

Supplementary Information

Mesofluidic platform integrating on-chip probe ultrasonication for multiple sample pretreatment involving denaturation, reduction, and digestion in protein identification assays by mass spectrometry

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Authors' contributions

J. L. Capelo and M. Miró designed the experimental setup. J. D. Nunes-Miranda, Cristina Núñez, G. Vale and H. M. Santos conducted the experimental work. J. D. Nunes-Miranda and C. Núñez prepared the figures. J. D. Nunes-Miranda, H. M. Santos, Cristina Núñez, Manuel Miró and J. L. Capelo wrote the manuscript. M. Reboiro-Jato and F. Fdez-Riverola wrote the statistics section. C. Lodeiro revised the manuscript and provided valuable suggestions. C. Lodeiro and J. L. Capelo and M. Miró provided financial support.

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EXPERIMENTAL SECTION

1.1. Protein standards and reagents

All reagents used were of HPLC grade or better. α -lactalbumin from bovine milk (α -lacta, $\geq 85\%$), bovine serum albumin (BSA, $>97\%$), trypsin from porcine pancreas (proteomics grade) and ovalbumin (OVA) were purchased from Sigma (Steinheim, Germany). α -cyano-4-hydroxycinnamic acid (α -CHCA, $\geq 99.0\%$) puriss for MALDI-MS obtained from Fluka (Buchs, Switzerland) was used as MALDI matrix. All materials were used as received without further purification.

The following reagents and solvents were used in protein digestion assays: acetonitrile (ACN), iodoacetamide (IAA) and DL-dithiothreitol (DTT) (99%) from Sigma; formic acid and ammonium bicarbonate (AMBIC, $\geq 99.5\%$) from Fluka and trifluoroacetic acid (TFA, 99%) from Riedel-de-Haën (Seelze, Germany).

1.2. Serum samples

Human serum samples were purchased from Patricell Ltd (BioCity Nottingham, UK). In this study, serum samples from five patients diagnosed with lymphoma and another five patients diagnosed with multiple myeloma were analyzed. Further sample information is provided in Table SI2. The US-assisted LOV method was first optimized using BSA, ovalbumin and α -lactalbumin as model proteins. The proof-of-concept assessment was then performed by fingerprinting of a complex proteome, namely human serum, of 10 individuals as pinpointed above. For comparative purposes, the same sample treatment was also performed in a batchwise mode.

1.3. Instrumentation

Mass spectrometric assays were performed using the Ultraflex II MALDI-TOF/TOF-MS instrument from Bruker Daltonics equipped with a 50 Hz Smart beam laser system. Data was acquired by Flex Control 3.3.92.0 (Bruker Daltonics). External calibration was performed with the $[M+H]^+$ monoisotopic peaks of angiotensin II (m/z 1046.5418), angiotensin I (m/z 1296.6848) substance P (m/z 1758.9326), ACTH clip 1–17 (m/z 2093.0862), ACTH18–39 (m/z 2465.1983) and somatostatin 28 (m/z 3147.4710). The mass spectrometer was operated with positive polarity in the reflectron mode. Spectra were acquired at each spot position at a constant power and in the mass range of 1000–3500 Da.

A vacuum concentrator centrifuge from UniEquip (model UNIVAPO 150ECH, Martinsried, Germany) with a refrigerated aspirator vacuum pump (model Unijet II) was used for sample drying and pre-concentration. 885 μL of depleted sera samples were transferred to new eppendorf tubes for sample drying and then pellet was reconstituted in 40 μL of 12.5 mM 1:1 (v/v) AMBIC/ACN or 100 mM 1:1 (v/v) AMBIC/ACN. After digestion in the LVOV system, 154 μL of sample were dried and then reconstituted in 10 μL of 0.1% (v/v) formic acid, mixed with the MALDI Matrix at a 1:1 ratio and spotted in the MALDI target prior to analysis.

1.4. Protein quantification

Standard protein solutions and sera samples for total protein assays were analyzed in triplicate using the NanoDrop® ND-1000 Spectrophotometer with the function protein ABS 280nm. An aliquot of 1 μL of each sample was pipetted to the pedestal and measured at 280 nm. For the multiple myeloma samples the protein concentration after depletion was $1.12 \pm 0.43 \mu\text{g}/\mu\text{L}$ (n=3) whilst for the lymphoma samples the protein concentration was $1.27 \pm 0.34 \mu\text{g}/\mu\text{L}$ (n=3).

1.5 Sample treatment

1.5.1. Chemical sequential protein depletion

To avoid the presence of the high abundant proteins, serum samples were depleted twice, first using DTT, and then ACN. This depletion protocol was previously developed by our group with success in the searching of biomarkers from patients with osteoarthritis.¹ Depletion is here recommended; otherwise the peptides belonging to the high abundant proteins would dominate the MALDI spectrum. As the abundant proteins would most likely occur in high levels in all sera, the classification of patients through profiling might be then jeopardized. To this end, 60 μL of individual serum samples were diluted up to 140 μL with milli-Q water. Sequential protein depletion was performed by adding 35 μL of 500 mM DTT to the serum followed by a 60 min incubation at room temperature in the dark until a viscous precipitate persisted. Then, the samples were centrifuged at 14000 g for 30 min; the supernatant was recovered and supplemented with 200 μL of milli-Q water followed by 450 μL of pure ACN. Samples were briefly vortexed and sonicated for 15 min in an ultrasonic bath. The protein precipitate was then pelleted by centrifugation at 14000 g for 30 min. The pellet was withdrawn and the supernatant recovered to a new eppendorf tube and then evaporated to dryness. Samples were resuspended

¹ C. Fernandez, H. M. Santos, C. Ruiz-Romero, F. J. Blanco and J. L. Capelo, *Electrophoresis*, 2011, **32**, 2966.

in 12.5 mM 1:1 (v/v) AMBIC/ACN or 100 mM 1:1 (v/v) AMBIC/ACN for subsequent protein digestion in both on-chip and batchwise (off-line) methods.

1.5.2. Batchwise (off-line) sample treatment

Data obtained previously in our laboratory^{2,3} suggested that the processing time for denaturation, reduction, alkylation and digestion of proteins is reduced considerably with the aid of ultrasonic energy.

Serum albumin, α -lacto-albumin and ovalbumin at the 1 μ g/ μ L level were used in method development. Proteins were dissolved in 12.5 mM 1:1 (v/v) AMBIC/ACN or in 100 mM 1:1 (v/v) AMBIC/ACN. For denaturation, samples were vortexed and sonicated in an ultra-sonic bath for 10 min. Reduction of proteins was undertaken as follows: 2 μ L of 110 mM DTT were added to 20 μ L of protein solution followed by activation of the ultrasonic probe (200 W, 50% amplitude, 0.5 mm tip, 1 min ultrasonication time). Then, 2 μ L of 600 mM IAA were added to alkylate the proteins using the very same ultrasonication procedure to that of reduction. Based on our expertise, protein denaturation, reduction and alkylation can be accomplished in merely 1 min each step, whilst a timeframe of 5 min is recommended for proteolytic digestion. The sample was then diluted 4-fold and protein digestion was performed over 10 μ L of sample with a trypsin to protein ratio of 1:20. Digestion was carried out using ultrasonic energy as described above but for 300 s. To stop trypsin activity 1 μ L of 50% (v/v) formic acid was added to the digested mixture.

1.5.3. Ultrasonic-based LOV system for sample treatment

The analytical procedure for automatic on-chip protein digestion exploiting US-assisted LOV is listed in Table SII and summarized as follows: First, the reagent and sample tubing lines are filled with the respective solutions. The syringe pump is then programmed to draw 40 μ L of sample (in 12.5 mM 1:1 (v/v) AMBIC/ACN or 100 mM 1:1 (v/v) AMBIC/ACN) into HC whereupon the flow is reversed and the sample is directed to the LOV chemical reactor (port 5) where it is subjected to high-intensity focused ultrasound for 60s for protein denaturation. A virtually identical process is followed to bring consecutively 40 μ L of 20 mM DTT and 40 μ L of 150 mM IAA towards the LOV reactor for protein reduction and alkylation, respectively. Likewise, the sonication probe is activated for 60 s in the course of each individual sample treatment step. The syringe pump is then set to deliver 80 μ L of AMBIC (carrier) to the reaction mixture. At this point, 60 μ L of the LOV reactor volume is preserved to subsequent protein

² F. M. Cordeiro, R. J. Carreira, R. Rial-Otero, M. G. Rivas, I. Moura and J. L. Capelo, *Rapid Commun. Mass Spectrom.*, 2007, **21**, 3269.

³ H. M. Santos, C. Mota, C. Lodeiro, I. Moura, I. Isaac and J. L. Capelo, *Talanta*, 2008, **77**, 870.

digestion, while the remaining volume is disposed. Afterward the HC is filled consecutively with 200 μL of air (to prevent dispersion in the HC) and 80 μL of trypsin where upon the protease is pumped to the LOV reactor for on-chip digestion. The sonication probe is then turned on and activated for 300 s. A metered volume of 140 μL of digested mixture is then drawn up in the HC and dispensed toward port 7 where it is collected in a vial. Protein digestion is stopped after the addition of 14 μL of 50% (v/v) formic acid and the mixture is subsequently dried in a speed vacuum for further mass spectrometric detection. The HC and chemical reactor (port 5) are finally thoroughly rinsed with carrier (AMBIC) prior to start a new analysis cycle. The total time needed is circa of 25 min.

1.6. MALDI-TOF-MS analysis

Prior to MALDI-TOF/TOF-MS analysis, the digested sample was resuspended in 10 μL of 0.1% (v/v) formic acid and then mixed with the matrix solution. α -CHCA was used as MALDI matrix throughout this work and was prepared as follows: 10 mg of α -CHCA was dissolved in 1.001 mL of Milli-Q water/ACN/TFA (500 μL + 500 μL + 1 μL). Then, 5 μL of the aforementioned matrix solution was mixed with 5 μL of sample and shaken in a vortex for 30 s. Finally, 1.5 μL of the mixture was hand-spotted onto the MALDI target plate and allowed to air dry at room temperature. Mass lists from each spectrum of the sera samples analysed were generated for the clustering analysis with the FlexAnalysis 3.3 software (Bruker Daltonics), using the peak detection algorithm SNAP (Sophisticated Numerical Annotation Procedure).

1.7. Clustering analysis

An agglomerative hierarchical clustering was used in order to compare the results obtained with the different methodologies. We decided to use only the peak presence information, discarding the use of the peaks intensity. For this reason, Hamming distance was selected as the distance metric for the hierarchical clustering construction. Additionally, the mean was selected as the linkage criteria between groups. The results of this clustering algorithm were presented as heat maps using a customized version of the Java Treeview software.⁴ The peaks from the mass lists previously created with a signal/noise value under 5 were discarded. Additionally, spectra were aligned and binned with a tolerance of 150 ppm and, finally, for each sample, peaks missing in two or more spectra were discarded.

⁴ A. J. Saldanha, *Bioinformatics*, 2004, **20**, 3246-3248.

Table SII. Operational sequence of the LOV method for US-assisted on-chip protein denaturation, reduction, alkylation and digestion.

Step	Selection valve position	Pump	Volume handled (μL)	Ultrasonic probe (min)
1	1	load	40	off
2	5	dispense	40	on (60 s)
3	8	load	40	off
4	5	dispense	40	on (60 s)
5	3	load	40	off
6	5	dispense	40	on (60 s)
7	5	dispense carrier	80	off
8	5	load	140	off
9	4	dispense	140	off*
10	2	load	200	off
11	6	load	80	off
12	5	dispense	80	on (300 s)
13	5	load	140	off
14	7	dispense	140	off

*This volume (140 μL) of each digested sample was stored to make replicates in case it was necessary.

1 **Table SI2.** Classification of the patients used in the study. F-female; M-male.

Patient	Diagnosis	Age	Gender	Medication	Stage/other comments
I	Lymphoma	90	F	-	Pretreatment
II	Lymphoma	77	F	-	Pretreatment
III	Lymphoma	69	M	-	Pretreatment
IV	Lymphoma	53	F	-	Pretreatment
V	Lymphoma	83	F	-	Pretreatment
VI	M. Myeloma	57	M	Revlimid, Velcade	-
VII	M. Myeloma	57	M	Revlimid, Velcade	-
VIII	M. Myeloma	57	M	Revlimid, Velcade	Stable
IX	M. Myeloma	57	M	Revlimid, Velcade	Stable
X	M. Myeloma /High Cholesterol	57	M	Aredia, Revlimid	Stable

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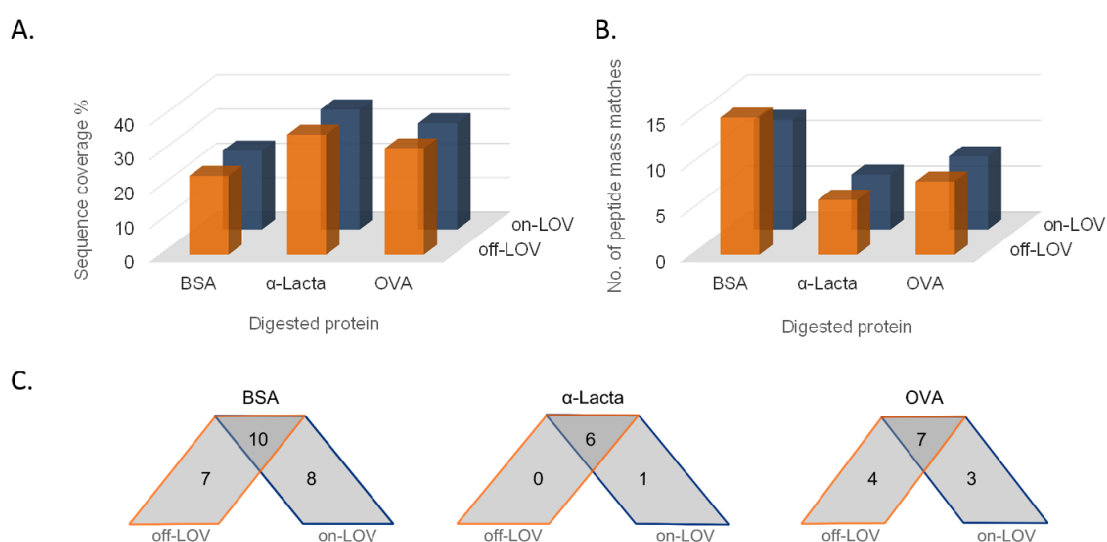
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20 **Figure S11.** Protein sequence coverage (A) and number of peptide mass matches (B) for
21 BSA, α -Lacta and OVA proteins in both the LOV and off-line methods. (C) Edwards'
22 Venn diagram representations of the number of unique/common peptide mass matches
23 identified between methods for each protein. To perform the analysis a unique peptide
24 mass list for each protein and method was created considering all replicas performed.
25 Buffer 3:1 (v/v) AMBIC 100 mM/ACN. 50% Ultrasonication Amplitude. The samples
26 were denatured, alkylated and reduced with the aid of ultrasonic energy using in each
27 step an ultrasonic probe (0.5 mm tip). Protein digestion was conducted for 5 min with
28 the ultrasonication settled at 50%.



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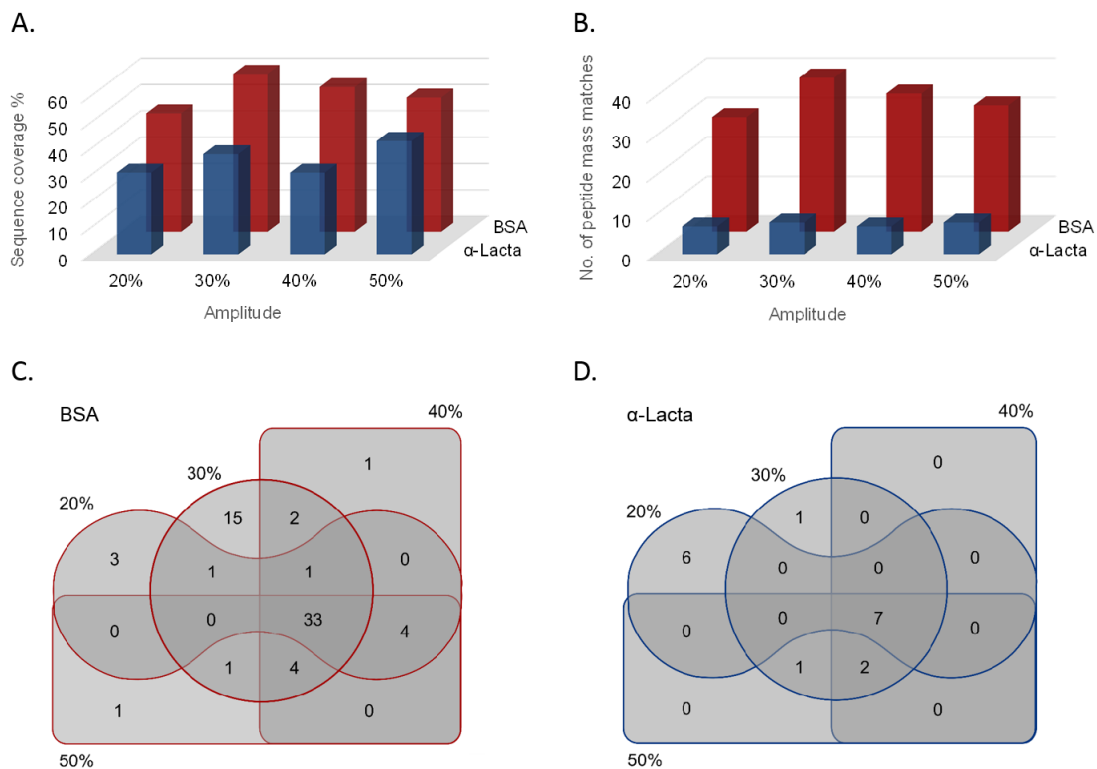
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42 **Figure SI2.** Protein sequence coverage (A) and number of peptide mass matches (B) for BSA
43 and α -Lacta proteins as a function of the amplitude in the LOV mesofluidic platform. (C)
44 Edwards' Venn diagram representation of the number of unique/common peptide mass matches
45 identified among the amplitudes studied for BSA (C) and α -Lacta (D) proteins. To perform the
46 analysis a unique peptide mass list for each protein and amplitude studied was created
47 considering all replicas performed.



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