Rapid fabrication of glass/PDMS hybrid μIMER for high throughput membrane proteomics†

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Mass spectrometry (MS) based proteomics has brought a radical approach to systems biology, offering a platform to study complex biological functions. However, key proteomic technical challenges remain, mainly the inability to characterise the complete proteome of a cell due to the thousands of diverse, complex proteins expressed at an extremely wide concentration range. Currently, high throughput and efficient techniques to unambiguously identify and quantify proteins on a proteome-wide scale are in demand. Miniaturised analytical systems placed upstream of MS help us to attain these goals. One time-consuming step in traditional techniques is the in-solution digestion of proteins (4–20 h). This also has other drawbacks, including enzyme autoproteolysis, low efficiency, and manual operation. Furthermore, the identification of α-helical membrane proteins has remained a challenge due to their high hydrophobicity and lack of trypsin cleavage targets in transmembrane helices. We demonstrate a new rapidly produced glass/PDMS micro Immobilised Enzyme Reactor (μIMER) with enzymes covalently immobilised onto polyacrylic acid plasma-modified surfaces for the purpose of rapidly (as low as 30 s) generating peptides suitable for MS analysis. This μIMER also allows, for the first time, rapid digestion of insoluble proteins. Membrane protein identification through this method was achieved after just 4 min digestion time, up to 9-fold faster than either dual-stage in-solution digestion approaches or other commonly used bacterial membrane proteomic workflows.

1. Introduction

Proteome characterisation has evolved into a process of systematic identification and quantitation of the proteins expressed in biological samples at a given time and under specific stimuli.[1] Proteome profiling represents a major challenge due to the large number, diversity, complexity, and concentration range of expressed proteins.[2,3] If throughput and automation in proteomics are to be improved, faster sample analysis, whilst increasing the coverage, reduced sample size, and coupling of devices directly to mass spectrometers (MS) are required.[4] Amongst the time-consuming steps in traditional bottom-up proteomic workflows is the digestion of soluble proteins (typically performed in solution over 4–20 h).[5] Other drawbacks of solution digestion are enzyme autoproteolysis, low digestion efficiency (10–40% are semi-cleaved peptides digested in-solution having one or more missed cleavages[6]), and non-automated sample handling.[7,8] The digestion and analysis of insoluble proteins further complicate this equation.

Membrane proteins represent 30% of the naturally occurring proteins and are of interest due to their functional diversity, their roles in the import/export of metabolites, the expulsion of toxic substances, and the conversion of energy.[9,10] The analysis of membrane proteins is complicated by the high hydrophobicity of such proteins.[11] This limits the digestion efficiency, and results in few analysable membrane peptides. Several approaches are currently directed on obtaining comprehensive membrane proteome coverage, with current specific focus on the development of techniques addressing difficulties of analysing these complex, low abundance, and highly hydrophobic proteins.[10,12–14] In contrast, considerably less attention has focussed on increasing analysis throughput.

In all forms of protein digestion for mass spectrometry, trypsin is the preferred enzyme due to its well-defined cleavage specificity, low cost, and peptide yield (~10 amino acids of length).[15] Several studies have sought to optimise proteins tryptic digestion, either in-gel or in-solution.[6,16–18] The most promising approaches involve micro immobilized enzyme reactors (μIMERs) and miniaturised reaction systems that take advantage of microfluidics. These systems allow for lower costs and enhanced analytical performance over traditional in-solution techniques.[19–23] Along with the improved digestion efficacy, μIMERs could be easily directly coupled with the separation and detection systems, leading to increased throughput for proteome profiling. At the time of writing, there were no published reports demonstrating a μIMER for rapid elucidation of the membrane proteomes.

Different supports have been used to immobilise enzymes inside microfluidic devices, including immobilization onto the channel wall. The enzyme support structure determines the substrate accessibility to the active sites, the quantity of loaded

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enzyme, and the substrate diffusivity.[24] Enzyme supports commonly used for μIMERs can be classified into beads, membranes, silicon microstructures in a microchannel, sol–gels, porous polymers/monoliths, or microchannel surfaces.[19,21,25] The enzyme can then be immobilised using physical adsorption, covalent binding or encapsulation/entrapment methods.[23,24] Almost all reported μIMERs have been fabricated in either glass or polymeric materials.[26] With advances in polymer technology, the use of polymeric materials has gained in popularity due to the relative ease, low cost, and increased design versatility/flexibility compared to glass-based systems.[27] The soft elastomeric polymer poly(dimethylsiloxane) (PDMS) has become a popular material due its high adaptability, excellent physical and chemical properties, ease of device sealing (covalently or conformally), and ease and speed of fabrication *via* soft lithographic moulding.[28] However, PDMS has several major drawbacks for many specific microfluidic applications, including a lack of surface functional groups, an intrinsically hydrophobic surface, and incompatibility with many organic solvents.[28] These properties, also present in the most used enzyme supports, are not advantageous for direct and controlled immobilization of enzymes needed in μIMERs. Furthermore, it has been concluded elswhere[29] that biomolecules present in most proteomic unit operations need to be effectively controlled by surface modification to obtain a globally reliable, robust, and reproducible microfluidic chip platform for complex proteome analyses. Therefore, surface modification is a critical design issue for PDMS microdevices for bioanalysis.

Here, we characterize a rapidly manufactured microfluidic PDMS/glass μIMER for proteomics and its ability to digest proteins for downstream MS identification. The novel μIMER comprises of a microchannel containing enzymes covalently attached to a plasma polymerised acrylic acid (ppAAc) surface. XPS, ELISA, and L-BAPA assays are used to characterise the immobilisation and activity of the enzyme. Peptide yield and the extent of confident protein identification are used to benchmark digestion throughput of this μIMER against conventional in-solution trypsin digestion. The most efficient and rapid in-chip digestion conditions are then further adapted for the analysis of the total bacterial membrane fraction from *Synechocystis* sp. PCC 6803, and a μIMER containing trypsin and chymotrypsin is tested for high throughput membrane proteomics. Finally, the results from the new μIMER with conventional in-solution digestion and literature membrane proteomic protocols are systematically compared.

2. Experimental

2.1 PDMS replica fabrication

PDMS devices with microfluidic channel networks were produced using standard soft lithography.[28,40] The master was produced with SU-8 photolithography protocols.[41] The photocurable polymer SU-8-100 (MicroChem Corp, USA) was spin coated onto a silicon wafer at a thickness of 120 μm, and exposed to UV light through a high transparency photomask (JD Photo-Tools, UK). The master had a straight-line 52 mm × 200 μm × 120 μm microfluidic channel. PDMS (Sylgard 184, Dow Corning, UK) base and curing agent were thoroughly mixed at a 10:1 weight ratio before being poured onto the master. This pre-polymer mixture was degassed and then baked at 85 °C for 45 minutes. Subsequently, PDMS replicas were peeled from the master and trimmed before having fluidic inlet/outlet reservoirs formed by a blunt gauge 13 needle tip. Finally, samples were placed in the oven at 100 °C for 72 hours to ensure optimal PDMS curing.[42]

2.2 Surface modification of PDMS/glass hybrid IMER production

PDMS replicas and glass microscope slides were placed within a custom-built stainless steel plasma polymerisation reactor. The samples were then coated with ppAAc, using a reported standard methodology.[43] The monomer used was acrylic acid, plasma power was 10 W, deposition time was 25 min, and the monomer flow rate was 2.5 sccm. Two separate coatings of ppAAc were deposited to ensure optimum coating. The μIMER was produced through clamping the PDMS replica onto the glass slide within a custom-built polymethylmethacrylate (PMMA) clamp, providing conformal pressure to seal the microfluidic channel. Finally, fluids entered/exited the microfluidic channel through tubing directly inserted into the bored out inlet/outlet entries.

2.3 Enzyme immobilisation

Fig. 1 depicts the general workflow for enzyme immobilization on ppAAc modified surfaces, and a representation of protein digestion. The μIMER channels were rinsed with PBS to clean and remove dust/debris. To activate the acid groups in the plasma polymer, an EDC/NHS solution [150 mM NHS and 1.6% (w/v) EDC in PBS buffer] was flowed into the channel at 5 μL min^{-1} and channels were filled with solution for 20 minutes before withdrawing at 5 μL min^{-1}. The channel was then rinsed with PBS before the digestive enzyme in PBS (0.5 mg mL^{-1}) was flowed into the channel at 5 μL min^{-1}. The solution was left in the channel for 20 minutes before excess enzyme solution was flowed through the channel (5 μL min^{-1}). The channel was thoroughly washed with 3× 250 μL PBS at 50 μL min^{-1} before subsequent protein sample introduction.

2.4 XPS analysis

XPS analysis used a Kratos Ultra DLD (Kratos Analytical Ltd, UK). Standard wide-scans were collected over a binding energy range of −5 to 1200 eV, at a pass energy of 160 eV and a step interval of 1 eV. High resolution C 1s spectra were collected using a binding energy range of 275 to 300 eV, at a pass energy of 20 eV and a step interval of 0.1 eV. Scans were obtained using a 90° take off angle (sampling depth ≈ 10 nm).

Charge neutralisation was employed using an electron flood gun. All peaks were fitted using the CasaXPS software using the relative sensitivity factors supplied with the instrument. All C 1s peaks were fitted symmetrically (70% GL) with a fixed full width half maximum (FWHM) value. Each result published has a minimum of 3 replicates analysed and averaged to the mean value.

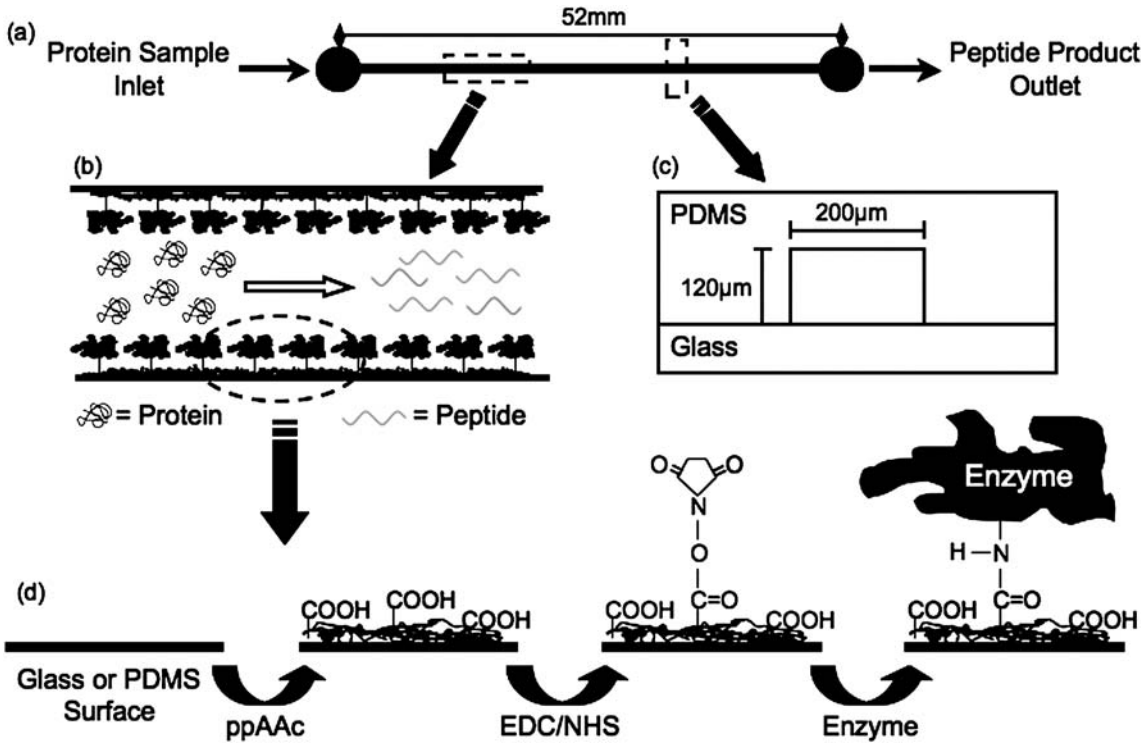

Fig. 1 (a) Schematic of in-chip protein digestion in a PDMS + glass μIMER. (b) Amplified view of the μIMER microchannel with immobilized enzyme on the modified surface (ppAAc) and digestion process. (c) Cross-sectional view and dimensions of microchannel. (d) Depiction of the covalent immobilization of the enzyme on the plasma polymerised surface using EDC/NHS chemistry.

2.5 Protein standards for in-solution and in-chip digestions

BSA or a six protein mixture (cytochrome, lysozyme, ADH, BSA, apo-transferrin, and β-galactosidase) was prepared for each experiment (Sigma-Aldrich, UK). Aliquots with 100 μg BSA or with 1500 pmol of each protein were prepared with PBS buffer (pH 7.6) and a total volume of 100 μL each. Samples were then reduced, denatured, and alkylated, based on a literature protocol.[44] Each aliquot was reduced and denatured with 5 μL 200 mM dithiothreitol (DTT) in PBS and 1 μL 10% SDS, 10 min heating at 70 °C, and then cysteine residues were blocked with 4 μL 1 M iodoacetamide at room temperature for 45 min. Residual iodoacetamide was neutralized with 20 μL 200 mM DTT, followed by incubation at room temperature for 45 min. The samples were then diluted 10-fold in the buffer required.

2.6 Tryptic digestion in-chip

Protein digestion was carried out in different μIMERs (Table S1, ESI†) to assess the stability of the different modifications and to perform negative controls. The reduced and alkylated samples were 10-fold diluted in PBS prior to digestion. Digestions were carried out at 37 °C. 100 μL of protein solution were flowed through the chip using a syringe pump. The digestion time (20 min – 30 seconds), determined by the flow rate, was assessed (Table S1, ESI†). After each digestion, digests were dried by vacuum concentration and stored at −20 °C for later MS analysis.

2.7 Enzyme activity assay

Immobilised trypsin activity was assessed using L-BAPA colorimetric analysis (Sigma-Aldrich, UK). Immobilized enzyme (immobilised onto the ppAAc surface *vs.* physical adsorbed onto untreated PDMS) stability and the number of washes needed to remove the free enzyme in the microchannel were also assessed. The protocol followed a previously reported one.[45] For each digestion, 100 μL of L-BAPA (1 mM) prepared in PBS were flowed through the trypsin-modified μIMER at 50 μL min^{-1} for 2 minutes. Digestion was monitored using an absorbance plate reader at 405 nm. The channel was washed in between each digestion with 250 μL of PBS at 50 μL min^{-1}. Removal of free enzyme from the channels was assessed by adding 27.7 μL of 10× L-BAPA solution to each wash (digest with a final 1 mM L-BAPA concentration). Readings were taken after each digestion and after 5 minutes of incubation time at 37 °C for the washes.

2.8 In-chip digestion of total membrane fraction

The two-staged in-chip digestion followed the same protocol as the in-solution digestion (see ESI†), but carrying out in-chip digestion using two different μIMER channels during a 4 min total digestion time (2 min each digestion). Trypsin was immobilized in microchannel 1 and a 1:1 mixture of both enzyme solutions was immobilized in microchannel 2. 50 μg of isolated membrane protein were diluted in 100 μL of 25 mM ammonium bicarbonate and digested in the trypsin-containing microchannel at a flow rate of 50 μL min^{-1} and at 37 °C in a water bath. After

digestion, the insoluble fractions were sedimented and cleaned as for the in-solution protocol. The sample was then resuspended in 100 μL of 60% methanol and 25 mM ammonium bicarbonate before digestion in the trypsin/chymotrypsin microchannel at a flow rate of 50 μL min^{-1} and at 37 °C. After cleavage, the insoluble membrane proteins were sedimented by centrifugation at 21 000*g* at 4 °C for 1 hour. The peptide-containing supernatant was dried by vacuum concentration and stored at −20 °C for later MS analysis.

2.9 MS analysis

Prior to HPLC-ESI-MS/MS sample analysis of soluble proteins, frozen samples were diluted with 300 μL 0.1% TFA and 3% ACN. 5 μL of each sample were injected and analysed (2500 fmol of each protein). Membrane proteins were diluted with 100 μL of the same solvent and 10 μL were injected (~5 μg). LC Packings Ultimate 3000 system (The Netherlands) coupled with a QStar XL Hybrid ESI Quadrupole TOF tandem mass spectrometer (Applied Biosystems, USA) were used for protein identification. Each sample was injected at 300 nL min^{-1}. Separation was performed using a 0.075 × 150 mm reverse-phase capillary column (C18 PepMap100, LC Packings) at a 300 nL min^{-1} flow rate. HPLC-ESI solvents contained 0.1% formic acid and either 3% ACN (solvent A) or 97% ACN (solvent B). Peptides separation was performed by two linear gradients from 5 to 55% solvent B: 30 min (soluble proteins) and 220 min (insoluble proteins). The electrospray fused silica PicoTip™ needle (New Objective, Inc., USA) was operated with a 5.5 kV voltage differential. Doubly and triply charged ions were selected in the full scan for MS/MS. The ion source gas was set to 50 psi, survey scans were acquired from 350 to 1800 *m*/*z*, MS/MS scans from 65 to 1800 *m*/*z*, and the collision energy and collision gas were set to zero volts and 4.0 psi respectively. MS profile data were acquired using Analyst QS 2.0 (Applied Biosystems).

3. Results and discussion

3.1 Characterisation of the trypsin μIMER

XPS surface chemical characterisation of the μIMER devices was performed at different stages in the fabrication cycle, and the results are shown in Table 1. The successful 3-stage surface modification was monitored *via* quantification of curve fitting of the C 1s spectra and the elements present in the survey spectra (Table 1). The main feature of interest in the C 1s spectra was the acid (%C–C*(O)=O) functional peak, situated at approximately 289 eV. After acrylic acid plasma polymer coating of the PDMS, there was a significant (~20%) peak shift in the spectra, indicative of acid functional groups, in contrast to the untreated PDMS surface, which contains only Si–C and C–H species (285 eV). An elemental quantification of these surfaces also reveals increases in carbon (%C 1s) and reductions in silicon (%Si 2p) due to attenuation associated with the deposition of the functional ppAAc layer on the surface. Both the PDMS and ppAAc coated surfaces contain negligible quantities of nitrogen (%N 1s), so the introduction of nitrogen (3.9%) after the EDC/NHS immobilisation stage was indicative of the immobilisation of the biofunctional crosslinking agent. Changes in the high resolution C 1s scan were also observed and are indicative of the introduction of imide

Table 1 Percentage high resolution C 1s components of surfaces after each stage of surface modification and trypsin immobilisation, and percentage atomic concentrations from XPS wide scan analysis. All thresholds represent one standard deviation of triplicate analyses

	High resolution C 1s components					Atomic concentrations				
Sample	%C–C/H/ Si 285 eV	%C*–C–(O)=O	%C–O/C–N	%C=O/O–C–O/O=C–N	%C–C*(O)=O	%C 1s	%O 1s	%N 1s	%Si 2p	O/C ratio
Pure PDMS	100.0	0.0	0.0	0.0	0.0	48.9 ± 0.7	28.0 ± 1.4	0.0	23.1 ± 1.0	0.57
PDMS + ppAAc	41.9 ± 0.1	19.9 ± 0.1	12.9 ± 0.1	5.4 ± 0.2	19.9 ± 0.1	65.0 ± 2.1	31.5 ± 1.5	0.0	3.5 ± 0.7	0.46
PDMS + ppAAc + EDC/NHS	44.1 ± 0.1	16.3 ± 0.7	16.6 ± 0.6	6.7 ± 0.7	16.3 ± 0.7	65.9 ± 0.5	26.8 ± 0.6	3.9 ± 0.4	3.4 ± 0.6	0.41
PDMS + ppAAc + EDC/NHS + trypsin	45.6 ± 0.2	6.3 ± 0.6	25.8 ± 0.4	16.0 ± 0.7	6.3 ± 0.6	64.3 ± 0.4	23.3 ± 0.3	8.1 ± 0.8	4.3 ± 0.5	0.36

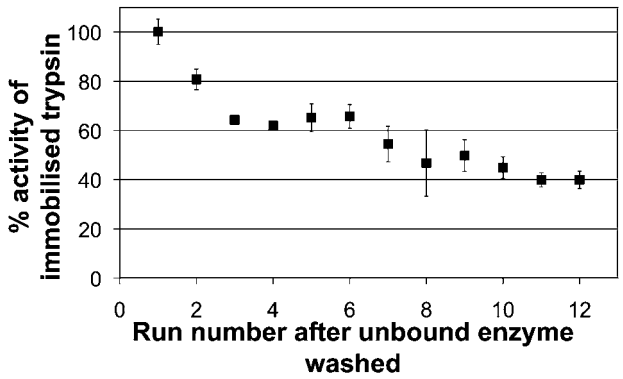

Fig. 2 Relative enzyme activity on L-BAPA substrate of covalently immobilised trypsin on PDMS surface modified with ppAAC + EDC/NHS, 12 consecutive assays with intermediate washes using the same μIMER, and after 3× 250 μL of PBS solution at 50 μL min^{-1} (to remove any unbound enzyme). Error bars represent one standard deviation of triplicate analyses. Note: background signal has been subtracted from the values reported in the figure.

species and the attendant reduction in the acid component that is consistent with the chemistry expected on the surface after EDC/NHS immobilisation. Subsequent exposure to trypsin resulted in further increases in the nitrogen content (from 3.9% to 8.1%), and the introduction of shifts in the binding energy within the C 1s profiles showing increases in the amine and amide components at 286.6 and 288 eV. Coupled with the extensive washing protocols, this indicates that the EDC/NHS surfaces had successfully immobilised trypsin. Overall, the XPS data clearly show that each stage of surface modification proceeded as desired.

Trypsin immobilisation on the ppAAc modified surface was studied using an anti-trypsin ELISA assay (see ESI†), where the ABTS-produced colour directly correlates to the amount of anti-trypsin–HRP antibody immobilised on the surface, which in turn directly correlates with the amount of immobilised trypsin. The results (see Fig. S1, ESI†) indicate that surface saturation was achieved when the trypsin solution concentration exceeded 100 μg mL^{-1}. To ensure that the maximum possible amount of trypsin was being bound in each case, all further studies were carried out on μIMERs 500 μg mL^{-1} trypsin solution concentration for the immobilization step. The results of the L-BAPA enzyme activity assay (Fig. S2, ESI†) demonstrate that the covalently immobilized trypsin retained activity, even after 15 runs (including a wash in between each run). In contrast, the physically adsorbed trypsin lost all activity after the third run. This suggests that covalent immobilization of the enzyme using EDC/NHS is more stable than physical adsorption, results that correlate with a previous study.[30] From these, further protein digestion studies were carried out using μIMERs previously washed with 3× 250 μL of PBS solution at 50 μL min^{-1} (to remove any unbound enzyme). The relative enzyme activity of the immobilised enzyme (after unbound enzyme is washed) is shown as a percentage in Fig. 2. Cleavage activity greater than 60% is achieved even after 6 runs using the same chip and with washes in between. Further experiments carried out herein equal

Table 2 BSA peptides identified in Fig. S3 (ESI†) after in-solution and in-chip digestions

				In-solution	In-chip
Sequence coverage (%)				16	35
Protein score				759	1209
Digestion time				4 h	30 s
Unique peptides matched				11	20
Accession no.				P02769	P02769
Label	*m/z*	Start–end	Peptide	In-solution	In-chip
A	487.7333	37–44	DLGEEHFK	✓	✓
B	582.3154	66–75	LVNELTEFAK	✓	✓
C	732.2558	76–88	TCVADESHAGCEK		✓
D	739.6945	106–117	ETYGDMADCCEK		✓
E	443.6835	131–138	DDSPDLPK		✓
F	788.8403	139–151	LKPDPNTLCDEFK		✓
G	464.2522	161–167	YLYEIAR	✓	
H	874.2943	184–197	YNGVFQECCQAEDK		✓
I	379.6965	198–204	GACLLPK		✓
J	461.7462	249–256	AEFVEVTK	✓	✓
K	395.2433	257–263	LVTDLTK	✓	✓
L	722.2726	286–297	YICDNQDTISSK		✓
M	536.7232	310–318	SHCIAEVEK		✓
N	978.4386	319–336	DAIPENLPPLTADFAEDK		✓
O	784.3665	347–359	DAFLGSFLYEYSR	✓	
P	751.7591	375–386	EYEATLEECCAK		✓
Q	653.3620	402–412	HLVDEPQNLIK	✓	✓
R	534.6906	413–420	QNCDQFEK		✓
S	769.3976	421–433	LGEYGFQNELIVR	✓	
T	756.4147	438–451	VPQVSTPTLVEVSR	✓	
U	449.7175	483–489	LCVLHEK		✓
V	569.7147	499–507	CCTESLVNR		✓
W	507.8123	549–557	QTALVELLK	✓	
X	700.3090	569–580	TVMENFVAFVDK		✓
Y	554.2357	588–597	EACFAVEGPK		✓

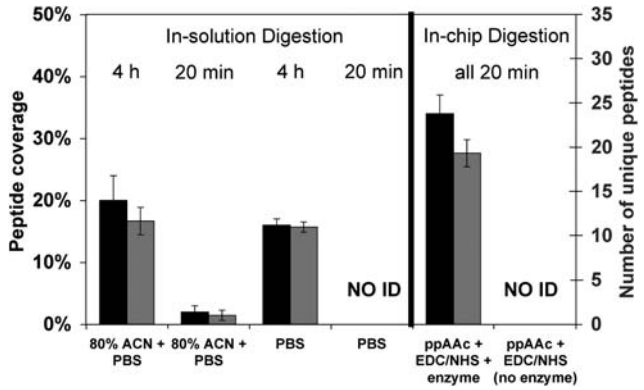

Fig. 3 Peptide coverage percentage (black bars) and number of peptides identified (grey bars) after digesting 10 μg of BSA at 37 °C under different methods: optimised in-solution (two different solvents and two incubation times) *versus* in-chip digestion (ppAAc modified surface and negative control). Error bars represent one standard deviation of triplicate analyses. MS analysis of 5 μL of 500 fmol μL^{-1} digested BSA, with a 30 min HPLC gradient. Mascot search allowed zero miss cleavages.

to 100% activity after washing unbound enzymes. The digestion of the same amount of protein of reused chips maybe estimated from these percentages.

3.2 Digestion of soluble proteins for MS based analysis using the μIMER

A BSA sample was digested in-chip for 20 min (5 μL min^{-1}) and compared with in-solution digestion (see ESI†). Fig. 3 demonstrates that a higher number of unique peptides (up to 58%

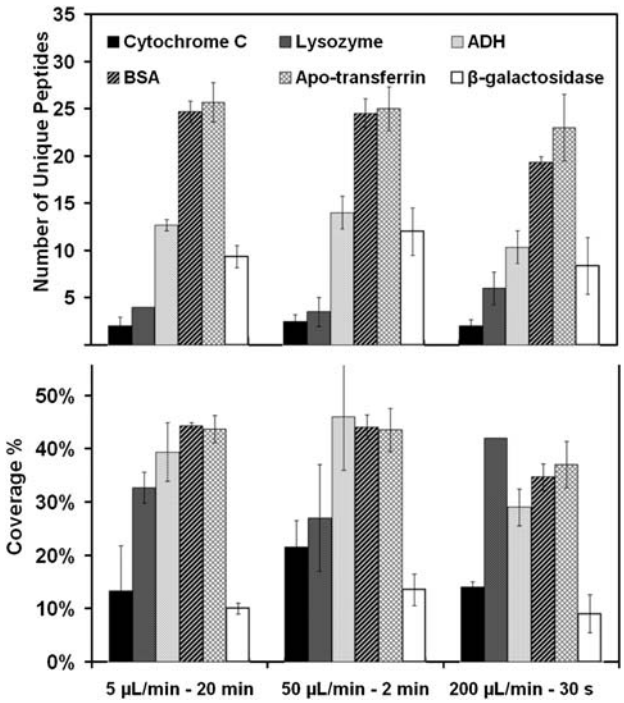

Fig. 4 Number of unique peptides and coverage percentage of the six proteins in the mixture after in-chip digestion at different flow rates. Error bars represent one standard deviation of triplicate analyses. MS analysis of 5 μL of 500 fmol μL^{-1} digested BSA, with a 30 min HPLC gradient. Results shown after Mascot search defining 2 missed cleavages.

Table 3 Tryptic digestion comparison of six protein mixture between conventional in-solution *vs.* in-chip protocols. Number of identified proteins and unique peptides after different digestion times and miss cleavages allowed

Type of digestion/device	Digestion time	No. of proteins identified (with ≥2 peptides)	Total no. of unique peptides	No. of proteins identified (with ≥2 peptides)	Total no. of unique peptides
In-house optimised protocols		*0 missed cleavage search*		*2 missed cleavage search*	
In-solution	4 h	6	72	6	82
Hybrid chip	2 min	6	73	6	82
Rapid digestion					
In-solution	30 s	5	41	5	70
Hybrid chip	30 s	5	60	6	69

higher) and coverage (up to 70% higher) was obtained with μIMERs when compared to conventional in-solution trypsin digestion, and that protein digestion was 12 times faster using the μIMER.

Table 2 and Fig. S3† show further digestion results and demonstrate improved MS information produced from the in-chip digestion (30 s) when compared to in-solution digestion (4 h), resulting in almost double the number of unique peptides (20 to 11 peptides, respectively), the peptide coverage (35 to 16%, respectively), and the Mowse score (1209 to 759 score, respectively). Only five peptides were common to both methodologies, and all of them showed higher counts for the in-chip method; for example peptide LVNELTEFAK (582.31 *m/z*) showed increased ion counts from 341 (in-solution) to 9157 (in-chip) (Fig. S3, ESI†).

A complex sample (six protein mixture) was digested in-chip under different flow rates. Fig. 4 shows that all proteins were confidently identified after all digestion times, even after 30 s. The maximum total number of peptides occurred for the 2 min in-chip protocol. The general digestion efficiency was analysed by comparing the total number of peptides identified with zero, one, and two enzymatic miss cleavages (MCs) (Fig. S4, ESI†). An increment in the total peptides identified was observed when allowing from zero to 1 MC (12 ± 2%), and a smaller increment (1%) was observed for 1 to 2 MCs, mainly due to the one additional peptide identified for the in-chip digestions of 1 min and 30 s. From these, the 2 min in-chip digestion was the optimal digestion protocol for highly complex samples after considering throughput and digestion efficiency. However, a 30 s in-chip digestion increases the throughput 4-fold if 1–2 MCs are allowed in the database search, as is a common practice in proteomics.[31]

A summary of the total number of proteins and unique peptides identified according to each optimised protocol is shown in Table 3. The in-chip (2 min) and in-solution (4 h) digestions identified all six proteins with a similar number of identified peptides either analysed with 0–2 MCs. To allow comparison, both protocols were also carried out at 30 s digestion time, but no 30 s protocol was able to identify all six proteins if MCs were not allowed. The only digestion protocol to identify all proteins was the in-chip digestion allowing 2 MCs. A greater peptide yield was obtained from the in-chip digestion in a considerably less time. To a large extent, and as previously reported,[23,25,32] this was likely due to: (1) the significant change of enzyme-to-protein molar ratio; (2) high digestion efficiency as the low level protein sample interacts more directly with the highly concentrated immobilised enzyme in the microchannel, due to the high surface-to-volume ratio; and (3) the lack of trypsin autoproteolytic fragments that may suppress the signal of the protein ions. Also, enhanced mobility of the molecules can be attributed to the significantly enhanced peptide yield, that is the proteins and the digests are rapidly removed from the vicinity of the active site of the immobilised trypsin, liberating the active site of the enzyme for further reaction.[33]

3.3 Membrane protein fraction proteomic analysis using the μIMER

Considering the results obtained from the new in-house μIMER, a new in-chip digestion workflow for rapid membrane

Table 4 Summary of the annotated and predicted protein localization, GRAVY values, transmembrane helices (TMH), and lipoproteins of identified proteins from the total membrane fraction after two-staged in-solution and in-chip digestions

Type of annotation/prediction	Percentage of proteins from in-solution workflow	Percentage of proteins from in-chip workflow
Total proteins identified	*158 proteins (100%)*	*105 proteins (100%)*
False Discovery Rate (FDR)	*4.2%*	*5.4%*
Uniprot annotation		
Thylakoid membrane	30	30
Other membrane/periplasmic	17	20
Cytoplasm/other	18	15
Not annotated	35	35
PSORTb bacterial localization prediction		
Cytoplasmic membrane	10	11
Outer membrane	4	5
Periplasmic	5	5
Cytoplasm	37	31
Unknown	44	48
GRAVY		
GRAVY > 0.0	27	31
GRAVY ≤ 0.0	73	69
Prediction of transmembrane helices in proteins prediction (1–17 TMH per protein)		
Predicted TMH	26	31
No TMH predicted	74	69
Lipoproteins prediction		
Predicted signal peptide (Sp I or Sp II)	15	19
No signal peptide predicted	85	81
Total proteins with either membrane annotation, membrane prediction, hydrophobic, predicted TMH, or predicted lipoprotein	*65*	*70*
Total proteins excluded from the above	*35*	*30*

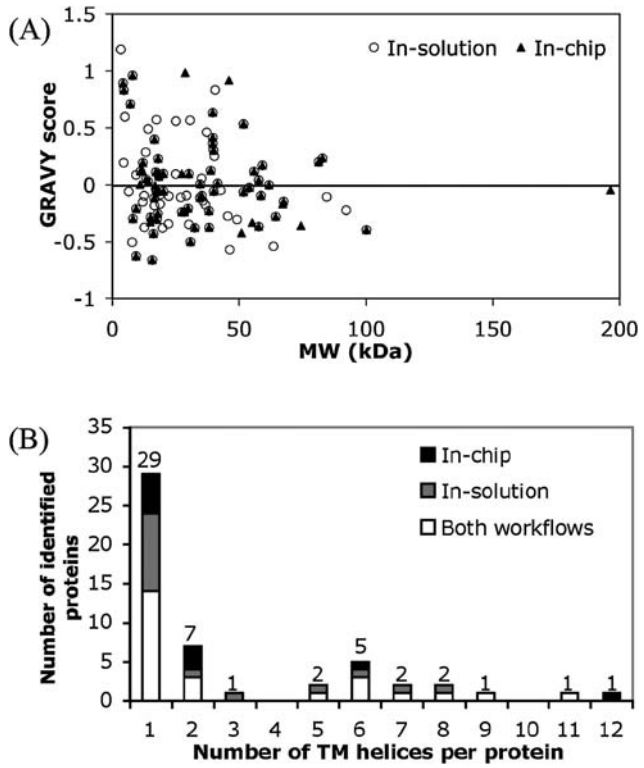

Fig. 5 Arrangement of the proteins identified from the total membrane fraction sample, after in-solution and in-chip workflows, with either membrane localization annotation, membrane localization prediction, one or more transmembrane helices, highly hydrophobic, or predicted lipoproteins, indicating: (A) relationship between molecular mass and GRAVY scores and (B) plotted against their number of TMH. A total of 51 proteins were identified with predicted TMH present. Note: numbers above the bars show the total number of identified proteins.

proteomics was developed. The efficient 2 min in-chip digestion protocol was used for the analysis of the total bacterial membrane fraction of *Synechocystis* sp. PCC 6803 (see ESI†), resulting in a digestion rate of 25 μg of protein mixture per minute. Initially, a two-staged in-solution digestion of the highly hydrophobic membrane proteins was carried out to obtain baseline results (see ESI†), based on a literature protocol.[34] This protocol comprised a two day digestion: firstly an overnight trypsin digestion in an aqueous buffer and a second overnight digestion with trypsin/chymotrypsin in a 60% methanol buffer of the purified insoluble fraction. The new μIMER-based workflow followed a variation of the reported protocol using a 2 min in-chip digestion instead of each overnight digestion.

The identified proteins of the total membrane fraction after two-staged in-solution and in-chip digestions are listed in Table S2 (ESI†). A summary of the identified proteins from both digestions and based on the annotation and prediction tools employed (see ESI†) are shown in Table 4. The False Discovery Rates (FDR) calculated were 4.2 and 5.4% for the in-solution and in-chip two-staged digestion workflows, respectively, also comparable to those in the literature.[35,36] After the two staged in-solution digestion, 714 unique peptides corresponding to 158 proteins were identified, from which 65% are highly hydrophobic, contain transmembrane helices, and/or are annotated to be present in the thylakoid/other membrane, following similar protein identification trends presented elsewhere.[34] For the in-chip workflow, 346 unique peptides corresponding to 105 proteins were identified for the total membrane fraction. The results show that 70% are highly hydrophobic, contain transmembrane helices, and/or are annotated to be present in the thylakoid/other membrane. Comparing the novel workflow (5 h turnaround digestion time) results with the in-solution digestion workflow (45 h turnaround digestion time), the first method identified 2/3 of the total proteins identified through the

Table 5 Summary of different proteomic studies of complex samples, comparing digestion time and estimated turnaround digestion time including type of sample and number of identified proteins for the two in-house digestion workflows studied and from previous bacterial membrane proteome and μIMER digestion studies

Type of digestion/device	Digestion time	Turnaround digestion time[a]	Fraction type – no. of proteins identified
Conventional/suggested protocols tested in-house			
Two staged in-solution digestion	20 h	45 h	Total membrane – 103 membrane/ hydrophobic[b] of 158 total
Two staged in-chip digestion	4 min	5 h	Total membrane – 73 membrane/ hydrophobic[b] of 105 total
Previously reported bacterial membrane proteome studies			
In-solution digestion[37]	12 h	12 h	Total membrane – 174 integral or associated membrane proteins of 410 total
In-solution digestion[38]	11 h	11 h	Inner membrane – 159 integral membrane proteins of 358 total
In-solution digestion[34]	24 h	45 h	Total membrane – 326 integral membrane proteins of 481 total
Solid-phase digestion in lipid-based protein immobilization (LPI) flowcell[39]	NI	NI	Plasma membrane vesicles – 92 membrane proteins of 313 total

[a] Digestion time starting after reduction and alkylation of sample and total duration of digestion including setup, digestion and collection of sample prior lyophilization, *i.e.* total digestion turnaround time. [b] Proteins with an annotation and/or prediction of membrane localization, TMH (1–17), GRAVY score grater than zero, or are predicted lipoproteins. NI: no information.

in-solution digestion workflow, but nine times faster. All annotations and predictions resulting from the two-staged in-chip digestion workflow reported an average of 2/3 of the number of proteins for each parameter/prediction when compared with the two-staged in-solution digestion annotations and predictions.

Particular physiochemical characteristics of the membrane proteins identified were compared between both methods, such as hydrophobicity, molecular weight, and isoelectric point. Fig. S5 (ESI†) shows a virtual 2D gel of the identified membrane proteins. Both the workflows identified proteins covering a wide p*I* range, including very basic proteins (up to 11 p*I*), and a wide range of protein molecular weights (M_w) (up to 200 kDa). Fig. 5A demonstrates that numerous very hydrophobic proteins were detectable with both methods, and that the number and size of hydrophobic and hydrophilic proteins were similar between methods. Fig. 5B shows that proteins with diverse predicted TMHs were identified, as also observed elsewhere.[34] Thus, there is similar performance between the in-solution and in-chip workflows for membrane protein identification. Reduction in coverage with the in-chip digestion was probably due to short (only 4 min) digestion time, and further optimisation of the in-chip digestion workflow is possible and is required to increase the proteome coverage. Potential optimizations include different membrane sample fractionation and purification techniques, and multidimensional HPLC fractionation approaches.

All the unique proteins identified using the two-staged in-solution and in-chip workflows were sorted into functional groups according to the CyanoBase (http://genome.kazusa.or.jp/cyanobase/Synechocystis/) genome annotation (Fig. S6, ESI†). The largest group identified are proteins involved in photosynthesis and respiration, which occur at the membrane-bound compartment, the thylakoid, indicating that membrane-related proteins are part of the photosynthesis and respiration. Another expected membrane function is transportation, also verified by the third largest group found in both samples. Other several functions occur in the total membrane fraction, highlighting the importance of increasing the membrane proteome profiling in order to answer numerous physiological questions and better understand the processes in a living cell.

Table 5 compares the digestion times and the number of identified proteins from the previously reported studies for bacterial membrane proteome profiling (highest number of reported membrane proteins identified, found to date in the literature). Comparing the in-solution and in-chip digestions carried out herein with three bacterial membrane proteome studies from the literature, the number of membrane/hydrophobic proteins identified here (73–103 proteins) was lower than previously reported (159–326).[34,37,38] However, these literature studies used a 2D HPLC fractionation unlike the 1D HPLC fractionation carried out here. Also, the samples are from different organisms, and this usually results in identification differences. Despite the lower number of identified membrane/hydrophobic proteins, the protocols presented here can be optimised further with changes to HPLC protocols. This, however, is beyond the scope of the work of using microfluidics chips.

The only other reported in-chip digestion of membrane proteins[39] (Table 5) used a Lipid-based Protein Immobilization Technology (LPI™, Nanoxis AB, Gothenburg, Sweden), where the proteoliposomes were immobilized and trypsin was digested in a flow cell, followed by elution of the tryptic peptides. 313 proteins were identified from plasma membrane vesicles, from which 92 were reported as membrane proteins. The disadvantage of the LPI is the time-consuming sample preparation for the processing of the plasma membrane vesicles before their injection into the flow cell for digestion. Also, it is impossible to compare the total turnaround digestion time, as this was not reported.

Wu *et al.*[33] reported a similar device using a PDMS chip and an UV-grafted polyacrylic acid surface modification. However, the μIMER produced in our study shows many distinct manufacturing advantages, as well as improved proteomic results. The manufacturing advantages include increased control over device fabrication using SU-8 photolithography, and the ability to simultaneously fabricate and ppAAc surface modify numerous μIMERs compared to the time-consuming UV graft polymerisation needed for each device. Finally, trypsin immobilisation *via* EDC/NHS in our novel μIMER was completed more rapidly (1 hour *vs.* 12 hours), while exhibiting high stability even after 16 protein digestion runs. Key proteomic advantages include the rapid digestion of complex soluble (6 protein mixture) and insoluble (total bacterial membrane protein fraction) samples.

4. Conclusions

The novel μIMER shows a substantial advantage in throughput for the proteomic analysis of complex soluble and, as a first, membrane protein samples, with comparable digestion efficiencies and proteome coverage over conventional in-solution digestion. A rapid 30 s digestion of soluble proteins was attained using the new PDMS/glass hybrid μIMER, up to 2400 times faster than in-solution digestion (20 h). The rapid enzyme biocatalysis time is comparable to the fastest μIMERs in a chip, previously reported for proteomics applications of soluble proteins, reporting 5 s to 20 min digestion times.[33,46–52] Furthermore, a 9-fold increase in membrane proteomic analyses throughput was demonstrated, an aspect of membrane proteome profiling that has been somewhat disregarded until now.[10,12–14] The novel device achieved greater peptide yield, thereby facilitating confident soluble and in-soluble protein identification. This increased yield was a result of the advantages of the μIMER design, including a significant change in the molar ratio of enzyme to protein, a high digestion efficiency resulting from a high surface-to-volume ratio, and the absence of trypsin autoproteolytic fragments.[23,25,32] Additionally, the ease and rapid fabrication of this hybrid μIMER, using plasma polymerisation to immobilise enzymes, provide a viable alternative device for a future large-scale manufacturing. This new μIMER is envisioned as a part of a complex shotgun proteomic workflow preceded by upstream fractionation employing LC-based methods or by on-line microchip protein fractionation.

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