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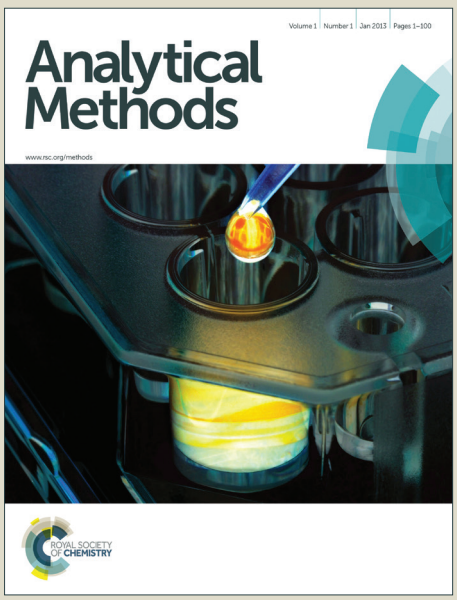

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ARTICLE

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based profiling as a step forward in the characterization of peritoneal dialysis effluent

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The aim of this study was to differentiate patients with glomerulonephritis and diabetic nephropathy using (i) peritoneal dialysate effluent, (ii) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and (iii) bioinformatics tools. Profiles of peritoneal dialysate effluent were obtained using (a) sample preparation consisting in protein concentration through centrifugal concentrators and chemical-assisted protein depletion using DL-dithiothreitol, and (b) MALDI-TOF MS. The free, open-source bioinformatics tool, Mass-UP, was used to classify such profiles using principal component analysis and hierarchical clustering. The methodology here proposed allows for classifying two different groups of patients with kidney failure, one with chronic glomerulonephritis and other with diabetic nephropathy.

Introduction

The utilization of profiles to classify samples has been used in analytical chemistry since decades ago [1–3]. In proteomics, mass spectrometry, MS, profiling of complex proteomes comprises three main steps. First, an appropriate sample treatment developed for each particular type of sample is needed. Second, an adequate mass spectrometer is necessary. High throughput, cost-effectiveness and robustness are characteristics of the Matrix-Assisted Laser Desorption Ionization time of flight, MALDI,-based MS [4] Third, bioinformatics tools are needed, either through commercial or free/open-source software to handle the large sets of data provided by MALDI-MS-based profiling. As both, bioinformatics and MALDI-MS have reached maturity, currently the bottleneck in proteome profiling remains sample treatment. Recently our group has proposed a number of fast, cheap and robust methods to deplete and/or to equalize the protein content of serum samples to speed disease profiling for patient classification and diagnostic purposes [5].

Renal insufficiency is a medical condition in which the kidneys fail to filter waste products adequately from the blood, eventually leading to dead. Therapy for renal failure can be done by effective methods such as peritoneal dialysis (PD) and haemodialysis, both of which effectively remove blood´s waste products [6–9]. Indeed, PD is a highly effective, convenient and reasonably safe treatment modality for patients with end-stage renal disease. PD is based on the use of the peritoneal membrane as a replacement of the kidney. This is possible because the peritoneal membrane can function as a dialyzing membrane, allowing mimicking the kidney capabilities for cleaning solutes and waste products from the blood [10, 11]. Although PD replaces the function of the kidney, pathologic damage to the peritoneum frequently results in decreasing of the dialysing capacity and then, the patient is forced to move to haemodialysis. At this stage, the patient´s condition gets worst slowly but constantly, leading to the death of the patient [11–13]. Peritoneal dialysate effluent (PDE) renders a sample reach in proteins and metabolites. This sample is a potential source of clinician information and, as such, it deserved to be interrogated for diagnosis and classification purposes. This work presents a novel approach to such aim based first on protein separation and concentration using centrifugal concentrators and then in protein equalization using DL-dithiothreitol, DTT. Then, the samples are trypsinated using a fast ultrasonic approach reported by us previously [14]. Finally the pool of peptides is profiled using MALDI-MS and then the sets of data generated are treated with free/open-source software by principal component analysis and hierarchical clustering (Mass-up: http://sing.ei.uvigo.es/mass-up/ last time accessed June, 2015). As proof-of-concept peritoneal dialysate

collected from patients with diabetic nephropathy and chronic glomerulonephritis was used.

2. Material and methods

2.1 Reagents

All reagents used were HPLC grade or electrophoresis grade. Albumin, from bovine serum (BSA), (N,N,N′,N′-tetramethylethylene-diamine (TMED), glycine, β-mercaptoethanol, glycerol 86–88%, acrylamide/bis- acrylamide 30% solution (37.5:1), mineral oil, the Bradford reagent, DL-dithiothreitol (DTT), iodoacetamide (IAA), Sodium Fluoride (NaF), trifluoroacetic acid (TFA), Trypsin (proteomics grade) and acetonitrile (ACN, LC-MS CHROMASOLV(R)), were purchased from Sigma-Aldrich (St. Louis, USA). EDTA and bromophenol blue were purchased from Riedel-de Haen (Seelze, Germany). Ammonium bicarbonate (Ambic) and formic acid were purchased from Fluka (Steinheim, Germany). α-Cyano-4-hydroxycinnamicacid puriss for MALDI-MS (Fluka, Germany) was used as MALDI matrix. Peptide Calibration Standard II from Bruker Diatonic GmbH was used for mass calibration standard for MALDI-TOF MS.

2.2 Peritoneal Dialysate Effluent Samples

Peritoneal dialysis effluent samples from ten anonymous patients from the Garcia de Orta Hospital, Portugal were collected in centrifuge sterile tubes supplemented with sodium fluoride and EDTA. Four patients with glomerular chronic nephritis, GNC, and six with diabetic nephropathy, DN, were enrolled in this study. For further details refers to Table 1 of supplementary information, T1SI †. The patients were informed about the project and their consent was requested in written. Once in the laboratory, the samples were centrifuged at 9,000g for 20 min to remove insoluble solids and stored at -80 ºC until use.

2.3 Apparatus

PDE samples were collected and aliquoted in centrifuge sterile tubes of 50 mL and 15 mL, respectively (Ratiolab, Germany). Protein concentration was done in Vivaspin centrifugal concentrators of 50 mL from Sartorius Stedim Biotech (Bohemia, U.S.A.) and protein digestion was done in safe-lock tubes of 0.5 mL from Eppendorf (Hamburg, Germany). A vacuum concentrator centrifuge model UNIVAPO 150 ECH SpeedVac and a vacuum pump model UNIJET II (Munich, Germany) were used for sample drying and sample pre-concentration. A mini incubator from Labnet (New Jersey, U.S.A.) was used for protein reduction and for protein alkylation steps. Centrifuge MPW-350 from MPW Med. Instruments (Warsaw, Poland), vortex models ELMI CM70M-09 Sky Line (Southern California, U.S.A.) and Labnet vortex mixer VX-200 (New Jersey, U.S.A.), were used throughout the sample treatment. An ultrasonic bath, Elma model Transsonic TI-H-5 (Singen, Germany), was used to facilitate protein depletion and peptide solubilization. A sonoreactor model UTR200 from Dr. Hielscher (Teltow, Germany) was used to accelerate enzymatic protein digestions. Protein identification was done in an Ultraflex II MALDI-TOF/TOF instrument from Bruker Daltonics.

2.4 Peritoneal dialysate concentration

PDE concentration and desalting was performed in centrifugal concentrators Vivaspin 15R MWCO 10 kDa. 10 mL of PDE were concentrated until a final volume ranging from 150 µL to 300 µL, by centrifugation at 5000×g for 20 min at 4ºC. The concentrated PDE was transferred into a clean safe-lock tube. The Vivaspin 15R MWCO 10 kDa membrane was washed with 50 µL of MQ-Water and then the water was added to the safe-lock tube with the concentrated PDE (Figure 1). The total protein content was determined using a Bradford protein assay. Once the samples were quantified, they were divided in aliquots of 20 µL and stored in 0.5 mL safe-lock tubes at -80 ºC.

2.5 Protein depletion with DTT

Protein depletion from sera samples was performed with DTT according to the protocol described by Warder *et al.* [15] with minor modifications as described by Fernández et al. [16]. In brief, to 20 µL of serum, 2.2 µL DTT 500 mM were added and the resulting mixture was vortexed for 20 s. The samples were then incubated for 1 h until a white precipitate was observed. Then the samples were pelleted by centrifugation at 14.000×g (2 × 20 min at 18ºC). This process was performed with five aliquots for each patient. Then, the supernatants were pooled in a clean safe-lock tube and total protein content was determined using a Bradford protein assay, using BSA as standard protein.

2.6 2D gel electrophoresis

Samples were resuspended in 8 M urea, 2% (w/v) CHAPS, 0.2% (v/v) IPG buffer pH 4–7, 50 mM DTT and traces of bromophenol blue. Samples were incubated on ice for 30 min and sonicated using a 1 mm diameter probe for 6×10 seconds on ice at 50% sonication amplitude. Insoluble mater was removed by centrifugation (20 minutes 14,000×g at 20 °C). Protein concentration was determined using a Bradford protein assay using BSA as protein standard. IPG stips pH 4-7, 7 cm were rehydrated overnight at 20 ºC with 135 µL of rehydration buffer containing 8 M Urea, 0.5% (w/v) CHAPS, 0.2% (v/v) IPG buffer pH 4–7, 10 mM DTT and traces of bromophenol blue. Sample loading onto the IPG Strip was carried out using the cup-loading method. IPG strips were removed from the rehydration tray; the oil was drained, and placed gel side up into the focusing tray's channels. The movable electrode assemblies were carefully positioned on top of the strips at the anode and cathode ends, after that, the sample loading cups were placed near the cathode. Afterwards, 50 µL of the protein-containing solution (2 mg/mL) were loaded in the sample-loading cup and then overlaid with mineral oil. The focusing tray was placed into the PROTEAN IEF Cell. The isoelectric focusing was performed in three steps as follows: step 1 250 Volts for 30 min, rapid voltage ramping; step 2: 4,000 Volts for 60 min, slow voltage ramping; Step 3: 4,000 Volts, 10,000 V-hr. For the three steps the current was limited to 50 µA/gel.

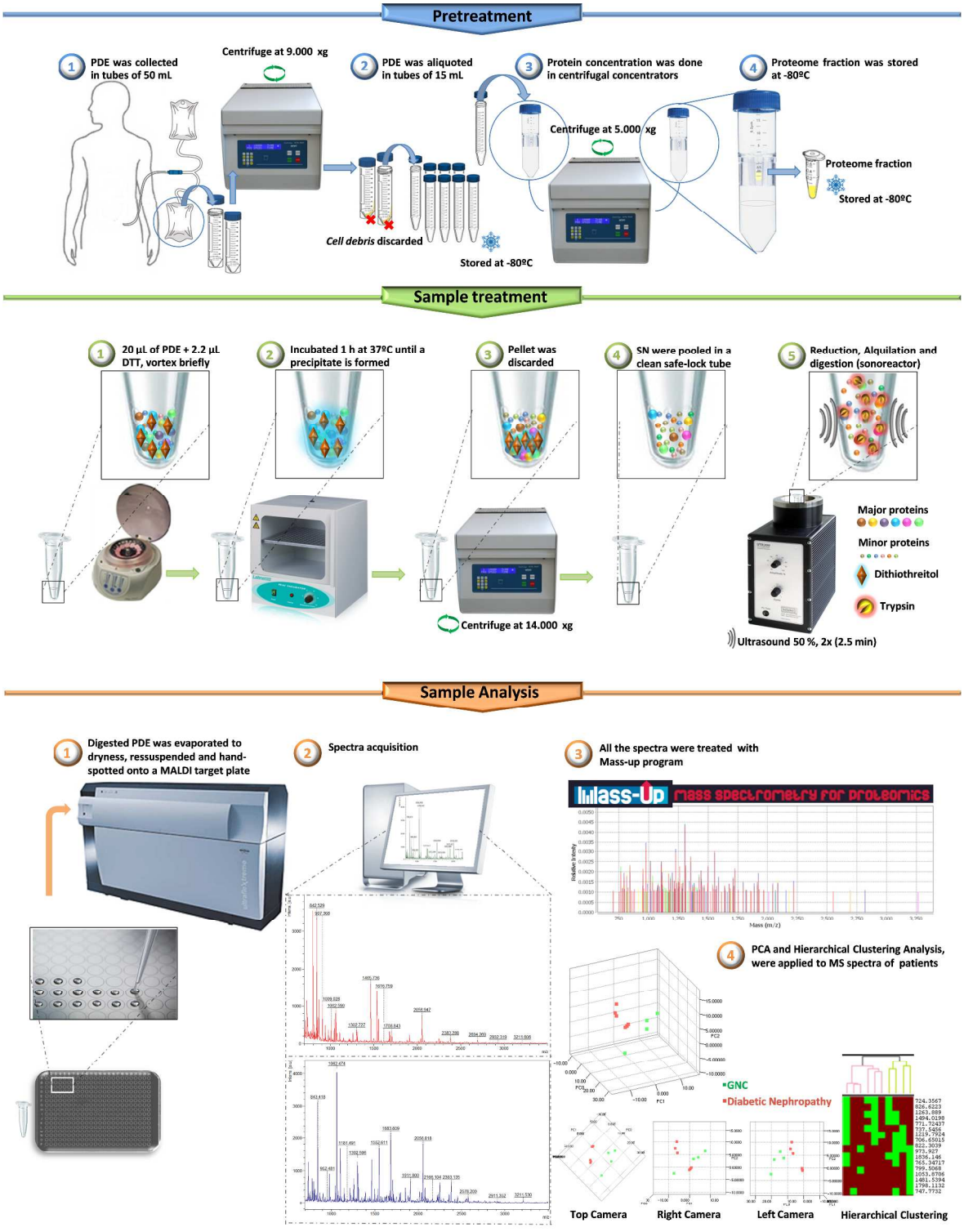

Figure 1- PDE sample treatment chart. **1- Concentration of proteins:** the protein content of 10 ml of PDE is concentrated to c.a. 150 µL using protein concentration tubes Vivaspin 15R MWCO 10 kDa. **2- Protein depletion:** The sample treatment to equalize the proteome content consists in DTT depletion. The resulting pellet is discarded and the supernatant (SN) is withdrawn and stored at -80 ºC until analysis. **3- Sample trypsination:** samples are digested using the ultrafast protein digestion, which was performed in a sonoreactor with the following operating conditions: 50% ultrasonic amplitude and 2.5 min ultrasonic time (twice). Then the sample was vacuum centrifuged till dryness. **4- MALDI Profiling:** For sample analysis the peptides were re-suspended, hand-spotted onto a MALDI target plate and analyzed by MALDI-TOF MS. Finally, the Mass-up program was used, as it was designed to support the pre-processing [(i) Baseline Correction; (ii) Normalization; (iii) Smoothing; (iv) Peak detection; (v) Peak matching)] and analysis of MALDI-TOF mass spectrometry data through principal component analysis and Hierarchical Clustering analysis.

After IEF, gel strips were removed and incubated with equilibration buffer (6 M urea, 75 mM Tris pH 8.8, 20% glycerol (v/v), 2% (w/v) SDS, traces of bromophenol blue) as follow 15 minutes incubation with 2.5 mL of equilibration buffer containing 2% (w/v) of DTT, followed by 15 minutes incubation with 2.5 mL of equilibration buffer containing 2.5% (w/v) of IAA. The IPG strips were removed from the equilibration tray and clip briefly into the graduated cylinder containing running buffer. The strip was placed side up and onto the back plate of the SDS-PAGE gel. The IPG well of the gel was overlay with agarose sealing solution (0.5% w/v prepared with 50 mL of Lammeli running buffer and traces of bromophenol blue). After agarose solidification, the electrophoresis was conducted at 200 V (constant voltage) for 55 minutes. Finished the gel electrophoresis, the gel was fixed for 30 minutes with 40% (v/v) ethanol and 10% (v/v) acetic acid and then stained overnight with colloidal coomassie blue G-250. Gels were rinsed 4 × 20 min with 100 mL of distilled water and further washed twice with 100 mL of 0.5 M sodium chloride until a clear background was observed. Gel imaging was carried out with a ProPicII-robot using 16 ms of exposure time and a resolution of 70 µm. Gel piking was done with the same equipment.

2.7 In-gel protein digestion

After spot piking the spots were transferred to 0.5 mL low adhesion tubes. Gel spots were washed twice with 200 µL of water and then with 3 × 200 µL of 50% (v/v) acetonitrile/25 mM ammonium bicarbonate and sonicated at 60% ultrasonic amplitude for 10 min using an ultrasonic bath. Then the gel pieces were dehydrated with 200 µL of acetonitrile. Subsequently, 15 µL of trypsin (20 ng/µL in ammonium bicarbonate 12.5 mM / 2% (v/v) acetonitrile) was added to the gel spots and incubated for 60 min on ice, then covered with 20 µL of 12.5 mM ammonium bicarbonate / 2% (v/v) acetonitrile and incubated 12 h at 37°C. Finally, 25 µL of 5 % (v/v) formic acid was added and the supernatants were transferred to new low adhesion tubes. Peptides were further extracted from the gel matrix with 2 × 50 µL of 50% (v/v) acetonitrile/0.1% trifluoroacetic acid and sonicated using at 60% ultrasonic amplitude for 5 min using an ultrasonic bath. Samples were dried-down and stored at -20°C until MALDI-TOF MS analysis.

2.8 In-solution protein digestion

Ultrasonic in-solution digestion was performed according to the ultrafast proteolytic digestion protocol previously developed in our laboratory [17]. Before protein digestion, the pH of the samples obtained in 2.5 were adjusted to 8.0 adding 1 µl of Ambic 0.5 M. Protein disulfide bonds were reduced with 2 µl 110 mM DTT, samples were then vortexed and incubated for 45 min at 37ºC. The resulting cysteines were then blocked with 2 µl IAA 400 mM. Samples were vortexed and incubated during 45 min at room temperature in the dark. For protein digestion, the reduced and alkylated samples were diluted to 0.04 µg/µL (2 µg of protein in a volume of 50 µL of AmBic 12.5 mM). Afterwards, trypsin was added according to the ratio 1:20 (w/w) twice (addition of 2.5 µL of trypsin, ultrasonic sonoreactor digestion, addition of another 2.5 µL of trypsin and then a final ultrasonic digestion with sonoreactor). Once the trypsin was added, the digestion was performed in the sonoreactor with the following operating conditions: 50% ultrasonic amplitude and 2.5 min ultrasonic time. Finally, 5µl of formic acid 50% (v/v) were added to stop the enzymatic activity (Figure 1), and the digested PDE was evaporated to dryness.

2.9 MALDI-TOF-MS analysis

Prior to analysis, samples were solubilized in 10 µL of formic acid 0.3% and 0.5 µL of sample was hand-spotted onto a MALDI target plate (384-spot ground steel plate) then 1 µL of a 7 mg/mL solution of a-cyano-4-hydroxycinnamic acid matrix in 0.1% (v/v) TFA and 50% (v/v) ACN was added and allowed to air dry. The mass spectrometer was operated in positive ion mode using a reflectron, and thus, spectra were acquired in the m/z range of 600–3500. A total of 500 spectra were acquired for each sample at a laser frequency of 50 Hz. External calibration was performed with the $[M+H]^+$ monoisotopic peaks of bradykinin 1–7 (m/z 757.3992), 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). Peptide mass fingerprints (PMF) were searched with MASCOT search engine with the following parameters: (i) SwissProt Database2012_04 (535698 sequences; 190107059 residues); (ii) molecular weight of protein: all; (iii) one missed cleavage; (iv) fixed modifications: carbamidomethylation (C); (v) variable modifications: oxidation of methionine and (vi) peptide tolerance up to 100 ppm after close-external calibration. The significance threshold was set to a minimum of 95% ($p \leq 0.05$). A match was considered successful when protein identification score is located out of the random region and the protein analysed scores first.

2.10 Principal component analysis (PCA)

Each sample was spotted in the MALDI plate five times. The corresponding raw-data spectrum of each sample was pre-processed with the Mass-Up open source program (http://sing.ei.uvigo.es/mass-up/) using the following parameters: (i) Intensity transformation (Square root), (ii) Smoothing (None), (iii) Baseline correction (Snip), (iv) Standardization (Total Ion Current), (v) Peak detection (MALDIquant: SNR (3), HalfWindowSize (60)), (vi) Minimum peak intensity (0.001). Peaks were matched with the following parameters: (i) Intra-sample matching (MALDIquant: tolerance (0.002)), without select the "generate consensus spectrum" box, (ii) Inter-sample matching (MALDIquant: tolerance (0.002). Then the PCA was run with the following parameters: (i) Max. Components (-1, for no maximum number of components), (ii) Variance Covered (0.95), (iii) Normalize, (iv) Discretize.

2.11 Hierarchical clustering analysis

An agglomerative hierarchical clustering analysis was applied as a complement to the PCA. Using the same handling of data as described above for the PCA, the clustering analysis operation of the Mass-up software was executed with the following parameters: (i) Minimum variance (0), (ii) Peptide List (Null), (iii) Distance Function Type Reference (Average), (iv) Conversion Values (Presence), Distance Function Type Function (Hamming), (v) Intra-sample Minimum Presence (0), Deep Clustering (No).

3. Results and discussion

3.1 Sample treatment

Unravelling the peritoneal dialysate effluent, PDE, proteome presents some challenges in terms of sample treatment, as PDE is a relatively diluted solution of proteins, containing hundreds of them, with their concentrations spanning several orders of magnitude. To overcome this problem, concentrating low-abundance proteins whilst diluting high abundance ones is mandatory. In our case such tasks were accomplished as follows. First, we focused in concentrating the proteins. Proteins can be separated and concentrated from a solution using different approaches such as precipitation of proteins [18], using magnetic beads [19] or centrifugal concentrator tubes [20]. In previous reported work [18], we have used DOC/TCA precipitation to concentrate proteins from PDE, however this method requires chaotropic agents like urea or thiourea and detergents to solubilize protein pellets. The use of such reagents is required to solubilize protein samples before 2D gel electrophoresis. On the other hand, high concentrations of chaotropic agents and detergents are contaminants for mass spectrometry analysis, requiring a further purification step before analysis. To overcome the use of urea and detergents, concentration of proteins was accomplished using centrifugal concentrators tubes with a cut-off membrane of 10 kDa. Through this process the protein content was concentrated from 10 mL to 150-300 µL.The second step was to overcome the problems related to high abundance proteins. For mass spectrometry-based applications the presence of major proteins makes difficult the detection of other less abundant proteins [16, 20, 21]. Different strategies are currently used to solve this problem, from which selective major protein depletion with chemical reagents, such as ACN or DTT, has been recently proposed as an economic and fast method to accomplish this task [5, 23, 24]. Indeed, as we have suggested, the use of ACN to deplete proteins in sera samples renders an extract rich in apolypoproteins whilst the use of DTT renders an extract rich in immunoglobulins. As a matter of fact, the use of DTT renders serum with a concentration in major proteins higher than when ACN is used [5, 23]. Because profiling is the main aim of this work, major proteins must be diluted from the target sample as much as possible but not totally. Through this way, the m/z signals originated from such proteins in the MALDI spectrum are drastically decreased, both in number and intensity, but are not totally eliminated. For the aforementioned reasons the application of DTT was selected for this work. To this end, first the samples were concentrated with the centrifugal concentrators and then they were depleted using DTT as described in section 2.5. The samples were then left to stand for about 1h [23]. We recommend making this process at temperatures above 25 ºC otherwise precipitation will take longer than 1h. After depletion the pellet was discarded and the supernatant was interrogated using 2D-gel electrophoresis. The result of this study is presented in Figure 2, where a typical gel image from a pooled sample can be seen along with the distribution of the type of proteins found. A complete list of the proteins identified is provided in Table SI2 †. As may be seen in Figure 2, the type of proteins present in the PDE clearly indicates a complex mechanism of membrane transportation through the peritoneum. It can be also noted that a significant fraction of those proteins are linked to regulation and response to stimulus. A total of 50 proteins were identified, of which 12 (24%) were included in the top-20 of the most abundant proteins. This was an excellent result and further confirmed that DTT selectively depletes high abundance proteins from complex proteomes. Furthermore, protein AMBP, vitamin D-binding protein, α1-antitrypsin and pigment epithelium-derived factor, identified in our experiments, have been associated with diabetic nephropathy [24]. Moreover, Ig mu chain C region and fibrinogen gamma chain, have been correlated to GNC [25].

3.2 Profiling PDE samples

Once confirmed the rationale of the method used for depleting major proteins from PDE samples, the next step was to apply the method to a number of samples from patients on PD treatment. The main aim of this work was to obtain for each patient a MALDI-MS profile of the pool of peptides belonging to the proteins obtained as described in 3.1. The number of patients as well as their individual characteristics is listed in Table SI1 †. The complete sample treatment is depicted in Figure 1. The approach presented therein takes advantage of the ultrasonic energy as a tool to boost the enzymatic cleavage of proteins. Such approach is fast, cheap and easy of handling [14, 17, 26]. An additional advantage for clinical applications is that ninety-six samples can be treated in one day. This sample treatment has been described previously by our team [14, 17, 26, 27]. A typical MALDI-MS profile obtained using this protocol is shown in Figure 1. The profiling method renders a number of MALDI-m/z signals, which in practical terms can be considered as a series of numbers. For each sample, a series is obtained. Such series are interrogated with bioinformatics programs using PCAs or hierarchical clustering analysis in our case, once all samples were profiled; a free/open-source software developed in collaboration with the SING group (http://sing.ei.uvigo.es) named mass-up was used. PCA and clustering analysis were the statistics tools used to study the samples.

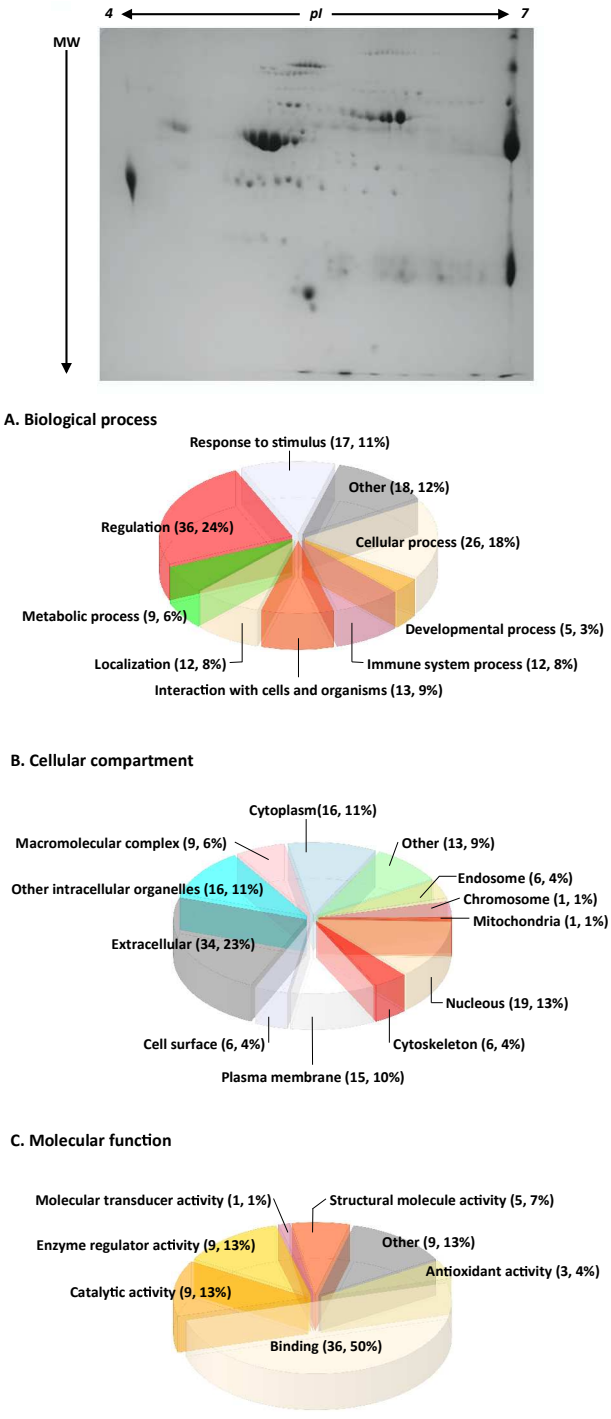

Figure 2. 2D-SDS-PAGE image from a pooled PDE sample of ten patients after protein concentration and depletion. Representative pie charts of: (A) Biological processes; (B) Cellular components; (C) Molecular function of the identified proteins. The pie charts were generated with the STRAP 1.5 software.

The Mass-up software is available free of charge at http://sing.ei.uvigo.es/mass-up/ (last time accessed June, 2015). The software is easy to use, and only requires to up-loading the m/z values corresponding to the profiles as an excel file. A tutorial is available in the same web page from which the program is downloaded. Figure 1 shows the PCA analysis (Figure SI1 †) obtained for the two groups of patients profiled in this work. As may be seen, the groups are well classified. The two diseases assessed (a) diabetic nephropathy, and (b) chronic glomerulonephritis alters the peritoneum membrane is a different way. As consequence the proteins that cross the membrane are also different. This difference is reflected in the pool of peptides obtained and thus the MALDI m/z values of each sample are also expected to have differences. The utility of the concept here proposed was further demonstrated by using hierarchical cluster analysis, which is shown in Figure 1 and in the Figure SI2 †. As may be seen, the groups are again well classified. The hierarchical clustering suggests that both diabetic nephropathy and chronic glomerulonephritis can be readily distinguished. This result opens new horizons in the research of the peritoneum degradation as a consequence of dialysis. Furthermore, it is anticipated also differences in the peritoneum of each patient as a result of a long period of dialysis treatment, thus helping physicians in preventing early peritoneum degradation.

Conclusions

We have demonstrated that the introduction of mass spectrometry-based profiling can provide a powerful, fast, cheap and accurate tool to classify patients with renal failure. It has been demonstrated that a workflow combining (i) sample preparation consisting in protein concentration through centrifugal concentrators and chemical-assisted protein depletion using DTT, (ii) MALDI-TOF MS and the Mass-UP software is an effective method to classify patients with glomerular chronic nephritis, and diabetic nephropathy. Therefore helping physicians in defining the aetiology of the kidney disease. Although the approach is promising, large international trials to provide extensive spectra databases are needed to make this kind of profiling methodology a useful tool for the nephrology community. We are currently working to address this issue.

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The authors declare no conflict of interest

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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