Variable Mixing with Theta Emitter Mass Spectrometry: Changing Solution Flow Rates with Emitter Position

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Abstract

Two solutions can be rapidly mixed using theta glass emitters with products measured using electrospray ionization mass spectrometry. The relative flow rates of the two emitter channels can be measured using different calibration compounds in each channel or the flow rates are often assumed to be the same. The relative flow rates of each channel can be essentially the same when the emitters are positioned directly in front of the capillary entrance of a mass spectrometer, but the relative flow rates can be varied by up to three orders of magnitude by moving the position of the emitter tip ±1 cm in a direction that is perpendicular to the inner divider. Results of emitter position on the different concentration of reagents in the initially formed electrospray droplets is demonstrated through protein denaturation using a supercharging reagent as well as two different bimolecular reactions. The average charge state of myoglobin changed from +7.8 to +13.8 when 2.5% sulfolane was mixed with a 200 mM ammonium acetate solution containing the protein when the position of the emitter was scanned in front of the mass spectrometer inlet. The conversion ratio of a bimolecular reaction was changed from 0.98 to 0.04 with varying emitter position. These results show that the relative flow rates must be carefully monitored because the droplet composition depends strongly on the position of theta glass emitters. This method can be used to measure the dependence of reaction kinetics on different solution concentrations using a single emitter and only two solutions.

Introduction

Mass spectrometry is widely used to study chemical reactions owing to its high sensitivity, specificity and analysis speed. Reactions can be initiated by mixing two solutions containing reagents prior to the electrospray ionization (ESI) process,[1] including with on-line mixing devices.[2,3] Reactions can also be initiated after droplet formation occurs, as is the case with droplet fusion,[4] reactive desorption electrospray ionization,[5,6] or through introduction of gaseous reactants.[7,8] Mixing has also been done immediately prior to where droplet formation occurs using microfabricated devices[9,10] or with theta glass emitters that have two channels formed by a center septum, the end of which looks like the Greek letter θ when viewed head on.[11–27] Theta glass emitters have been used in conjunction with mass spectrometry to measure reaction intermediates,[11,12] noncovalent complexation formation,[14,15] reduction-oxidation reactions,[15] hydrogen-deuterium exchange,[18,19] and initiate protein folding[16,20,27] or unfolding.[17,21,22] Supercharging reagents have been used with theta-glass emitters to increase protein charge states.[23,28] Theta glass emitters have also been used to extract material from cells into one channel and react the cellular extract with acid or derivatizing reagents contained in the other channel prior to ESI.[24]

One advantage of using theta glass emitters for mixing experiments is that mixing times are fast and the time for a solution reaction to occur can be varied by changing initial droplet size[16,25] or by mixing solutions at the end of the capillary prior to initiation of electrospray ionization.[21,26] The droplet size and lifetime depends on the solution flow rate. The flow rate can be varied either by changing the backing pressures on the ESI emitter[16,25] or by changing the diameter of the emitter tip.[16,19,25] Droplet lifetimes have also been varied by changing the distance of the emitter to the mass spectrometer inlet.[18] Droplet lifetimes have been obtained by

measuring kinetics of protein folding that is initiated upon mixing using proteins with known folding time-constants.[11,16,17,19,25] Folding of a protein initially in an acidified solution is induced by a pH increase upon solution mixing and the lifetime of the droplet is determined from the extent of folding that occurs.[11,16,17,19,25] Droplet lifetimes between 1 and 100 μs[16,25] have been measured through protein refolding kinetics[11,17,19,29] making it possible to obtain reaction kinetics on a time scale much faster than is achieved using conventional mixing devices. Droplet lifetimes have also been estimated by the transit time of larger droplets that can be optically imaged (typically many microns in diameter). The time for a large droplet to reach the capillary inlet of a mass spectrometer has been used as an estimate of the droplet lifetime by assuming that ions are formed at the inlet. [4,18,30] However, these large droplets survive well into heated metal capillary inlets prior to gaseous ion formation[25] and large droplets may not be the ones that form ions observed in the mass spectrometer. Longer reaction time scales with theta glass emitters on the order of 50-500 ms can be obtained by electroosmosis mixing that is initiated by applying different potentials between the two electrodes in the channels of the theta glass emitter resulting in migration of solution from one channel into the other.[21]

Imaging of theta glass emitters during electrospray have shown that both channels can form a single, shared Taylor cone[13] or they can form separate Taylor cones.[18] There is evidence for complete mixing through a fast equilibrium complexation reaction with 18-crown-6 and Na^+,[15,16] as well as fluorescent imaging showing mixing in a Taylor cone formed from an emitter with a 4 μm outer diameter tip.[13] Optical imaging of an electrospray plume generated from a theta emitter with a 4 μm inner diameter showed dual Taylor cone jets initially repelling each other. The average initial droplet size was 4.3 μm,[18] which is much larger than typically

generated from electrospray emitters of this size[29,31,32] and it was reported that some droplets collided about 0.2 mm from the tip of the emitter.[18]

In mixing experiments with theta glass emitters, the mixing ratio is often assumed to be 1:1. However, if the emitter is off center or rotated while off center, the flow rates between the channels can differ,[15] and this difference has been reported to be pronounced in z-spray sources.[19] The relative flow rates of the two channels can be measured using standards. This has been done with two similar peptides or other calibration compounds, one in each channel, and the flow rates are obtained from the relative abundances of the calibrant signals.[15,16,18,19,29] Here, we show that the relative flow rates of the different channels can be varied by 3 orders of magnitude controllably by changing the position of the theta glass emitters with respect to the mass spectrometer entrance. The advantage of this method for rapidly and easily changing mixing ratios is demonstrated with a supercharging reagent and for measuring the concentration dependent reaction yields for two different bimolecular reactions.

Methods

Borosilicate theta capillaries (1.5 mm outer diameter, Sutter Instruments, Novato, CA) were pulled using a Flaming/Brown P-87 micropipette puller (Sutter Instruments, Novato, CA) to produce theta glass emitters that have tips with inner diameters of 1.87 ± 0.48 μm. Tip diameters were imaged using a Hitachi TM-4000 (Schaumburg, IL) microscope in the Electron Microscope Laboratory at the University of California, Berkeley. The solution flow rate for the theta glass emitters is 36 ± 3 nL/min.[29] All mass spectra were acquired using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with a capillary temperature of 100 °C and a maximum injection time of 20 ms, All mass spectral data were an average of 69

spectra, with the exception of data acquired at distances more than 8 mm offset from the inlet, which were an average of 138 spectra. The theta glass emitters were mounted on a custom-built stage, and a voltage of 0.7-1.2 kV was applied to platinum wires inserted into both barrels to initiate electrospray. The emitter tips were positioned 3 mm away from the instrument inlet with the dividing barrier between the two channels oriented in the vertical direction. The theta glass emitters were moved horizontally, perpendicular to the vertical dividing barrier, using a micrometer that was adjusted in 1.0 mm increments (Figure 1).

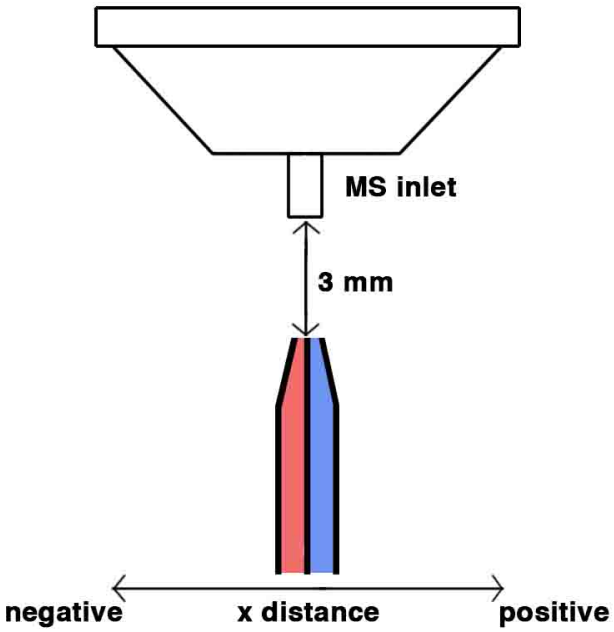


Figure 1. Schematic diagram of the theta glass emitter relative to the heated metal capillary of the mass spectrometer inlet. The centered position was set as 0 mm, movement to the left and right of the inlet is defined as negative and positive, respectively.

The peptides, M-enk and L-enk, were individually added to solutions contained in the two barrels of the theta glass emitters in order to measure the relative flow rates of the two

solutions in these experiments. The relative flow rates between the two channels in mixing experiments were determined from the relative abundances of the protonated peptides normalized for their different ionization/detection efficiencies as described previously.[15,29] To determine if the different solution conditions for the chemistries investigated here affected the relative ionization efficiencies, solutions containing the different constituents for each set of experiments were mixed 1:1. The resulting mass spectra from these mixed solutions showed that the relative abundances of protonated L-enk to protonated M-enk was 0.8:1, indicating that the different solution compositions used to investigate different chemistry does not affect the relative ionization efficiency.. To determine if the relative ionization efficiency depends on the relative concentrations of the two peptides, mixtures of 1:100, 1:10, 1:1, 10:1, and 100:1 L-enk and M-enk in water were prepared so that the concentration of either peptide was no more than 1 μM. The ratio of the protonated peptide abundances as a function of relative concentration are shown in Figure S1, These data show that the relative ionization efficiency of the two peptides does not change over four orders of magnitude of relative concentrations. To determine if the position of the emitter affects relative ionization efficiency, solutions of the two peptides mixed at a ratio 1:1, 1:100, and 100:1 L-enk and M-enk were prepared. Mass spectra were acquired with the emitters centered with the capillary inlet of the mass spectrometer and displaced both +5 mm and -5 mm from this position in the direction perpendicular to the center divider. These data indicate that the relative ionization efficiency of these two peptides do not change with tip position within this range (Supporting Information).

All chemicals were from Sigma-Aldrich (St. Louis, MO) except for 2,6-dichlorophenolindophenol sodium salt (DCIP) that was obtained from Fisher Scientific (Fair

Lawn, NJ). All chemicals were used without further purification. 18.2 MΩ water from a Milli-Q integral water purification system was used to prepare solutions (Millipore, Billerica, MA).

Results and Discussion

Effects of ESI Emitter Position on Supercharging. Myoglobin, a 17.6 kDa protein with a noncovalently bound heme group, is often used in studies on supercharging[33–36] because both the higher extent of charging and the loss of the noncovalently bound heme group are indicators of the extent to which the conformation of the protein changes as a result of the supercharging reagent concentration. Although several factors may ultimately contribute to the supercharging phenomenon,[37,38] including droplet surface tension,[39] the primary factor is the destabilizing effect of the supercharging reagents that causes proteins to denature in late stages of droplet evaporation when the supercharging reagent is most concentrated.[34,35,40–43] The effect of the supercharging reagent sulfolane on myoglobin charging are illustrated in Figure 2, which shows mass spectra obtained from 5 μM myoglobin in 200 mM aqueous ammonium acetate with 0%, 1.25% and 2.5% sulfolane (Figures 2a-c, respectively).

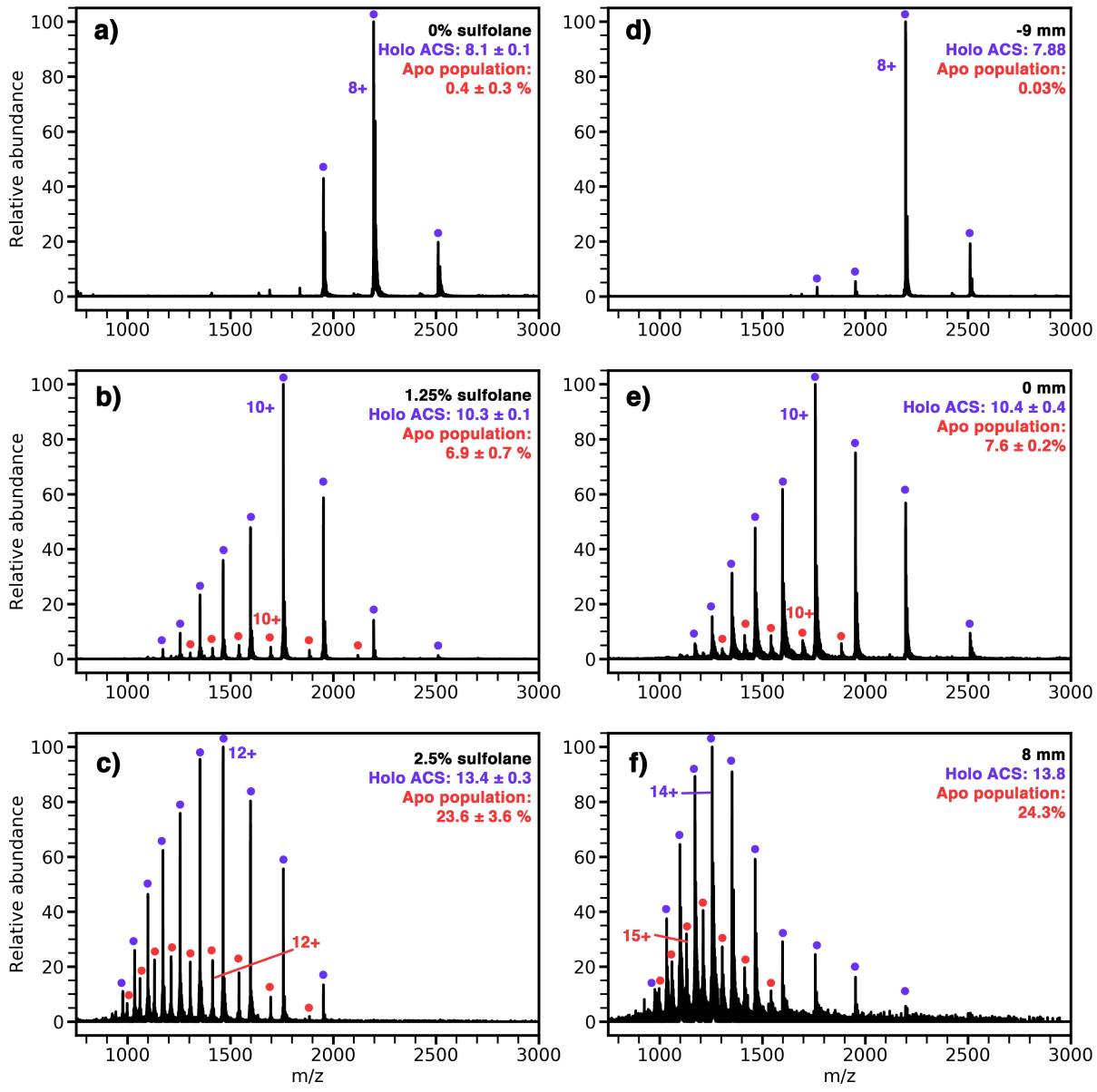


Figure 2. Representative mass spectra of premixed solutions containing 5 μM myoglobin with (a) 0%, b) 1.25%, and c) 2.5% sulfolane. Representative mass spectra using theta glass emitters to mix two solutions, one containing 10 μM myoglobin, the other containing 2.5% sulfolane at emitter positions d) -9 mm, e) 0 mm, and f) 8 mm from the inlet. At 0 mm (e), the flow rate of the two channels is equal and the mixed concentration is the same as that shown in (b).

These data were obtained using theta glass emitters with both channels containing this solution along with 2 μM of the peptides L-enk and M-enk as internal standards used for subsequent mixing experiments. The spray potential of 0.7 kV was close to the threshold for ion formation. The average charge state from these three solutions is 8.1 ± 0.1, 10.3 ± 0.1 and 13.4 ± 0.3, respectively. The apo myoglobin population as a percentage of total myoglobin population is 0.4 ± 0.3 %, 6.9 ± 0.7% and 23.6 ± 3.6%, respectively. Both the increasing charge state and the increasing apo-myoglobin population indicate that holo-myoglobin undergoes increased extents of unfolding with increasing sulfolane concentration. Little further increase in average charge state (13.9 ± 0.3) and apo myoglobin population (22.7 ± 4.5%) occurs with up to 5% sulfolane in solution (Figure S2). The abundance ratio of protonated L-enk to protonated M-enk is 0.8:1 in these pre-mixed solutions. The ratio of these two ions is used to measure the relative flow rates of each channel in subsequent mixing experiments, with a value of 0.8:1 indicating a 1:1 flow between the two barrels.

Results from a theta glass emitter containing 10 μM myoglobin in 200 mM AA with 2 μM M-enk in one barrel and 2.5% of the supercharging reagent sulfolane in 200 mM AA and 2 μM L-enk in the other barrel are shown as a function of emitter position in Figures 2d-f. The emitter was initially centered with the heated metal capillary entrance of the mass spectrometer. In this position, the abundance ratio of protonated L-enk to protonated M-enk is 0.8 ± 0.1 indicating that there is an equal flow of solution from each of the two barrels. Prior studies using theta capillaries under nearly identical conditions indicate that complete mixing occurs.[16] Assuming that is true here, then the initial droplets that are formed contain 5 μM myoglobin and 1.25% sulfolane. The average charge state for the holo form of the protein is 10.4 ± 0.4 and the apo form is 7.6% ± 0.2% of the myoglobin population (Figure 2e). This mixing ratio produces

the equivalent solution to the premixed myoglobin solution containing 1.25% sulfolane, shown in Figure 2b. These results show that mixing two solutions with the theta glass emitters produces similar results as those obtained from mixing the two solutions and loading the premixed solutions into both barrels.

The emitter was oriented so that the divider between the two channels was vertical. Moving the emitter horizontally in the direction that is perpendicular to the divider between the two channels changes the ratio of the protonated ion abundances of the two peptides. The protonated peptide abundance ratio at a -9 mm offset from center is 1:1470 indicating that the ratio of the flow rates between the channels is 1:1180. At these flow rates, the concentration of sulfolane in the initial droplets is 0.002%. The mass spectrum (Figure 2d) is similar to the native protein mass spectrum obtained with no sulfolane (Figure 2a), with an average charge state of 7.9 and 0.03% apo myoglobin population. At this negative offset, the barrel containing myoglobin is closest to the mass spectrometer inlet and it has the higher flow rate. In striking contrast, the peptide ratio is 19.6:1 when this same emitter is positioned +8 mm from center. This ratio indicates a relative flow rate of 24.5:1 between the two channels corresponding to a sulfolane concentration of 2.4% in the initial droplets if complete mixing occurs. The average charge state is 13.8 (Figure 2f), similar to that obtained from the premixed solution with 2.5% sulfolane (13.4 ± 0.3; Figure 2c). The fraction of apo-myoglobin population is 24.3%, which is similar to the value of 23.6% ± 3.6% in the pre-mixed solution with nearly the same sulfolane concentration. At this position, the barrel containing supercharging reagents is closer to the mass spectrometer inlet, again reflecting that the channel closest to the inlet has a significantly higher flow rate. These results indicate that the droplet composition and flow rate of each channel can be significantly changed by the emitter position.

There is a consistent systematic increase in the average charge state of both forms of myoglobin, the population of apo-myoglobin and the peptide ratio as the emitter is moved from –9 mm to +8 mm from the center of the mass spectrometer entrance. These data are shown as a function of position (Figure 3a,b) and as a function of sulfolane concentration in the droplet obtained from the peptide ratios that were used as internal standards of flow rate (Figure 3c,d).

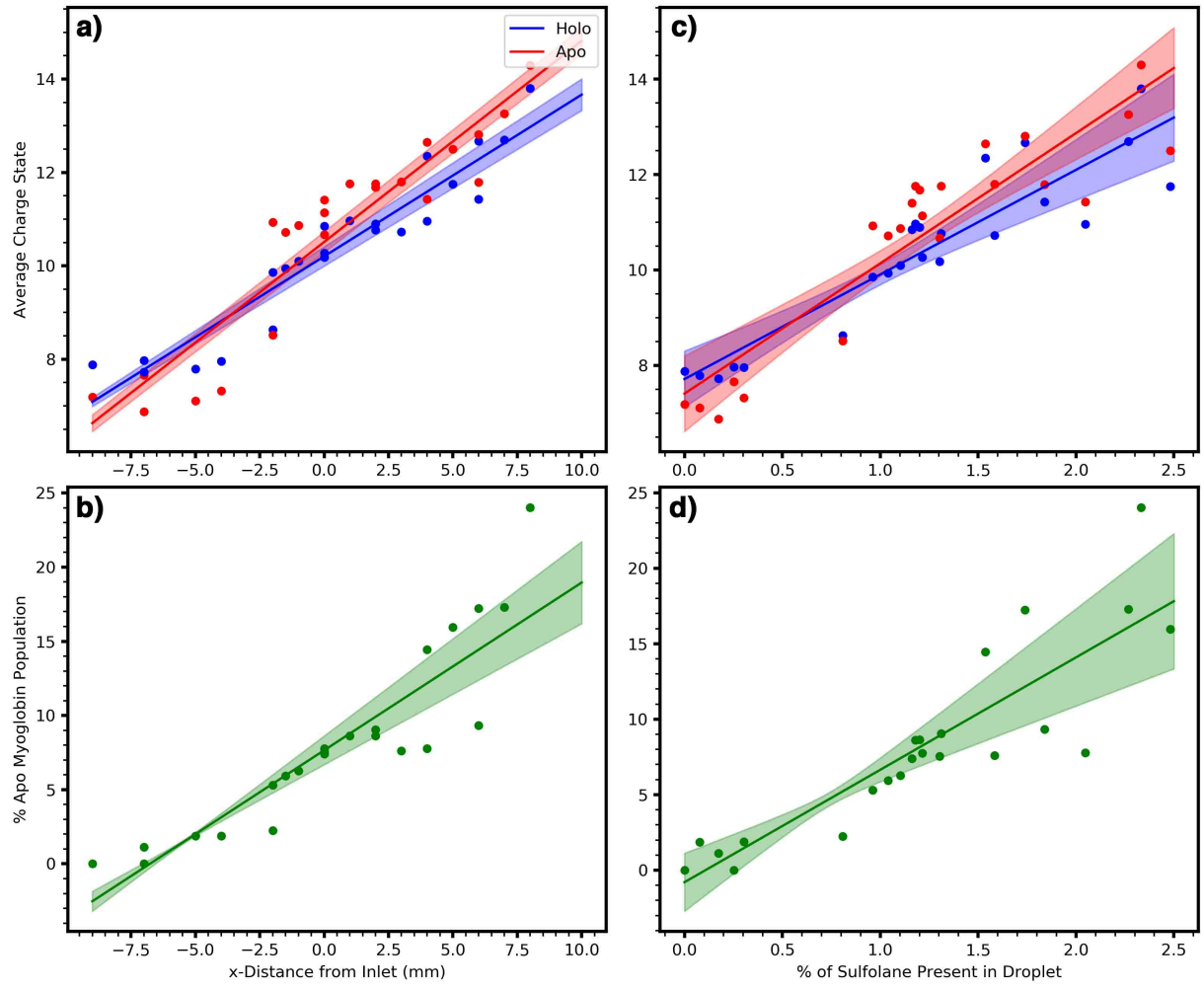


Figure 3. (a) Average charge state of the holo (blue) and apo (red) forms of the protein and (b) the % apo myoglobin population (green) of 10 μM myoglobin and 2.5% sulfolane in 200 mM AA as a function of distance from the instrument inlet center and (c) (d) as a function of % sulfolane present in the droplet determined from the M-enk:L-enk ratio. The solid lines show a

linear line of best fit; the shaded regions correspond to one standard deviation from the line of best fit.

The signal decreases with increasing offsets from center so spectra were acquired for longer time at the positions significantly off center. The lower signal is due in part to the lower concentration of the protein (more than 10x lower protein concentration in Figure 2f than in Figure 2e), as well as a reduction in the number of ions entering the mass spectrometer when the emitter is significantly off center.

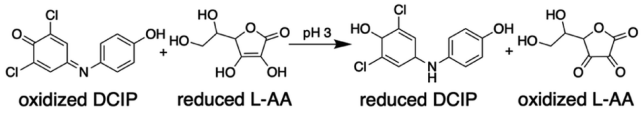
All of these results indicate that moving the emitter tip in a direction that is orthogonal to the theta glass divider results in differences in flow rates between the channels that can be readily measured using the peptide internal standards in each barrel. In contrast, moving the emitter at a constant potential of 0.7 kV up to 4 mm vertically in the direction of the divider results in no change in the peptide ratio or appearance of the mass spectra indicating that the relative flow rates of the two channels are not significantly affected when the emitter is moved in this direction (Figure S3). Further vertical movement resulted in reduced signal. Ion formation continued when the emitter tip was moved an additional 2 mm by increasing the potential to 1.4 kV. The average charge states at these potentials were slightly higher even though the peptide ratio did not change (Figure S4). The slightly higher charging may be due to electrothermal supercharging at the higher electrospray potential.[44,45]

A recent report by Brown et al. of protein supercharging with water/ methanol/1,2-butylene carbonate/ acetic acid using theta glass emitters indicated that the charge-state distributions from theta glass mixing were different than those formed with premixed solutions.[28] From the subtle differences in charge state, the authors concluded that the two barrels formed

independent Taylor cones and that mixing occurs by merging collisions of the progeny droplets that are both highly charged and have the same polarity. Multiple Taylor cones can occur at high electrospray potentials and with very large diameter theta glass emitters.[18] Some collisions between relatively large droplets have been reported.[18] Droplet fusion has also been observed for relatively large droplets formed by two separate emitters.[4] While it is possible for some droplets to fuse after their initial production, there is an alternative explanation that more likely accounts for the prior results on supercharging with theta glass emitters. In the prior experiments, the relative flow rates of the two channels were not apparently monitored.[28] As our results demonstrate, the relative flow rates of the two channels are a strong function of emitter tip position. This suggests that the subtle differences in charge states observed in the prior mixing experiments may be a result of emitter position and differences in flow rates of the two channels. The orientation of the emitter relative to the inlet of a z-spray source was also shown to change the relative signal intensities of these same two peptides used to monitor flow rates,[19] although this may not always affect the extent of supercharging observed.[28] If two independent Taylor cones were formed in our experiments, we would expect a bimodal charge state distribution when the emitter is at +8 mm (Figure 2f) if droplet recombination was not 100% efficient. If some droplets containing myoglobin do not recombine, then myoglobin should not become denatured in these droplets, and some 7+ ions should be observed. Based on the signal-to-noise ratios, we conclude that droplet recombination would have to be at least 94% efficient in order to not observe a 7+ charge state of holo myoglobin when the emitter was positioned at +8 mm. An even more dramatic demonstration that droplet recombination almost certainly does not occur in these theta emitter experiments with supercharging reagents is indicated by results of Donald and coworkers.[23] In these experiments, the supercharging reagent vinyl ethylene carbonate in a

denaturing solution was mixed with proteins in ammonium acetate. Solutions with theta glass emitters showed significant increases in the protein charge-state distributions (from 7.6 ± 0.1 to 23.1 ± 0.1 for cytochrome *c*).[23] The two charge-state distributions are widely separated and the signal-to-noise ratio was excellent. If two separate Taylor cones were formed, the recombination efficiency of the droplets would need to be 99+% in order to not observe low charge state proteins that are formed from protein containing droplets that do not recombine. Optical imaging of electrospray plumes show strong evidence that the vast majority of droplets formed by electrospray repel each other and do not recombine.[46] It is implausible that 99+ % of highly charged droplets that have the same polarity recombine under standard electrospray conditions, even with theta glass emitters. Thus, we conclude that mixing occurs in the Taylor cone and in the resulting electrospray droplets that are formed in these supercharging experiments with theta glass emitters.

Effects of emitter position and bimolecular reactions. 2,6-dichlorophenolindophenol (DCIP) and L-ascorbic acid (L-AA) undergo a reduction-oxidation reaction with a rate constant of 5.6×10^4 M^{-1} s^{-1} at pH 3 in solution.[47] The reaction is shown in Scheme 1:



Scheme 1. Reaction between DCIP and L-AA.

A theta glass emitter was filled with an aqueous solution at pH 3 containing 1.0 mM DCIP and 2 μM L-enk in one barrel and a pH 3 aqueous solution containing L-AA with 2 μM M-enk in the

other. Electrospray mass spectra were acquired as a function of distance from the center of the entrance capillary of the mass spectrometer in the direction perpendicular to the channel barrier. A conversion ratio of oxidized to reduced DCIP was determined from eq. 1

$$Conversion\ ratio = \frac{A_{rDCIP}}{A_{oDCIP}+A_{rDCIP}} \quad (1)$$

where A_{rDCIP} and A_{oDCIP} are the abundances of the reduced and oxidized forms of protonated DCIP, respectively. The conversion ratio values as a function of horizontal distance from the center of the mass spectrometer inlet (with the center divider vertical) and the concentration of DCIP in the droplet determined from the peptide ratios are shown in Figure 4a and 4b, respectively.

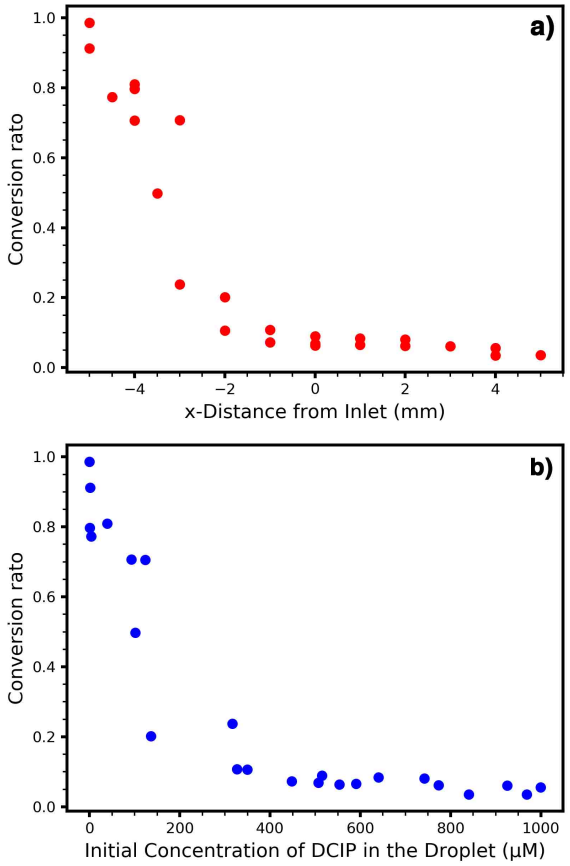


Figure 4. Conversion ratio for the DCIP and L-AA reduction-oxidation reaction determined from eq. 1 as a function of (a) horizontal distance from the inlet with the divider vertical and (b) initial concentration of DCIP in the droplet determined from the M-enk:L-enk ratio.

When the emitter is centered (0 mm), the peptide ratio indicates that the flow rates of the two channels are approximately equal, and the conversion ratio is 0.07 ± 0.01 (triplicate measurements, one shown in Figure 5b).

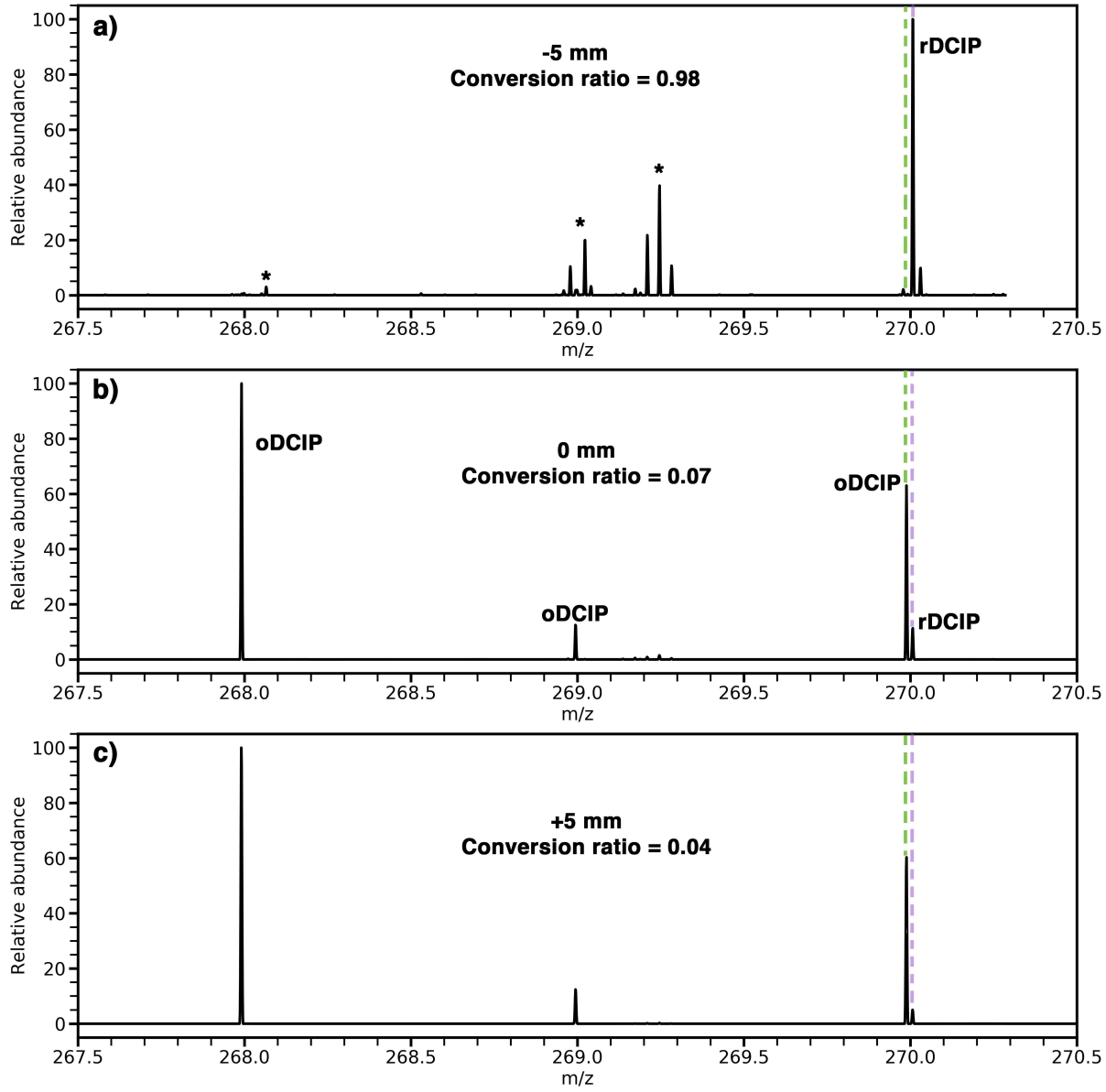


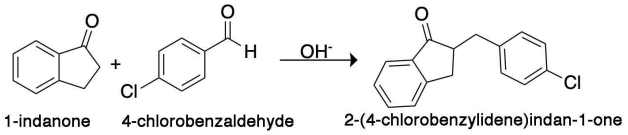
Figure 5. Representative mass spectra obtained using theta glass emitters loaded with 1 mM DCIP in one channel and 1 mM L-AA in the other channel, both in pH 3 aqueous solution at (a) -5 mm, (b) 0 mm, and (c) +5 mm at the region around protonated oxidized DCIP (*m/z* 267.99). The ^{37}Cl isotope of DCIP is at *m/z* 269.96 and the reduced DCIP product is at *m/z* 270.08. Starred peaks are background contaminants in the pH 3 water.

The relatively low conversion rate is due to the short lifetimes of the droplets that limits the time for reaction to occur. The droplet lifetime is 23.0 ± 4.6 μs determined from separate measurements of protein folding kinetics in pH jump mixing experiments in same size emitters.[29]

When the emitter is moved to the right (positive displacement from center), the flow rate of the left channel containing oDCIP is greater than that containing L-AA. This leads to a steady decrease in concentration of rDCIP relative to oDCIP concentration with increasing displacement. At +5 mm (Figure 5c), the conversion ratio is 0.04 and the initial oDCIP concentration is ~30x of that of L-AA. In this region, the reaction has an apparent pseudo-first order behavior where the conversion is nearly independent of increasing oDCIP concentration, i.e., L-AA is the limiting reagent. For negative displacements, oDCIP becomes the limiting reagent. At -5.0 mm, the protonated L-enk could not be distinguished above the noise level, indicating that little or no oDCIP was consistently present in the droplets. The initial average concentration of oDCIP is estimated to be below 0.15 μM based on the noise level, and reduced L-AA is nearly 1.0 mM. The conversion ratio is 98% indicating that most oDCIP is reduced (Figure 5a). The near linear conversion ratio with oDCIP concentration at larger positive displacement values indicates that the reaction again becomes pseudo-first order. These data show that it is possible to change the relative concentrations of both reagents by several orders of

magnitude simply by moving the position of the theta emitter with respect to the mass spectrometer over a distance of ~1 cm. Thus, monitoring the flow rates from each barrel of the theta glass emitters is critically important to accurately quantify chemical reactions. Reaction rates can be accelerated in droplets and the extent of acceleration can depend on the initial concentration of reactants.[29,48,49] Thus, this effect must also be taken into account in order to fully quantitate reaction kinetics.

Base Catalyzed Claisen-Schmidt condensation of 1-indanone and 4-chlorobenzaldehyde. The reduction-oxidation reaction in the previous section follows an A + B → C + D bimolecular reaction scheme where the products and reactants are similar. The Claisen-Schmidt synthesis of 1-indanone and 4-chlorobenzaldehyde follows the reaction scheme A + B → C where the product has a significantly distinct structure from that of either reactant.[1] This base catalyzed condensation reaction is shown below in Scheme 2:



Scheme 2. Reaction scheme of 1-indanone and 4-chlorobenzaldehyde

The first step of the reaction is the alpha hydrogen deprotonated 1-indanone attacking the aldehyde carboxyl carbon on 4-chlorobenzaldehyde. A series of subsequent rearrangements occurs to produce the final product 2-(4-chlorobenzylidene)indan-1-one. This reaction has been reported to have an acceleration rate of 10^4 in droplets.[1]

The product conversion for this reaction[1] was defined as

$$Conversion\ ratio = \frac{A_{2-(4-chlorobenzylidene)indan-1-one} \times 1.4}{A_{4-chlorobenzaldehyde} + A_{2-(4-chlorobenzylidene)indan-1-one} \times 1.4} \quad (2)$$

where $A_{2\text{-(4-chlorobenzylidene)indan-1-one}}$ is the abundance of protonated 2-(4-chlorobenzylidene)indan-1-one, and $A_{4\text{-chlorobenzaldehyde}}$ is the abundance of protonated 4-chlorobenzaldehyde. This definition of a conversion ratio was used because the signal of protonated 1-indanone is ~190x higher than that of protonated 4-chlorobenzaldehyde from an equimolar mixture of these two compounds. In contrast, the signals of protonated 2-(4-chlorobenzylidene)indan-1-one and protonated 4-chorobenzaldehyde formed from an equimolar mixture is 1:1.4. To account for differences in ionization efficiency, the abundance of protonated 2-(4-chlorobenzylidene)indan-1-one was multiplied by 1.4 in Eq. 2.

A methanol solution containing 250 μM of 4-chlorobenzaldehyde, 2 μM L-enk and 3 μM NH_4OH was added into one barrel and another methanol solution containing 250 μM of 1-indanone, 2 μM M-enk and 3 μM NH_4OH was loaded into the other. With the emitter centered (x = 0), the flow rates of the two channels were equal indicating a concentration of ~125 μM of each reactant upon mixing. The conversion ratio was 29.8 ± 2.5% (four replicates). The conversion ratio as a function of emitter tip displacement from center and initial concentration of 4-chlorobenzaldehyde in the droplet are shown in Figures 6a and 6b, respectively.

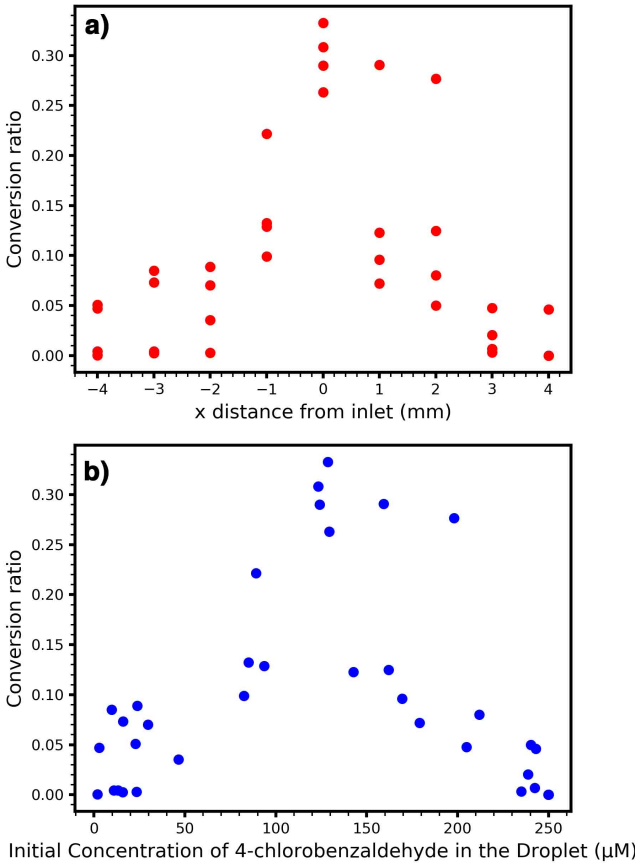


Figure 6. Conversion ratio for the base catalyzed Claisen-Schmidt condensation reaction determined from eq. 2 as a function of (a) horizontal distance from the inlet with the divider vertical and (b) initial concentration of 4-chlorobenzaldehyde in the droplet determined from the M-enk:L-enk ratio.

The conversion ratio is highest when the emitter is centered and decreases rapidly when moved to either side of the inlet, with only 1% conversion observed at -4 mm and +4 mm. The flow rates measured at these positional offsets indicate an average relative flow rate of 1:61 at -4 mm and 3700:1 at +4 mm. These data show significantly more scatter for reasons that are not readily apparent. These results show that the absolute product ion yield for a bimolecular reaction is maximized when the reactants are mixed in equal concentration and that the yield is reduced as

the emitter is moved off center in either direction due to an unequal concentration of reactants in the initial droplets that are formed.

Conclusion

Theta glass emitters can be used to rapidly mix solutions and can be used to investigate fast reactions that occur over a wide range of timescales that can be as short as one microsecond.[15,16] The relative flow rates of the two channels depend strongly on the emitter position in the direction perpendicular to the center divider. The relative flow rates of the two channels can be varied by three orders of magnitude by moving the emitter over a range of less than 2 cm in the direction that is perpendicular to the center divider. Thus, the use of internal standards in each channel of the emitter is essential to measure the relative flow rates of the two channels. The peptides used in this study and in previous studies to ensure that two solutions are equally mixed appear to work well for this purpose, although virtually any internal calibrant system should work equally well. Concentration studies can be made with a single emitter and two solutions consisting of a single reagent concentration by simply moving the position of the emitter. This eliminates effects of emitter variability when solutions are prepared and loaded into individual emitters. This also eliminates the need for externally regulated flow rates in experiments that use mixing apparatus prior to electrospray. Moreover, this method enables accurate mixing ratios of two solution with overall flow rates that are in the low nL/min range, making this method well suited for measurements of concentration dependent reaction rates where only trace amounts of reactants are available.

Prior differences in theta mixing vs premixing solutions when one solution contains a protein in a native-like conformation and the other solution contains a supercharging reagent has

been attributed to the formation of two different Taylor cones and independent electrospray plumes of the two different solutions with droplets merging to initiate reaction.[28] Our results, along with prior results on supercharging with theta glass emitters,[23] show that if droplets combine to form mixed solutions, the efficiency of the droplet recombination would have to be 99+ % in order to not observe low charge states of proteins formed from aqueous droplets containing protein that do not recombine. Although droplet recombination can occur, this extraordinarily high efficiency of droplet mixing is implausible for droplets with the same charge that repel each other due to Coulombic forces. An alternate explanation for results where theta mixing is not the same as pre-mixed solutions is that there can be differences in the relative flow rates of two solutions depending on the exact position of the emitter. These results further emphasize the importance of monitoring the flow rates through the use of internal standards. Significant differences in reaction time-scale can also lead to differences between results from theta-glass mixing and premixing solutions.[15-17,19] This method for significantly changing protein structure and charge state just by moving the position of the emitter may be useful for native mass spectrometry experiments in which the emitter can be positioned to produce low charge state ions from buffered solution with minimal supercharging reagent to obtain information about noncovalent complex stoichiometry and then rapidly moved to a position where there is significant supercharging reagents to generate high charge state ions that can be more efficiently dissociated in tandem MS experiments to obtain information about protein identity and posttranslational modifications through top-down experiments.

Author Contributions

CJC performed experiments and ERW directed the investigations. CJC and ERW designed experiments, analyzed experimental data and wrote the manuscript.

Conflicts of Interest

There are no conflicts to declare

Supporting Information

Ratios of protonated L-enk to M-enk at different relative concentrations, mass spectra of mixtures of myoglobin and 0-5% sulfolane, total ion current and mass spectra for vertical displacement of the emitter, tabulated data for the average charge states of myoglobin, percent apo-myoglobin population, reaction conversion ratios, and solution concentrations in initially formed droplets are shown in the Supporting Information.

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