CHARACTERIZATION OF A MICROFABRICATED ELECTROSPRAY IONIZATION (ESI) DEVICE FOR SOLUTION-PHASE H/D EXCHANGE IN NON-DENATURING MEDIA

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INTRODUCTION

The need to study the protein conformations using ESI mandates simulating conditions under which proteins exist in real life. Meanwhile, the ESI experiments demand deviations from biological conditions such as using acetic acid, methanol, acetonitrile, *etc.* to improve the quality of the electrospray. Addition of solvents and ionization reagents result in protein denaturation. To date, direct addition of ionization reagents such as acetic acid has been necessary but rationalized by suggesting that the gas phase behavior of proteins is not determined by the ESI spray conditions. However, a recent study showed the existence of solution phase "memory effect" in ESI [1]. We have developed a a microfabricated ESI device to study the solution-phase H/D exchange (HDX) patterns of proteins under non-denaturing conditions that can address the question of solution-phase "memory" effect. To the best of our knowledge, this is the first time to use pure HPLC water as the ESI media and study solution-phase H/D reactions in the absence of other potentially interfering reagents.

EXPERIMENTAL

Instrumentation: Mass spectra were acquired with an FT-ICR mass spectrometer equipped with an open-ended cylindrical Penning trap (IonSpec Corp., Irvine, CA) and a 9.4 tesla superconducting magnet (Cryomagnetics Inc., Oak Ridge, TN).

ESI Device Fabrication: The ESI device was microfabricated using photolithography technique on silicon wafer. The device has a mixing chamber (4 mm × 8 mm × 1 mm) with two square-shaped inlets and one outlet (220 μm in length). Fused silica capillaries (i.d. ~ 50 μm) were attached to the outlet (as a spraying tip) and two inlets to carry the protein and deuterating solution(s) into the mixing chamber of the device (~ 50 nL volume).

Sample Preparation: Ubiquitin, D_2O, and other reagents were purchased from Sigma-Aldrich, Inc. (St Louis, MO) and used without further purification. Ubiquitin solution (~1 μM) was prepared in HPLC water or in 1% acetic acid (AA) in methanol:water (50:50) and infused through one inlet of the ESI device; the D_2O solution (~10% in HPLC water) was infused using the second inlet of the microfluidic device. The "mixing" of the analyte solution {*i.e.*, ubiquitin in water or in 1% acetic acid in methanol:water (50:50)} and the deuterium exchange reagent (*viz.*, 10% D_2O in HPLC water) occurred inside the microfluidic device (Figure 1). A second "partner" spray solution (containing either ~20% acetic acid in methanol or 1% acetic acid in methanol:water (50:50)) was utilized to provide a spray plume that yielded protonated ubiquitin ions. Electrospray voltage was applied to both the sample and the partner spray solutions; ubiquitin in pure water required higher ESI voltage than the conventional approach (*viz.*, ~+4 kV for all experiments shown herein). The flow rate ranged from 0.25 μL/min to 2 μL/min.

Data Analysis: For qualitative comparisons between the observed deuteration levels (DLs) for ubiquitin dissolved in two different media (Figure 5), we used the following simplified formula:

$$DL = [m/z_{\text{ion peak}}\,(\text{after HDX}) - m/z_{\text{ion peak}}\,(\text{before HDX})] \times \text{Charge state} \qquad \text{equation 1}$$

Where, $m/z_{\text{ion peak}}$ is the m/z value for the most abundant peak in the isotopic envelope of $[\text{ubiquitin} + 7H]^{+7}$ or $[\text{ubiquitin} + 8H]^{+8}$.

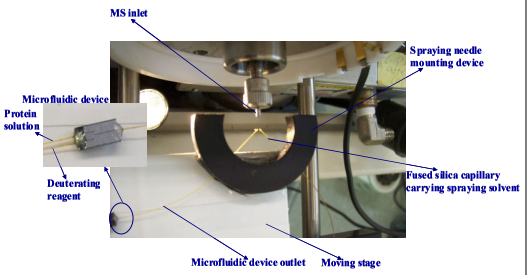

Figure 1. Electrospray setup and microfluidic device configuration.

RESULTS

Spray Characterization: To study the solution-phase H/D exchange of proteins and peptides, it is important to have a stable spray during the course of H/D exchange reactions. Different spraying angles (SA) between microfluidic device outlet capillary and spraying solvent outlet capillary were examined. Figure 1 shows the stability of spray for ubiquitin in water when SA is 90 degrees (in the same horizontal plane).

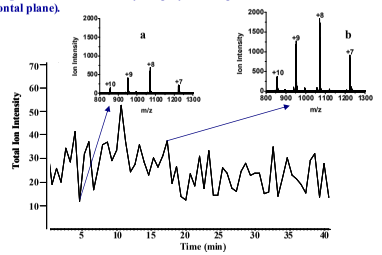

Figure 2. Demonstration of spray stability for ubiquitin under solution-phase H/D exchange simulated conditions {one inlet of microfluidic device (see Figure 1) was used to deliver ubiquitin solution in water and the second inlet delivered pure water into the mixing chamber. A 20% AA in methanol solution was used as the spraying "partner"}.

Note: Electrospray for longer than 60 min caused condensation in the outer section of the metal capillary of MS inlet (see Figure 1) and therefore prevented ion transfer through the capillary. To improve the spray quality, we plan to use a flow of hot N_2 gas around the spraying capillaries and metal capillary of MS inlet. Our preliminary results indicate that the gas-phase post-spray "back-exchange" can be significantly minimized using N_2 screening and that back exchange can account for up to 25% at low infusion rates (*e.g.*, 0.3 μL/min).

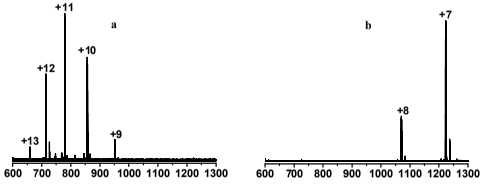

Figure 3. Appearance of different charge states of ubiquitin under (a) direct mixing (at the tip) of the outlet solution from microfluidic device and outlet solution from the fused silica capillary carrying the spraying "partner" solvent (*i.e.*, the two spray tips physically touching) and (b) mixing the two outlets after forming the "plumes" – *i.e.*, the two spray tips ~1 mm apart {20% acetic acid in methanol was used as spraying solvent}.

Note: By inspecting the MS patterns (observed charge states) the direct mixing or mixing at the "plumes" can be distinguished. We have noticed that the distance between the spraying capillary and metal capillary (SC-MC) of MS inlet (see Figure 1) can affect the charge state distribution of ubiquitin. For example, Figure 3b and Figure 2b show the MS patterns of ubiquitin at two different SC-MC distances.

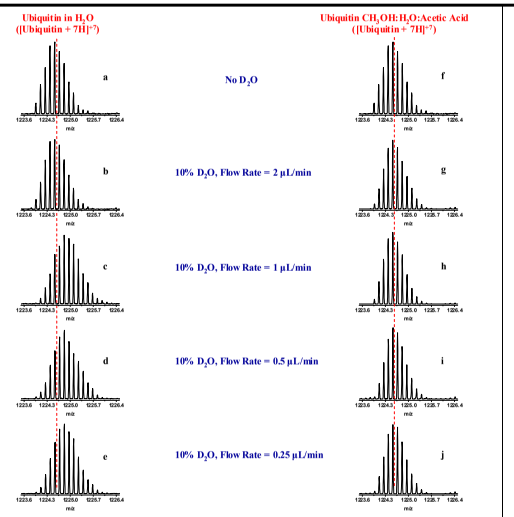

Figure 4. The observed mass spectral patterns for $[\text{ubiquitin} + 7H]^{+7}$ after the solution-phase H/D exchange of ubiquitin in (a-e) HPLC H_2O and (f-j) CH_3OH:H_2O:Acetic Acid (49.5:49.5:1) at different flow rates. A 10% D_2O in H_2O was used as the deuterating solvent. The spraying "partner" solvent was CH_3OH:H_2O:Acetic Acid (49.5:49.5:1). Red dashed vertical lines are positioned at the same m/z value to use as a guide.

Note: Our preliminary H/D exchange results suggest that ubiquitin in highly hydrophilic solvent {*e.g.*, pure water} has higher exchange rate than ubiquitin in presumably less hydrophilic solvent {*e.g.*, CH_3OH:H_2O:Acetic Acid (49.5:49.5:1)}. Such experimental approaches can be utilized to characterize protein hydrophilicity and/or hydrophobicity.

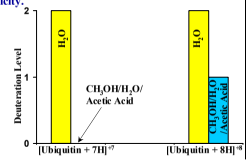

Figure 5. Deuteration level of ubiquitin dissolved in H_2O and ubiquitin dissolved in CH_3OH:H_2O:Acetic Acid (49.5:49.5:1) {deuteration levels were calculated using equation 1}.

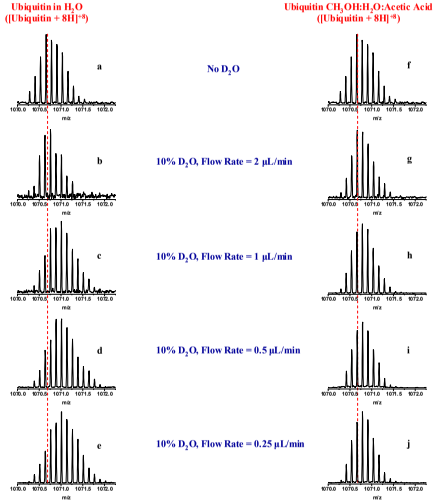

Figure 6. The observed mass spectral patterns for $[\text{ubiquitin} + 8H]^{+8}$ after the solution-phase H/D exchange of ubiquitin in (a-e) HPLC H_2O and (f-j) CH_3OH:H_2O:Acetic Acid (49.5:49.5:1) at different flow rates. A 10% D_2O in H_2O was used as the deuterating solvent. The spraying "partner" solvent was CH_3OH:H_2O:Acetic Acid (49.5:49.5:1). Red dashed vertical lines are positioned at the same m/z value to use as a guide.

CONCLUSIONS

- We were able to spray ubiquitin dissolved in pure water using second "partner" spraying solution.
- Our results for solution-phase H/D exchange of $[\text{ubiquitin} + 7H]^{+7}$ and $[\text{ubiquitin} + 8H]^{+8}$ generated from ubiquitin in water showed higher exchange levels than the same charge state generated from ubiquitin in CH_3OH:H_2O:Acetic Acid.
- We plan to utilize ECD to compare the fragmentation patterns of different charge states of ubiquitin generated from ubiquitin in water and ubiquitin in CH_3OH:H_2O:Acetic Acid.

LITERATURE CITED

[1] Jiang, H.; Kong, X.; Brenker, K.; Hossain, M.; McLafferty, F. W., "Folding Inhibition by Electrospray Additives for Top-Down Mass Spectrometry of Larger Proteins," *Proceedings of the 56th ASMS Conference on Mass Spectrometry and Allied Topics*, 2008, Denver, CO, WPV 600.

ACKNOWLEDGEMENTS

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