Integration of monolithic frit into the particulate capillary (IMFPC) column in shotgun proteome analysis

Fangjun Wang, Jing Dong, Mingliang Ye, Ren'an Wu, Hanfa Zou*

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1. Introduction

To increase the detection sensitivity, nano-flow liquid chromatography (μLC) using capillary column with diameter less than 100 μm is directly coupled with MS for peptide mixtures analysis in shotgun proteome analysis [1–8]. Among all of the separation materials, reversed phase (RP) materials are most widely used to pack into capillary columns due to its powerful separation capability and compatibility with ESI-MS detection [9,10].

There are usually two strategies to pack the separation materials into the capillary columns. The first one is positioning a filter assembly containing a 2-μm (or less) pore size stainless steel frit at one end of the capillary, and then the packing materials suspended in slurry solvent are flushed and retained into the capillary by a LC pump. After capillary column packed, one replaceable ESI emitter is connected with the filter assembly, on which the high voltage is applied for ESI [11]. Shen et al. packed 3-μm C18 particles into capillary columns with inner diameter from 15 to 150 μm to obtain high efficiency separation with a peak capacity at about 1000 by this packing method [12–15]. The other method is using a fritless capillary to pack μLC separation columns. Briefly, one ~5 μm i.d. tip is pulled from one end of the capillary by a flame or laser puller, and then the separation materials suspended in slurry solvent are packed into the fritless capillary and confined by the tip using LC pump or gas pressure. This type of column eliminates the post column void volume, which is benefit for peptides separation and identification [16,17]. Yates and co-workers packed RP and SCX materials into a fritless capillary column in sequence and developed multidimensional protein identification technology (MudPIT), which had high separation capability and detection sensitivity for identifying thousands of proteins in one analysis [18–20].

Monolithic column is a good alternative to particulate column due to its good permeability and fast analytes’ transferring rate [21–23]. When the monolithic columns are coupled with ESI-MS, an integrated ESI emitter tip is usually created onto the front of the monolithic column by several of methods [24–27]. Furthermore, comparing to the fritless packed capillary column, the ESI emitter tip on monolithic column will not be clogged by the packing materials and can be fabricated at smaller inner diameter (~2 μm) with thinner wall, which is benefit for the ESI quality and stability [25,27].

The development of LC analytical column with high separation capability and reproducibility should be one of the key issues in shotgun proteome analysis. Particles packed fritless capillary column developed by Yates and co-workers is widely applied in μLC–MS/MS analysis for high throughput qualitative and quantitative proteome analysis [16–20]. However, the fine tip of the fritless capillary is easily to be clogged during packing procedures and separation process, which is relied on randomicity. Furthermore, it is difficult to pack particles less than 5 μm into the fritless column and the packed particles would be flushed out of the tip.
during the separation process. In this study, we developed an integrating monolithic frit particulate capillary (IMFPC) column, which combined the separation capability of packed column and the advantages of monolithic ESI emitter. Briefly, ~1-cm C12 monolith was prepared in one end of the capillary column, and then 12-cm C18 separation stationary phase was packed and retained by the monolith, finally an integrated ESI emitter tip was fabricated on the monolith section. Comparing to the classical fritless packed capillary column, higher average peak capacity and proteome coverage were obtained. As the high quality monolithic emitter was integrated, high performance electrospray and good run-to-run and column-to-column reproducibility could also be achieved in shotgun proteome analysis.

2. Experimental

2.1. Materials

Fused silica capillaries with 75 and 50-μm i.d. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Daisogel ODS-AQ (3 or 5 μm, 120 Å pore) was purchased from Daiso (Osaka, Japan). All the water used in the experiments was purified using a Mill-Q system from Millipore Factory (Bedford, MA, USA). Lauryl methacrylate, ethylene dimethacrylate and γ-methacryloxypropytrimethoxysilane (γ-MAPS) were obtained from Sigma (St. Louis, MO, USA). Azobisisobutyronitrile (AIBN) was obtained from Shanghai Fourth Reagent Plant (Shanghai, China). Trypsin was obtained from Promega (Madison, WI, USA).

2.2. Sample preparation

The mouse liver protein extract was prepared in a denaturing buffer containing 50 mM Tris/HCl (pH 8.1) and 8 M urea as before [28]. The protein concentration was determined by BCA assay. The protein sample was reduced by DTT at 37 °C for 2 h and alkylated by iodoacetamide in dark at room temperature for 40 min. Then the solution was diluted to 1 M urea with 50 mM Tris/HCl (pH 8.1). Finally, trypsin was added with weight ratio of trypsin to protein at 1/25 and incubated at 37 °C overnight. Then, the tryptic digest was purified with a homemade C18 solid phase cartridge and exchanged into buffer A (0.1% formic acid water solution). Finally, the samples were stored at −20 °C before use.

2.3. Preparation of IMFPC column

The preparation procedures for IMFPC column were shown in Fig. 1. At first, the 75-μm i.d. capillary was pretreated with γ-MAPS as described elsewhere [29,30]. Second, the polymerization mixture of 100 μL lauryl methacrylate, 100 μL ethylene dimethacrylate, 170 μL 1-propanol, 130 μL 1,4-butanediol, 20 μL water, and 2 mg AIBN was mixed, sonicated for 20 min to obtain a homogeneous mixture and then purged with nitrogen for 10 min. After the pretreated capillary was filled with ~2-cm of this C12 polymerization mixture by siphon, it was sealed at both ends with rubber stoppers. The sealed capillary was submerged into a water bath and allowed to react for 12 h at 60 °C. The resultant capillary column with ~2-cm C12 monolith was washed with methanol using an LC pump to remove unreacted monomers and porogens followed by drying by nitrogen gas. Third, the ODS-AQ materials suspended in methanol were packed into capillary column from the open end and retained by the 2-cm C12 monolith using a homemade pneumatic pressure cell at constant nitrogen gas pressure of about 580 psi. When 12-cm ODS-AQ materials were packed, the capillary column was connected to a LC pump and flushed with water at 3000 psi for 30 min. Fourth, an integrated electrospray emitter tip was directly pulled from the end of 2-cm C12 monolith by a flame as described by Xie et al. and only ~1-cm C12 monolith was remained. The inner diameter of the tip was controlled at 2–3 μm [25].

To prepare the fritless packed capillary column, one end of a 75-μm i.d. capillary was first manually pulled to a fine point of ~5 μm with a flame torch, and then the ODS-AQ particles were packed until the packing section reached the length of 12 cm [29].

2.4. μLC–MS/MS system

The LC system (ThermoFinnigan, San Jose, CA) consisted of a degasser, a quaternary Surveyor MS pump. The three buffer solutions used for the quaternary pump were 0.1% formic acid aqueous solution (buffer A), ACN with 0.1% formic acid (buffer B), 1000 mM NH₄Ac aqueous solution at pH 2.7 (buffer C). Capillary with 50 μm i.d. was used for splitting, and the flow rate after splitting was adjusted to 200 nL min⁻¹. The LTQ linear ion trap mass spectrometer equipped with a nanospray source, a six-port/two-position valve (Thermo, San Jose, CA). The temperature of the ion transfer capillary was set at 200 °C. The ESI voltage was set at 1.82 kV and the normalized collision energy was set at 35.0%. One microscan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data dependent mode. The mass spectrometer was set that 1 full MS scan was followed by 6 MS/MS scans on the 6 most intense ions. The dynamic exclusion function was set as follows: repeat count 2, repeat duration 30 s, and exclusion duration 90 s. System control and data collection were done by Xcalibur software version 1.4.

2.5. Sample injection and LC separation

The configuration of automated sample injection system was the same to previous work [29]. During sample injection, the switching valve was switched to close the splitting flow and the flow through from the phosphate monolithic trap column was switched to waste. Sample was automatically injected at a high flow rate to ensure three sample volumes of buffer A were passed through for removing contaminants. After sample injection, the sample enriched onto the phosphate monolithic column was flushed to ODS-AQ separation column by buffer C, followed with buffer A equilibrium as described previously [29]. Then, the sample was separated by a binary gradient with buffer A and buffer B, which was set as from 0 to 10% buffer B for 2 min, from 10 to 35% for 90 min, and from 35 to 80% for 5 min.
After flushing 80% buffer B for 10 min, the separation system was equilibrated by buffer A for 15 min. The data acquisition of MS was begun immediately after the start of RP gradient separation.

### 2.6. Data analysis

The acquired MS/MS spectra were searched on the database using the Turbo SEQUEST in the BioWorks 3.2 software suite (Thermo). Reversed sequences were appended to the mouse International Protein Index (IPI) protein database (v3.17) for the evaluation of false positive rate. Cysteine residues were searched as static modification of 57.0215 Da, and methionine residues as 3.75 for triply charged peptides. The mass tolerances were considered as positive identification if the Xcorr were higher than 1.9 for singly charged peptides, 2.2 for doubly charged peptides, and 3.75 for triply charged peptides. $\Delta C_\text{cutoff}$ value was set to control the false positive rate of peptides identification <1%.

### 3. Results and discussion

#### 3.1. Separation capability of IMFPC column in $\mu$L–MS/MS analysis

In order to evaluate the performance of IMFPC column packed with 3 $\mu$m ODS-AQ particles, tryptic digest of 1 $\mu$g mouse liver extract was used as standard sample for $\mu$L–MS/MS analysis and the obtained base peak chromatogram was shown in Fig. 2 A. Then, the single ion currents (SICs) of the six peptides, R.VSQEHPVVLTK.F, K.VITAFNEGLK.N, K.VITAFNEGLK.N, K.VITAFNEGLK.N, K.INEAFDLLR.S, R.GFLDVVAALR.W, K.AFAISGPFNVQFLVK.G, and K.GEFQILLDALDKIK.T were extracted from the base peak chromatogram as shown in Fig. 3 A–F. It can be seen that the peak profile of tryptic digestion of mouse liver proteins on IMFPC column (shown in Fig. 2A) is narrower and sharper than that on the fritless capillary column, which demonstrates that the separation capability of fritless capillary column is inferior to IMFPC column. In order to compare the performance of those columns more quantitatively, SICs of the same six peptides separated on the fritless capillary column were extracted from the base peak chromatogram as shown in Fig. 3 G–L. The measured average values of the $W_{0.613}$ for the six peptides by three consecutive analyses were varied from 0.22 to 0.35 min as the data shown in Table 1, and the average value of the $W_{0.613}$ for all the six peptides is 0.30 min. Then the average peak capacity calculated for fritless capillary column is 154. By comparing to the IMFPC column, the peak capacity of this fritless capillary column decreased 26.7%.

The column-to-column reproducibility was also investigated for the two types of columns. At first, 3 $\mu$g tryptic digest of mouse liver extract as testing sample was separated on three IMFPC columns (1 $\mu$g tryptic digest of mouse liver extract for each column), respectively. The obtained $W_{0.613}$ values of the extracted six peptides on different IMFPC columns were given in Table 2. The average val-

### Table 1

<table>
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<tr>
<th>Peptides</th>
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<th>2</th>
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<th>4</th>
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<th>6</th>
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<td></td>
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<tr>
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<td>0.34</td>
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<td>16.30</td>
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Similarly, a fritless capillary column packed with 5 $\mu$m ODS-AQ particles was also applied for the separation of tryptic digest of 1 $\mu$g mouse liver extract and the obtained base peak chromatogram was shown in Fig. 2B. It can be seen that the peak profile of tryptic digest of mouse liver proteins on IMFPC column (shown in Fig. 2A) is narrower and sharper than that on the fritless capillary column, which demonstrates that the separation capability of fritless capillary column is inferior to IMFPC column. In order to compare the performance of those columns more quantitatively, SICs of the same six peptides separated on the fritless capillary column were extracted from the base peak chromatogram as shown in Fig. 3 G–L. The measured average values of the $W_{0.613}$ for the six peptides by three consecutive analyses were varied from 0.22 to 0.35 min as the data shown in Table 1, and the average value of the $W_{0.613}$ for all the six peptides is 0.30 min. Then the average peak capacity calculated for fritless capillary column is 154. By comparing to the IMFPC column, the peak capacity of this fritless capillary column decreased 26.7%.

The column-to-column reproducibility was also investigated for the two types of columns. At first, 3 $\mu$g tryptic digest of mouse liver extract as testing sample was separated on three IMFPC columns (1 $\mu$g tryptic digest of mouse liver extract for each column), respectively. The obtained $W_{0.613}$ values of the extracted six peptides on different IMFPC columns were given in Table 2. The average val-
Fig. 3. Single ion currents (SICs) for six peptides R.VSQEHPVVLTK.F, K.VITAFNEGLK.N, K.INEAFDLLR.S, R.GFLDVVAALR.W, K.AFAISGPFNVQFLVK.G, and K.GEFQIDLDALDIKT by using (A–F) IMFPC column and (G–L) classical fritless capillary column ($W_{0.613}$, peak width at 0.613 height of the peak, which was an average value of three replicate injections).

ues of $W_{0.613}$ of the six peptides on three IMFPC columns were varied from 0.14 to 0.29 with an average value at 0.24. Therefore, the average peak capacity of the three IMFPC columns was 193, which was determined by the same way as described above. Then, three fritless columns were applied to analysis of same standard sample. The obtained $W_{0.613}$ values of the same six peptides were also given in Table 2 and the average $W_{0.613}$ value of the three fritless columns were varied from 0.19 to 0.33 for the six peptides with an average value at 0.27. Thus, an average peak capacity 173 could be obtained for the three fritless columns, which decreased 10.6% comparing to the three IMFPC columns. Moreover, the average RSD of $W_{0.613}$ values for the six peptides in three columns' analysis was 17.51% for IMFPC columns and 27.49% for fritless columns, which demonstrated that better column-to-column reproducibility could be obtained for IMFPC columns. Therefore, the IMFPC column has better separation capability and reproducibility in column-to-column analysis, which was consistent with the results in run-to-run analysis as described above.

3.2. Performance of IMFPC column in shotgun proteome analysis

Both the fritless capillary column and the IMFPC column were applied to proteome analysis of mouse liver by injection of 1 µg tryptic digests of mouse liver proteins. The obtained MS/MS spectra were searched against mouse IPI database v3.17 by SEQUEST, and the criteria described above were applied to filter the results. When IMFPC column packed with 3 µm ODS-AQ particles was applied, 461 distinct proteins (RSD = 2.14%, $n = 3$) and 1564 unique peptides (RSD = 2.28%, $n = 3$) could be identified from one LC–MS/MS analysis. And totally 640 proteins and 2287 unique peptides could be
Column-to-column reproducibility of W\textsubscript{0.613} for six peptides RVSQEHQVLTKF, K.VITAFNGLK.K, K.INEAFLILRS, R.GFHDMYVAALK.W, K.AFSFQFNVQIVK.K, and K.GEIQILIADLKK.K by using IMFPC column and classical fritless capillary column.

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obtained in three consecutive analyses as shown in Fig. 4A, among which the percentages of proteins and peptides identified at least 2 or 3 times were 67.5, 48.6% and 63.4, 39.3%, respectively. When fritless capillary column packed with 5 \( \mu \)m ODS-AQ particles was used, 384 distinct proteins (RSD = 5.40%, \( n = 3 \)) and 1286 unique peptides (RSD = 5.95%, \( n = 3 \)) could be identified from one \( \mu \)LC–MS/MS analysis. And totally 537 distinct proteins and 2110 unique peptides were obtained by the three fritless capillary columns as shown in Fig. 4B, among which the percentages of proteins and peptides identified by at least 2 and 3 columns were 67.0, 47.7% and 60.6, 35.0%, respectively. Thus, the total number of proteins and peptides identified by fritless column in three consecutive analyses decreased 16.1 and 13.8% by comparing to those obtained on the IMFPC column packed with 3 \( \mu \)m ODS-AQ particles.

The column-to-column reproducibility in shotgun proteome analysis was also investigated for the two types of columns. When three IMFPC columns were applied, 467 distinct proteins (RSD = 1.26%, \( n = 3 \)) and 1549 unique peptides (RSD = 3.38%, \( n = 3 \)) could be identified from one \( \mu \)LC–MS/MS analysis, and totally 672 distinct proteins and 2382 unique peptides were identified by the three IMFPC columns as shown in Fig. 5A, among which the percentages of proteins and peptides identified by at least 2 and 3 columns were 64.1, 44.2% and 61.0, 36.7%, respectively. When three fritless capillary columns packed with 5 \( \mu \)m ODS-AQ particles were used, 397 distinct proteins (RSD = 4.60%, \( n = 3 \)) and 1311 unique peptides (RSD = 4.53%, \( n = 3 \)) could be identified from one \( \mu \)LC–MS/MS analysis, and totally 585 distinct proteins and 2110 unique peptides were obtained by the three fritless columns as shown in Fig. 5B, among which the percentages of proteins and peptides identified by at least 2 and 3 columns were 62.0, 41.5% and 56.2, 30.2%, respectively. Obviously, the total number of proteins and peptides identified by three fritless columns decreased 12.9 and 11.4% comparing to those on the three IMFPC columns packed with 3 \( \mu \)m ODS-AQ particles. Therefore, the IMFPC column has better performance in shotgun proteome analysis than fritless capillary column by column-to-column analysis, which was consistent with the results of run-to-run analysis as described above.

The main advantage of IMFPC column is the easiness for packing of separation materials. As a monolithic frit is integrated at one end of the capillary, the packing process becomes much easier and faster comparing to fritless capillary column due to the high permeability of the monolithic frit. Furthermore, smaller packing particles (less than 5 \( \mu \)m) could be easily packed and would not be clogged during...
Fig. 5. The column-to-column reproducibility of proteome analysis of tryptic digest of 1 μg mouse liver extract by (A) IMFPC column packed with 3 μm ODS-AQ particles and (B) fritless column packed with 5 μm ODS-AQ particles.

the packing process, which increases the separation capability evidently. After column packing, a high quality ESI tip can be directly pulled from the end of monolithic section, which eliminates the void volume between the analytical column and ESI emitter and improve the quality of electrospray as described by Xie et al. [25]. It was demonstrated that the peptides elution profiles were better by using IMFPC column than fritless column, and the peak profile of peptide R.GFLDVVAALR.W was even branched when fritless column was used (see Fig. 3). Furthermore, if the fine tip onto the monolithic emitter is broken during separation process, it can be regenerated by a flame under water flushing, which is extremely helpful for long-time μLC–MS/MS analysis with MudPIT approach. In our experiments, one IMFPC column could be continuously used over 1 month. Very recently, Facarro et al. fabricated a silica monolithic frit integrated with an ESI tip on the front of the particulate column, and they demonstrated that this monolithic emitter could improve electrospray ionization efficiency greatly [31]. The only difference of IMFPC column used in this study is that the frit is prepared by organic polymer-based monolith. Comparing to the column fabricated by Facarro et al., the IMFPC column can be prepared more facilely due to the easiness of prepa-ration of organic polymer-based monolith; however, the separation efficiency of capillary column with polymeric monolith frit may be some lower than that with silica monolith frit.

4. Conclusions

High quality capillary analytical column is essential to high separation capability in μLC–MS/MS for shotgun proteome analysis. In this study, we developed an IMFPC column that has a monolithic frit fabricated into one end of the capillary column, from which an integrated ESI tip was pulled out. This type of column can be packed by stationary phase particles with small diameter (ca.1.5 μm) more easily and would not be clogged during packing and separating process comparing to fritless capillary column. The monolithic emitter integrated on the packed capillary column can improve the quality and efficiency of electrospray ionization and can be regenerated. When 3-μm packing particle was used, the IMFPC column had much higher average peak capacity and proteome coverage comparing to conventionally fritless capillary column packed with 5 μm particles. Considering the easiness in column packing and the good run-to-run and column-to-column reproducibility in proteome analysis, this type of IMFPC column may be superior to fritless column in shotgun proteome analysis, and it is worth investigating the performance of this type of column by packing more fine particles into the capillary in near future.

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References