A cyclic-olefin-copolymer microfluidic immobilized-enzyme reactor for rapid digestion of proteins from dried blood spots

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A critical step in the bottom-up characterization of proteomes is the conversion of proteins to peptides, by means of endoprotease digestion. Nowadays this method typically uses overnight digestion and as such represents a considerable bottleneck for high-throughput analysis. This report describes protein digestion using an immobilized-enzyme reactor (IMER), which enables accelerated digestion times that are completed within seconds to minutes. For rapid digestion to occur, a cyclic-olefin-copolymer microfluidic reactor was constructed containing trypsin immobilized on a polymer monolithic material through a 2-vinyl-4,4-dimethylazlactone linker. The IMER was applied for the rapid offline digestion of both singular protein standards and a complex protein mixture prior to liquid chromatography–electrospray ionisation-tandem mass spectrometry (LC–ESI–MS/MS) analysis. The effects of protein concentration and residence time in the IMER were assessed for protein standards of varying molecular weight between 11 and 240 kDa. Compared to traditional in-solution digestion, IMER-facilitated protein digestion at room temperature for 5 min yielded similar results in terms of sequence coverage and number of identified peptides. Good repeatability was demonstrated with a relative standard deviation of 6% for protein-sequence coverage. The potential of the IMER was also demonstrated for a complex protein mixture in the analysis of dried blood spots. Compared to a traditional workflow a similar number of proteins could be identified, while reducing the total analysis time from 22.5 h to 4 h and importantly omitting the sample-pre-treatment steps (denaturation, reduction, and alkylation). The identified proteins from two workflows showed similar distributions in terms of molecular weight and hydrophilic character.

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1. Introduction

Mass spectrometry (MS) has become an indispensable tool for large-scale proteome analysis. In almost all workflows, proteins are extracted from their environment by a sample-specific procedure. After this they are often denatured before performing a reduction/alkylation step, which is immediately followed by enzymatic digestion that leads to a complex peptide mixture. The whole process typically consumes a complete day. Following digestion, the peptide mixture is mostly separated by liquid chromatography (LC) and analyzed by (tandem) mass spectrometry (MS/MS or MS) [1]. Enzymatic digestion of proteins is a critically important step in sample preparation prior to MS analysis. It is usually performed in solution by mixing a proteolytic enzyme (e.g. trypsin, Lys–C, chymotrypsin) with proteins in a specific ratio (typically between 1:100 and 1:20) [2]. The digestion is conventionally carried out overnight, and as such remains a bottleneck for high-throughput analysis [3–5]. Numerous protocols have been reported to accelerate digestion, including the use of elevated temperatures and pressures, microwave and infrared irradiation, ultrasound, micropin columns, and solvent-facilitated digestion. These methods may significantly reduce digestion times down to a range of 30 s to several hours [6–8], yet 18 h remains the most-commonly used digestion duration. However, they still suffer from disadva-
tages such as the inability to re-use (costly) enzymes and challenges or incompatibility with automated or in-line LC–MS workflows [9].

Immobilized-enzyme reactors (IMERs) offer an principle that is fundamentally different from in-solution digestion and subsequently lead to several advantageous characteristics, (i) enzymes are immobilized in a confined space, allowing much higher enzyme-to-protein ratios due to prevention of auto-digestion [10], (ii) shorter diffusion distances, which lead to shorter digestion times in the order of minutes to seconds [11,12], and (iii) the immobilized enzymes are typically more stable than free enzymes [13]. Enzymes can be immobilized through matrix entrapment/encapsulation, adsorption (through hydrogen bonds, hydrophobic interactions, ionic bonds), or covalent bonding. The surface of an open microfluidic channel can be used as a substrate for enzyme immobilisation [14,15]. However, this approach offers a very small surface-to-volume ratio. Therefore, immobilisation on membranes [16,17], particles [18] or porous monolithic structures [19,20] is typically preferred.

The use of polymer-based rigid macroporous monoliths as a stationary phase for liquid chromatography has been pioneered in the early 1990s [21]. These structures consist of a single monolithic entity with interconnected microglobules and micrometer-sized flow-through pores. As a support for the immobilisation of enzymes, their advantages lie in a large surface-to-volume ratio and rapid mass transfer, which is based on convection rather than diffusion [22]. Monolithic materials can be prepared in a wide range of chemistries and morphologies and the surface can be functionalised after polymerization. Enzyme immobilisation on monolithic supports has been demonstrated in different formats, including disks [23], columns [24], and microfluidic devices made from fused-silica, glass, polydimethylsiloxane, polyethylene terephthalate, and thiol-ene polymers [25–30].

In the present contribution, we discuss the construction of a cyclic-olefin-copolymer (COC) microfluidic reactor containing trypsin immobilized on a polymer monolithic material via azlactone chemistry. Vinyl azlactone readily react with amine and thiol groups of enzymes, forming covalent bonds through a dipeptide spacer through a ring-opening reaction [31–33]. COC was chosen due to its low material costs, compatibility with mass-replication methods, chemical resistance to a wide range of solvents and chemicals, and good transparency in the ultraviolet and visible-light regions [34]. The immobilized-enzyme reactor was applied for the rapid offline digestion of both singular protein standards and a complex protein mixture prior to LC–MS analysis (protein identification). The effect of a range of residence times and concentrations on protein-sequence coverage and number of identified peptides were assessed for singular proteins of varying molecular weight from 11 to 240 kDa. Finally, the potential of the IMER was demonstrated for the analysis of a complex protein mixture obtained from dried blood spots (DBS). Protein analysis from DBS has a multitude of advantages over whole blood and other biological matrices, including less-invasive sample collection, easy storage and shipping, and reduced costs [35]. In a clinical setting the typical workflow for bottom-up proteomics analysis of DBS consists of extraction from bloodstain cards, sample pretreatment (reduction/alkylation), overnight in-solution digestion, and analysis of the generated peptides by LC–MS [36]. In a clinical setting speed is a key issue, because results are typically required within 24–36 h. In this context use of an IMER may significantly shorten the overall analysis time, allowing to monitor the clinical status of patients in a timeframe useful for clinicians.

2. Experimental

2.1. Materials and chemicals

Sodium hydroxide (NaOH, American Chemical Society reagent, ≥97.0%) was purchased from Merck (Hohenbrunn, Germany). Hydrochloric acid (HCl, 37% in water) was purchased from Acros Organics (Geel, Belgium). Aluminum oxide (Al₂O₃), butyl methacrylate (BMA, 99%), ethylene glycol dimethacrylate (EDMA), ethylene glycol diacyrlate (EDA, 90%, technical grade), methyl methacrylate (MMA, 99%), 1-propanol (≥99.8%), 1,4-butanediol (ReagentPlus quality, ≥99%), 2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%), 4,4’ bis(diethylamino) benzophenone (DEBP, >99%), poly(ethylene glycol) methacrylate (PEGMA, number-average molar mass Mₑ 500 g/mole), t-butanol (American Chemical Society reagent, ≥99%), calcium chloride dihydrate (American Chemical Society reagent, ≥99%), ethanolamine (≥98%), tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), albumin from bovine serum (BSA, ≥96%), albumin from chicken egg white (OVA, ≥98%), cytochrome c from equine heart (CYC, ≥95%), myoglobin from equine heart (MYG, ≥90%), transferrin from human serum (THS), catalase from bovine liver (CAT), α-casein from bovine milk (CAS, ≥70%), and trypsin (European Pharmacopoeia (EP) reference standard), ammonium bicarbonate (NH₄HCO₃, BioUltra, ≥99.5%), sodium deoxycholate (SDC, BioXtra, ≥98%), iodoacetamide (IAA, ≥99%), dithiothreitol (DTT, ≥99.0%), trifluoroacetic acid (TFA, ≥98%), and tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP, 0.5 M, pH 7) were purchased from Sigma–Aldrich (Leipzig, The Netherlands). Formic acid (FA, ULC/MS–SFC–CC grade, ≥99%), acetonitrile (ACN, LC–MS grade) and water (ULC/MS–CC/SFC) were purchased from Biosolve (Valkenswaard, The Netherlands). 2-Vinyl-4,4-dimethylazlactone was purchased from Pure Chemistry Scientific (Warterton, MA, USA). TOPAS cyclic-olefin-copolymer substrate material (grade 8007) was purchased from Kunststoff-Zentrum Leipzig (Leipzig, Germany). BMA and EDMA were passed over activated basic alumina to remove hydroquinone inhibitors. Other monomers were used as received. Water was purified in-house using an Arium 611 UV water-purification system (Sartorius, Göttingen, Germany).

2.2. Prototyping of the microfluidic reactor

Channel layouts for the microfluidic reactor were designed in AutoCAD (Autodesk, San Rafael, CA, USA) and machined using a computer-controlled micromilling robot (Datron M7 Compact, Datron, Mühltal, Germany). Ball-nose end mills were used to machine semi-circular channels in each of the two cyclic-olefin-copolymer (COC) layers. Subsequently, the layers were cleaned with 2-propanol followed by solvent-vapor-assisted bonding to seal the channels. During the bonding process, the top layer was positioned above a reservoir of cyclohexane and exposed to solvent vapor for 5.5 min. After aligning the two layers, they were pressed together for 10 min applying 2.5 kN of force. The applied procedure was based on our previous study [37]. Finally, holders were manufactured in-house, consisting of two aluminum plates bolted together with controlled torque, connecting the reactor with flat-bottom NanoPort connections (Upchurch Scientific, Oak Harbor, WA, USA) for 360-μm o.d. capillary fused-silica tubing.

2.3. Prototyping of the immobilized-enzyme reactor (IMER)

A multi-step enzyme-immobilisation procedure was adapted from a publication by Logan et al. [38]. The procedure consisted of (i) surface modification of COC and in situ polymerization, (ii)
photographing of poly(ethylene glycol)methacrylate and 2-vinyl-4,4-dimethylazlactone, and (iii) trypsin immobilisation.

2.3.3. Surface modification of cyclic-olefin copolymer and in situ polymerization

The empty microfluidic channel was flushed with acetone and dried with a flow of nitrogen gas. Subsequently, a mixture of DEBP, EDA, and MMA (0.1, 49.95, and 49.95%, by weight, respectively) was introduced into the channel, which was then exposed to 365-nm UV light for 4 min (XL-1500 UV Crosslinker, Distrilab, Leusden, The Netherlands). The reactor was again flushed with acetone and dried with a flow of nitrogen gas. A homogeneously stirred and degassed polymerization mixture of BMA (24% by weight), EDMA (16%), 1,4-butanediol (32%), 1-propanol (28%) and DMPA (10 mg per gram of monomers) was then introduced into the reactor and exposed to UV irradiation (365 nm, 10 min), yielding the polymer monolithic material. Finally, the reactor was flushed with methanol (overnight, 10 µL/min).

2.3.2. Photografting of poly(ethylene glycol) methacrylate and 2-vinyl-4,4-dimethylazlactone

A 5% solution by weight of DEBP in methanol was pumped through the reactor (30 min, 0.5 µL/min) using a syringe pump (KDS 210 model, KD scientific, Zoeterwoude, The Netherlands), followed by UV irradiation (365 nm, 2 min) and a washing step with methanol (20 min, 0.5 µL/min). Subsequently, a solution of 0.1 M PEGMA in water was pumped through the reactor (30 min, 0.5 µL/min). The reactor was then irradiated (365 nm, 2 min) and flushed with water (overnight, 0.5 µL/min). Finally, 2-vinyl-4,4-dimethylazlactone was photografted on the polymer substrate by flushing the reactor with a mixture of 2-vinyl-4,4-dimethylazlactone (15% by weight) and DEBP solution (0.22%) in 75:25 (% by weight) t-butanol:water solution, exposing the reactor to UV irradiation (365 nm, 5 min) and flushing with acetone (1 h, 0.5 µL/min).

2.3.3. Trypsin immobilisation

Trypsin was immobilized on the polymer monolithic material by flushing a 2000 mg/L trypsin solution in 50 mM Tris–HCl containing 20 mM CaCl₂ (pH = 8) through the reactor (2 h, 0.5 µL/min), followed by 1 M ethanolamine (1 h, 0.5 µL/min) to quench unreacted azlactone groups, and finally a 50 mM Tris–HCl, 20 mM CaCl₂, (pH = 8.0) buffer solution (1 h, 1.0 µL/min). The reactor was stored at 4 °C until further use.

2.4. Sample preparation

2.4.1. IMER-facilitated digestion

Stock protein solutions (1000 mg/L) were prepared in 50 mM Tris–HCl containing 20 mM CaCl₂ (pH = 8.0), and a range of concentrations were obtained by subsequent dilution. Protein solutions were introduced into the reactor using a syringe pump at set flow rates, allowing to control the residence time in the IMER. Digested samples were collected after discarding the first two reactor volumes. After each digestion, the reactor was flushed with 15 reactor volumes of 50 mM Tris–HCl containing 20 mM CaCl₂ (pH = 8.0) buffer solution.

For the digestion of proteins in dried blood spots (DBS), capillary blood was collected from the fingertips of a healthy male volunteer using a contact-activated lancet (Becton Dickinson, Breda, The Netherlands). The first drop of blood was wiped away and 10 µL of blood were spotted directly on a Whatman Human ID Bloodstain Card BFC 180 (Sigma–Aldrich, Zwijndrecht, The Netherlands). Blood spots were left to dry for 2 h in freely circulating air in the dark at room temperature, cut out and placed into 2 mL Eppendorf tubes. Subsequently 800 µL of 25 mM NH₄HCO₃ were added and samples were vortexed for 5 min at 1400 rpm to extract blood and its proteins from the cards. After digestion of the samples, digests were collected and desalted using Empore C18 solid-phase extraction (SPE) cartridges (4 mm/1 mL, Sigma–Aldrich). Finally, samples were evaporated to dryness and peptides were reconstituted in water prior to LC–ESI-MS/MS analysis.

2.4.2. In-solution digestion

Stock protein solutions (1000 mg/L) were prepared in 50 mM Tris–HCl containing 20 mM CaCl₂ (pH = 8.0) and 6 M urea, and a range of concentrations were obtained by subsequent dilution. 5 µL of DTT (200 mM in 25 mM NH₄HCO₃) were added to 300 µL of protein solution and samples were reduced for 1 h at 37 °C. Samples were then alkylated with 20 µL of IAA (200 mM in 25 mM NH₄HCO₃) for 1 h in the dark at room temperature and subsequently an additional 20 µL of DTT were added. Samples were diluted with 50 mM Tris–HCl containing 20 mM CaCl₂ to bring the urea concentration below 1 M. Trypsin was added to the protein solution with an enzyme-to-protein ratio of 1:30. Samples were incubated at 37 °C for 18 h and finally 45 µL of 5% TFA were added to stop digestion.

For DBS, capillary blood was collected as described for the IMER-facilitated digestion (see Section 2.4.1). After collection, drying and extraction of the blood from the cards, a pre-treatment was carried out before starting overnight in-solution protein digestion. First, proteins were denaturated by adding 100 µL of 10% DDC and 10 µL of 0.5 M TCEP followed by incubation for 1 h at 60 °C. Next, 50 µL of 200 mM IAA were added and the samples were alkylated 1 h in the dark at room temperature. To consume any remaining IAA, 55.4 µL of 200 mM DTT were added, followed by incubation for 30 min at 37 °C. For digestion 3.5 µL of trypsin (1000 mg/L) were added and samples were incubated at 37 °C for 16 h. 40 µL of 2% FA were added to the digests to precipitate DDC. Digests were centrifuged at 4000×g for 20 min and supernatant was desalted using Empore C18 SPE cartridges. Finally, collected samples were evaporated to dryness and stored at −20 °C until further analysis. Prior to LC–ESI-MS/MS analysis, the samples were reconstituted in water.

2.5. NanoRPLC-ESI-MS/MS

Samples were loaded onto an Eksigent trap column (nano LC trap set, ChromXP C18, 120 Å, 350 µm i.d.) and desalted at 2 µL/min for 10 min with 3% ACN and 0.1% TFA. After desalting, peptides were separated on an in-house packed analytical column (Magic C18 resin, 100 Å pore size, 5 µm particles, 75 µm i.d., 100 mm column length) at 300 nL/min. Peptides were eluted using a 60 min linear gradient composed of solvent A (0.1% FA in H₂O) and solvent B (0.1% FA in ACN). The 60 min gradient ran from 5% to 40% B (0–45 min), 40%–100% B (45–50 min), 100% B (50–59 min), and 100–5% B (59–60 min). Eluted peptides were analyzed using an Eksigent Ekspekt nanoLC 425 system (Sciex, Singapore) coupled to a nano-electrospray interface (ESI) installed on a TripleTOF 5600+ mass spectrometer (Sciex, Singapore) and spectra were acquired in information-dependent-acquisition (IDA) and high-sensitivity mode. TOF-MS scans were performed in the mass range m/z 400–1250 Da and the top 30 precursor ions with charge states from +2 to +4, exceeding a threshold of 100 cps, were selected for MS/MS analysis. Product-ion spectra were formed using collision-induced dissociation (CID) in rolling-collision-energy mode and collected across the mass scan range m/z 100–1800 Da. Both the nanoLC and TripleTOF 5600+ instruments were operated using Analyst 1.7 software (Sciex, Singapore).
2.6. Data analysis

Proteins were identified using Protein Pilot Software v 5.0 (Sciex, Singapore) using identification at 1% false-discovery rate (FDR). Results for protein standards were obtained by searching against the Uniprot sprot database (www.uniprot.org). DBS results were searched against Uniprot human database. For IMER-digested samples, trypsin was chosen as a digestion enzyme without prior cysteine alkylation. For in-solution digestion, trypsin was also selected as the digestion enzyme and iodoacetamide as the alkylation reagent. GRAVY indices were calculated using the GRAVY calculator [39] and molecular weights were retrieved using the Expasy website tool [40].

3. Results and discussion

3.1. Prototyping of the microfluidic immobilized-enzyme reactor (IMER)

The microfluidic device (Fig. 1A) consists of two bonded layers of micro-machined cyclic-olefin copolymer (COC), and features a microchannel with a 300 μm diameter circular cross-section and a channel length of 60 mm. In order to connect a syringe pump to the IMER and to collect digested samples, an aluminum holder was manufactured connecting the reactor with flat-bottom NanoPort connections for 360 μm o.d. capillary fused-silica tubing (Fig. 1B).

For our research, the photo-initiator 4,4′-bis(diethylamino) benzophenone was used as an alternative to the commonly-used benzophenone during the multi-step enzyme-immobilisation procedure, due to differences between the UV-transmission of cyclic-olefin copolymer and glass-type materials used in previous reports. 4,4′-bis(diethylamino) benzophenone features a much higher absorbance at 365 nm compared to benzophenone, while COC has an excellent transmission at this wavelength. Assuming a complete conversion of monomers to polymer, the porosity of the polymer-monolithic support in the microchannel was approximately 60% and the interstitial volume of the reactor was 2.8 μL.

3.2. Evaluation of IMER-facilitated digestion of protein standards

The IMER was applied for the offline digestion of singular protein standards prior to protein identification by LC–MS analysis and results in terms of sequence coverage, number of identified peptides, and number of missed cleavages were compared with in-solution digestion. The data underlying Figs. 2–5 can be found in Tables I–IV in the Supplementary information.

α-S1 casein (23 kDa) is a protein consisting of 199 amino acids and was used as a substrate to evaluate IMER-facilitated digestion. IMER digestion was performed at room temperature with α-casein protein concentrations of 1, 5, 10, 25, 50, 75, and 100 mg/L using a residence time of 59 s. No denaturation or protein
pre-treatment was used prior to IMER-facilitated digestion. Fig. 2 shows the relation between \( \alpha \)-casein protein concentration and \( \alpha \)-S1 sequence coverage (2A) and the number of identified peptides (2B) for IMER-facilitated and in-solution digestion. Generally, IMER-facilitated digestion resulted in a higher sequence coverage compared to overnight in-solution digestion. For \( \alpha \)-casein concentrations higher than 25 mg/L, IMER-digested samples resulted in sequence coverages of 75–80%, while in-solution digested samples resulted in sequence coverages of 72–76%. For \( \alpha \)-casein concentrations lower than 25 mg/L, in-solution digestion resulted in a low number of identified peptides and sequence coverage, which might be attributed to insufficient sensitivity or a matrix-related effect. Overall, an increase in \( \alpha \)-casein concentration resulted in a higher number of identified peptides both for IMER-facilitated and in-solution digestion.

Subsequently, the efficiency of the IMER was assessed for digestion of 100 mg/L \( \alpha \)-casein with varying residence times, i.e. 39, 47, 59, 78, 117, 167 and 293 s. The shortest residence time of 39 s resulted in 81% sequence coverage for \( \alpha \)-S1 with 196 assigned peptides (as shown in Fig. 3). In comparison, overnight in-solution digestion resulted in a sequence coverage of 76% and 182 identified peptides. Further increasing the IMER residence time did not result in an increased sequence coverage or a higher number of identified peptides. Pressure limitations of the syringe pump, syringe, and connection leading to the IMER used for this experiment prevented us from further decreasing the residence time in the IMER below 39 s.

In order to assess the repeatability of IMER-facilitated digestions, the same digestion was performed 9 times using the same reactor. For an \( \alpha \)-casein concentration of 100 mg/L and an IMER residence time of 59 s, the average \( \alpha \)-S1 casein sequence coverage was 84% with RSD of 6%, and the average number of identified peptides was 195 with RSD of 15% (Fig. 4). An average of 58 tryptic sites (K/R) were missed for cleavage with an RSD of 19%, and the number of peptides resulting from missed cleavage sites was 25% of the total number of identified peptides. For in-solution digestion a higher number of missed cleavage sites was observed, 90 with an RSD of 13%, while the number of peptides resulting from missed cleaved sites was 24% of the total number of identified peptides.

Finally, IMER-facilitated digestion was compared to overnight in-solution digestion for proteins with different molecular weights (MW), specifically cytochrome c (11.7 kDa), myoglobin (17 kDa), \( \alpha \)-S1 casein (23 kDa), ovalbumin (42.8 kDa), bovine serum albumin (66.5 kDa), transferrin (75.2 kDa), and catalase (240 kDa). The proteins were digested in the IMER at room temperature for 293 s and no denaturation or protein pre-treatment was performed. Fig. 5 shows that the IMER performance was slightly better than in-solution digestion for cytochrome c, myoglobin, \( \alpha \)-S1 casein, and ovalbumin. Lower sequence coverages were observed for bovine serum albumin (55% and 95% for IMER-digested and in-solution digested, respectively) and transferrin (56% and 78% for IMER-digested and in-solution digested, respectively). The higher number of disulfide bridges in these proteins may be shielding cleavage sites from the protease, as no reduction/alkylation was performed prior to digestion. Lower sequence coverage was also observed for catalase (75% and 88% for IMER-digested and in-solution digested, respectively), possible due to the large size of this protein.

3.3. Application of the IMER for digestion of proteins from dried blood spots (DBS)

As shown in Fig. 6A, the typical workflow for bottom-up proteomics analysis of DBS consists of 6 steps: extraction from bloodstain cards (10 min), sample pre-treatment (reduction/alkylation) (2.5 h), in-solution digestion (16.3 h), solid-phase extraction (10 min), and LC-MS analysis of the generated peptides (1.5 h), summing up to a total analysis time of 22.5 h. Fig. 6B shows the novel IMER-facilitated workflow which omits the pre-treatment step and reduces the digestion step from overnight to 5.6 min. The total analysis time for the IMER-facilitated workflow is 4 h, thus representing a total reduction in DBS analysis time by a factor of 6. For an IMER-facilitated digestion time of only 5.6 min, 142 proteins could be identified from pooled triplicate measurements, compared to 156 proteins from pooled triplicate measurements using the traditional workflow. The identified proteins from both workflows were compared in terms of molecular weight and hydrophobic character (using the GRAVY index [39]). As shown in Fig. 7A, the identified proteins displayed a similar MW distribution, with majority of the proteins (26% and 29% for IMER-facilitated and in-solution digestion, respectively) distributed in the region of 20–30 kDa, followed by proteins in the region of 30–60 kDa. Additionally, the results were evaluated in terms of the GRAVY index where positive and negative scores are associated with hydrophobic and hydrophilic properties, respectively. As shown in Fig. 7B, most identified proteins for both workflows had GRAVY indices in the range from −0.8 to 0, with a majority of them in the region of −0.4 to −0.2 (33% and 29% for in-solution and IMER-facilitated digestion, respectively). A smaller percentage of identified proteins had a GRAVY indices >0, which might be associated with their presence in a polar matrix such as blood. The
Fig. 6. Comparison of duration of traditional workflow including in-solution digestion (A) with the novel IMER-facilitated workflow (B). IMER-facilitated workflow consists of 5 steps, omitting the pre-treatment step, and reduces the total analysis time to 4 h.

Fig. 7. Distribution of proteins identified from DBS extracts \((n = 3)\) according to (A) their molecular weight and (B) GRAVY index \([39]\) using IMER-facilitated (grey) or in-solution (white) digestion.

similarity in GRAVY indices of identified proteins for both workflows indicates that unwanted adsorption of proteins to the inner surface of the IMER did not play a significant role.

4. Concluding remarks

A cyclic-olefin-copolymer-based microfluidic reactor was created containing trypsin immobilized on a polymer monolithic substrate through an azlactone chemistry. 4,4'-bis(diethylamino)benzophenone was used as an alternative photo-initiator instead of benzophenone, due to the UV cut-off of COC under 300 nm and the respective absorbance of the photo-initiators. Evaluation of IMER-facilitated protein digestion revealed good repeatability in terms of sequence coverage and number of identified peptides. Compared to in-solution digestion, use of the IMER reduced the digestion time drastically, by more than three orders of magnitude, viz. from 18 h to below one minute (39 s). The application of the IMER for the analysis of complex protein mixtures was demonstrated on dried blood spots. Compared to a traditional overnight workflow a comparable number of protein identifications could be achieved with similar distributions in terms of molecular weight or hydrophobic character, while reducing the total reduction in DBS analysis time by a factor of 6, and omitting sample pre-treatment steps. For future applications, flow-through immobilized-enzyme reactors will be implemented inline in a high-throughput LC–MS workflow.
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Appendix A. Supplementary data

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