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Article

Proteomic study provides new clues for complications of hemodialysis caused by dialysis membrane

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ABSTRACT

The complications of hemodialysis accompanied the hemodialysis and threaten the patients' life. Besides the loss of nutrient substance, such as amino acid and vitamin, we found new clues that the adsorbed proteins on common-used polysulfone-based dialysis membrane might be the reason according to the qualitative proteomic study by ionic liquid assisted sample preparation method. Our results indicated that the adsorbed proteins on the membrane were related with complement activation, blood coagulation, and leukocyte-related biological process. The quantitative proteome further demonstrated some significant changes of signal proteins in the post-dialysis plasma after the hemodialysis, such as beta-2-microglobulin and platelet factor-4, which would further verify these new clues.

1. Introduction

Various complications, such as coagulation, accelerated atherosclerosis, malnutrition and thrombotic diathesis, accompanied the hemodialysis and weaken the patients' life quality [1,2]. Currently, the loss of nutrient substance, such as amino acid and vitamin, during the dialysis is usually taken as the important factor related to these complications. And, their compensation, for example L-carnitine supplementation, is also usually applied as the therapeutic strategy after the hemodialysis [3]. Although this strategy, in some extent, decreased the complications of hemodialysis, it still threaten the patient's life seriously, especially in the long-term dialysis therapy [4–6].

To solve this problem, the improvement of biocompatibility of the dialysis membrane have been conducted for decades [7,8]. Big data also regarded the close relationship between the dialysis membrane and the complications: the more biocompatible materials are more favorable to decrease the systemic effects [7,9]. But, its molecular substrates remain obscure. Recently, some studies demonstrated that the membrane could adsorbed some proteins [4,5,10–12]. Some studies even find that these proteins associated with the coagulation cascade and platelet activation [13,14]. In our opinion, the proteome analysis of protein adsorption onto dialysis membrane materials with depth coverage and high accuracy might uncover the mechanism of the complications of hemodialysis caused by dialysis membrane.

Recently, we have developed ionic liquid assisted sample preparation (ILSP) method to extract both hydrophilic and hydrophobic proteins with wide concentration ranges to promote significantly the large-scale proteome profiling [15,16]. Herein we employed ILSP method to pretreat the hemodialysis membrane for the proteomic analysis qualitatively. Meanwhile, proteomic comparison of pre-dialysis and post-dialysis plasma were conducted by mass defect-based pseudo-isobaric dimethyl labeling (pIDL) method for the further quantitative analysis [17]. Finally, we found the adsorbed proteins trigger a variety of biological pathways, related to the complications of hemodialysis (Scheme 1).

2. Materials and methods

2.1. Patients and treatment

Patients were recruited at the Hemodialysis Center of the Second Hospital of Dalian Medical University, and the study protocol was approved by the Institutional Ethics Committee. Inclusion criteria were as follows: regular dialysis for more than one year, established 4-h dialysis three times a week with Fresenius Polysulfine® Hemofilter (1.4 m^2, Fresenius Medical Care, Bad

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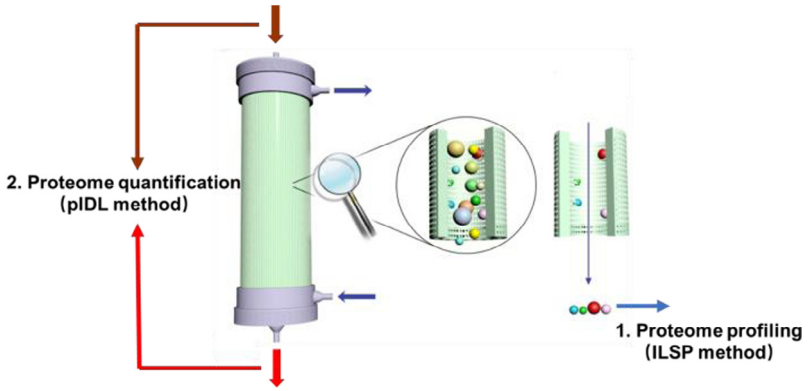


Scheme 1. (Color online) Schematic diagram of the proteomic study on dialysis membrane (1) ionic liquid assisted sample preparation (ILSP) method for the proteome profiling analysis; (2) mass defect-based pseudo-isobaric dimethyl labeling (pIDL) method for the proteome quantification analysis.

Homburg, Germany). Patients meeting the following criteria were excluded from the study: history of thrombotic complication or known coagulation disorder, diabetes mellitus, current or past anticoagulation treatment other than during the dialysis session, active inflammation, malignancy, or liver disease. Finally, four patients treated with hemodialysis were included in the study. All hemodialysis sessions were performed routinely; that is, blood flow (300 mL/min), dialysate flow (500 mL/min). As soon as the patient was disconnected from the system and the dialyser was flushed immediately with normal saline till no residual blood was left on the dialyser. Meanwhile, before and after the hemodialysis session, 2 mL of plasma were collected respectively.

2.2. Reagents and materials

The ionic liquid 1-dodecyl-3-methylimidazolium chloride (C_{12}Im-Cl) was purchased from Shanghai Cheng Jie Chemical (Shanghai, China). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA). Tris(hydroxymethyl) aminomethane (Tris), 1,4-dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA) and trifluoroacetic acid (TFA) were from Sigma (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade) and methanol were from Merck (Darmstadt, Germany). The filtration device with a relative molecular mass cut-off of 10,000 (10k filter) was from Sartorius AG (Goettingen, Germany). Deionized water was purified using a Milli-Q system from Millipore (Milford, MA, Germany). Other chemicals were of analytical grade.

2.3. Sample preparation of adsorbed proteome on the dialysis membrane

Four dialysers were incubated with C_{12}Im-Cl overnight to elute proteins adsorbed on the dialysis membrane. Then the eluted liquid containing abundant proteins was collected in the centrifuge tubes, after which 100 μL was transferred to filtration devices with a relative M_W cut-off of 10,000 Da for each 15 mL eluent. The samples were washed three times with 200 μL 1 × phosphate-buffered saline (1 × PBS) to remove the C_{12}Im-Cl, followed by centrifugation at 16,000 r/min for 10 min at 4 °C. The proteins were denatured at 95 °C for 5 min with 90 μL 1 × PBS and 10 μL 1 mol/L DTT, and then washed with 200 μL 1 × PBS. Next, the proteins were subjected to alkylation with 20 mmol/L IAA in the dark at room temperature for 40 min, followed by centrifugation at 16,000 r/min for 10 min. Finally, the samples were put in new collection tubes and 6 μg trypsin was added to the four samples at 37 °C for 12 h, followed by washing with 100 μL 1 × PBS and centrifugation at 16,000 r/min for 10 min. The digested peptides were desalted by reverse-phase liquid chromatography with a homemade C18 trap column (4.6 mm × 10 mm) at a flow rate 1 mL/min (A: 98% H_2O + 0.1%TFA; B: 98% ACN + 0.1% TFA).

2.4. Sample preparation of plasma

The plasma was diluted 100 times. Then, 100 μL diluted plasma was transferred to filtration devices. The sample pretreatment followed the abovementioned protocol till the digested peptides were desalted for the further usage. The digested peptides from the pre-dialysis and post-dialysis plasma were labeled with $^{13}CD_2O$ and $NaCNBD_3$ (light labeling, 32L) and with CD_2O and $NaCNBH_3$ (heavy labeling, 32H) respectively as our reported protocol.

2.5. Liquid chromatography-tandem mass spectrometry analysis

The resultant peptides were analyzed using a Q-Exactive Mass Spectrometer (Thermo, CA, USA) equipped with an Accela 600 HPLC system (Thermo) and an ESI probe Ion Max Source with a microspray kit, controlled by Xcalibur software version 2.1.0 (Thermo Fisher, Waltham, MA, USA). The mixture of labeled peptides was separated on a C18 capillary column (75 μm i. d.×15 cm) with C18 silica particles (5 μm, 100 Å) under acidic conditions. The two eluent buffers were 98% H_2O with 2% ACN and 0.1% FA (A), and 98% ACN with 2% H_2O and 0.1% FA (B). The gradient of the mobile phase was set as follows: 5%–22% B for 120 min, 22%–35% B for 30 min, and 35%–80% B for 5 min; maintenance with 80% B for 15 min at 300 nL/min. The resultant peptides were detected using a Q-Exactive Mass Spectrometer in data-dependent acquisition mode. Full scan mass spectrometry (MS) spectra were acquired from *m/z* 300 to 1,800 with a resolution of 70,000 at *m/z* 200. The AGC target was 1,000,000 and maximum IT was 50 ms for MS scans. 10 most intensive ions were subjected to high-collision fragmentation at a AGC target of 100,000 and maximum IT of 100 ms for MS/MS. The tandem mass spectra (MS/MS) were acquired in the orbitrap mass analyzer with a resolution of 17,500 at *m/z* 200 for the adsorbed protein identification and 35,000 at *m/z* 200 for the quantification between pre-dialysis and post-dialysis plasma. The dynamic exclusion time was set to 20 s. Normalized collision energy was 28% for the second stage of MS (MS2). The raw data had been published online (http://www.iprox.org/page/PDV014.html?projectId=IPX0000996000).

2.6. Database searching

The acquired raw files were searched with the pFind3.0 against the Human UniProtKB database (Download: Dec. 12, 2016; 71242 proteins), and the parameters were set as follows. Peptides were

searched using fully tryptic cleavage constraints, and up to two cleavage sites were allowed for tryptic digestion. Cysteine carbamidomethylation was set as a static modification, whereas protein N-terminal acetylation and methionine oxidation were set as variable modifications. Peptide identification was based on a search with an initial mass deviation of up to 10 ppm for the precursor ions and an allowed fragment mass deviation of 20 ppm. Heavy- and light-labeled samples were searched independently. The false discovery rate of 0.01 for both proteins and peptides levels was required to filter the results. Total intensities of the heavy and light paired fragment ions were used to calculate the ratio by pFind 3.0 software.

2.7. Bioinformatics analysis

The protein classes and biological processes based on the Gene Ontology (GO) Consortium were assigned with Panther tools (http://www.pantherdb.org/).

3. Results and discussion

3.1. Protein adsorbed on the dialyser

In order to analyze the proteins adsorbed on the dialysis, the proteins from each dialyser were analyzed in triplicate experiments on the LC-MS. Therefore, 12 shots of experiments were conducted for 4 samples. If the proteins were detected at least in 6 shots, the protein were considered as the adsorbed proteins on the dialysis with high confidence.

As shown in Table S1 (online), 462 protein groups were detected on the dialysis with high confidence. These proteins do not only include the well-known high abundance proteins, such as serotransferrin, IgG and apolipoprotein [18], but also some significant low abundance proteins such as complement factor H-related protein 5 (complement activation, alternative pathway, usually ~11 ng/mL) and Ficolin-2 (complement activation, lectin pathway, usually ~27 ng/mL). The distributions of molecular weight, GRAVY values and PI values were shown from Figs. S1 to S3 (online), which indicated that these 462 proteins span across a wide range of size, electrification, hydrophobicity. We deduced that the dialyser membrane could adsorb some proteins due to two reasons. On one hand, the $S(=O)_2$ group in the Polysulfone is capable of accepting electronic charge by the resonance effect, which lead to the adsorption of charged protein on the membrane [19]. On the other hand, the exposure of blood to an artificial surface results in the nearly instantaneous deposition of a layer of plasma proteins. Then, more proteins could be deposited on the first layer of plasma proteins through protein-protein interaction (Protein Corona Effect) [14].

3.2. Biological process of protein adsorbed on dialyser

The biological processes of these 462 proteins were analyzed by the Gene Ontology (GO). As shown in Table S2 (online), 206 biological processes were related to these proteins with high confidence (fold Enrichment > 5). It is obvious that the complement activation related biological process occupied 2 processes (Complement activation-alternative pathway and Complement activation-lectin pathway) in the top 5 biological processes.

Three categories of biological process should be discussed specially because its significant relationship with the complications of hemodialysis. The first category is the family of complement activation. Three pathways of complement activations (complement activation-alternative pathway, complement activation-lectin pathway and complement activation-classical pathway) are all included with high ranking (2, 5 and 23) in the biological process list. For the complement activation-alternative pathway, 11 proteins were detected on the dialysis membrane, while 13 proteins were reported in the database. For the complement activation-lectin pathway, 5 proteins were detected on the dialysis membrane, while 7 proteins were reported in the database. These proteins included Ficolin-2, Mannan-binding lectin serine protease 2, Ficolin-3, Mannan-binding lectin serine protease 1, and Keratin type II cytoskeletal 1. Their theoretical concentrations ranged from 27 ng/mL to 28 μg/mL. Because of their low abundance, these proteins could not be detected in the plasma control, with the identical sample pretreatment protocol.

The second category is the family of the blood coagulation. Blood coagulation (GO:0007596) is one of the obvious biological process in this family, as high as 61 proteins of the adsorbed proteins were attributed to this biological process.

The third category is the family of Leukocyte-related biological process. Nine biological processes were included in this family. As shown in Table 1, large number of proteins adsorbed on the membrane could attributed to each biological process in this family. Because of their low abundance in the plasma, these proteins could not be detected in the control sample.

Table 1
The biological processes of the adsorbed proteins on the dialyzer membrane.

Family of biological processes	Biological processes	Counts of adsorbed proteins on the dialyzer membrane	Counts of proteins in control plasma
Complement activation	Complement activation, alternative pathway (GO:0006957)	11	9
	Complement activation, lectin pathway (GO:0001867)	5	0
	Complement activation, classical pathway (GO:0006958)	21	30
Blood coagulation	Blood coagulation, fibrin clot formation (GO:0072378)	10	11
	Blood coagulation, intrinsic pathway (GO:0007597)	6	8
	Positive regulation of blood coagulation (GO:0030194)	7	6
	Negative regulation of blood coagulation (GO:0030195)	13	13
	Regulation of blood coagulation (GO:0030193)	17	15
	Blood coagulation (GO:0007596)	61	28
Leukocyte related biological processes	Leukocyte migration involved in inflammatory response (GO:0002523)	5	0
	Leukocyte mediated immunity (GO:0002443)	29	31
	Myeloid leukocyte migration (GO:0097529)	16	0
	Leukocyte chemotaxis (GO:0030595)	18	0
	Regulation of leukocyte chemotaxis (GO:0002688)	12	0
	Leukocyte migration (GO:0050900)	30	0
	Regulation of leukocyte migration (GO:0002685)	15	0
	Regulation of leukocyte proliferation (GO:0070663)	18	0
	Regulation of leukocyte cell-cell adhesion (GO:1903037)	20	0

3.3. Molecular function and Panther pathway of protein adsorbed on dialyzer

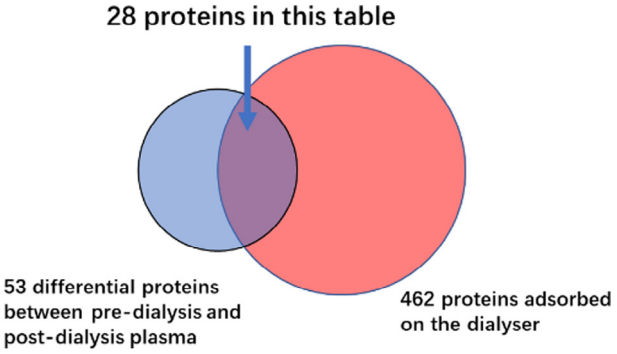
The Molecular function of these 462 proteins were also analyzed by the Gene Ontology (GO). As shown in Table S3 (online), 48 molecular functions were related to these proteins with high confidence (fold Enrichment > 5). When these 462 proteins were analyzed in the form of the Panther pathway, as shown in Table S3 (online), 4 Panther pathways were obtained, including pentose phosphate pathway (P02762), glycolysis (P00024), blood coagulation (P00011) and plasminogen activating cascade (P00050).

3.4. Comparative proteome analysis between pre-dialysis and post-dialysis plasma

Discovering differentially expressed proteins in various biological samples requires proteome quantification methods with accuracy, precision, and wide dynamic range [19–21]. The novel proteome quantification methods, pIDL method, satisfied these demands and was applied to the comparative proteome analysis of real sample with highly accurate and precise quantification results [17]. Herein, the comparative proteome analysis between pre-dialysis and post-dialysis plasma were conducted by pIDL methods.

To obtain the differential proteins, significant regulations of proteins were defined by outlier significance scores for log protein ratios (significance A, P value < 0.05). Then all the significant regulation proteins were combined. As shown in Table S4 (online), 53 differential proteins were obtained for the pre-dialysis and post-dialysis plasma, including 28 proteins which could be also detected in the eluted proteins on the dialysis membrane (Table 2). As shown in Table S5 (online), 77 biological processes were related to these 28 differential proteins with high confidence (fold enrichment > 5), while 19 molecular functions were related to these proteins with high confidence (fold enrichment > 5). It is should be mentioned that all these proteins attributed only one panther pathways, blood coagulation (P00011). These differential proteins provide additional evidence that the adsorbed protein lead to the proteomic change of plasma, which would lead to series of complications of hemodialysis.

Table 2
Differential proteins of the pre-dialysis and post-dialysis plasma which could be also detected on the dialyzer membrane.



Protein	Description	Significance[a]
sp|P80748|LV302_HUMAN	Ig lambda chain V-III region LOI OS	++
tr|V9GYM3|V9GYM3_HUMAN	Apolipoprotein A-II OS	+
sp|P02654|APOC1_HUMAN	Apolipoprotein C-I OS	++
sp|P02656|APOC3_HUMAN	Apolipoprotein C-III OS	+
sp|P55056|APOC4_HUMAN	Apolipoprotein C-IV OS	+
sp|P02649|APOE_HUMAN	Apolipoprotein E OS	+
sp|Q13790|APOF_HUMAN	Apolipoprotein F OS	+
sp|P61769|B2MG_HUMAN	Beta-2-microglobulin OS	++
sp|O43866|CD5L_HUMAN	CD5 antigen-like OS	++
sp|P00746|CFAD_HUMAN	Complement factor D OS	+
sp|P01034|CYTC_HUMAN	Cystatin-C OS	+++
sp|Q15828|CYTM_HUMAN	Cystatin-M OS	+
sp|P02679-2|FIBG_HUMAN	Isoform Gamma-A of Fibrinogen gamma chain OS	+
sp|P69905|HBA_HUMAN	Hemoglobin subunit alpha OS	++
sp|P68871|HBB_HUMAN	Hemoglobin subunit beta OS	++
sp|P24592|IBP6_HUMAN	Insulin-like growth factor-binding protein 6 OS	+
sp|Q14624-4|ITIH4_HUMAN	Isoform 4 of Inter-alpha-trypsin inhibitor heavy chain H4 OS	+
sp|P61626|LYSC_HUMAN	Lysozyme C OS	++
sp|P02776|PLF4_HUMAN	Platelet factor 4 OS	+
sp|P05109|S10A8_HUMAN	Protein S100-A8 OS	+
sp|P0DJI8|SAA1_HUMAN	Serum amyloid A-1 protein OS	+
sp|P0DJI9|SAA2_HUMAN	Serum amyloid A-2 protein OS	+
tr|A0A096LPE2|A0A096LPE2_HUMAN	Protein SAA2-SAA4 OS	+
sp|P01008|ANT3_HUMAN	Antithrombin-III OS	+
sp|P08697-2|A2AP_HUMAN	Isoform 2 of Alpha-2-antiplasmin OS	+
sp|P05155-3|IC1_HUMAN	Isoform 3 of Plasma protease C1 inhibitor OS	++
sp|P10451-5|OSTP_HUMAN	Isoform 5 of Osteopontin OS	+
sp|P02766|TTHY_HUMAN	Transthyretin OS	+
sp|P80748|LV302_HUMAN	Ig lambda chain V-III region LOI OS	++

[a] The amount of "+" in significance indicates that number of replications in the patients.

3.5. Significant proteins in the comparative proteome analysis

Although it is beyond our ability to mine all the 28 differential proteins, some proteins were really significant to be emphasized here.

Beta-2-microglobulin is a kind of small molecule globulin produced by lymphocytes, platelets and polymorphonuclear leukocytes [22]. It is composed of a single chain polypeptide of 99 amino acids, with molecular weight of 11,800. In patients with long-term hemodialysis, it can aggregate into amyloid fibers that deposit in joint spaces, a common complications, known as dialysis-related amyloidosis [22]. Therefore, it is significant to remove the redundant Beta-2-microglobulin during the hemodialysis. From the results of the protein adsorbed on the dialysis, it can be noted that at least certain amount of Beta-2-microglobulin was trapped on the membrane. From the results of the comparative proteome analysis between the pre-dialysis and post-dialysis plasma, Beta-2-microglobulin was also the differential protein. But, it is should be mentioned that this protein was significantly improved in one patients. As the signal protein of the inflammation and rejection, the improvement of Beta-2-microglobulin in the post-dialysis plasma demonstrated that the dialyzer triggered the high expression of the Beta-2-microglobulin, which would lead to the high risk of complications of hemodialysis. This deduction should be verified in the large number of clinical samples in the further studies.

Platelet factor-4 is a 70-amino acid protein that is released from the alpha-granules of activated platelets [23]. Its major physiologic role appears to inhibit local antithrombin III activity and promote coagulation [23]. It should be noted that the platelet factor-4 is not only adsorbed on the dialyzer membrane, but also overexpressed in the post-dialysis plasma. This indicated that the certain amount of platelets were activated during the hemodialysis due to the contact with the dialyzer membrane to release this protein, which would increase the risk of blood coagulation.

4. Conclusion

To find the clues for the complications of hemodialysis, we focused our study on the adsorbed proteins on the dialyzer membrane and the differential proteins between pre-dialysis and post-dialysis plasma by the novel proteomic methods, ILSP method and pIDL method. The qualitative proteome study of the adsorbed proteins revealed that the adsorbed proteins on the membrane were related with complement activation, blood coagulation, Leukocyte-related biological process. The quantitative proteome further demonstrated that some significant changes of signal proteins would lead to the complement activations and blood coagulation. Therefore, the adsorbed proteins should be one of the causes of the complications of hemodialysis. The application of novel proteomic techniques in this study also warned that the current dialysis membrane is still not biocompatible enough to favor the benefit of the patients.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scib.2017.08.026.

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