Characterizing Theta-Emitter Generation for Use in Microdroplet Reactions

Jacob B. Hatvany, Emma-Le P. Olsen, Elyssia S. Gallagher*

Department of Chemistry & Biochemistry, Baylor University, One Bear Place

#97348, Waco, TX 76798

*Corresponding author:

Elyssia S. Gallagher

Elyssia_gallagher@baylor.edu

Abstract:

Theta emitters are useful for generating microdroplets for rapid-mixing reactions. Theta emitters are glass tips, containing an internal septum that separates two channels. When used for mixing, the solutions from each channel are sprayed with mixing occurring during electrospray ionization (ESI) with reaction times on the order of microseconds to milliseconds. Theta emitters of increasing size cause the formation of ESI droplets of increasing size, which require longer times for desolvation and increase droplet lifetimes. Droplets with longer lifetimes provide more time for mixing and allow for increased reaction times prior to desolvation. Because theta emitters are typically produced in-house, there is a need to consistently pull tips with a variety of sizes. Herein, we characterize the effect of pull parameters on the generation of distinct-sized theta emitters using a P-1000 tip puller. Of the examined parameters, the Velocity value had the largest impact on the channel diameter. This work also compares the effect of pulling parameters between single-channel and theta capillaries to examine how the internal septum in theta capillaries affects tip pulling. We demonstrate the utility of using theta emitters with different sizes for establishing distinct reaction times. Finally, we offer suggestions on producing theta emitters of various sizes while maintaining high repeatability. Through this work, we provide resources to establish a versatile and inexpensive rapid-mixing system for probing biologically relevant systems and performing rapid derivatizations.

Introduction:

Rapid mixing during electrospray ionization (ESI) has been used to label peptides and carbohydrates,[1-4] create derivatives of small molecules for enhanced detection,[5] digest proteins,[6] rapidly synthesize phenols and amine-containing compounds,[7-9] and perform several other chemical reactions,[10] including condensation[10] and redox reactions,[11] as well as noncovalent complexations.[11] These mixing experiments typically occur on the timescale of microseconds to milliseconds, matching the lifetime of the ESI process and often undergo rate acceleration.[12-14] By starting mixing immediately before and/or during ESI, reactions can continue until either the reactants desolvate from the droplets or enter the vacuum in the mass spectrometer.[1, 2, 15] Mixing can occur prior to ESI using microfluidic chips or rapid fluid mixing where two solutions are combined at a T-junction and mix as they travel to an ESI emitter.[4, 16-18] In contrast, droplets can be mixed by nebulization[19, 20] or during ESI by tandem ESI,[1, 21-23] desorption ESI (DESI),[24, 25] or via exposing an ESI plume to a reactive vapor that condenses within ESI droplets.[26-28] Mixing during ESI enables shorter reaction times compared to mixing prior to ESI.

One method to establish tandem ESI for rapid mixing is with theta emitters. Theta emitters are borosilicate capillaries containing an internal septum, resulting in the presence of two distinct channels.[1, 23, 29] Tandem ESI occurs when two solutions are sprayed simultaneously and mixed between Taylor Cone formation and the entrance to the mass spectrometer. With theta emitters, the proximity of the two channels maximizes mixing throughout droplet lifetimes.[29] However, reports differ on where mixing is initiated, whether that be in the Taylor cone or as sprayed droplets collide.[1, 22, 23] Theta emitters can also be used to adjust reaction times by changing either the distance between the emitter tip and the mass spectrometer[1, 8] or the initial size of the emitter-tip opening.[2] For reactions that occur both in solution and the gas phase,

increasing the distance from the tip to the mass spectrometer allows for longer reaction times.[1] While for reactions that occur solely in solvated conditions, increasing the emitter-tip size increases the initial ESI-droplet size, leading to longer times for desolvation, and allowing for longer reaction times.[2] Thus, theta emitters are an inexpensive method to achieve rapid mixing with a range of reaction times.

Theta emitters are typically generated using theta capillaries and a micropipette puller. Because these emitters alter droplet lifetimes and thus mixing times, there is significant need to consistently generate emitters of various size. Jordan *et al*. optimized pulling parameters for single-channel capillaries that can be used for native-like mass spectrometry (MS), focusing on generating repeatable, submicron tips.[30] The goal of native-like MS is to preserve the noncovalent interactions within a protein's structure throughout ESI and MS detection. Proteins exist in buffered solutions with high conductivity; thus, emitters with submicron openings have been shown to minimize nonspecific adduct formation, improving analyte signal.[31-34] Compared to these single-channel emitters, theta emitters are used for different applications, specifically rapid mixing. Certain properties – such as repeatability – are also desirable for theta emitters, though the ideal emitter-opening size may vary compared to single-channel emitters based on the desired reaction time.[2] For rapid-mixing ESI experiments, labeling reactions have used theta emitters with 2 μm openings,[1] while synthesis in microdroplets have used emitters with 70 μm openings.[8, 35] Kim and Gallagher used theta emitters ranging from submicron to tens of microns to perform hydrogen/deuterium exchange (HDX) of carbohydrates.[2] The use of emitters of different sizes shows the importance of repeatably generating theta emitters of specific sizes for different reactions. Herein, we investigate how to repeatably create a range of theta emitters of different sizes. In addition, we compare how common pulling parameters that are used to create

single-channel emitters can be adapted to generate theta emitters of various sizes. Then, with emitters of different size, we show how these tips can be used for in-ESI mixing experiments prior to MS detection.

Methods:

Throughout this paper, we use the terms 'capillary', 'emitter', 'tip', and 'channel' to reference unpulled borosilicate tubes, the entire pulled electrospray emitter, the end of the emitter that tapers to an opening where Taylor Cone(s) are generated during ESI, and the fluid-filled region within the emitter, respectively.[30] Theta capillaries (1.5 mm outer diameter, 1.17 mm inner diameter, 0.165 mm septum thickness, Sutter Instrument, Novato, CA) and single-channel capillaries (1.5 mm outer diameter, 1.17 mm inner diameter, Sutter Instrument) were pulled using a P-1000 Micropipette Puller (Sutter Instrument) containing a box filament (FB255B). While specific values for pulling parameters differ between pullers, filaments, and glass thicknesses,[36] general trends in emitter formation are expected to remain consistent. The analyzed P-1000 parameters include orientation of theta-capillary septum (horizontal or vertical), Heat (Ramp-40 to Ramp+10), Pull (0-100), Velocity (0-100), Time (200-250), and Pressure (100-500). The channel diameter(s) for each tip were measured using a TM3030Plus Scanning Electron Microscope (Hitachi, Tokyo, Japan) with 15 KV applied to the filament and utilizing the secondary electron detector.

Statistical tests to compare sample means generally assume that each value in the sample set is independent of the other values. However, each pulled theta capillary results in two emitters, with each tip containing two channels with opening diameters that can be measured

(**Figure 1**). Thus, statistical analyses were performed to determine the independence of emitter and channel-opening size to enable statistical comparisons between the tested parameters. Because each pulling event produces two tips with a total of four channels, paired-difference *t*-tests and ANOVA were used to determine appropriate degrees of freedom. Paired-difference *t*-tests revealed that there were no significant differences in the average opening size between the channels of an individual emitter (e.g., between the left and right channels). Additionally, Pearson coefficients showed a strong correlation between the size of both channels on an emitter (*i.e.* an emitter with an above average opening in the left channel was correlated with an above average opening in the right channel). There was also a correlation in the sizes of the two emitters that were pulled from the same capillary based on an ANOVA. An example of these statistical correlations is shown in **Figure S1**. However, each emitter is used independently in a mixing experiment, even though two emitters are generated from one capillary. Due to these correlations and considerations, for statistical comparison, only one opening on each theta emitter was included in the data set, though both emitters from the same capillary were measured and included in the data set. The sample size was kept at eight emitters for analyzing each pulling parameter. Further statistical analyses were performed using F-tests, two-tailed Student's *t*-tests, and/or two-tailed Welch's *t*-tests at the 95% confidence interval. Error bars in figures represent standard deviation.

All chemicals were from Sigma-Adrich (St. Louis, MO) unless specified otherwise. Nanopure water was acquired from a Purelab Flex 3 water purification system (Elga, Veolia Environment S. A., Paris, France).

For rapid-mixing experiments, 18-crown-6 was prepared at 100 μM with 500 μM NaCl and loaded in one channel of a theta emitter, while the opposite channel was loaded with 500 μM

KCl. Leucine enkephalin and methionine enkephalin were used as internal standards, with one peptide being added to each channel to ensure equal mixing from both channels, as has been reported previously.[2, 23] For rapid HDX experiments, glycopeptide (KVANKT-A2G2S2, Ludger, Oxfordshire, UK) solutions were prepared at 10 μM in water and loaded in one channel of a theta emitter. D_2O (99.96% purity, Cambridge Isotope Laboratories, Tewksbury, MA) was loaded in the opposite channel of the emitter. All mixing experiments used a custom open source based on designs of a nano-ESI source from the University of Washington's Proteomics Resource,[37] which enabled spray from emitters 3-5 mm in front of the MS inlet. No sheath, auxiliary, or backing gas was used. All reactions were detected using an Orbitrap Fusion Tribrid MS (Thermo Fisher Scientific, Waltham, MA). Reported values of MS experiments represent spectra generated by three tips (n = 3) for each reported size with numerical values indicating the average ± the standard deviation. Comparative ratios are based on the peak height for the primary, monoisotopic peak for each species.

Results and Discussion:

Effect of Tip-Puller Parameters.

Unlike single-channel capillaries, theta capillaries have an internal septum that creates two distinct channels, thus for every pulled theta capillary, two emitters are generated, each with two channels (**Figure 1**). Depending on the rotation of the capillary when inserted into the puller, the septum can either be parallel (horizontal) or orthogonal (vertical) to the floor (**Figure 1C & 1D**). This orientation in loading the capillary could affect how the septum is heated when pulled to form emitters because the box filament will apply more heat to the glass nearer to the filament.

Thus, the heat dispersion may vary if the septum is parallel versus perpendicular to the box filament. Capillaries were pulled into emitters with the septum in both orientations using the following pull parameters; Heat: Ramp-20, Pull: 0, Velocity: 70, Time: 235, and Pressure: 300. These parameters were selected to test the effect of theta-capillary orientation as well as provide a starting point for comparing pulling parameters because they produced repeatable single-channel tips.[30, 36] Capillaries pulled in the horizontal or vertical orientations did not produce emitters with significantly different channel-sizes at the tip, (1.1 ± 0.2) μm and (0.94 ± 0.08) μm, respectively (n = 8). However, emitters that were generated from a capillary with a vertical orientation did have a significantly lower standard deviation, which represents a higher repeatability. Thus, theta emitters were generated with the capillary septum in a vertical orientation for all further comparisons.

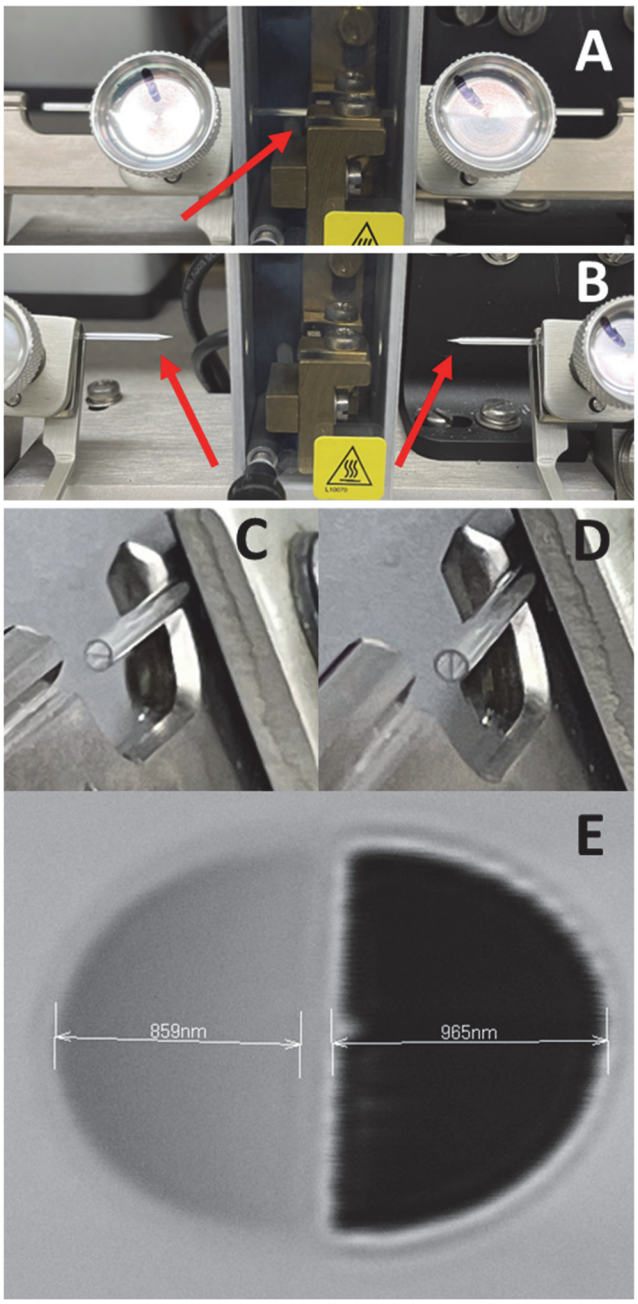


Figure 1. P-1000 Micropipette puller showing placement of capillary (red arrow, A) and pulled emitters (red arrows, B). Theta capillaries loaded in puller with septa in the horizontal (C) or vertical (D) orientation. Electron micrograph of theta-emitter tip showing two channels (E).

Five parameters were individually varied on the P-1000 micropipette puller to assess their effects on emitter-tip size and repeatability (**Figure 2, Table S1**). (1) 'Heat' varies the current applied to the box filament. (2) 'Pull' varies the force applied to the pulley arms when generating emitters. (3) 'Velocity' controls the speed of the pull by changing the start time after the glass is heated and treated with cooling gas. (4) 'Time' is the amount of time in ½ millisecond increments that the cooling gas (dry air) is applied during emitter generation. (5) 'Pressure' represents the pressure of the cooling gas applied during pulling.[36] All parameters are represented in arbitrary units.

Of the tested parameters, Velocity was found to have the largest effect on the size of each channel opening at the tip. At a low Velocity (value 10), theta emitters were generated consistently with large channel openings , (36 ± 2) μm, while at higher Velocity (values 50-90), smaller channel openings, (340 ± 40) nm, were generated (**Figure 2A**). At intermediate Velocity (value 20-30), two distinct sizes, both large (jagged) and small (smooth) channel openings were formed (**Figure S2**), leading to an intermediate average size with a large standard deviation. We hypothesize that the formation of two distinct sizes is due to the capillaries being near a threshold value for looping at this pull parameter. Looping is where the capillary is not pulled into an emitter in a single cycle of the tip-puller parameters and thus the program runs repeatedly over several iterations (up to ten times). The smaller channel openings at the tip were generated after one or two loops while larger channel openings were generated between seven and ten loops.

Single-channel emitters pulled at a low Velocity (value 10) consistently yielded large tip sizes, (64.1 ± 0.7) μm, while higher Velocity (values 70-90) generated smaller tips (680 ± 10) nm. This trend was consistent to that observed for theta emitters, though the average tip sizes differed. Intermediate Velocity (values 20-50) also generated both large and small single-channel

tips, as observed with theta tips (**Figure 2B, Table S2**). Yet, the range of Velocity values that generated two populations of emitter sizes was narrower for theta emitters compared to single-channel emitters. We attribute this difference to the presence of the internal septum within theta capillaries. We hypothesize that when the Velocity is between the high and intermediate regime, the septum stabilizes the capillary, leading to a smooth 'pulling' that produces smaller tips compared to the jagged 'snapping' that generates larger tips (**Figure S2**). As the Velocity is further reduced, more loops are required to generate emitters. Each loop involves the heating of the filament before the capillary has fully cooled from the previous loop. Thus, the capillary undergoes several rapid cycles of heating and partial cooling due to the applied cooling gas for each looping cycle, leading to the eventual snapping of the emitter and creating the larger, jagged tips. Looping is frequently used to generate patch pipettes in the larger size regime for single-channel emitters.[36]

The next most impactful parameter for generating theta-emitter channels of various opening size at the tip was Heat. When discussing Heat, the value is in reference to that obtained during a ramp test, which heats the filament until the glass within the capillary softens. Because the ramp-test value can shift over the course of a filament's lifetime and between different filaments, it is crucial to select Heat values based on a current ramp test.[30] For theta tips, sizes ranged from (1.7 ± 0.2) μm to (300 ± 30) nm when Heat was varied from Ramp–40 to Ramp+10, respectively (**Figure 2C**). Values above Ramp+10 were found to seal the theta-emitter tip, leaving no opening. Single-channel emitters pulled from Ramp-40 to Ramp+20 generated channel openings at the tip between (9 ± 9) μm to (500 ± 30) nm, respectively (**Figure 2D**). Similar to the Velocity parameter, single-channel emitters pulled at Ramp-20 and below produced a mixed population of both small and large tips due to capillaries being either pulled or

snapped, respectively. We hypothesize that Heat values lower than Ramp-40 could yield similar dual, sample populations for theta emitters, but due to the manufacturer recommendations, we did not test Heat values lower than 10% of the Ramp value (e.g., if the Ramp value is 500, the manufacturer recommendations advise not using a Heat value below 450). Overall, similar trends were observed for both theta and single-channel emitters, with higher Heat yielding smaller tip sizes. Yet, Heat values around 20 arbitrary units lower were required to generate similar-sized channel openings for theta emitters compared to single-channel emitters due to the difference in the ramp test for each type of emitter.

Pull values from 0 to 100 were used to generate theta- and single-channel emitters with values above 100 leading to sealed theta tips. From low to high Pull values, theta emitters had openings from (1.7 ± 0.2) μm to (340 ± 30) nm, respectively, while single-channel emitters had openings from (2.3 ± 0.2) μm to (330 ± 20) nm, respectively. For single-channel emitters, high pull values yielded the smallest, repeatable tips compared to any other tested parameter (**Figure 2E, 2F, Table S2**). In contrast, no one parameter produced uniquely small theta emitters, with the minimal-sized theta-tip openings being achieved using high Velocity, high Heat, high Pull, low Pressure, and low Time values.

While the Pull parameter was previously reported to have the largest impact on tip size for single-channel emitters,[30] this did not hold true for either single-channel emitters or theta emitters generated in our lab. Many factors could contribute to this difference, including differing capillary-glass thickness and ambient lab conditions, such as temperature and/or humidity.[36] In this case, we hypothesize that glass thickness is the leading factor for differences in the observed trends. Capillaries used here had a glass thickness of 0.33 mm, while work performed by Jordan *et al.* used capillaries with a glass thickness of 0.28 mm.[30] As glass

thickness increases, more force is needed to fully separate a capillary into emitters. Thicker glass capillaries have more mass than thinner glass capillaries and take longer for the filament to distribute heat through the thicker glass, meaning the thicker glass will be less malleable when pulling occurs. Thus, pull is expected to have a diminished effect for capillaries of increasing glass thickness and produce smaller tips overall.[36]

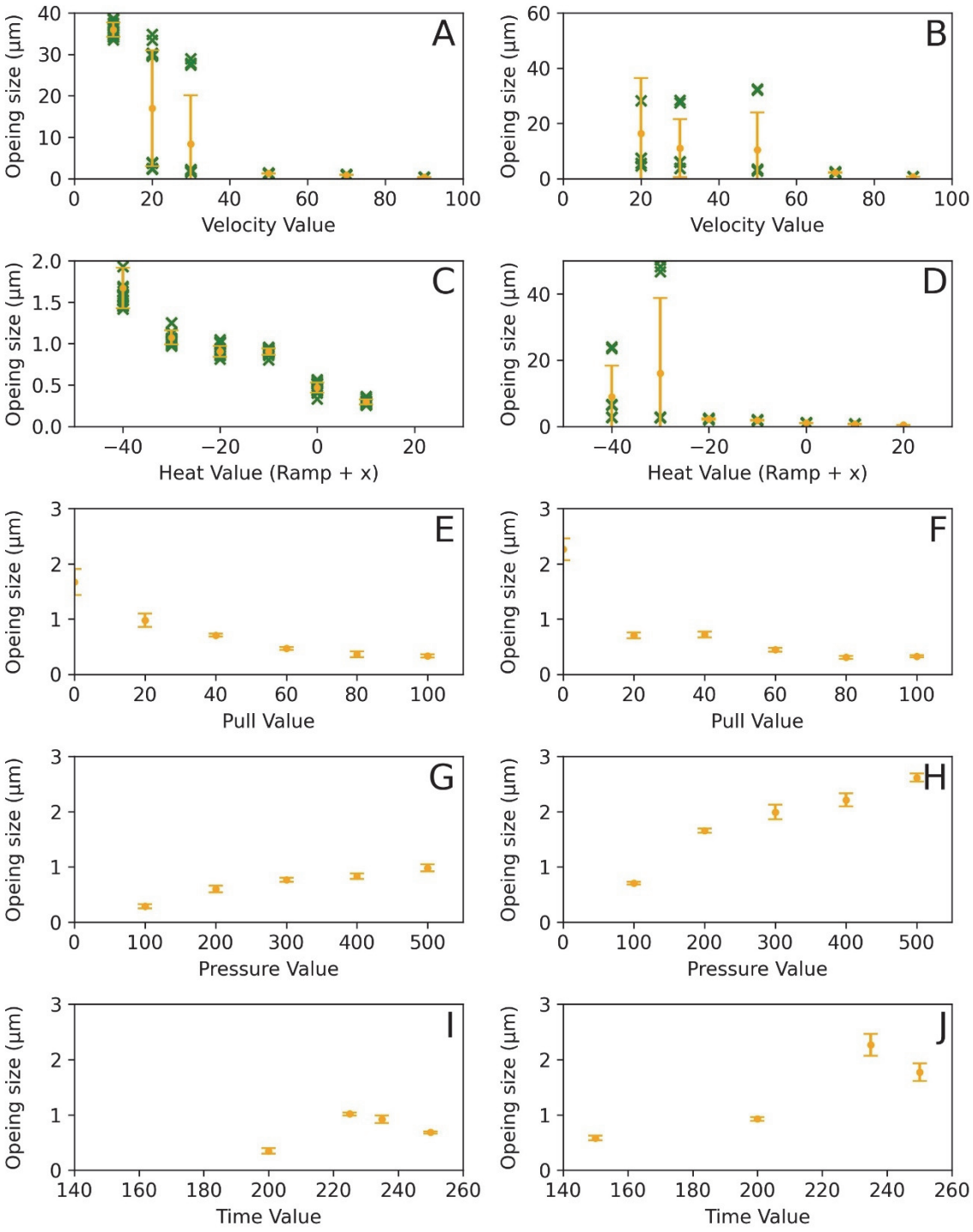
Both Pressure and Time had minor effects on the size of tips pulled from both theta and single-channel capillaries. Pressure was varied from 100 to 500, and the size of the theta emitters varied from (310 ± 30) nm to (990 ± 90) nm, respectively (**Figure 2G**). For the same range of Pressure values, the size of the single-channel emitters varied from (710 ± 20) nm to (2.61 ± 0.07) µm, respectively (**Figure 2H**). In contrast to the other tested parameters, channel diameter increased as the Pressure increased. Finally, Time values ranged from 200 to 250 for the generation of theta emitters with Time values under 200 forming sealed emitters. Time 200 generated the smallest theta tips at (320 ± 50) nm and Time 225 generated the largest tips at (1.03 ± 0.03) µm (**Figure 2I**). This lack of a consistent trend in tip size as the Time changed suggests that a minimal Time is needed to successfully pull capillaries with an opening but that there is limited utility in optimizing this parameter. Single-channel emitters exhibited a similar trend to theta emitters but could be generated from lower Time values (150-250) without the tips sealing (**Figure 2J**).

The repeatability of pulled tips was compared as the parameters were changed. While larger tips had larger absolute standard deviations, relative standard deviations (RSD) were compared to examine the variation in relation to the channel size at the tip across multiple size regimes. Excluding the parameters that showed different breaking patterns (both pulling and snapping), the average RSD for all generated theta emitters was ~10% with the largest channel

openings, (36 ± 2) μm, and smallest channel openings, (300 ± 30) nm, having an RSD of 5% and 11%, respectively (**Table S1**). Tips with an opening of 0.6 μm or larger typically had an RSD under 10%, while tips smaller than 0.6 μm had an RSD between 10-20%. We attribute this difference in RSD to the scale of the tip opening diminishing faster than the scale of the tip-to-tip variation.

In comparison to work performed by Jordan *et al.*,[30] we measured similar RSD for single-channel emitters with sizes less than 2.5 μm. In our measurements for single-channel emitters, the average RSD, when excluding parameters near different breaking patterns was 5.9%. The largest tips at (64.1 ± 0.7) μm had a low RSD of 1% (**Table S2**). Tips with an opening of 0.6 μm and larger had an RSD under 10%, similar to that of theta-emitters. Small tips (<0.6 μm) had a significantly lower RSD (~6%) compared to theta emitters (~14%). We attribute this difference in RSD for small tips to the presence of the septum in theta emitters being at a different heat/malleability compared to the outer capillary wall, leading to greater variation during pulling. Additionally, these repeatability values are based on using one tip puller and performing frequent Ramp Tests.

For theta emitters, tips with smaller openings were generated with longer, narrower tapers between the body of the emitter and the tip (**Figure S3**). This is similar to trends observed for single-channel emitters.[30, 36] Long tapers in single-channel emitters have been associated with increased non-specific adduction of analytes to the glass walls and can result in protein unfolding.[38, 39] If theta emitters are used for mixing experiments involving proteins, we expect more extensive adduction in emitters with long-tapers due to the septum, which yields narrower channels and increases the surface area-to-volume ratio of fluid near the tip.


A
Opeing size (µm)
Velocity Value
B
Opeing size (µm)
Velocity Value
C
Opeing size (µm)
Heat Value (Ramp + x)
D
Opeing size (µm)
Heat Value (Ramp + x)
E
Opeing size (µm)
Pull Value
F
Opeing size (µm)
Pull Value
G
Opeing size (µm)
Pressure Value
H
Opeing size (µm)
Pressure Value
I
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Time Value
J
Opeing size (µm)
Time Value

Figure 2. Opening diameters for theta-emitter channels (A, C, E, G, I) and single-channel emitters (B, D, F, H, J) when varying pull parameters: (A, B) Velocity, (C, D) Heat, (E, F) Pull, (G, H) Pressure, and (I, J) Time. When testing the Velocity and Heat parameters, some values resulted in two sample populations with different sizes. Tip sizes from individual channel measurements are represented by green X (A-D), while average tip sizes are shown with orange circles (A-J).

Utility of Theta Emitters for Rapid Mixing Reactions.

Once generated, theta emitters are used for rapid-mixing reactions with MS detection. Each channel of the theta emitter is filled with a different solution that mixes during ESI. One such system involves placing 18-crown-6 (18C6) with NaCl in one channel of a theta tip and KCl in the other channel. 18C6 in the presence of NaCl adducts Na^+ to form $[18C6 + Na]^+$ (287.1464 *m/z*) with a forward rate constant (k_F) of 2.2×10^8 $M^{-1}s^{-1}$ and an equilibrium constant of 6.5.[40, 41] Comparatively, in the presence of potassium, $[18C6 + K]^+$ (303.1202 *m/z*) forms with a forward rate of 2.5×10^9 $M^{-1}s^{-1}$ and an equilibrium constant of 116.4.[40] The forward rate constants are indicative of the rapid reaction, which can occur on a timescale compatible with ESI. Because the equilibrium constant of potassium binding to 18C6 is nearly 2 orders of magnitude larger than that of sodium binding to 18C6, complexes of 18C6 with K^+ are expected to dominate over those with Na^+ when both cations are present at equal concentrations.[23]

In **Figure 3**, theta emitters with different tip sizes were used to spray 18C6 with NaCl from one channel and KCl from the other channel. These spectra are compared to spectra in which 18C6, NaCl, and KCl were premixed prior to spraying. To assess whether the spray from

both channels of the theta emitter was consistent, leucine-enkephalin and methionine-enkephalin were used as internal standards with one peptide added to each channel (**Figure S4**). For the premixed solution, the peak for $[18C6 + K]^+$ is more intense, with a ratio of peak heights for $[18C6 + Na]^+$ to $[18C6 + K]^+$ of 0.010 ± 0.001. Spraying from separate channels in submicron tips (330 nm) resulted in the sodiated peak remaining dominant and the peak-height ratio for $[18C6 + Na]^+$ to $[18C6 + K]^+$ being 14.7 ± 0.9, indicating that minimal mixing has occurred. Alternatively, micron-dimension tips (4.2 μm) resulted in the potassiated peak becoming dominant with the peak-height ratio of $[18C6 + Na]^+$ to $[18C6 + K]^+$ being 0.1 ± 0.2. Compared to the 330 nm emitters, this value is approaching the ratio that was observed for the premixed solution. However, because the ratio is an order of magnitude larger than that of the premixed solution, the sample has not yet reached equilibrium. Finally, large emitters, in the tens of microns (37.2 μm), showed that the intensity of the sodiated peak diminished significantly in comparison to the intensity of the potassiated peak, leading to a peak-height ratio of 0.009 ± 0.009 for $[18C6 + Na]^+$ to $[18C6 + K]^+$. This ratio of peak heights is most similar to that observed for the premixed solution. As the tip size increases, the droplet-mixing time increases, enabling 18C6 to approach equilibrium conditions when mixing with Na^+ and K^+.

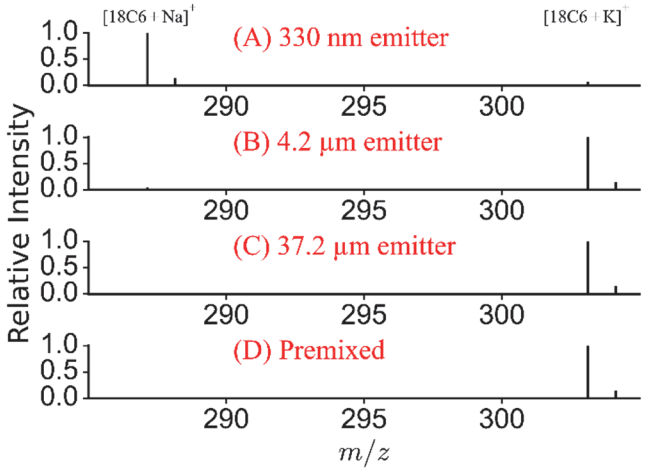

Figure 3. Representative mass spectra showing the ratio of $[18C6 + Na]^+$ and $[18C6 + K]^+$ when solutions of (1) 18C6 and NaCl and (2) KCl were sprayed from separate channels of theta emitters with opening sizes of 330 nm (A), 4.2 µm (B), and 37.2 µm (C) with applied voltages of 1100 V, 1600 V, and 3000 V, respectively. All voltages represent onset values for the different sized tips. Representative mass spectrum for a premixed solution of 18C6, NaCl, and KCl sprayed from a 4.2 µm emitter at 1600 V (D).

Rapid mixing ESI, and thus theta emitters, are also useful for labeling with reaction times between microseconds and tens of milliseconds.[1, 2] **Figure 4** shows the use of theta emitters with different opening sizes at the tip to label a glycopeptide, which is sprayed from one channel while a deuterating reagent (D_2O) is sprayed from the other channel. Rapid HDX using theta tips has previously been used to label carbohydrates and peptides during ESI.[1, 2] Over the course of ESI, solutions from the two channels mix and labile hydrogen atoms exchange with deuterium. The mass increase between hydrogen and deuterium can be detected as an increase in the *m/z* of the glycopeptide. When sprayed from emitters of different size (12 µm versus 1 µm), both

emitters resulted in deuterium exchange for the glycopeptide as observed by the isotopic peaks shifting to higher *m/z* values. This is in comparison to the glycopeptide that is sprayed without deuterating reagent, where both channels of the theta emitter were loaded with glycopeptide in H_2O. The glycopeptide sprayed from theta tips with small channel opening (1 μm) experienced HDX, as indicated by the *971.048 m/z* peak (3rd peak) showing higher intensity compared to the *970.379 m/z* peak (1st peak) in contrast to the spectrum for the glycopeptide without HDX. The glycopeptide sprayed from the theta tip with large channel opening (12 μm) experienced significant exchange as indicated by the increase in the number of isotopic peaks with increasing *m/z*. We attribute the presence of multiple peak distributions in the data from the 12-μm tip to the model glycopeptide having multiple conformations in solution that undergo HDX at different rates. Multiple peak distribution have previously been correlated to multiple conformations for both carbohydrates and proteins during HDX.[44, 45] The initial droplets formed during ESI are estimated to be between 1/10 and 1/17 of the size of the theta-tip opening.[23, 31] Thus, emitters with different opening sizes (1 μm versus 12 μm) are anticipated to form droplets with different initial sizes, but travel consistent distances between the emitter and inlet to the mass spectrometer (5 mm). Differences in HDX for the glycopeptides samples sprayed from either the 1 μm or 12 μm tips are therefore attributed to the initial ESI droplets having different sizes, which influence droplet lifetimes and exchange times for HDX before the glycopeptides enter the mass spectrometer. This is consistent with prior carbohydrate labeling using theta tips of different size.[2]

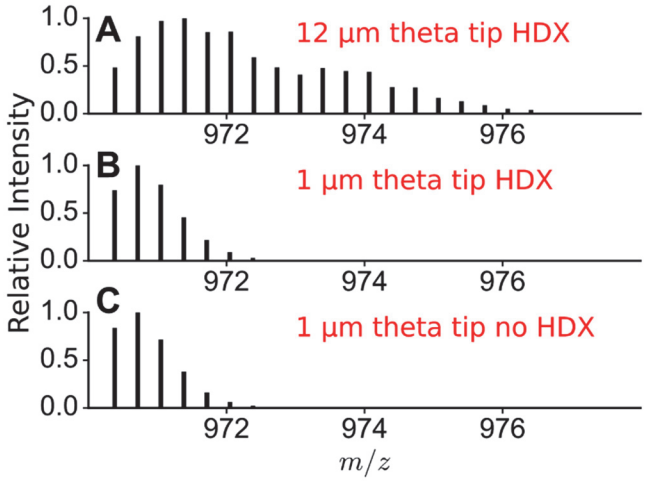

Figure 4. Representative spectra showing the effect of theta-emitter channel size on rapid H/D exchange reactions. A glycopeptide (KVANKT-A2G2S2) and $D_2O_{(l)}$ were sprayed from separate channels with a 5 mm distance from the emitter tip to the inlet of the mass spectrometer. Spectra were collected from emitters with a 12 µm tip with HDX (**A**), 1 µm tip with HDX (**B**), and 1 µm tip without HDX (**C**). Spectra show the $[H+2Na+glycopeptide]^{3+}$ adduct.

Conclusions:

Throughout this work, by comparing emitter-tip generation between single-channel and theta capillaries, we provide insight into tip-puller parameters that need adjustment to generate emitters in different size regimes. When making theta emitters, repeatability is a more realistic goal than reproducibility. This can be attributed to a wide variety of factors causing variability between different laboratories or experimental setups; including laboratory environment, such as temperature and humidity, capillary thickness, capillary material, type of puller filament, and point in puller-filament lifetime.[36] Thus, here we offer suggestions for 'dialing-in' the desired theta-tip size. In addition, compared to single-channel emitters, we show the utility of theta tips

for performing rapid mixing and labeling reactions from theta emitters with multiple tip sizes, ranging from submicron to tens of microns.

1. When pulling theta capillaries, orientation of the septum in comparison to the puller and filament should be considered. We observed that with a box filament on a P-1000, a vertical orientation improved the repeatability. Initial tips should be imaged to verify that the filament is appropriately centered to produce equal sized tips between emitters and equal sized channel openings on each tip.
2. A ramp test should be performed often for pulling theta capillaries. The filament is a consumable and the performance will change over the filament lifetime as well as if it is bumped or damaged. Typically, a ramp test once every three months is sufficient to monitor and adjust for differences in the Heat value,[30] but when instrumentation is shared between multiple users, more frequent testing could be beneficial due to increased use and wear on the filament. For initial emitter generation, set the Heat value to 30 units below the Ramp value, Pull 0, Pressure near 500, and Time near 225 because these values produced repeatable tips.
3. If large channel openings are desired (>20µm), a low Velocity, between 5-15, should be selected. For small channel openings (<1µm), a high Velocity, near 70-90, should be used. For a mid-size channel opening (2-10µm) a Velocity near 40 - or slightly higher than the pulling/snapping threshold velocity - should be utilized.
4. Once a rough size is established for the theta emitter being pulled, Pull, Heat, and Pressure should be optimized sequentially to obtain emitters with the desired opening size. Imaging several emitters is recommended to ensure consistency of

both channel sizes on a single emitter, emitter sizes across a capillary, and capillary-to-capillary differences.

These guidelines will assist in the fabrication, and encourage the use of, theta emitters with various sizes to generate microdroplet reactions in ESI-MS, including rapid HDX, protein folding and unfolding, digestions, rapid synthesis, and derivatization reactions with multiple, rapid timepoints.

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Supporting Information.

Tables characterizing emitter tip sizes and repeatability, electron micrographs of theta emitters, and representative mass spectra

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Abstract Graphic: