Picoflow Liquid Chromatography-Mass Spectrometry for Ultra-sensitive Bottom-up Proteomics using 2-µm i.d. Open Tubular Columns

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Experimental Procedures

Materials and reagents

Fused-silica capillaries used for making the NOT columns (2-μm inner diameter, 150-μm outer diameter) were purchased from Polymicro Technologies, a subsidiary of Molex (Phoenix, AZ). Trypsin was purchased from Promega (Madison, WI). Sodium hydroxide, ammonia bicarbonate, acetonitrile, toluene and trimethoxy(octadecyl) silane were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were prepared via ultrapure water (Nanopure ultrapure water system, Barnstead, Dubuque, IA) and filtered through a 0.22-μm filter (VWR, TX), degassed before use.

NOT column and spray tip fabrication

Figure S1 presents the apparatus configuration for NOT column preparation. Two septa were placed between the stainless-steel pressure chamber cap and base to make the chamber air-tight. To prepare a NOT column, an 85-cm-long, 2-μm-i.d. capillary (Polymicro Technologies, a subsidiary of Molex (Phoenix, AZ)) was cut. The polyimide coating on the beginning of the capillary was removed. A 25 G X 7/8" hypodermic needle was used as a guide to facilitate the insertion of this capillary through the septa into the reagent solution vial in the pressure chamber. First, a vial with piranha solution (3 parts of concentrated H_2SO_4 and 1 part of 30% H_2O_2) and 1000 psi N_2 was applied. The end of the capillary was inserted into a waste vial with DI water. The whole setup was then moved into an oven @ 75 ℃. After an hour, the piranha solution vial was replaced with a DDI water vial with 1000 psi N_2 applied. The setup was reinserted in the oven for 30 min. Then the setup was removed from the oven to cool to room temperature. An empty vial was then placed inside the chamber with 1000 psi N_2 applied at room temperature. This procedure lasted for an hour to dry the inside of the capillary.

A syringe delivered a mixture of premixed 15 μL trimethoxy(octadecyl)silane (C18, Sigma-Aldrich (St. Louis, MO)) and 5 μL toluene (Sigma-Aldrich (St. Louis, MO)) into the reagent vial. The capillary inlet was inserted into the pressure chamber via a needle guide and 1000 psi nitrogen was then applied to the chamber. The water waste vial was replaced with another vial with isopropanol. The whole setup was moved inside an oven at 75 °C. After 18 h, the coating reagent vial was replaced with an empty vial with 1000 psi N_2 in the pressure chamber to remove the coating reagent inside of the capillary.

Figure S2 presents the setup for making the emitter. The uncoated end was inserted into a pressure chamber with a water vial inside. 1000 psi N_2 was applied to flush the capillary with water to prevent etching inside the channel. The end with polyimide coating was dipped inside 49% HF for 90 min. The polyimide coating outside of the tip was then removed with hot H_2SO_4.

Cell culture and proteomic sample preparation

Shewanella oneidensis MR-1 was cultured under fed-batch mode using a Bioflow 3000 fermentor (New Brunswick Scientific, Enfield, NC) as described previously.[1] The horse blood agar (HBa) culture media was supplemented with 0.5 mL/L of 100 mM ferric NTA, 1 mL/L of 1mM Na_2SeO_4, and 1 mL/L of 3 M $MgCl_2 \cdot 6H_2O$ as well as vitamins and amino acids. For proteomic preparation, bacterial cells were lysed by homogenizing with 0.1 mm zirconia/silica beads in a Bullet Blender (Next Advance, Averill Park, NY) at speed 8,000 rpm for 3 min. Proteins were denatured with 8 M urea and reduced with 10 mM DTT. The denatured proteins were digested with Trypsin at a protein/Trypsin ratio of 50:1 and incubated at 37 °C for 3 hours. The digested peptides were purified by C18 solid-phase extraction column and aliquoted at a concentration of 100 ng/μL for long-term storage.

LC-MS/MS analysis

A Dionex NCP3200RS UPLC gradient pump (ThermoFisher) was employed for both sample injection and reversed-phase separation. Mobile Phase A (Fisher Scientific, Hampton, NH) consists of 0.1% (v/v) formic acid in water and Mobile phase B (Fisher Scientific, Hampton, NH) consists of 0.1% formic acid in acetonitrile. For sample injection, the VICI 6-port valve was switched to loading position. Protein digests were diluted using Mobile Phase A and manually injected into a 2.65-µL sample loop. The sample was loaded into the column head by infusing the sample at a flow rate of 700 nL/min for 5 min with a 10-µm-i.d., 25-cm-long restriction capillary. After sample loading, the 6-port valve was switched to separation position. A flow rate of 700 nL/min and a linear 40-min gradient from 5–35% mobile phase B were used for LC separation. The column was washed by increasing to 60% mobile phase B for 12 min and finally equilibrated with 5% mobile phase B for 15 min before the next injection.

For electrospray ionization, a potential of 2 kV was applied to the capillary tip via the cross union-based flow splitter as shown in Figure 1. A nitrogen sheath flow maintained at 50 psi was employed to stabilize the picoliter-scale electrospray. An Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher, San Jose, CA) was employed for data acquisition under data dependent acquisition mode with a cycle time of 1.2 s. The ion transfer tube was set at 150 °C for desolvation and RF lens level was set at 30%. For MS1 acquisition, a mass range of 375 to 1575, a scan resolution of 120 k, an AGC target of 1E6, and a maximum injection time of 100 ms were employed. Precursor ions with charges of +2 to +7 and intensities >8,000 were selected for MS/MS fragmentation in ion trap. Selected precursor ions were isolated with a m/z window of 2 Da and fragmented by collision induced dissociation (CID) at an energy level of 35% and an activation time of 10 ms. Rapid ion trap scan rate and an AGC target of 10,000 were used for MS/MS scan. For 0.75 pg and 7.5 pg peptide loading samples, a maximum injection time of 300 ms was used. For the highest 75 pg peptide loading samples, a maximum injection time of 80 ms was used.

Data Analysis

All protein identification and quantification were performed using MaxQuant (version 1.6.2.6).[2] MS/MS spectra were searched against a UniProtKB/Swiss-Prot human database for *Shewanella oneidensis* MR-1 (Downloaded in 2/23/2017 and containing 645 reviewed and 3426 unreviewed sequences). Methionine oxidation and N-terminal acetylation were selected as variable modification. The minimal and maximal peptide lengths were set as 6 and 25, respectively. The maximal missed cleavage was set as 2. Both peptides and proteins were filtered with a maximum FDR of 0.01. Match between run (MBR) algorithm was activated with an alignment time window of 20 min and a match time window of 0.5 min. Note MBR identifications were only used for protein quantification studies as shown in Figure 4.

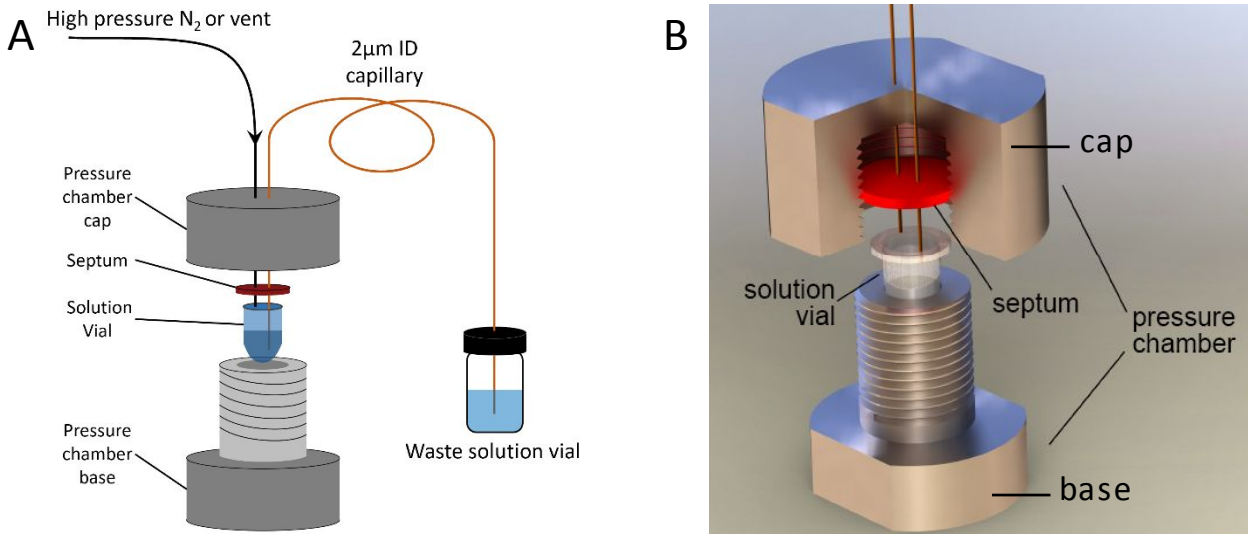


Figure S1. Apparatus for coating the NOT column. (A) The whole setup for NOT column coating. (B) The rendered 3D diagram for the pressure chamber.

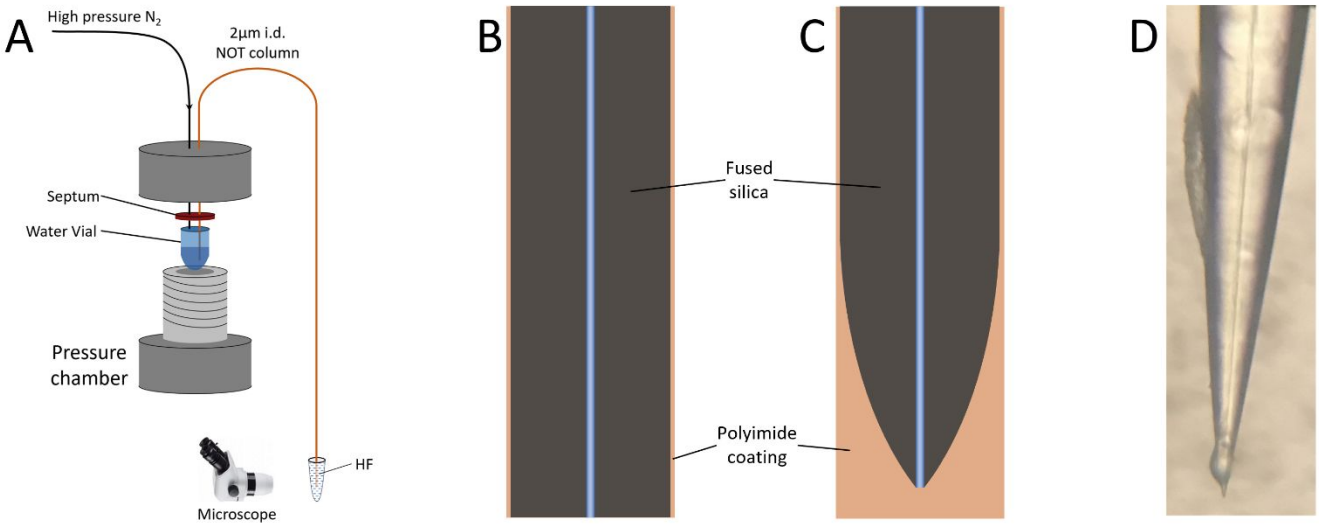


Figure S2. Experimental setup and procedures for the fabrication of externally tapered electrospray emitter tip on the NOT column. (A) One end of the NOT column was inserted into HF solution (49%) while flushing water from the capillary to prevent the etching of the inner capillary. (B) Illustration of the capillary end before (B) and after (C) HF etching. (D) A photograph of the etched emitter tip after removing the polyamide coating using heated sulfuric acid.

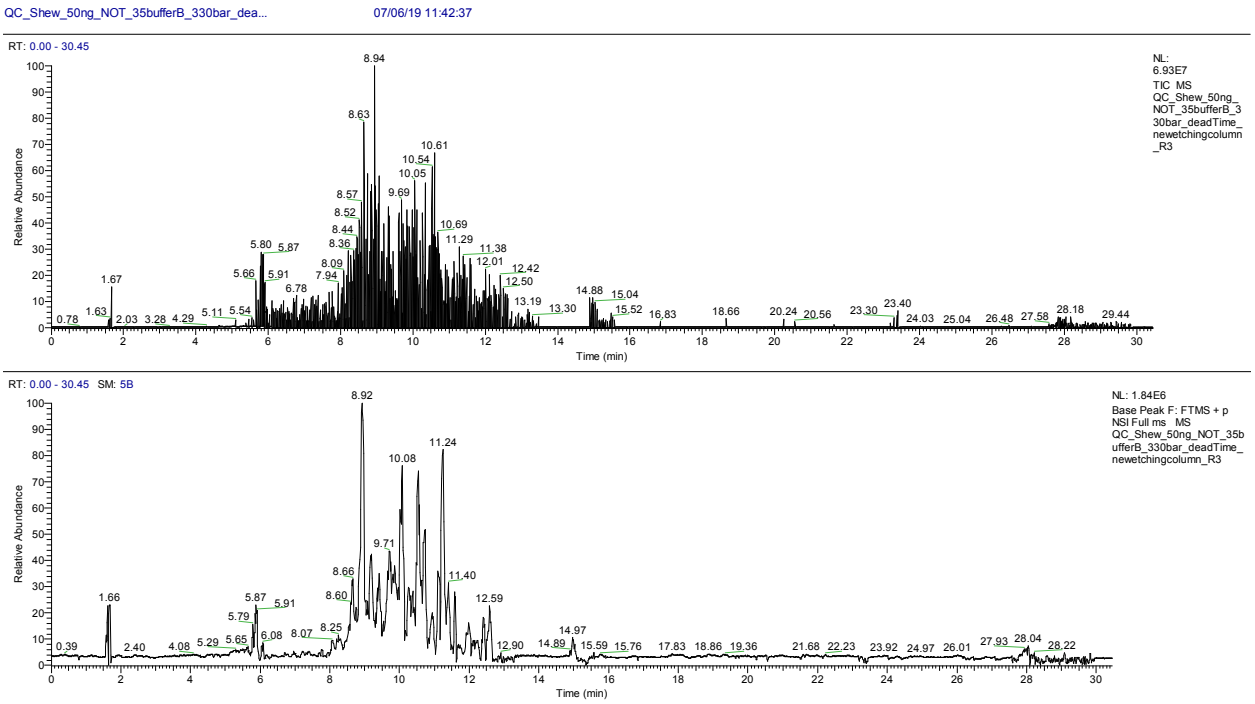
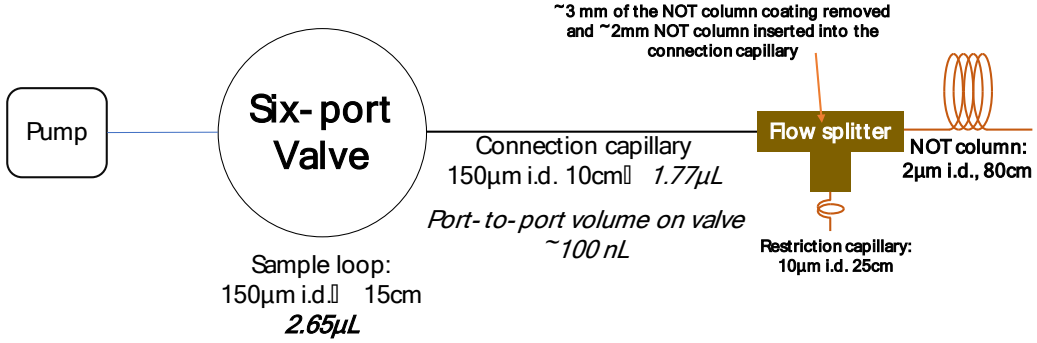


Figure S3. The base-peak and TIC chromatograms showing the dead time of the NOT column by eluting the peptide mixtures with 35% Buffer B. From the TIC chromatogram, unretained peptides were eluted out at ~5.87 min. After subtracting the dead time in the connection capillary (2.53 min), the actual dead time is 3.34 min, corresponding to a linear flow rate of 3.98 mm/s and volumetric flow rate of 790 pL/min.

This page denotes the data of ***sample injection*** for the NOTLC- MS experiment



Pump rate during sample injection: 0.7µL·min^{-1}.
Time that the first non retained peptide came out: T = 5.87 min
Connection cap. dead time: $t = \frac{1.77 \times 0.1}{0.7} = 2.67 min$
NOT column linear flow velocity: $v = \frac{800}{(5.87 - 2.67) \times 60} = 4.17\ mm \cdot s^{-1}$
NOT column flow rate during sample injection: $u = \frac{2512}{5.85 - 2.67} = 790\ pL \cdot min^{-1}$
Sample injection splitting ratio: $\frac{790}{700000} = 1:886$

Sample concentration*: $25 ng \cdot \mu L^{-1}$
Sample in injection loop: $25 \times 2.65 = 66.25 ng$
Sample loaded on NOT column:
$\frac{66250}{886} = 75\ pg$

Figure S4. Step-by-step calculation of the splitting ratio and on-column peptide amounts.

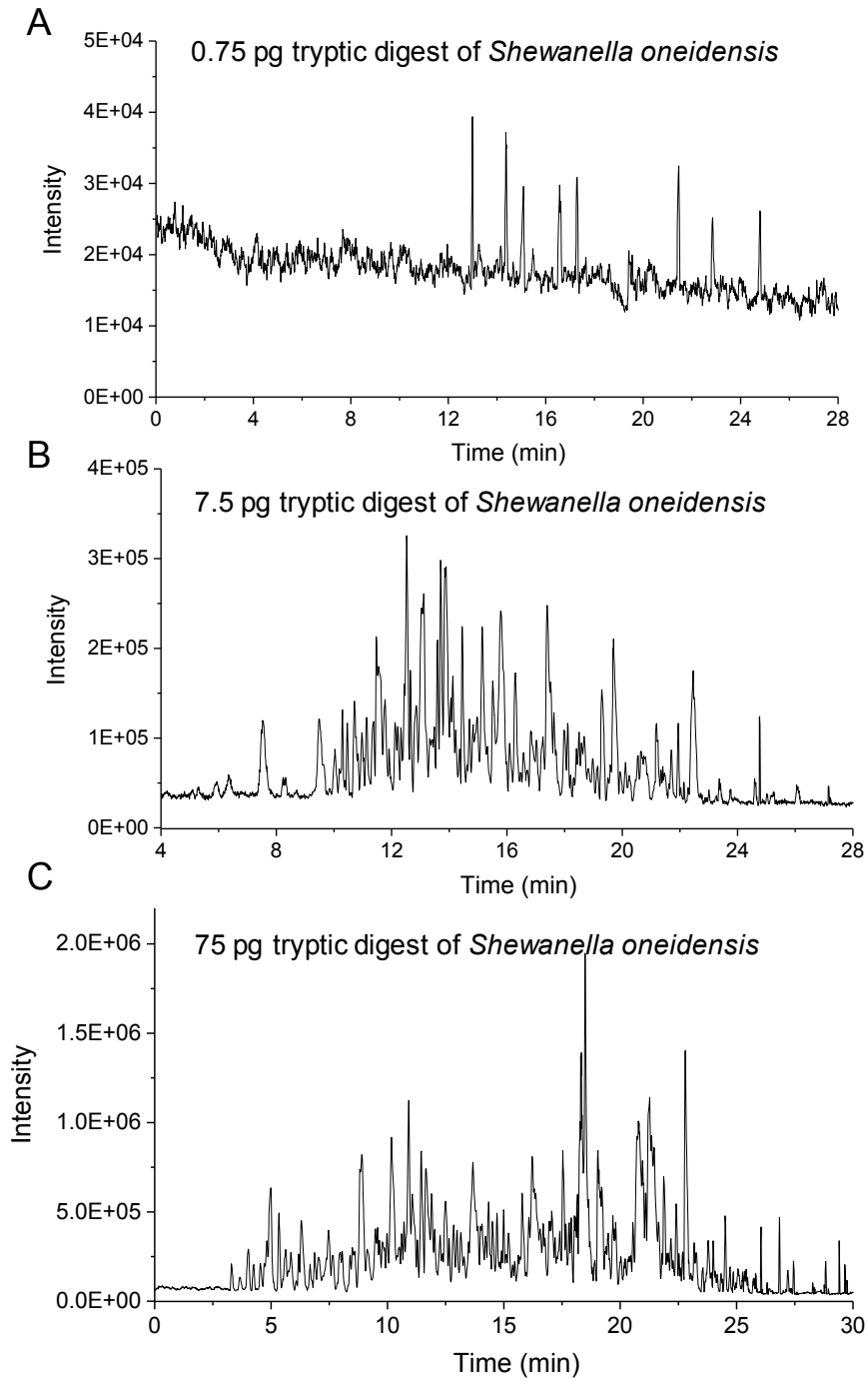


Figure S5. Base peak chromatograms of (A) 0.75 pg, (B) 7.5 pg, and (C) 75 pg tryptic digest of *Shewanella Oneidensis*. The difference in peptide retention times can be attributed to inconsistent start times for MS acquisition, because all the LC-MS experiments were performed manually.

References

[1] Y. Zhu, R. Zhao, P. D. Piehowski, R. J. Moore, S. Lim, V. J. Orphan, L. Paša-Tolić, W.-J. Qian, R. D. Smith, R. T. Kelly, *Int. J. Mass Spectrom.* **2018**, *427*, 4-10.

[2] S. Tyanova, T. Temu, J. Cox, *Nat. Protoc.* **2016**, *11*, 2301.