by 5 times with PBS (1x). 20 µL of mouse IgG HRP polymer were then incubated with the material for 30 min to bind anti-BSA Ab (raised in mouse). The material was then washed with 10 cycles of PBS $(1\times)$ to remove excess mouse IgG HRP polymer before adding 200 µL of ABTS liquid substrate for visualisation (see Fig. 1a). In addition, a second colorimetric visualisation using HRP was used to ensure Ab-Ag complex binding. BSA was covalently bound to silica-CMD material, incubated with anti-BSA and mouse IgG HRP polymer and visualised using the ABTS substrate (see Fig. 1b).



reaction

Antibody immobilisation onto µSPEed cartridges for protein isolation

Antibodies were covalently immobilised onto silica-CMD µSPEed cartridges in-situ using the digiVOL® digital syringe driver. An immobilisation buffer was prepared using MES to a final concentration of 10 mM. Tris buffer was prepared from tris base at a concentration of 500 mM and adjusted to pH 8 with HCl. Tris buffer was used to prepare two buffers, a running buffer to be used as a diluent for protein samples and cartridge conditioning (25 mM Tris with 1 mM NaCl) and a salt wash buffer (25 mM Tris with 20 mM NaCl). The cartridge was conditioned twice with 500 µL of 10 mM MES buffer activated with 0.1 M EDC/NHS in 10 mM MES, loaded with 20 ng/µL of antibody in 10 mM MES and end-capped with 0.1 M ethanolamine pH 8 with a 500 µL wash with 10 mM MES between each step. The cartridge was then washed three times with 500 µL wash buffer to remove any non-specifically bound antibody and conditioned twice with 500 µL running buffer. The sample prepared in running buffer was loaded and cycled twice, washed three times with 500 µL wash buffer to remove all non-specifically bound components and eluted with 80 µL of 0.2% HCl into an LC vial. 20 µL of 500 mM Tris 2.5 mM CaCl₂ pH 8 were add to the elute for compatibility with trypsin digestion. The cartridge was immediately reconditioned to pH 8 twice with 500 µL of wash buffer to prevent antibody denaturation for further use.

Instrumentation

The immobilisation of trypsin using the BAEE substrate was confirmed conducting *HPLC-UV* separations (Thermo Fisher Ultimate 3000, North Ryde, NSW, Australia). A Zorbax 300SB-C8 column (2.1×100 mm, 1.8μ m) (Agilent Technologies, Santa Clara, CA, USA) was used for separation. The mobile phase was 85% water and 15% acetonitrile containing 0.1% formic acid. The flow rate was 0.2 mL/min, and the injection volume 1 μ L. UV detection was set at 254 nm.

Liquid chromatography quadruple time-of-flight mass spectrometry (LC-QToF-MS) experiments for protein analyses were performed using a 1290 Infinity II LC equipped with an Accucore C18+ column ($2.1 \times 100 \text{ mm}$, $1.5 \mu\text{m}$) and coupled to a 6510 series QToF-MS (Agilent Technologies, Santa Clara, CA, USA). The mobile phases were water (A) and acetonitrile (B) both containing 0.1% formic acid. The gradient program (A:B v/v) was 95:5 from 0 to 20 min followed by a linear gradient until 40:60 at 25 min, which was held for 5 min. The flow rate was 0.2 mL/ min, and the injection volume was 20 μ L. MS parameters were optimised in positive electrospray ionisation (ESI). The optimised values for capillary and fragmentor voltages were 3500 V and 175 V, respectively, and the drying gas flow was set to 5 L/min at 325 $^{\circ}$ C. The mass range analysed was 400–2000 m/z.

MS data obtained by LC-QToF-MS was processed using Agilent MassHunter B.07.00 (Agilent Technologies) to obtain a peptide list of charge state $\geq 2+$. The mass list was searched against the contaminants database using MAS-COT Peptide Mass Fingerprint (Matrix Science). Within the MASCOT search engine, the enzyme selected was trypsin, and experimental ion peptide tolerance was set to ± 1.2 Da. The criteria also allowed two missed cleavages to account for incomplete digestion.

Liquid chromatography LTQ-Orbitrap mass spectrometry (LC-LTQ-Orbitrap MS) experiments for protein analyses were carried out using a Waters ACQUITY M-Class Nano LC (Waters, Rydalmere, NSW, Australia) coupled to Thermo Fisher Q Exactive Plus (Thermo Fisher Scientific, North Ryde, NSW, Australia). Chromatographic separation was performed using a nanoEase Symmetry C18 trapping column in line with a PicoFrit column (300 mm \times 75 μ m (ID); New Objective, Woburn, MA) packed with Magic C18AQ resin (3 µm, Michrom Bioresources, Auburn, CA). Elution solvents were 2% water (A) and 98% acetonitrile containing 0.2% formic acid (B). 5 μ L of sample were loaded at 15 μ L/ min for 3 min onto the trap column and then washed into the PicoFrit column using the following program, 0–15 min, 5 to 30%B, and 15-28 min, 30-80%B, held for 5 min at 80%B followed by a re-equilibration step for 2 min with 5%B. The eluting peptides were ionised at 2400 V. A data-dependant MS/MS (dd MS2) experiment was performed, with a survey scan of 350-1500 Da performed at a mass resolution of 70,000 for peptides of charge state 2+ or higher with an automatic gain control (AGC) target of 3e⁶ and maximum injection time of 50 ms. The top 12 peptides were selected and fragmented in the higher-energy collisional dissociation (HCD) cell using an isolation window of 1.4 m/z, an AGC target of 1e⁵ and maximum injection time of 100 ms. Fragments were detected with a mass resolution of 17,500, and the product ion fragment masses were measured over a mass range of 120-2000 Da.

MS/MS data files obtained by LC-LTQ-Orbitrap MS were searched using Peaks Studio X against either the human or bovine proteome and a list of common contaminants with the following parameter settings. Within the Peak Studio X search engine, the enzyme selected was trypsin, variable modifications were specified as carbamidomethyl (C), deamidation (N and Q) and oxidation (M), and the precursor and fragment ion peptide mass tolerance was set to 10ppm and ± 0.05 Da, respectively. The criteria also allowed three missed cleavages to account for incomplete digestion. The results of the search were then filtered to include peptides with a $-\log 10P$ score that was determined by the false discovery rate (FDR) of <1%).



Fig. 2 FTIR absorption spectra for unmodified silica (a), CMD (b) and the CMD coated silica (c). A combination of absorption band visible in unmodified silica and CMD confirms the formation of the desired silica–CMD material

Results and discussion

Characterisation of the support material

Organic-inorganic hybrid support materials for IMERs have excellent stability and compatibility with appropriate

functional groups for facile biomolecule immobilisation. We fabricated 3-um carboxymethylated dextran (CMD) covered silica support particles for covalent binding of biomolecules exploiting an EDC/NHS coupling chemistry. This polysaccharide-coated surface is also known to suppress non-specific binding [16]. The silica–CMD material was characterised by FTIR, SEM and conductometric titration to verify the integrity of the CMD coating and to estimate binding capacity. FTIR was employed to ensure successful coating of CMD by interrogation of the absorption bands of functional groups associated with both the silica support material and the CMD. Figure 2a shows the FTIR absorption spectra of unmodified silica with two absorption bands at wavenumbers of 1050 cm⁻¹ and 800 cm⁻¹ corresponding to the Si-O-Si stretch and trend vibrations [27]. Figure 2b shows the absorption bands for CMD (O-H stretch 3400 cm^{-1} , C-H stretch 2920 cm⁻¹, C=O stretch 1580 cm⁻¹, C-O-H bend 1410 cm⁻¹, C–O stretch 1000 cm⁻¹, α -glucopyranose ring 916 cm^{-1} [28]). The successful formation of the CMD coated silica support material was apparent in Fig. 2c, where a combination of all absorption bands was observed.

SEM was employed to confirm the integrity and shape following coating. This was critical as particle shape influences surface area, packing density and therefore efficiency. Figure 3 shows magnified images of the silica particles' size and shape, showing no substantial differences in their physical appearance before and after modification with CMD.

A conductometric titration was performed to quantify the number of -COOH groups available for enzyme conjugation, as shown in Fig. 4a. The titration curve of silica–CMD material consisted of three linear areas. The first area of linearity decrease in conductivity corresponded to the neutralisation of H⁺ ions from excess HCl. The second area increased in conductivity and represented the neutralisation the CMD carboxyl groups, whilst the third area of linearity demarcated the point of complete neutralisation of the CMD material by a continued increase in conductivity due to addition of excess NaOH. The ΔV for NaOH in the second part of the titration was used to calculate the number of moles of available -COOH and was determined via





Fig. 4 a Conductometric titration of silica-CMD material showing three stages of the titration: (1) neutralisation of HCl by NaOH, (2) neutralisation of acidic -COOH groups on the silica-CMD material and (3) excess additions of NaOH following neutralisation of all acidic groups. The first derivatives of the titration show the **b** starting and c end point of the neutralisation of -COOH groups on the silica-CMD which is observed in the second stage of the titration



computing the first derivatives of the titration curve seen in Fig. 4b, c where ΔV for NaOH was calculated from V_2 to V_1 . The titration of seven individual batches coated CMD silica resulted in an average of 0.60 ± 0.01 mmol/g, moles of -COOH groups per gramme of material (2% RSD). For comparison, a control conductometric titration of aminopropyl silica was performed which produced an expected "v"-shaped conductivity graph (see Fig. S3).

µSPEed trypsin reactor

The compatibility and covalent immobilisation of biomolecules on the CMD material were evaluated using horse radish peroxidase (HRP) as a model enzyme. HRP was immobilised in situ (within the μ SPEed cartridges) to the CMD/ silica support material harnessing EDC/NHS crosslinking chemistry [29, 30]. HRP catalyses the reaction of azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the presence of H₂O₂ to a radical cation with absorbance at 620 nm. The formation of the product was therefore monitored via spectroscopy to investigate the stability and conversion rate of the IMER. Figure S2 shows the absorbance of the ABTS radical cation, confirming the activity of HRP after immobilisation. The same IMER was employed for ten individual reaction cycles demonstrating that the conversion of ABTS was stable with an average value of 0.226 ± 0.018 AU and a RSD of 8%. Similarly, a non-covalent immobilisation was performed using the above colorimetric assay resulting in a decreasing signal across the ten measurements, indicating leaching of HRP from the support material (see Fig. S2). Between each of the ten readings, a mild wash buffer of 10 mM MESS was used. However, after the tenth reading, the cartridges were washed with a PBS (1×) pH 7.4 buffer solution, resulting in no absorbance signal at 620nm for the non-covalently bound HRP indicating complete removal of HRP from the cartridge. The same washing buffer was used for the covalently bound HRP without detrimental effects.

The same crosslinking chemistry was used to immobilise trypsin onto the generic CMD μ SPEed cartridge. The immobilised trypsin activity and repeatability within the cartridge was evaluated using BAEE as a model compound [31, 32]. In the presence of trypsin, BAEE produced N_{α}-benzoyl-l-arginine (BA) and ethanol via cleavage at the carboxyl side of arginine as seen in Fig. 5. This cleavage was monitored using high-performance liquid chromatography (HPLC) with a UV detector at 254 nm. The incubation



Fig. 5 a HPLC-UV chromatograms at 254 nm for BAEE digestion to BA for a 2-min digest (black line) and 5-min digest (red line). b Calibration curve constructed for BAEE analysis after 2-min digestion by HPLC-UV

time was evaluated at 2-min and 5-min digestions of 1000 μ M BAEE. Chromatographic signals for the undigested BAEE and the digest product (BA) were detected at 4 and 2 min, respectively. The peak ratio area of BA/BAEE was calculated for each digest where the 2-min digest resulted in a ratio of 0.10 compared to 0.33 for the 5-min digest, showing that increased incubation time resulted in more product formation. Similarly, the influence of flow rate was also assessed. Whilst keeping the BAEE substrate incubation time the same between two cartridges, two flow rates of 300 and 600 μ L/min were evaluated. The obtained BA/BAEE peak area ratio was 0.10 and 0.11 for 300 μ L/min and 600 μ L/min, respectively, demonstrating that the digestion efficiency was not affected by flow rates but incubation time.

The trypsin cartridge digestion reproducibility was evaluated by preparing a calibration curve (see Fig. 5b) to calculate the concentration of BAEE remaining after a 2-min incubation digest of 1000 μ M BAEE within six trypsin cartridges. The concentration of BAEE for the six samples averaged 513 ± 26 mol/L with a RSD of 5.2% indicating reproducible digestion.

Following trypsin activity experiments, preliminary protein loading capacity and digestion incubation times were investigated by loading Cyt c and BSA from 1 to 10 μ g for 5 and 10 min, respectively. Figure 6 shows the total ion chromatograms (TICs) from LC-QToF-MS analysis after tryptic digestion within the IMER. Initially, a 5-min room temperature digestion of Cyt c was attempted with 10 μ g of Cyt c loaded onto the cartridge. There was a large peak at 16 min representing undigested Cyt c (Fig. 6a). Loading of 1 μ g of Cyt c under the same conditions resulted in complete digestion of the protein with no evidence of residual intact Cyt c (Fig. 6b). Similarly, loading of 10 μ g of BSA and after 10 min of incubation, a large peak of undigested BSA was observed at 18 min (Fig. 6c). Reducing the protein load to 1 µg resulted in improved digestion and adequate peptide coverage (Fig. 6d). Therefore, efficient digestion of these two proteins required \leq 1 µg, and higher amounts should be avoided to prevent IMER overloading.

µSPEed IMER reproducibility and application to model proteins

Following optimisation of the μ SPEed workflow for protein digestion, three trypsin cartridges were prepared to assess the repeatability of the μ SPEed reactor for BSA digestion. As before, each cartridge was loaded with 1 μ g of reduced and alkylated BSA with a 10-min incubation time (30 cycles of 100 μ L sample at 300 μ L/min) and analysed using LC-LTQ-Orbitrap MS. The average sequence coverage of BSA was 79 \pm 4% demonstrating that the IMERs were suitable for incorporation into proteomic workflows.

Additionally, the performance of the IMER to digest and identify proteins by their peptide fragmentation fingerprint was further evaluated by digestion of Cyt c. The IMER digestion was compared to the common 18-h in-solution digestion method at 37 °C and both samples analysed by LC-LTQ-Orbitrap MS. Prior to analysis, all samples were diluted appropriately to ensure the same starting protein amount for digestion was performed across both methods. The IMER performance for the digestion of Cyt c was comparable with in-solution digestion obtaining protein sequence coverages of 80% and 87%, respectively.

Preparation of the immunosorbent for protein isolation

Anti-BSA and BSA protein were used to develop the immunoaffinity extraction μ SPEed cartridge. To ensure covalent Fig. 6 Total ion chromatograms (TIC) acquired by LC-QToF-MS after tryptic digestions of Cyt c and BSA. Different protein loads and incubation times were tested to determine the optimal working range of the developed IMER. **a** A 5-min digest of 10 μ g of Cyt c, **b** a 5-min digest of 1 μ g Cyt c, **c** a 10-min digest of 5 μ g BSA and **d** a 10-min digest of 1 μ g BSA



immobilisation of the antibody onto silica–CMD material and Ab–Ag formation, a colorimetric visualisation using HRP and ABTS was performed following the scheme described in Fig. 1a, b, respectively. A turquoise colour was observed for the covalently bound anti-BSA indicating successful immobilisation to the silica–CMD material. Similarly, the Ab–Ag formation was assessed by covalently immobilised BSA onto the support material. This resulted in a turquoise colour solution demonstrating the successful formation of the anti-BSA and BSA complex (see Table S1). For both experiments, control analyses were performed where no colour change was observed.

Immunoaffinity extraction followed by enzymatic digestion

The μ SPEed immunoaffinity cartridge and trypsin microreactor were combined for an automated method for protein isolation and digestion. Using the ePrep Workstation, samples, reagents and buffers for both cartridges were placed on the workstation bed and the immunoaffinity extraction and digestion methods setup to run sequentially, thus creating an automated workflow method combining the two cartridges. The respective cartridges were prepared according to Fig. S4 where following immunoaffinity isolation, the eluted fraction was diluted and buffered to pH 8 for compatibility with trypsin digestion. BSA in a concentration of 20 ng/ μ L was used as model protein, and once isolated and digested, the obtained peptide fragments were analysed by LC-LTQ-Orbitrap MS. The average sequence coverage for BSA was 87 ± 1% (n=3) demonstrating the efficiency of the developed workflow for protein isolation followed by tryptic digestion (see Table 1, 1st use of affinity cartridge).

After immunoextraction, the cartridge was immediately reconditioned to pH 8 twice with 500 μ L of wash buffer to prevent antibody denaturation for further use. The regeneration of the antibodies allows reuse of the immunosorbent reducing the cost of the extraction procedure. The regeneration of the immunosorbent was assessed by using the same affinity cartridge twice where a BSA protein coverage of 86 ± 8% (*n*=3) was observed (see Table 1, 2nd use of same affinity cartridge led to similar protein coverages, demonstrating that the immunosorbent may be reused to limit waste and expenses.

To further assess the performance of the designed automated workflow, 20 ng/ μ L of BSA were spiked into a human serum sample. This sample was reduced and alkylated prior to analysis. The affinity μ SPEed cartridge was used for the isolation of the spiked BSA followed by digestion and analysis by LC-LTQ-Orbitrap MS. BSA average sequence coverage was 25 ± 1% (*n*=3) (see Fig. S5). The sequence coverage obtained was sufficient for the identification of the BSA present in the serum sample, clearly demonstrating

 Table 1
 BSA protein coverage after immunoaffinity extraction followed by tryptic digestion using the affinity cartridge one and two times

IMER	Protein coverage (1st use of affinity cartridge)	Protein coverage (2nd use of same affinity cartridge)
1	88%	86%
2	86%	96%
3	86%	81%

the potential of the workflow to isolate the protein of interest from a complex matrix followed by a successful tryptic digestion of the isolated protein.

The developed IMERs present easy handling, automation and high reproducibility. Moreover, in comparison with the commonly used pre-immobilisation strategy, our modified silica–CMD material is packed into the μ SPE cartridges allowing in-situ immobilisation. This offers new avenues for the immobilisation of other enzymes (e.g. pepsin or chymotrypsin) and antibodies which will become useful for various micro-reactor strategies.

Conclusions

In this study, we developed a new customised support material based on silica particles coated with carboxymethylated dextran (CMD) using an EDC/NHS crosslinking chemistry. This new material was characterised using FTIR, SEM, conductometric titrations and enzymatic colorimetric assays. The developed trypsin IMER method showed high repeatability and low relative standard deviation (RSD) of 4% protein coverage. Moreover, the IMER was suitable for rapid protein digestion within 10 min at room temperature, with similar digestion performance of Cyt c to that of an in-solution digestion performed for 18 h at 37 °C. The same functionalised support material was employed to develop an accurate and reproducible automated method for the isolation, pre-concentration and tryptic digestion of trace level proteins using customised µSPEed cartridges. BSA was used as model protein, and once isolated and digested, the solution containing the peptide fragments was analysed by LC-LTQ-Orbitrap MS. The average sequence coverage obtained for BSA was $87 \pm 1\%$ demonstrating the efficiency of the developed workflow for protein isolation followed by tryptic digestion. We also demonstrated that it is possible to reuse the immunosorbent reducing the cost of the extraction procedure. Finally, we assessed the performance of the designed automated workflow for the analysis of BSA spiked in a human serum sample, being able to successfully identify the target protein within this complex matrix.

The developed IMERs are compatible with existing sample preparation workflows and technologies and will improve sample throughput and comparability. Furthermore, the automated in-situ immobilisation offers further opportunities for immobilisation of other enzymes and biomolecules and has potential as platform for targeted protein quantification in clinical settings.

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Data availability All data generated and/or analysed during this study are included in this published article (and its supplementary information files).

Declarations

Conflict of interest K. D. received financial support from ePrep Pty Ltd. A. M. is an employee of ePrep Pty Ltd. The other authors do not have any conflict of interest to declare.

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References

- Leon IR, Schwammle V, Jensen ON, Sprenger RR. Quantitative assessment of in-solution digestion efficiency identifies optimal protocols for unbiased protein analysis. Mol Cell Proteomics. 2013;12:2992–3005. https://doi.org/10.1074/mcp.M112.025585.
- Zheng YZ, DeMarco ML. Manipulating trypsin digestion conditions to accelerate proteolysis and simplify digestion workflows in development of protein mass spectrometric assays for the clinical laboratory. Clin Mass Spectrom. 2017;6:1–12. https://doi.org/10. 1016/j.clinms.2017.10.001.
- Moore S, Hess S, Jorgenson J. Characterization of an immobilized enzyme reactor for on-line protein digestion. J Chromatogr A. 2016;1476:1–8. https://doi.org/10.1016/j.chroma.2016.11.021.
- Safdar M, Sproß J, Jänis J. Microscale immobilized enzyme reactors in proteomics: latest developments. J Chromatogr A. 2014;1324:1–10. https://doi.org/10.1016/j.chroma.2013.11.045.
- Hoos JS, Damsten MC, de Vlieger JSB, Commandeur JNM, Vermeulen NPE, Niessen WMA, Lingeman H, Irth H. Automated detection of covalent adducts to human serum albumin by immunoaffinity chromatography, on-line solution phase digestion and liquid chromatographymass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2007;859:147–56. https://doi.org/10.1016/j.jchromb.2007.09.015.
- 6. Freije R, Klein T, Ooms B, Kauffman HF, Bischoff R. An integrated high-performance liquid chromatography-mass

spectrometry system for the activity-dependent analysis of matrix metalloproteases. J Chromatogr A. 2008;1189:417–25. https://doi.org/10.1016/j.chroma.2007.10.059.

- Bonichon M, Combès A, Desoubries C, Bossée A, Pichon V. Development of immunosorbents coupled on-line to immobilized pepsin reactor and micro liquid chromatography–tandem mass spectrometry for analysis of butyrylcholinesterase in human plasma. J Chromatogr A. 2017;1526:70–81. https://doi.org/10.1016/j.chroma.2017.10.033.
- Bonichon M, Valbi V, Combès A, Desoubries C, Bossée A, Pichon V. Online coupling of immunoextraction, digestion, and microliquid chromatography-tandem mass spectrometry for the analysis of sarin and soman-butyrylcholinesterase adducts in human plasma. Anal Bioanal Chem. 2018;410:1039–51. https://doi.org/10.1007/s00216-017-0640-z.
- Li Y, Yuan H, Dai Z, Zhang W, Zhang X, Zhao B, Liang Z, Zhang L, Zhang Y. Integrated proteomic sample preparation with combination of on-line high-abundance protein depletion, denaturation, reduction, desalting and digestion to achieve high throughput plasma proteome quantification. Anal Chim Acta. 2021;1154:338343. https://doi.org/10.1016/j.aca.2021.338343.
- Wei ZH, Zhang X, Zhao X, Jiao YJ, Huang YP, Liu ZS. Construction of a microfluidic platform integrating online protein fractionation, denaturation, digestion, and peptide enrichment. Talanta. 2021;224:121810. https://doi.org/10.1016/j.talanta.2020.121810.
- Zdarta J, Meyer AS, Jesionowski T, Pinelo M. A general overview of support materials for enzyme immobilization: characteristics, properties, practical utility. Catalysts. 2018;8:1–27. https://doi. org/10.3390/catal8020092.
- Peng G, Zhao C, Liu B, Ye F, Jiang H. Immobilized trypsin onto chitosan modified monodisperse microspheres: a different way for improving carrier's surface biocompatibility. Appl Surf Sci. 2012;258:5543–52. https://doi.org/10.1016/j.apsusc.2012.01.071.
- Piehler J, Brecht A, Valiokas R, Liedberg B, Gauglitz G. A highdensity poly(ethylene glycol) polymer brush for immobilization on glass-type surfaces. Biosens Bioelectron. 2000;15:473–81. https://doi.org/10.1016/S0956-5663(00)00104-4.
- Paribok IV, Solomyanskii AE, Zhavnerko GK. Patterns of the adsorption of bovine serum albumin on carboxymethyl dextran and carboxymethyl cellulose films. Russ J Phys Chem A. 2016;90:466–9. https://doi.org/10.1134/S0036024416020242.
- Markovic G, Mutschler T, Wöllner K, Gauglitz G. Application of surface acoustic waves for optimisation of biocompatibility of carboxymethylated dextran surfaces. Surf Coat Technol. 2006;201:1282–8. https://doi.org/10.1016/j.surfcoat.2006.01.062.
- Piehler J, Brecht A, Geckeler KE, Gauglitz G. Surface modification for direct immunoprobes. Biosens Bioelectron. 1996;11:579– 90. https://doi.org/10.1016/0956-5663(96)83293-3.
- Sassolas A, Blum LJ, Leca-Bouvier BD. Immobilization strategies to develop enzymatic biosensors. Biotechnol Adv. 2012;30:489– 511. https://doi.org/10.1016/j.biotechadv.2011.09.003.
- Shen M, Rusling J, Dixit CK. Site-selective orientated immobilization of antibodies and conjugates for immunodiagnostics development. Methods. 2017;116:95–111. https://doi.org/10. 1016/j.ymeth.2016.11.010.
- Jung Y, Jeong JY, Chung BH. Recent advances in immobilization methods of antibodies on solid supports. Analyst. 2008;133:697– 701. https://doi.org/10.1039/b800014j.

- Welch NG, Scoble JA, Muir BW, Pigram PJ. Orientation and characterization of immobilized antibodies for improved immunoassays (Review). Biointerphases. 2017;12:02D301. https://doi.org/ 10.1116/1.4978435.
- Lord HL, Rajabi M, Safari S, Pawliszyn J. A study of the performance characteristics of immunoaffinity solid phase microextraction probes for extraction of a range of benzodiazepines. J Pharm Biomed Anal. 2007;44:506–19. https://doi.org/10.1016/j.jpba.2007.01.040.
- Vashist SK. Comparison of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide based strategies to crosslink antibodies on aminefunctionalized platforms for immunodiagnostic applications. Diagnostics. 2012;2:23–33. https://doi.org/10.3390/diagnostics2030023.
- Vashist SK, Lam E, Hrapovic S, Male KB, Luong JHT. Immobilization of antibodies and enzymes on 3-aminopropyltriethoxysilanefunctionalized bioanalytical platforms for biosensors and diagnostics. Chem Rev. 2014;114:11083–130. https://doi.org/10.1021/cr500 0943.
- Płotka-Wasylka J, Szczepańska N, de la Guardia M, Namieśnik J. Miniaturized solid-phase extraction techniques. TrAC Trends Anal Chem. 2015;73:19–38. https://doi.org/10.1016/j.trac.2015.04.026.
- Wu HX, Wang TJ, Duan JL, Jin Y. Effects of -COOH groups on organic particle surface on hydrous alumina heterogeneous coating. Ind Eng Chem Res. 2007;46:4363–7. https://doi.org/10. 1021/ie070376m.
- Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem. 2005;53:4290–302. https://doi.org/10.1021/jf0502698.
- Jodeh S, Hanbali G, Warad I, Lgaz H. Synthesis of 1- (Pyrrol-2-yl)) imine modified silica as a new sorbent for the removal of hexavalent chromium from water. Appl J Environ Eng Sci. 2015;1:66–84.
- Purama RK, Goswami P, Khan AT, Goyal A. Structural analysis and properties of dextran produced by leuconostoc mesenteroides NRRL B-640. Carbohydr Polym. 2009;76:30–5. https://doi.org/ 10.1016/j.carbpol.2008.09.018.
- Hlaváček A, Peterek M, Farka Z, Mickert MJ, Prechtl L, Knopp D, Gorris HH. Rapid single-step upconversion-linked immunosorbent assay for diclofenac. Microchim Acta. 2017;184:4159–65. https://doi.org/10.1007/s00604-017-2456-0.
- Carl P, Ramos II, Segundo MA, Schneider RJ. Antibody conjugation to carboxyl-modified microspheres through N-hydroxysuccinimide chemistry for automated immunoassay applications: a general procedure. PLoS ONE. 2018;14:1–18. https://doi.org/10. 1371/journal.pone.0218686.
- Šlechtová T, Gilar M, Kalíková K, Moore SM, Jorgenson JW, Tesařová E. Performance comparison of three trypsin columns used in liquid chromatography. J Chromatogr A. 2017;1490:126– 32. https://doi.org/10.1016/j.chroma.2017.02.024.
- Meller K, Pomastowski P, Szumski M, Buszewski B. Preparation of an improved hydrophilic monolith to make trypsinimmobilized microreactors. J Chromatogr B Anal Technol Biomed Life Sci. 2017;1043:128–37. https://doi.org/10.1016/j. jchromb.2016.08.032.

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