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APPLICATION NOTE

Membrane-Based Continuous Remover of Trifluoroacetic Acid in Mobile Phase for LC-ESI-MS Analysis of Small Molecules and Proteins

Zhigui Zhou, Jialing Zhang, Jiawei Xing, Yu Bai, Yiping Liao, Huwei Liu

Beijing National Laboratory for Molecular Sciences, the Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, Institute of Analytical Chemistry, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China

Abstract
We developed a "continuous" trifluoroacetic acid (TFA) remover based on electrodialysis with bipolar membrane for online coupling of liquid chromatography (LC) and electrospray ionization mass spectrometry (ESI-MS) using TFA containing mobile phase. With the TFA remover as an interface, the TFA anion in the mobile phase was removed based on electrodialysis mechanism, and meanwhile, the anion exchange membrane was self-regenerated by the hydroxide ions produced by the bipolar membrane. So the remover could continuously work without any additional regeneration process. The established LC-TFA remover-MS system has been successfully applied for the qualitative and quantitative analysis of small molecules as well as proteins.

Key words: Membrane, Remover, Trifluoroacetic acid, Liquid chromatography, Mass spectrometry

TFA is a widely used mobile phase modifier in LC because of its excellent ion-pairing ability that could bring about better resolution and peak shapes for basic compounds and biomolecules on reversed-phase LC columns. Compared with UV detection, MS coupled to LC has proven to be a more powerful analytical strategy in biological and pharmaceutical fields due to the availability of structure information and better sensitivity. However, the coupling of LC with TFA-containing mobile phase to ESI-MS has shown some problems because the existence of TFA would suppress the electrospray ionization and reduce the MS signal. The suppression effect is mainly caused by the spray instability due to the high conductivity and surface tension of TFA solution [1, 2]. Additionally, the ion pairing between TFA anions and protonated analyte ions also inhibits the ionization process [1, 2]. So the incompatibility of TFA and MS has greatly restricted the full demonstration of both LC and MS advantages.

To overcome these problems, the post-column addition of propionic acid and isopropanol, so called "TFA-Fix" method, has been widely used [1, 2]. The TFA anion is removed by the high concentration of propionic acid, so the strong interaction of ion pair between TFA anion and analyte ion is eliminated. The direct addition of acetic acid or propionic acid to the TFA-containing mobile phase was reported to have a similar effect [3]. Also, the use of capillary chromatography instead of conventional column combined with low surface tension sheath liquid and sheath gas has shown good potential to improve MS sensitivity [4]. In addition, New et al. [5] presented an anion-exchange micro-membrane suppressor; TFA was removed as a result of concentration difference. The suppressor was applied for

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Correspondence to: Huwei Liu; *e-mail:* hwliu@pku.edu.cn

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the detection of fluorocinnamic acid in negative ion polarity, and sensitivity enhancement was observed. Recently, a strategy based on post-column electrophoretic mobility control was also reported to enhance the signal of peptides [6]. However, the limitation of these solutions could still be a problem, such as sample dilution caused by post-column addition or no suitability for conventional column. Hence, a more efficient and universal solution or interface for this purpose is desired.

In this study, we developed a novel "continuous" TFA remover based on electrodialysis with bipolar membrane. Based on electrodialysis mechanism, TFA was removed with high efficiency, and the remover could easily be coupled with conventional LC and ESI-MS (see Figure 1a for instrument setup). The remover, serving as a universal interface of LC to MS, has provided a simple and efficient solution to eliminate the influence of TFA on MS detection sensitivity while maintaining the good separation with TFA as modifier. The setup of the remover is shown in Figure 1b (see Figure S1 in Supplemental Data for the expanded view), containing an anion exchange membrane, a bipolar membrane, and a poly(tetrafluoroethylene) film with a micro-channel. The TFA-containing mobile phase was delivered through the micro-channel directly or after being split to a proper flow rate. Under electric field, TFA anion crossed the anion exchange membrane and was delivered to waste as shown in Figure 1c. Meanwhile, the anion exchange membrane could be regenerated by the produced OH^{-} from water dissociation that occurred in the boundary region of the anion and cation exchange layers of the bipolar membrane [7–10]. Hence, the TFA remover could work continuously without any additional regeneration process.

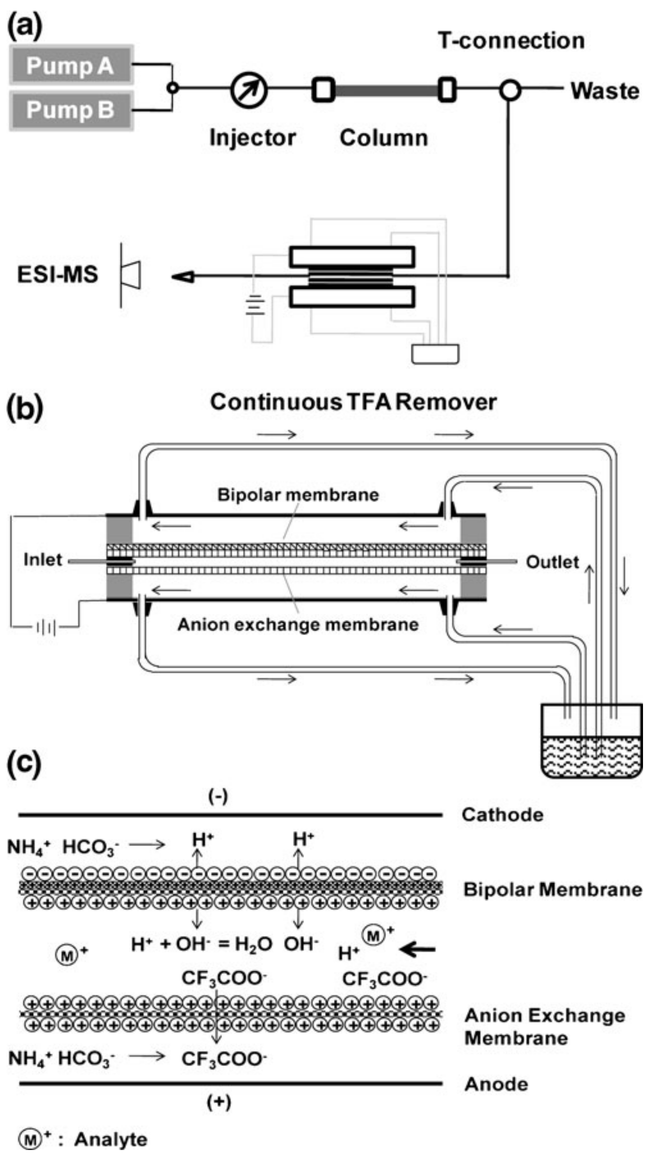

Figure 1. Schematic of the LC-TFA remover-MS system **(a)**, the TFA remover **(b)**, and the working mechanism of the remover **(c)**

The TFA-removal efficiency of the remover was systematically evaluated using water and ACN as mobile phase, which were widely used for biomolecule separation by reversed phase LC. Under electric field ranging from 0 to 25 V, the total conductivities of water and ACN/water (50/50, vol/vol) containing 0.5 % and 0.1 % TFA flowing through the remover at different flow rates were recorded by a conductivity detector, respectively (see Figure S2 of Supplemental Data for the results). The results showed that all of their conductivities could be reduced to a value that was comparable with that of pure water or ACN/water mixture when the voltage increased to a certain value, proving the high efficiency of TFA removal. However, when the voltage was increased beyond the optimal TFA-removal potential, the conductivity began to increase instead. That was because water dissociation was also enhanced with a higher voltage as shown in Figure S3 (Supplemental Data), and the produced OH^{-} began to dominate the conductivity. The facilitated water dissociation could accelerate the regeneration of anion exchange membrane. The whole evaluation process was continuously performed without any additional regeneration procedure, and no decline of TFA-removal ability was observed. However, it should be noted that mobile phase pH also increased with enhanced water dissociation (see Figure S2 of Supplemental Data for the pH changing), which would influence analyte ionization in positive mode. Additionally, high pH might cause analytes negatively charged and removed or adsorbed by the bipolar and anion exchange membranes. So the applied voltage should be selected based on the TFA-removal efficiency as well as pH of mobile phase. Typically, the voltage under which the conductivity began to increase was selected due to the complete removal of TFA and proper pH value under which the analyte was positively charged. Besides, to minimize the possible peak band broadening caused by the TFA remover with a channel volume of 2 μL, a relatively higher flow rate was preferred. However, considering the pressure limit of the membranes, the maximum flow rate was set to 0.1 mL min^{-1}. So for conventional column, the mobile phase needed to be split before entering the remover.

Table 1. The Calibration Equations, Correlation Coefficients, LODs, and RSDs of Peak Area for the Analysis of BAEE, BA, and Proteins using LC-TFA Remover-MS System

Compound	Extracted ion (*m/z*)	Calibration equation[a]	Linear range[b]	R^2	LOD[c]	RSD%[d]
Small molecules						
BAEE	307.4	y=0.855x+8.46	1.00–100	0.995	0.200	0.7
BA	279.3	y=0.869x+8.23	0.500–50.0	0.998	0.200	0.3
Proteins						
RNase A	1053.5	y=0.904x+5.66	10.0–500	0.992	5.00	10.7
CYC	816.3	y=1.12 x+6.77	5.00–500	0.997	1.00	1.9
LYS	1101.3	y=0.788x+5.44	10.0–500	0.973	5.00	6.0
MYG	848.6	y=0.995x+7.18	0.500–500	0.998	0.200	0.8

[a, b] y=log(peak area), x=log(concentration); for BAEE and BA, the concentration unit is μM; for proteins, the concentration unit is μg/mL
[c] LOD was evaluated on the basis of a signal-to-noise ratio of 3
[d] n=5; for BAEE and BA, C=5 μM; for proteins, C=50 μg/mL

With the TFA remover as interface, a LC-TFA remover-MS system was constructed and its performance was evaluated by the analysis of small molecules and proteins. As mentioned above, the produced OH^- for the self-regeneration of anion exchange membrane would cause the increase of pH, which might lead to the ionization of acidic analytes. The negatively charged analytes might be removed or absorbed by the remover. Therefore, the remover was more suitable for the analysis of neutral and basic compounds. In our study, two basic compounds, including *N*-α-benzoyl-L-arginine ethyl ester (BAEE) and *N*-α-benzoyl-L-arginine (BA), were first selected for the evaluation. These two molecules showed weak retention on C8 column and could not be separated (see Figure S4a, Supplemental Data for the result). However, with the addition of 0.5 % TFA in ACN/water (30/70, vol/vol) as mobile phase, a relatively good separation could be achieved (see Figure S4b, Supplemental Data). After being split at a ratio of 1:4 (0.05 mL/min in the remover), the mobile phase was delivered to the remover (voltage 10.5 V) and then detected using UV detector and MS, respectively. For the UV result as shown in Figure S4c (Supplemental Data), obvious band broadening was observed for two compounds. This might be caused by the lower flow rate through the relatively large UV detector cell (volume 13 μL) after splitting in order to maintain the efficiency of the remover. In addition, the nonspecific adsorption of analytes on the membranes could also result in some band broadening. For the LC-TFA remover-MS system, the outlet of the TFA remover was directly connected to the inlet of MS source without UV detector, and the result showed that the peaks only expanded a little (see Figure S4d, Supplemental Data). A more interesting result was that the peak area of each compound (2 mM, injection volume 2 μL) using UV detector was comparable or even larger than that obtained without TFA removal when the sample amount was reduced to 1/4 after splitting (see Table S1, Supplemental Data), indicating that the response of UV detector was enhanced after the removal of TFA; without the remover, the mobile phase containing 0.5 % TFA could not be directly delivered to MS due to the serious contamination to ESI source. Therefore, the remover has provided a feasible solution to eliminate the influence of TFA on detection sensitivity. The LC-TFA remover-MS system was further utilized for the quantitative analysis of BAEE and BA (see Table S2, Supplemental Data, for details). The calibration equation, limit of detection (LOD), and repeatability of the proposed method were tested, and the results are summarized in Table 1, showing that the correlation coefficients (R^2) were greater than 0.99, and the RSDs (n=5) were lower than 1 %. The feasibility of the developed system for the qualitative and quantitative analysis of small molecules was demonstrated.

The designed system was further applied to analyze proteins including ribonuclease A (RNase A), cytochrome *c* (CYC), lysozyme (LYS), and myoglobin (MYG) (50 μg/mL each, injection volume 5 μL) separated on a C18 column using mobile phase containing 0.1 % formic acid (FA) or 0.1 % TFA, showing that much better separation was obtained with TFA as modifier (see Figure S5a and S5b, Supplemental Data, for the results). To remove 0.1 % TFA, the mobile phase split at a higher flow rate of 0.1 mL/min

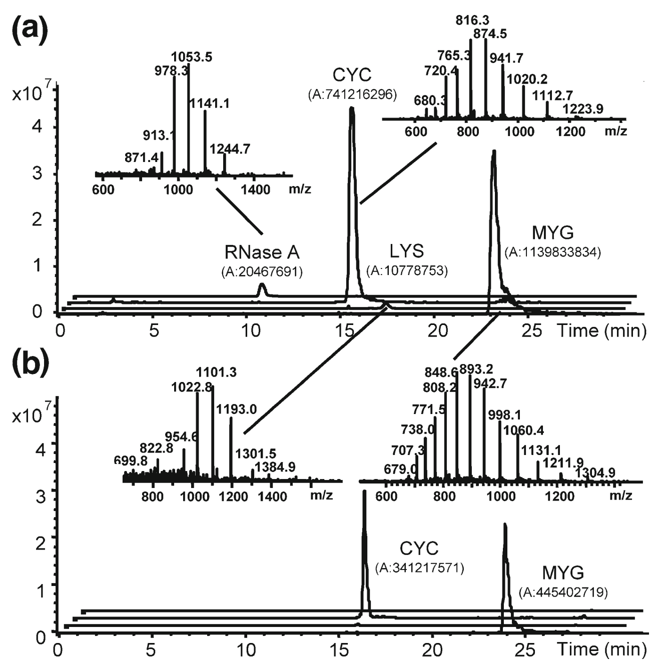

Figure 2. Combined EICs of four proteins obtained by LC-TFA remover-MS system **(a)** and LC-MS without TFA removal **(b)**, (MS spectra and peak areas in the brackets included)

was delivered to the remover and MS. Under the voltage of 5 V, all four proteins were detected (see Figure 2a). For the direct coupling of LC to MS without splitting and TFA removal, only CYC and MYG could be detected as shown in Figure 2b, and the peak areas (listed in the brackets of Figure 2) of CYC and MYG were just ~1/2 and 1/3 of that obtained with TFA remover, proving the significant sensitivity enhancement effect using the remover despite the fact that the mobile phase and injected sample had to be split for conventional column. The quantitative analysis results of the four model proteins are listed in Table 1 (see Table S3, Supplemental Data, for the details), indicating the usefulness of this proposed TFA remover. It should be noted that gradient elution was used for protein separation. Differing from isocratic elution, the conductivity of the mobile phase would decrease with the higher percent of organic solvent, requiring a higher voltage for complete removal of TFA. Besides, the pH of mobile phase, related to the formation of multiple charged proteins during ionization process, had a more significant effect on the detection sensitivity for protein analysis than for small molecules. When the voltage increased to 11 V, no protein was detected because of the poor ionization under high pH value (see Figure S6, Supplemental Data). Therefore, the remover voltage for protein analysis should be better optimized. Typically, 2~4 V higher voltage was required for complete removal of TFA at the end of the elution program (50 %~60 % ACN) than that for the beginning (1 %~5 % ACN), and the required voltage in the middle of the elution was generally applied to make a balance between the TFA removal efficiency and detection sensitivity. In addition, the membranes we used were based on polystyrene-divinylbenzene (PS-DVB). Although the ion exchange groups modified on the membranes played a key role in our design, the hydrophobic interaction between the hydrophobic proteins and the residual unmodified PS-DVB surface might result in the adsorption of proteins. Typically, this phenomenon was not observed as a result of the existence of organic solvent (e.g., ACN) in the mobile phase. But for highly hydrophobic proteins, high percent of organic solvent was required to avoid the adsorption, which was typically not applied in the separation of proteins. To make the remover more suitable for the analysis of highly hydrophobic proteins, the enhancement of the coverage of ion exchange groups on the PS-DVB surface or other surface modification methods to eliminate hydrophobic adsorption would be a solution.

In summary, a "continuous" TFA remover based on electrodialysis with bipolar membrane was developed that can continuously work without any additional regeneration process. With the TFA remover as an interface, LC using TFA-containing mobile phase could be coupled on line with MS. The proposed LC-TFA remover-MS system has been successfully applied for the qualitative and quantitative analysis of basic small molecules as well as proteins. The designed device has provided a novel solution for the incompatibility of TFA-containing mobile phase with MS.

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