Exploring Modifications to a Microfluidic Device and their Effect on Hydrogen Deuterium Exchange Rates

Cool Name

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Abstract

The structure and dynamics of a protein are fundamental to its biological function. One emerging technology that allows for elucidation of protein dynamics is hydrogen deuterium exchange (HDX) in conjunction with mass spectrometry-based analysis. A novel approach developed utilizing this technique, time-resolved hydrogen deuterium exchange (TRESI-HDX), utilizes a microfluidic device that allows the analysis of millisecond timescale exchange reactions. The TRESI-HDX microfluidic setup enables the exchange of backbone amide hydrogens followed by rapid quenching and injection into the MS. The accuracy and quality of the data obtained from utilizing TRESI is dependent on the proper functioning of the microfluidic setup, with inconsistencies in the flow rates of solutions or efficiency of quenching leading to altered exchange dynamics. Modifications were made to the microfluidic setup in an attempt to improve the consistency of deuterium uptake between experiments as well as ease of use. Two proteins were used as models to study the effect of the alterations, cytochrome C, a structured protein, as well as tau, a highly disordered protein. Through investigation of several modifications, the TRESI setup was found to be extremely sensitive to changes in flow rates, the inclusion of a filter, or substitutions of mechanical components. No modification tested provided a conclusive improvement to the quality of data, but discoveries were made regarding what components most often lead to mechanical failures. This study has also highlighted how this approach is much better suited to the study of disordered proteins, such as tau.

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List of Abbreviations

Alzheimer's Disease (AD)

Drift Tube Ion Mobility Spectrometry (DTIMS)

Electrospray Ionization (ESI)

Field-Asymmetric Ion Mobility Spectrometry (FAIMS)

Hydrogen/Deuterium exchange (HDX)

Intrinsically Disordered Protein (IDP)

Ion mobility spectrometry (IMS)

Liquid Chromatography (LC)

Mass Spectrometry (MS)

Matrix Assisted Laser Desorption/Ionization (MALDI)

Poly-Methyl Methacrylate (PMMA)

Stacked Ring Ion Guides (SRIG)

Time-of-Flight (TOF)

Time-Resolved Electrospray Ionization HDX-MS (TRESI-HDX)

Travelling Wave Ion Mobility Spectrometry (TWIMS)

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Chapter 1: Introduction

1.1 Hydrogen/Deuterium Exchange Mass Spectrometry, A Tool for Studying Protein Structure and Dynamics

1.1.1 Mass Spectrometry in Proteomics

Mass spectrometry (MS) is a technique whereby molecules are ionized and measured by their mass-to-charge ratio (m/z)[1]. To accomplish this, a mass spectrometer consists of an ion source, a mass analyzer that measures the m/z of the ionized analytes, and a detector that records the number of ions at each m/z value[1]. This allows for identification and quantification of a wide range of analytes, from small molecules to peptides and intact proteins[2].

Early forms of MS could only analyze small molecules, as no methods existed to ionize and transfer larger biomolecules into gas phase in order to analyze them[3]. The development of two methods that allow for ionization of proteins and peptides, Electrospray Ionization (ESI) or Matrix-Assisted Laser Desorption/Ionization (MALDI) revolutionised the field of MS based proteomics[1]. Both of these methods are a form of "soft" ionization, whereby the molecular ions remain intact with fewer resultant fragmentations than "hard" forms of ionization[4]. These soft forms of ionization are invaluable in the MS based study of proteomics as minimal fragmentation allows for detection of non-covalent interactions, like protein-protein interactions[4].

1.1.2 Electrospray Ionization

Electrospray Ionization is achieved through flowing analyte solution through a metal capillary with the tip held at a high voltage (2-6kV)[5]. This electrical potential creates a Taylor Cone of dispersed solution of positively charged droplets[6]. As the droplets move toward the mass spectrometer, the solvent evaporates, reducing droplet size until the analyte transfers into the gas phase as charged molecules. Once in the charged gas phase, the analyte can enter the mass spectrometer and be analyzed[5]. **Figure 1.1** shows

a schematic detailing this process. Nitrogen gas flows around the capillary to both increase nebulization and enhance drying of the droplets to better eject the analyte into the gas phase[7].

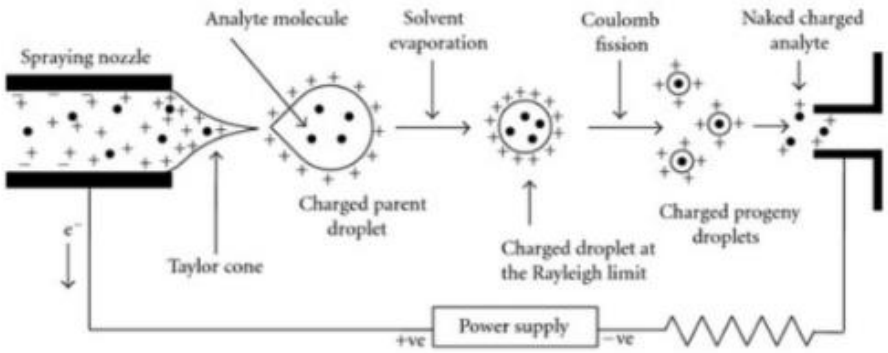

Figure 1.1 Schematic of positive Electrospray Ionization. High voltage applied to capillary tip produces electric potential resulting in formation of a Taylor Cone due to dispersal of sample solution. Positive charged drops reduce in size during transit to the mass spectrometer due to solvent evaporation. When droplet sizes reach a threshold, positive charge transfers onto the analyte to form naked charged molecules in gas phase that are transferred into the mass spectrometer. Figure adapted from Banerjee & Mazumdar, 2012[7].

1.1.3 Ion Mobility Spectrometry

In order to further improve characterization of sample ions, Ion Mobility Spectrometry (IMS) is often used in conjunction with mass spectrometry[8]. IMS is a technique where a collection of ions in gas phase are separated based on their charge and cross-sectional area. There are several methods that employ different techniques to achieve separation, with the common versions used being drift tube ion mobility spectrometry (DTIMS), travelling wave ion mobility spectrometry (TWIMS), and field-asymmetric ion mobility spectrometry (FAIMS).

The method used by a Waters G2-S HDMS TOF-MS is TWIMS, which contains three travelling wave cells. A TWIMS cell is comprised of stacked ring ion guides (SRIG) operating at low pressure and filled with a drift gas (usually N_2)[8]. A travelling wave is generated by a voltage pulse from the SRIG which propels the swarm of ions forward through the gas. The separation of ions in a travelling wave IMS cell is depicted in **Figure 1.2**. Ions with lower mobility have higher cross-sectional area and low charge,

and have their motion impeded through collisions with the drift gas. This will cause these ions to "slip over" the wave and migrate out of the cell slower than ions with higher mobility which will move forward with the wave, less impeded by the drift gas. In this way, two ions with the same m/z ratio that would be indistinguishable to MS analysis can be differentiated by IMS if the ions differ in cross-sectional area[9]. This technique can act as a form of pre-filtration before MS analysis, granting greater ability to distinguish between isomers or similar weight peptide fragments[8].

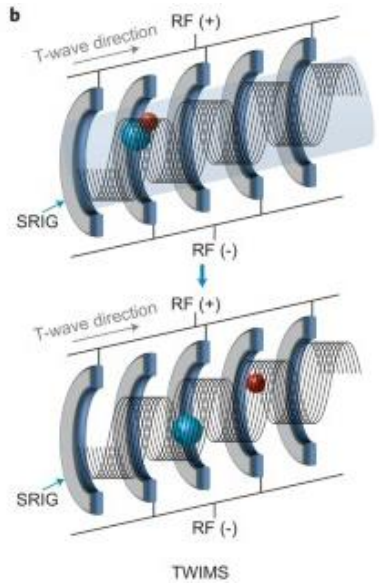

Figure 1.2 Separation of ions in a Travelling wave IMS cell. Ions in swarm "surf" the travelling wave generated by stacked ring ion guides. Ions separate based on charge and size, ions with high mobility have high charge and low cross-sectional area. Figure adapted from Lanucara *et al.* (2014)[9].

1.1.4 Hydrogen Deuterium Exchange

Hydrogen Deuterium Exchange (HDX) is a chemical process whereby a covalently bound labile hydrogen atom is substituted with deuterium. A protein in deuterium oxide (D_2O) solvent would exchange hydrogen on chemically labile groups such as S-H, O-H and N-H with deuterium[10]. Typically, only N-H exchanges are considered in the final analysis as they are present on every amino acid. HDX reactions can be coupled to MS to study aspects of protein structure and dynamics[11]. This is because hydrogen/deuterium exchange can be detected as a mass shift via MS due to the increased weight of

deuterium compared to hydrogen. To limit HDX exchange to amides only and to prevent further exchange after a desired time point, the reaction is rapidly quenched with acid to a pH of 2.5. This halts the exchange of hydrogen with deuterium and prevents further exchange before analysis. At this pH, H to D conversion is at a minimum, with the amide hydrogens on the protein backbone locked from further exchange with deuterium[10]. HDX is dependent on the fact that back exchange of deuterium to hydrogen is rare, meaning that once exchanged, the deuterium remains on the amide until detection[12]. The mechanism of hydrogen deuterium exchange is depicted in **Figure 1.3.**

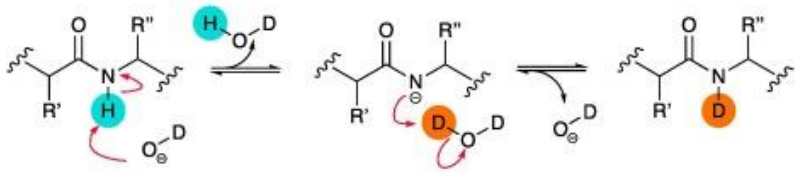

Figure 1.3. Mechanism of base catalyzed hydrogen/deuterium exchange. While HDX reactions can be acid or base catalyzed, base catalysis is dominant at pH 7. Figure adapted from Oganesyan *et al* (2018)[13].

The way that HDX is used to study protein dynamics is through determining the rate and location of deuterium uptake by amide hydrogens[14]. Amides in regions that are more solvent exposed or flexible will exchange at higher rates than amides that are rigid or hidden in the protein core. In this way, the dynamics and structure of a protein can be detected through examination of the mass shift due to the uptake of deuterium. This can be done in a "global" manner, whereby the intact protein is analyzed through native mass spectrometry, and the average deuterium uptake is measured over time. An alternative is analysis in a "bottom-up" approach, where the protein is digested with enzymes into peptides following deuterium uptake[14]. In this way, information can be gained about deuterium uptake on the peptide level to locate sites of high or low exchange in order to map regions of interest in relation to peptide sequence. The general outline of a "bottom-up" HDX approach is illustrated in **Figure 1.4.**

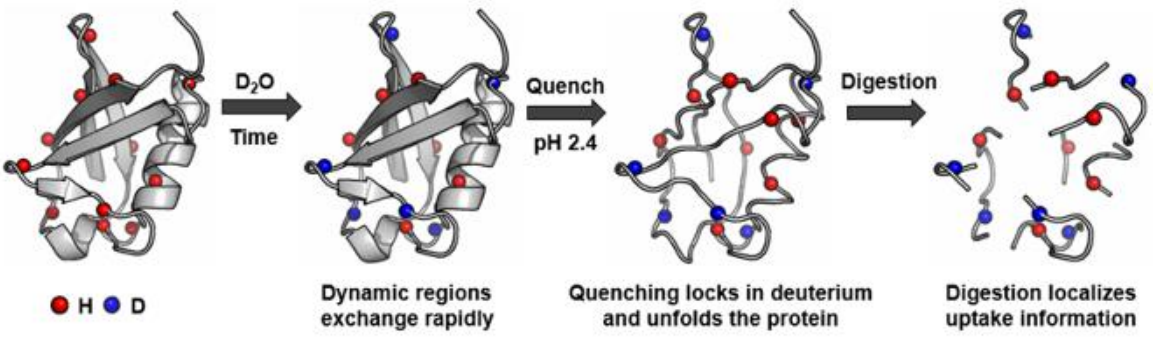

Figure 1.4. General outline of a bottom-up HDX approach. Dynamic and solvent exposed regions of a protein exchange rapidly relative to other regions. Quenching prevents further HDX reactions, and limits back exchange of amide hydrogens. Digestion of the protein into peptides allows for localization of deuterium uptake to identifiable regions. Figure adapted from Lento *et al.* (2017)[15].

1.1.4.1 Conventional HDX

The common workflow of conventional HDX-MS is as follows; the protein sample is labelled with deuterium oxide at pH 7 and quenched with chilled (0˚C) acid to pH 2.5 after a determined period as a single time point. Multiple replicates of this reaction are conducted with different quenching delay times to obtain several different timepoints to obtain deuterium uptake levels as a function of time. If the workflow is a top-down approach, the intact quenched protein would be analyzed by ESI-MS to determine uptake over time. Often HDX workflows operate in the "bottom-up" manner, whereby information gained at the peptide level is used to inform about or reconstruct the structure and dynamics of the native protein[14]. In the bottom-up approach, the quenched protein is digested by an enzyme, such as trypsin, producing peptide fragments before being analysed via ESI-MS. Liquid chromatography (LC) is often coupled to ESI-MS for its ability to desalt and separate the peptides which can greatly enhance resolution and signal quality[16].

1.1.4.2 Time Resolved HDX

Despite the power of conventional HDX as a tool for observing protein dynamics, it is limited in the timescales it can analyze. Conventional HDX can study protein dynamics on the minute to second time scale, with the lower bounds being around 10 seconds[17]. This is because the reaction is not able to be

quenched before this point. A new method of MS-HDX was first developed in 1996 whereby a shorter reaction time was achieved using a quench-flow system, where deuterium uptake time could be varied by altering flow rates[18]. This was expanded upon by the Konermann lab[19] and further developed by the Wilson lab into a technique called Time-Resolved Electrospray Ionization Mass Spectrometry (TRESI-MS)[20]. Time resolved refers to the ability to observe dynamics at the millisecond timescale in order to characterize pre-steady state dynamics[21]. The current form of TRESI-MS utilizes an online labelling and digestion system on a microfluidic device with an adjustable reaction time[21]. The basic structure of the TRESI system is illustrated in **Figure 1.5.**

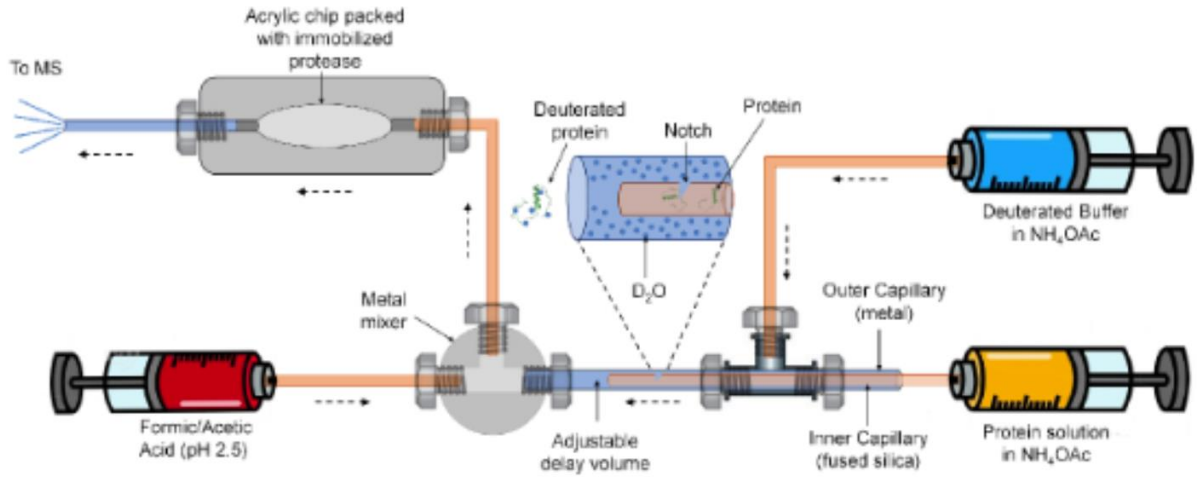

Figure 1.5. Experimental setup for TRESI-HDX-MS. Schematic of capillary-based rapid mixer with adjustable reaction chamber volume. The reaction chamber consists of a fused-silica capillary inserted into an outer metal capillary through a three-way union. Protein solution flows through the fused silica capillary, while deuterium oxide flows into the outer metal capillary through the three-way junction. The protein solution exits the fused silica capillary through a notch and exchanges with the deuterated buffer for the length of the reaction chamber volume. The reaction is quenched in a metal mixer through addition of acid solution flowing through a fused silica capillary. Quenched protein then exits the mixer into an acrylic chip with immobilized pepsin. Digested protein exits the acrylic chip into the metal capillary and is ionized by ESI before MS analysis. Figure provided by Dr. Moorthy Shenbaga.

The capillary-based kinetic mixer, which is comprised of an inner glass capillary within an outer metal capillary, is the key component of the TRESI microfluidic setup that allows for adjustable millisecond timescale labeling times. The end of the inner glass capillary is sealed, with a notch cut 2mm

from the end. Protein flows through the inner glass capillary and exits thorough this notch. Deuterium oxide also flows through a glass capillary into a tri-union and into the outer metal capillary. This allows protein that exits the notch to contact and exchange with the deuterium oxide in the intracapillary space in the volume after the notch. This volume can be adjusted through manual pull back of the inner capillary effectively increasing the labelling time through increasing the delay volume.

Following labelling, the exchange reaction is quenched in the mixing chamber to pH 2.5 through introduction of acid. Unlike conventional HDX, this is done at room temperature not 0˚C, but the short interval between quenching and entry into the MS removes the risk of significant back exchange before analysis. Quenched protein then flows into a PMMA proteolytic chip containing pepsin linked agarose beads and digested into peptides. Pepsin is immobilized to beads in order to remain in the proteolytic chamber. The peptides then flow through a metal capillary and are ionized via ESI and analyzed via IMS-MS.

While TRESI-MS provides a greater ability to observe dynamics at millisecond to second time points, the lack of liquid chromatography reduces the separation of peptides, as well as limiting buffer choices due to the inability to desalt the sample before ESI[21]. The online chip proteolysis also limits digestion efficiency compared to offline digestion due to the limited digestion interval.

1.2 Cytochrome C

Cytochrome C is a nuclear encoded mitochondrial protein that both functions as a key component in ATP energy generation as well as an apoptotic signalling protein if released into the cytosol[22]**. Figure 1.6** shows the structure of cytochrome C. This highly conserved protein is 104 amino acids long in mammals and was one of the first mammalian proteins to be subjected to X-ray crystallography[23]. The structure consists of 5 alpha helices and contains a characteristic CXXCH amino acid motif at the N-terminus responsible for binding heme[24]. This protein is soluble and at physiological pH has a charge of +8[22].

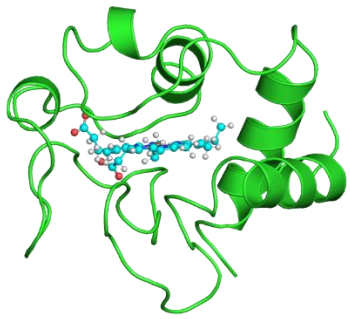

Figure 1.6. Structure of reduced horse heart cytochrome C. Figure adapted from Banci *et al* (1999)[25].

Cytochrome C has been previously studied using TRESI-HDX by the Wilson lab[21] in order to demonstrate the ability for TRESI-HDX to observe millisecond scale biochemical processes. This protein is well suited to MS analysis used due to its solubility, small size, and high digestion efficiency by pepsin. The solubility of cytochrome C is particularly useful for the TRESI-HDX methodology, as the absence of LC before MS analysis severely limits what solvents can be used. As cytochrome C can be diluted in MS-pure water, LC is not required to desalt the injected sample. The small size of cytochrome C also simplifies the number of peptides, and in combination with its previously established digestion profile, makes this protein a good choice as a standard protein to test aspects of a MS methodology.

1.3 Tau Protein

Tau protein is an intrinsically disordered protein (IDP), meaning that the protein lacks a well defined secondary structure, behaving primarily as a random coil[26]. **Figure 1.7** displays the structure of tau. Tau is a microtubule-associated protein, and it plays a role in axonal stabilization, neuronal development, and neuronal polarity. The biological activity of tau is dependant on its degree of phosphorylation, with the hyperphosphorylation of tau in the brain linked to neurodegenerative diseases such as Alzheimer disease (AD)[27].

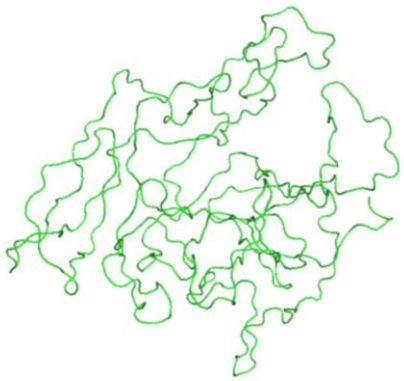

Figure 1.7. Structure of Tau protein. The pdb coordinate file for the 'native' tau ensemble predicted from NMR was provided by Dr. Markus Zweckstetter.

The highly unstructured nature of tau results in more uniform deuterium uptake across the entire protein since uptake in regions of protein secondary structure, such as helices and sheets, are reduced. The use of tau was intended to serve as a comparison to the more structured cytochrome C, for which the maximal uptake of deuterium is reduced by its secondary structure. In this way, more gradual and uniform increase of deuterium uptake across the peptides in tau was expected, allowing for greater ability to examine alterations in uptake due to modifications in the microfluidic setup.

1.4 Research Objective

In order to further improve the TRESI-MS technique as a powerful method to observe millisecond timescale protein dynamics through HDX, aspects of the microfluidic setup were altered in an attempt to further optimize performance. While the TRESI setup works well to provide adjustable timepoints and rapid quenching, there are still components that could be altered that would improve this technique. The current TRESI setup tends to exhibit a rapid uptake in deuterium after the first timepoint, followed by limited uptake after each following time point. Through altering the flow rates and mixing efficiency of the microfluidic setup, a more stable and gradual deuterium uptake could potentially be achieved. Another improvement of the setup that could be achieved would be an increase in consistency of deuterium uptake, requiring less replicates to be performed to acquire results that are significantly

similar. Finally, the changes in the setup will be evaluated on how they increase the complexity and relative ease of use, with the goal to keep the setup user friendly.

Chapter 2: Experimental Methods

2.1 Chemicals and Supplies

Deuterium oxide (D2O, 99.9%) and high purity acetic acid (>99.7%) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade ammonium acetate and water were purchased from Fisher Chemical (Fair Lawn, NJ). Pepsin (from porcine gastric mucosa) was purchased from Sigma-Aldrich (MO, USA), NHS-Activated beads were purchased from Thermo Fisher Scientific (MA, USA). Cytochrome C from equine heart was purchased from Sigma-Aldrich (MO, USA). Purified tau was obtained from Banafsheh Mehrazma from the Wilson lab.

2.2 Original Microfluidic Setup

Microfluidic chip and other components were constructed as detailed in Brown et al.[28] The microfluidic chip was comprised of a poly methyl methacrylate (PMMA) substrate (dimensions: 0.83” x 2” x 0.25”), with a proteolytic chamber and capillary channels engraved into it. A second PMMA substrate of the same dimensions was welded together with the first substate to seal the chamber. A glass capillary with an outer diameter of 151 µm and a 33-gauge stainless steel metal capillary screwed into the chip via double-winged nuts and were used as the input and output channel respectively.

The TRESI mixer was made by inserting a glass capillary (Outer Diameter = 151 µm) inside a metal capillary (inner diameter = 177.8 µM) creating an inter-capillary space of 26 µm. The end of the glass capillary was sealed, and a notch was cut 2 mm from the sealed end with the use of a laser. A metal three-way mixing device was used to connect the TRESI mixer, the acid line, and the capillary leading to the microfluidic chip. The device was placed at the front end of a modified NanoSpray platform on a quadrupole-ion mobility-time-of-fight mass spectrometer instrument (Synapt G2-S HDMS, Waters,

Milford, MA). The output channel of the microfluidic device also served as the electrospray ionization probe.

The HDX reaction occurred within the TRESI mixer in which gas tight Hamilton syringes were used to transport the reagents through the capillaries using syringe infusion pumps (Harvard Apparatus). Cytochrome C at a concentration of 5µM was flowed at 2µL/min through the inner capillary, while deuterium flowed through the outer capillary at 2 µL/min. 10% Acetic acid was introduced into the three-way junction mixing chamber at a rate of 16µL/min. The HDX reaction times were achieved by increasing the space between the end of the inner capillary and the outer capillary by 1, 2, 5, and 10 mm.

Online digestion was achieved using pepsin linked beads contained within the proteolytic chamber of the PMMA chip. Pepsin with NHS-activated agarose beads (2:5 w/w) were suspended in coupling buffer (0.1 M sodium phosphate and 0.15 M NaCl, pH 4.5) and rotated overnight at 4°C. The aspirated beads were then suspended in blocking buffer (1 M Tris–HCl, pH 4.5) and rotated at 4°C for 1 h. The beads were then stored in acetic acid (pH 2.4) at 4°C until use.

2.3 MS Parameters and Data Analysis

The Waters G2-S HDMS TOF-MS instrument was operated with a capillary voltage of 2.5 kV in positive ion mode with a sampling cone voltage of 20 V. The samples were scanned over a range of 400 to 1200 m/z., with data acquired in ion mobility acquisition mode. Data analysis for deuterium uptake was carried out using MS studio software. Mass Lynx was used for peptide identification.

2.4 Alterations to Microfluidic Setup Utilizing Cytochrome C

Alterations to microfluidic setup are presented in **Figure 2.1**. There were two main sites where alterations were made in the TRESI microfluidic setup. The first set of alterations were centered around altering the mixing tee, where the deuterated protein encounters the acid and quenches the reaction. The alterations to this region were intended to improve mixing at this junction, and therefore increase the efficiency of quenching. This would allow for the HDX reaction to be stopped as soon as it reached the mixing tee and protein would not continue to exchange while flowing into the proteolytic chip and to the

output channel. The alterations to the mixing tee included: 1) utilizing a four-way mixing tee instead of a three-way with an additional acid line, 2) using a different mixing tee with an integrated frit, and 3) using the original mixing tee, but inserting a loose frit where the quenched reaction would exit.

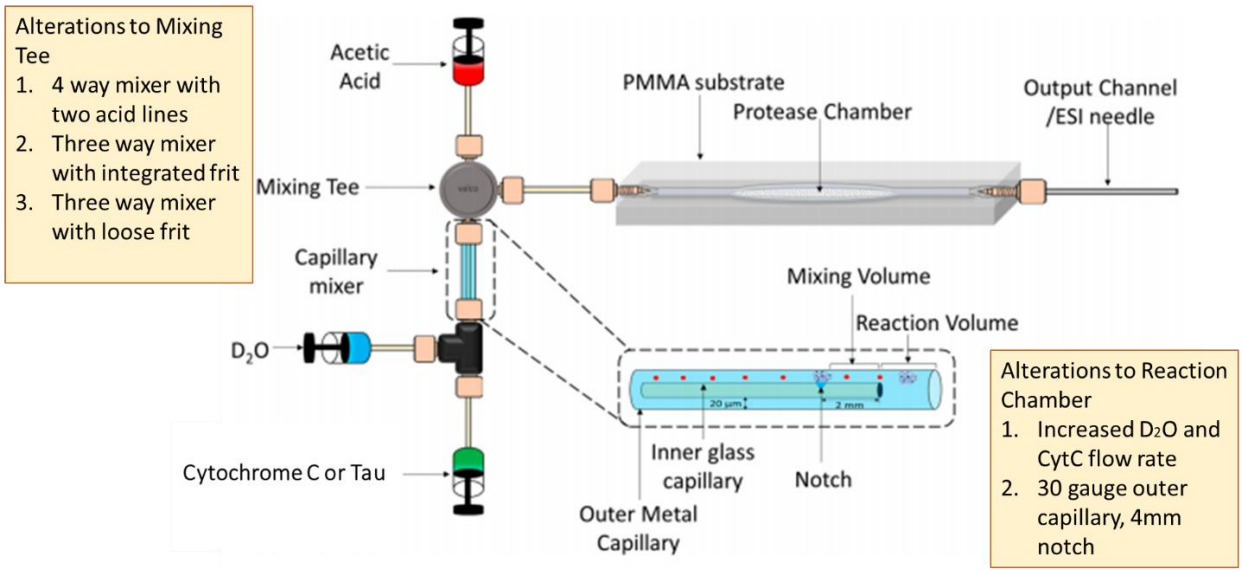

Figure 2.1. Summary of microfluidic setup alterations. Alterations to the microfluidic setup were centered around either the mixing tee or the reaction chamber. Figure adapted from Brown *et al* (2020)[28].

The other location of alteration was the reaction chamber, where the protein encounters deuterium, and where the HDX reaction takes place. The percent uptake of deuterium by the protein when using the microfluidic setup can be inconsistent, so alterations were made to the reaction chamber to observe if the repeatability of the length of reaction time could be improved. This was done by: 1) increasing the flow rate of deuterium and protein, and 2) by decreasing the inner diameter of the outer metal capillary of the reaction chamber while simultaneously increasing the distance of the notch from the sealed end.

2.4.1 Four Junction Mixing Chamber Utilizing Two Acid Lines

The metal three-way mixing junction of the original setup was replaced with a PEEK four-way junction. The two inlets across from each other were acid lines with 10% acetic acid flowing at a reduced

rate of 8µL/min. The third inlet perpendicular to the two acid lines was the TRESI line, with the fourth outlet across from the TRESI line connected to the proteolytic chip and ESI output capillary. All other aspects remained unchanged. Two replicates were conducted, with only the second replicate presented in the results since the first was an obvious outlier.

2.4.2 Three Junction Mixing Chamber Utilizing Integrated Frit

The metal three-way mixing junction of the original setup was replaced with a PEEK three-way junction with an integrated 10um pore size frit at the outlet connecting to the proteolytic chip. Other aspects were unchanged. Two replicates were performed, with both used in the final results.

2.4.3 Three Junction Mixing Chamber Utilizing Loose Frit

A 2µm pore size frit was inserted into metal three-way mixing junction, facing the proteolytic chip. One replicate was performed, the 10mm timepoint was not viable (over 100% uptake) and was omitted.

2.4.4 Increased Flow Rate and Acid Concentration

The acetic acid concentration was increased to 20%, and the flow rates of cytochrome C and deuterium were raised to 4ul/min. The flow rate of acetic acid remained unchanged at 16µL/min. A proteolytic chip with a larger chamber was used. One replicate was performed, the 10mm timepoint was not viable due to loss of the MS signal and was omitted.

2.4.5 Smaller Gauge Outer Capillary with Altered Notch Position

The inner diameter of the metal capillary encasing the TRESI line was reduced by using 30-gauge tubing. The notch in the TRESI line was cut 4mm from the sealed end rather than 2mm. Other aspects remained unchanged; one replicate was performed.

2.5 Alterations to the Microfluidic Setup Utilizing Tau

A similar microfluidic setup implemented in **Section 2.2** was utilized to study tau. Purified tau was obtained from Banafsheh Mehrazma from the Wilson lab. Tau was buffer exchanged into 100mM ammonium acetate, and further diluted to 3uM. Tau protein was introduced at a rate of at 2µL/min

through the inner capillary, with the flow rates of deuterium and acid maintained the same as the original setup. Peptide digestion was performed with either 100% pepsin beads or 100% Protease XIII beads, depending on the experiment.

A 2µm frit was inserted into the output of the proteolytic chip to prevent aggregated tau from entering and clogging the ESI capillary. All setups utilizing tau contained a frit, expect for the method in **Section 2.5.5**, where the modification tested was the exclusion of the frit. The modifications employed using tau served one of three purposes:

1. The three-junction mixing chamber with loose frit implemented a method previously used with cytochrome C to observe if similar trends exited.
2. The use of FEP tubing was an attempt to solve pressure issues by using a larger gauge connection from the mixing tee to the proteolytic chip.
3. Finally, the frit in the proteolytic chip was removed to see how its absence would impact data and the running of the setup.

2.5.1 Original Setup Utilizing Pepsin

The setup was identical to the original setup for cytochrome C, except for the use of tau, and the inclusion of a frit in the proteolytic chip. Pepsin beads were used for proteolytic digestion. One replicate was performed.

2.5.2 Three Junction Mixing Chamber Utilizing Loose Frit with Pepsin

A loose frit was inserted into the metal three-way in the same manner as section **2.4.3.** One replicate was performed, with the 5mm and 10mm timepoints unable to be collected due to loss of signal.

2.5.3 Original Setup Utilizing Protease XIII

This setup was performed using Protease XIII beads instead of pepsin beads. All other aspects remained unchanged. One replicate was performed.

2.5.4 FEP Tubing Utilizing Protease XIII

A 1/16 x 0.008 FEP tube was used in place of a fused silica capillary at the junction between the three-way mixing chamber and the proteolytic chip. Other aspects remained unchanged; one replicate was performed.

2.5.5 No Proteolytic Frit Utilizing Protease XIII

The in-chip 2µm frit was removed, with all other aspects unchanged. Only 8 peptides instead of 9 were analysed, as the 9th peptide was too low abundance to accurately determine uptake for. One replicate was performed.

Chapter 3: Results and Discussion

3.1 Modifications using Cytochrome C

3.1.1 HDX Profile of Original Setup

Four replicates of the original setup were performed, and while each replicate individually produced gradual percent deuterium uptake with increasing timepoints, the differences in the actual uptake percentages between replicates produced high error values. As presented in **Figure 3.1**, there are some subtle differential uptakes between different peptides, with the peptide that consistently exchanged the most deuterium being (101-105), KATNE, which resides at the C-terminal end. The 0mm (0.2s) timepoint consistently resulted in much lower uptake, with the following timepoint showing a jump in uptake. The timepoints following 1mm tended to increase in uptake with the longer reaction time, although in nearly all the peptides there was a drop in uptake from 2mm to 5mm. This was likely the result of one replicate experiencing an unusually high uptake for 2mm, skewing the average higher than what was expected.

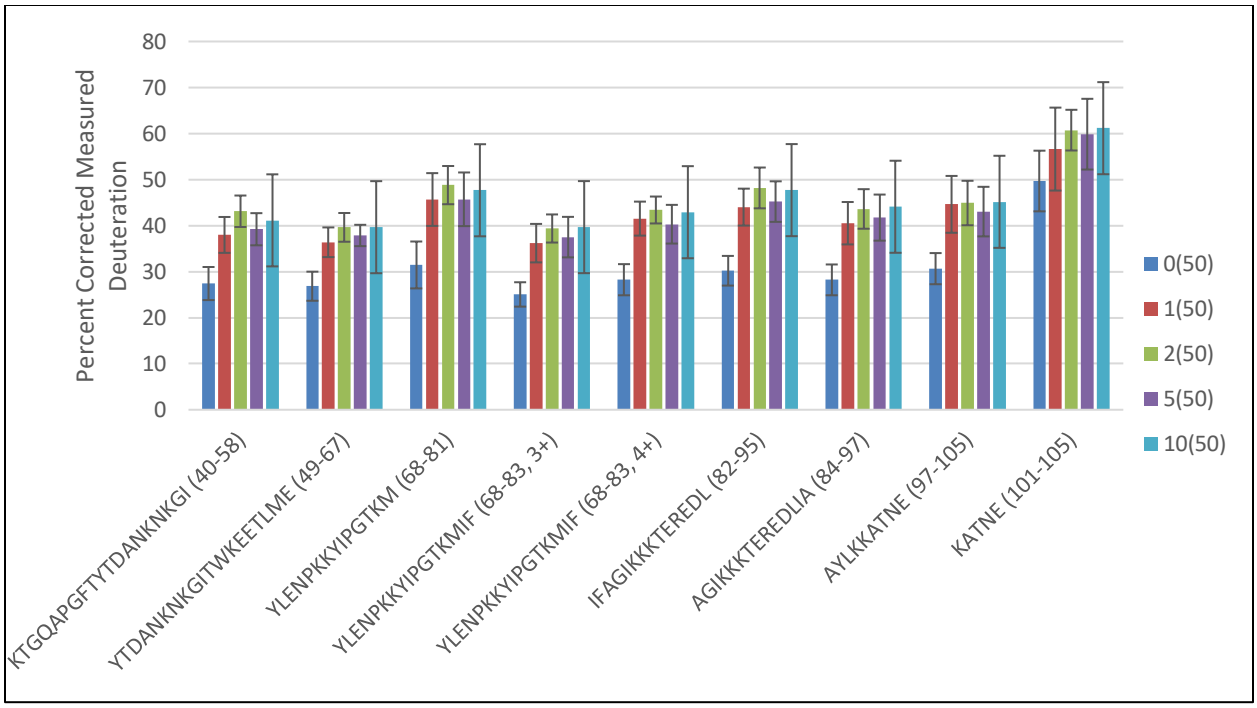

Figure 3.1. HDX analysis of cytochrome C utilizing the original setup. Representative kinetic plots of % corrected deuterium uptake vs. time for 9 peptides from cytochrome C. Error bars represent standard deviation values of four replicates.

As visible in the peptide map in **Figure 3.2,** the protein experienced a jump in uptake after the first timepoint, with little change in uptake following this.

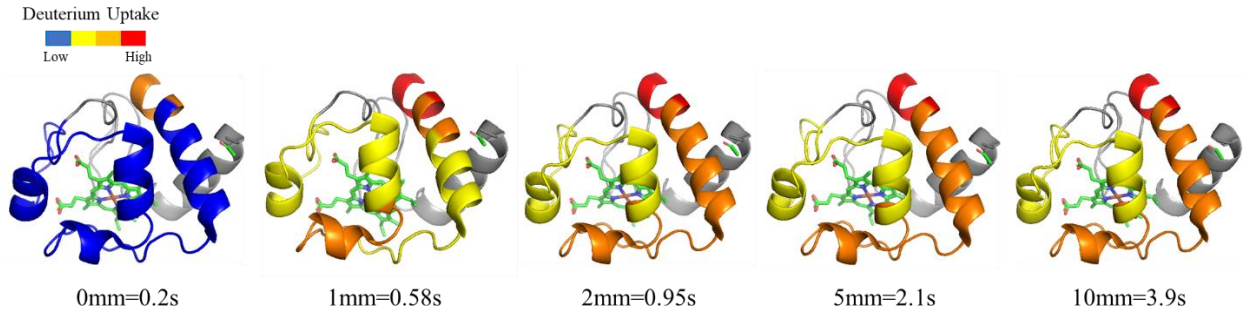

Figure 3.2 Deuterium uptake profiles of cytochrome C peptides mapped onto protein structure utilizing the original setup. The measured profiles are coloured according to total deuterium uptake: red (58-66%), orange (47-57%), yellow (36-46%), and blue (25-35%) Regions for which no peptides were observed are coloured in grey.

As the proper functioning and ease of use of the setup was also an important outcome that was evaluated, any technical difficulties experienced with each setup were noted. The original setup generally

resulted in lower instances of technical issues in comparison to the other setups. There were still some mechanical issues that complicated the running of experiments, as there was occasional clogging of the microfluidic lines, as well as leaks due to high pressure issues.

While the original setup often produced gradual uptake, it suffers from a lack of consistency, with replicates performed on different days producing different uptakes. Even within a single replicate, one timepoint would often produce unexpectedly high or low uptakes that did not fit with the uptake of the other timepoints. One common problem when running the microfluidic setup were high pressure issues, where a clog or blockage of some type prevented the constant low flow of deuterium and protein into the chip and out through the ESI capillary. These pressure issues could be the reason for the plateauing of uptake, as protein may not have been flowing at the intended rate, leading to increased uptake times even at low timepoints. The increased time spent in the microfluidic setup could allow for the hydrogen deuterium exchange to continue for much longer, reducing the effect of increasing the reaction time by pulling back the fused silica capillary. The other possible reason for unexpected increase or decrease in uptake could be due to leaking. Leaking results in less protein or deuterium flowing through the setup, altering the ratio of protein to D_2O, which changes the dynamics and deuterium uptake of the HDX reaction. The sudden decrease in uptake during the 5mm timepoint in one of the replicates could be a result of deuterium leaking somewhere in the setup, reducing the amount of D_2O available to exchange with the protein.

There was relatively “flat” uptake, where increasing the reaction volume seems to have little effect on uptake. This may be due to the nature of cytochrome C, as it is highly structured, and may reach a plateau of uptake early in the timepoints, with no easily accessible sites for deuteriation after the first few timepoints. This was one of the reasons that tau was used in the second half of the experiments, as its larger size and unstructured nature could allow for a less rapid plateau of deuterium uptake.

3.1.2 HDX Profile of Four Junction Mixing Chamber Utilizing Two Acid Lines

Utilizing two acid lines with a four-way mixing junction resulted in little change in deuterium uptake with increasing timepoints. Data collected for this setup is presented on **Figure 3.3**. At 0mm, the

average uptake across all the peptides was 49.6%, while at 10mm, the average uptake across all peptides was 51.8%, showing only a few percent change by the final timepoint. At 5mm, all peptides experienced reduced uptake from the 2mm time point, the most significant reduction occurring in the last residues of the C-terminal end, 97-105.

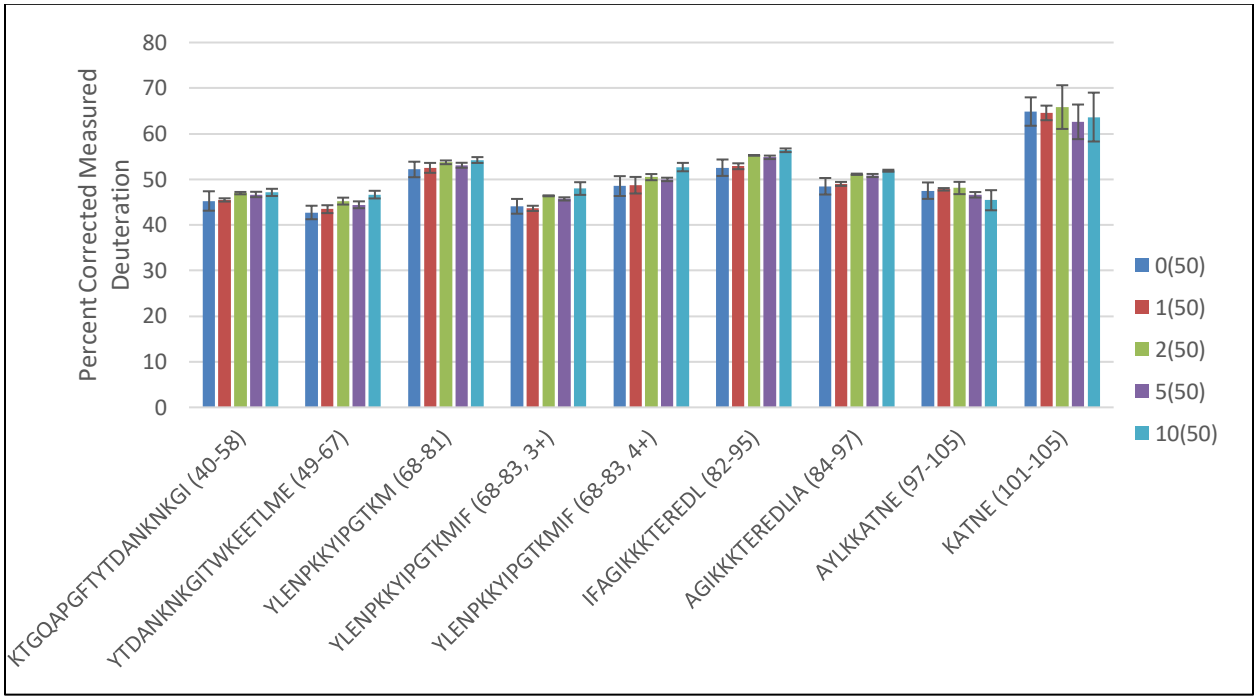

Figure 3.3. HDX analysis of cytochrome C utilizing four junction mixing chamber with two acid lines. Representative kinetic plots of % corrected deuterium uptake vs. time for 9 peptides from cytochrome C. Error bars represent standard deviation values of one replicate.

As can be seen in the peptide map in **Figure 3.4**, the majority of the peptides were maintained at a specific uptake level through all the timepoints, with only two peptides that increased in uptake, while a third experienced a significant reduction in uptake.

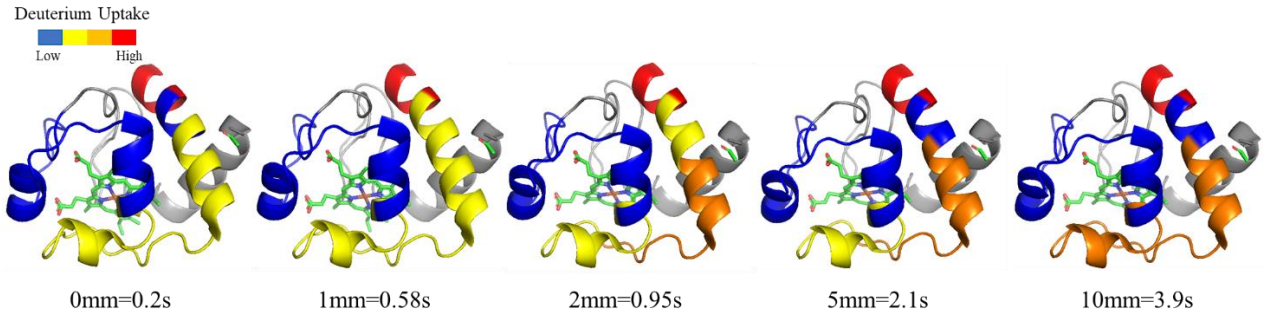

Figure 3.4. Deuterium uptake profiles of cytochrome C peptides mapped onto protein structure utilizing four junction mixing chamber with two acid lines. The measured profiles are coloured according to total deuterium uptake: red (59-65%), orange (53-59%), yellow (47-53%), and blue (42-47%) Regions for which no peptides were observed are coloured in grey.

In terms of difficulties using the setup, there were no major errors, although the altered orientation of the setup due to the TRESI capillary being in-line with the chip presented some issues due to the limited size of the platform that the setup rests upon. The setup could fit onto the platform, but for further use the size of the platform may need to be increased to reduce the chance of the setup falling off, as attempting to manipulate the system threatens to knock the whole setup off its platform.

The purpose of using two acid lines was to increase the mixing of acid with the deuterated protein, and therefore quench uptake more efficiently. This was not seen in the result, as even at the shortest timepoints, uptake was high. The distance the capillary was pulled back seemed to have little effect on how much deuterium exchanged with the protein. Instead of increasing the efficiency of quenching, there seemed to be longer than expected reaction times.

While the flow rate of the acid for each individual acid line were halved, the use of two acid lines was intended to compensate for this lower flow rate. This may not have had the intended effect of improving mixing, as while there was acid flowing at the protein from two directions, the lower acid flow rate may not have been able to sufficiently disrupt the laminar flow of protein and deuterium to efficiently quench the reaction. With the use of a three-way mixer, the protein meets the acid flow head-on, which may more efficiently disrupt laminar flow versus the protein encountering the acid perpendicularly in a four-way junction.

In summary, the flat uptake profile of this method may have been a result of inefficient quenching, with the use of a three-way mixer better suited to efficiently quench the HDX reaction.

3.1.3 HDX Profile of Three Junction Mixing Chamber Utilizing Integrated Frit

Utilizing the three-way junction with the integrated frit produced the most inconsistent results of all the methods tested. Data collected for this setup is presented on **Figures 3.5**. No gradual uptake was seen, as the deuterium uptake drastically dropped from the 2mm timepoint to the 5mm and 10mm timepoints. The 0mm timepoints of some peptides even produced higher uptake than the 10mm timepoints. Two replicates were performed, each producing varied and inconsistent uptake, which could be seen in the large error bars across the majority of the peptides.

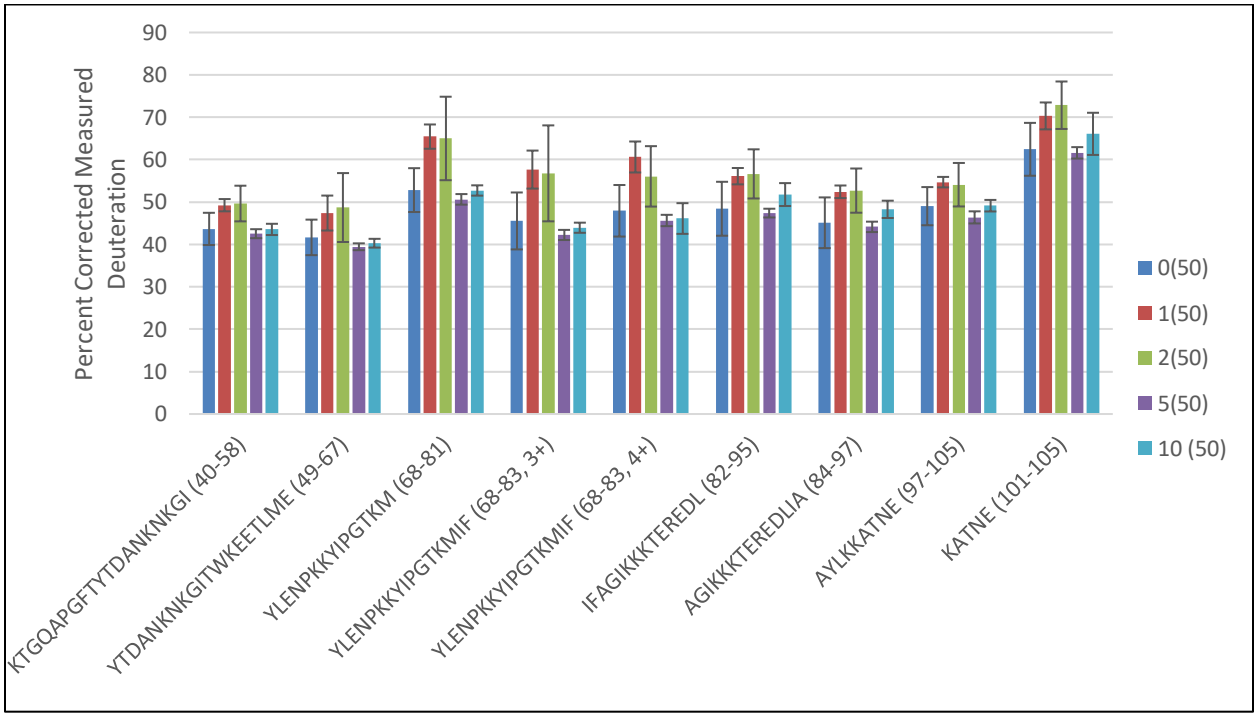

Figure 3.5. HDX analysis of cytochrome C utilizing three junction mixing chamber with integrated frit. Representative kinetic plots of % corrected deuterium uptake vs. time for 9 peptides from cytochrome C. Error bars represent standard deviation values of two replicates.

The peptide map for this experiment, presented in **Figure 3.6**, demonstrates the strange uptake pattern of this method, with peptides increasing and decreasing in uptake at random timepoints. The

uptake seemed to increase until the 2mm timepoint, before drastically dropping at the 5mm timepoint, followed by a slight increase in the 10mm timepoint.

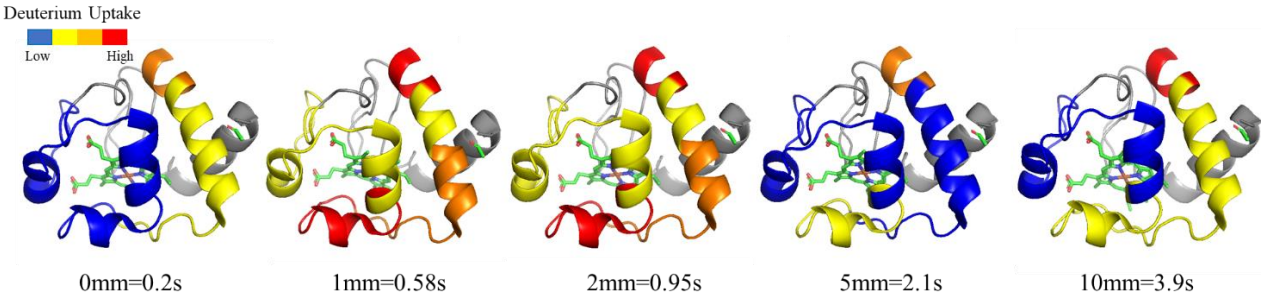

Figure 3.6. Deuterium uptake profiles of cytochrome C peptides mapped onto protein structure utilizing three junction mixing chamber with integrated frit. The measured profiles are coloured according to total deuterium uptake: red (63-72%), orange (55-63%), yellow (47-55%), and blue (39-47%) Regions for which no peptides were observed are coloured in grey.

Not only did this method produce inconsistent results, the mechanical issues and troubleshooting required were the greatest of all the setups. There was constant leaking and pressure issues using the integrated frit junction, with the pressure issues being so great it caused leaking back in three glass syringes (which had to be replaced). Using this modification would take twice as long as any other method to run, as the setup had to be constantly repaired due to these issues. A second replicate was performed implementing changes in the wingnuts that connected to the three-way junction an attempt to improve upon the first replicate, and while it resolved some leaking issues, there was still more troubleshooting and repair required than any other setup.

The data collected from the two replicates of this method do not at all represent what should occur in a HDX reaction. Uptake is unrelated to the reaction time of each timepoint, with intermediate timepoints such as 1mm and 2mm producing higher uptake than the final timepoints, and the 0mm timepoint resulting in higher uptake than the longest timepoints for some peptides. There was certainly something occurring in this setup that was preventing the proper functioning of the HDX reaction. Candidates for the source of error were pressure issues and leaking. Pressure issues could lead to inefficient quenching of the deuteriation reaction, leading to longer than intended reaction times

producing unexpectedly high uptake. Leaking can also result in inconsistent flow rates, which could affect the final concentrations of protein, acid, and deuterium, impacting the HDX reaction. Leaking of deuterium out of the system can cause random drops of uptake, which could have been what occurred in the 5m and 10mm timepoints.

In summary, the data collected from this experiment is not consistent with what would be expected of a HDX experiment, demonstrating that something went significantly wrong when using this setup. If this setup were to be used again, major testing needs to be done to attempt to determine and solve the source of the pressure issues, as the current operation of this setup is highly prone to mechanical failure.

3.1.4 HDX Profile of Three Junction Mixing Chamber Utilizing Loose Frit

Utilizing the three-way mixing tee with a loose frit inserted in front of the output of the mixer resulted in only very gradual of uptake over time. Data collected for this setup is presented in **Figure 3.7**. Aside from the 0mm time point of peptide 101-105, uptake values between timepoints were typically only a few percent different from each other. Excluding this peptide, the average percent uptake across all other peptides at 0mm was 47%, while the average uptake across all peptides at 5mm was 49.6%.

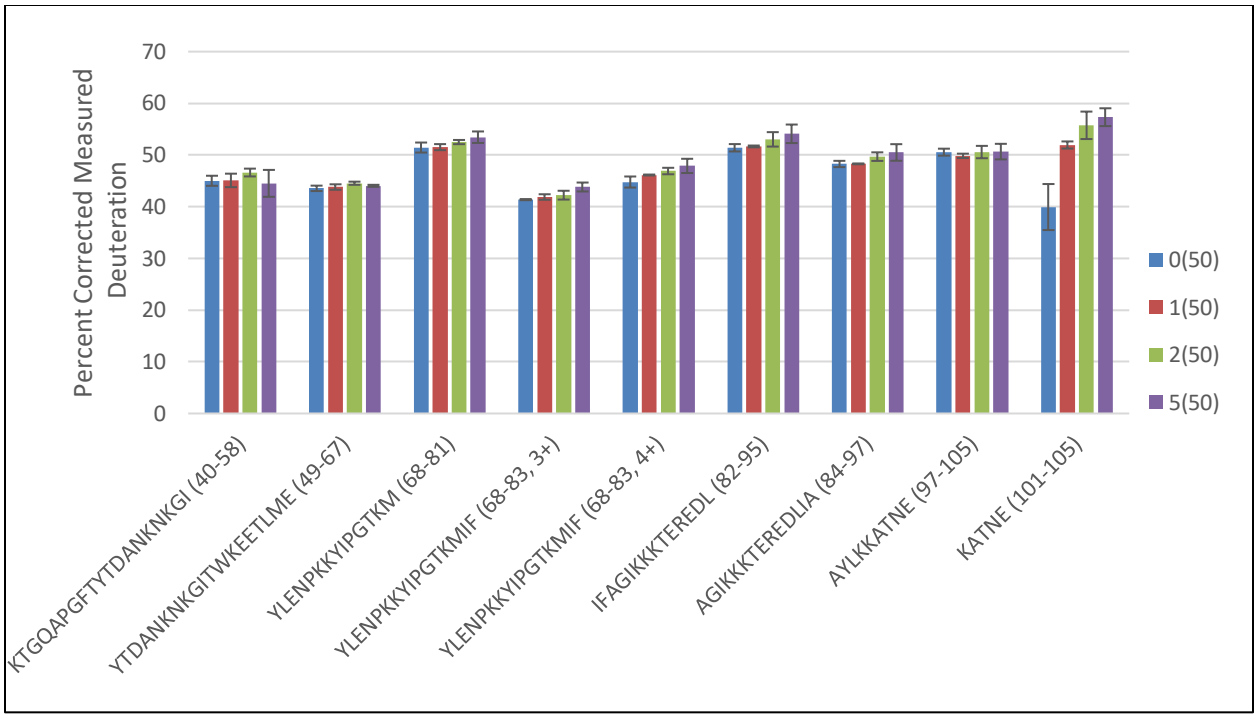

Figure 3.7. HDX analysis of cytochrome C utilizing three junction mixing chamber with loose frit. Representative kinetic plots of % corrected deuterium uptake vs. time for 9 peptides from cytochrome C. Error bars represent standard deviation values of one replicate.

The lack of significant change in uptake can be seen in the peptide map in **Figure 3.8**, as only two peptides showed any increase in uptake.

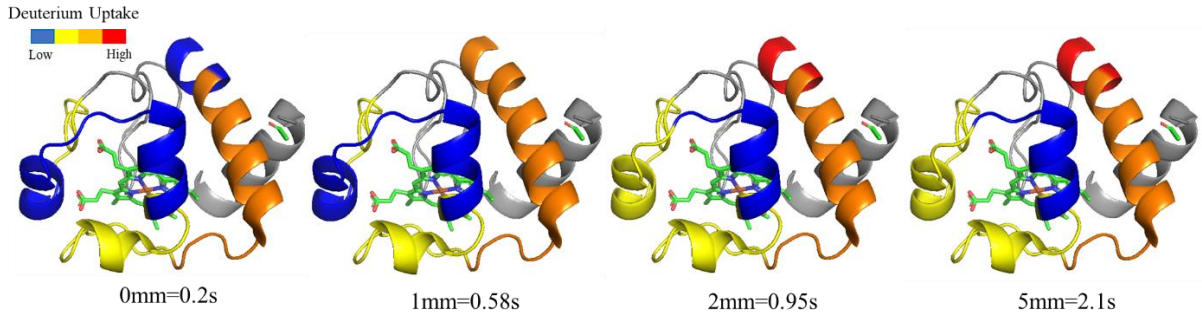

Figure 3.8. Deuterium uptake profiles of cytochrome C peptides mapped onto protein structure utilizing three junction mixing chamber with loose frit. The measured profiles are coloured according to total deuterium uptake: red (52-57%), orange (48-52%), yellow (44-48%), and blue (40-44%) Regions for which no peptides were observed are coloured in grey.

This setup experienced some technical issues, but most issues were due to other components of the setup unrelated to the three-way mixer with the loose frit. There was significant troubleshooting

required for the 10mm timepoint, and the results from that timepoint produced over 100% uptake for some peptides. The timepoint was omitted because of this, as it was the result of a mechanical issue that was unable to be resolved.

The purpose of the addition of the frit in front of the three-way was to improve mixing and thereby improve the efficiency of quenching. The introduction of the frit was meant to provide a barrier for the liquids to be perturbed by, disrupting laminar flow to produce turbulent mixing. Turbulent mixing was thought to increase the efficiency of quenching by the acid. However, the implementation of the frit appeared to cause pressure issues and produced another "flat' uptake profile seen in the other methods. The main cause for this trend in uptake was believed to be a result of the reaction time of HDX being much longer than intended, due to the protein and deuterium not flowing at the correct rate because of increased pressure caused by the frit. The implementation of the frit did not seem to improve mixing and quenching of the reaction, as even at the low timepoints the deuterium uptake is high, which could be a sign of inefficient quenching. In summary, this method did not achieve what it was designed to do and did not improve the quality of mixing and therefore the efficiency of quenching.

3.1.5 HDX Profile of Increased Flow Rate and Acid Concentration

Of all the setups tested using cytochrome C, this setup produced the highest uptake percentages, and most similar uptake profiles between peptides. Data collected for this setup is presented in **Figure 3.9.** The extremely high uptake of this method peaked at 98% for the peptide 101-105, with the average uptake of the 5mm timepoint across all the peptides being 84%. There was a sudden drop in uptake in the 1mm timepoint for the 101-105 peptide.

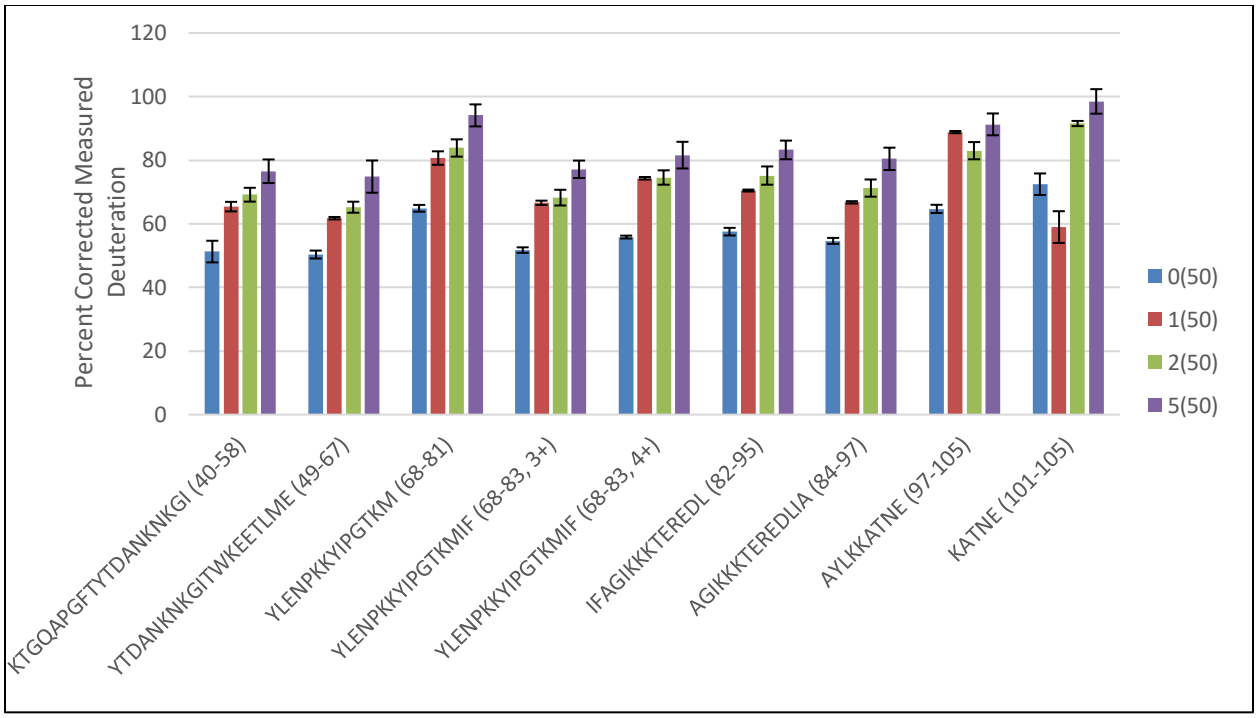

Figure 3.9. HDX analysis of cytochrome C utilizing increased flow rate and acid concentration. Representative kinetic plots of % corrected deuterium uptake vs. time for 9 peptides from cytochrome C. Error bars represent standard deviation values of one replicate.

As it can be seen in the peptide map in **Figure 3.10**, uptake is fairly uniform across the peptides, with all peptides exhibiting high uptake at the final timepoint. The increased flow rate of the protein decreased the reaction time, which is reflected in the lower values of the reaction times in **Figure 3.10**.

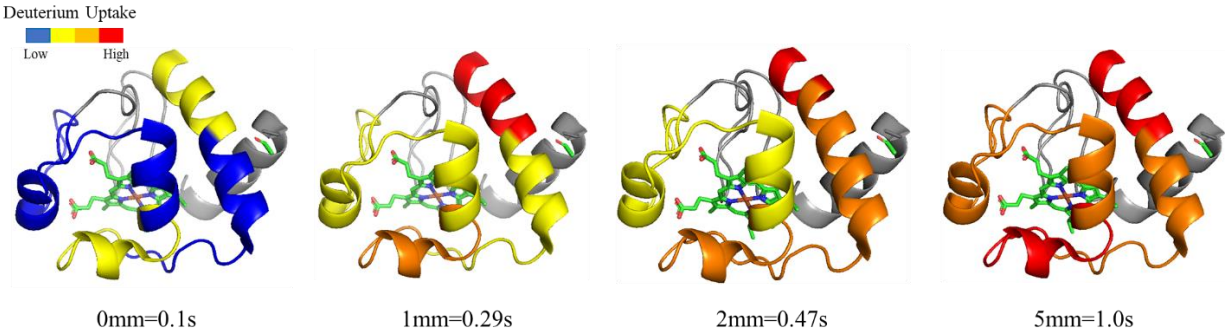

Figure 3.10. Deuterium uptake profiles of cytochrome C peptides mapped onto protein structure utilizing increased flow rate and acid concentration. The measured profiles are coloured according to total deuterium uptake: red (87-98%), orange (74-87%), yellow (62-74%), and blue (50-62%) Regions for which no peptides were observed are coloured in grey.

Only minor technical issues were experienced using this setup, however there was a pressure issue at one point, which caused stalling of the syringe pumps. Due to technical issues the 10mm timepoint was unable to be obtained.

As the reaction times for this method were halved compared to the original setup due to the increased flow rate, it was assumed that the uptake values would be decreased. This is the opposite of what was seen, as this experiment had increased or at least comparable uptake to all other experiments. The increased flow rate of the protein and deuterium was intended to improve the mixing of deuterium and protein within the reaction chamber, with the concentration of the acid doubled to quench the additional volume of the HDX reaction.

This method did not have the common issue of a "flat" uptake profile, meaning that the reaction time was changing in accordance with pull-back of the capillary. However, the overall reaction time of this setup should be reduced, as the higher flow rate should result in the protein and deuterium encountering and being quenched by the acid more rapidly. This discrepancy in magnitude of uptake may be a result of the larger chip size that was used for this experiment. If the protein spent more time in the proteolytic chip due to the increased size, this could result in a longer period where the protein could uptake deuterium. If the reaction was fully quenched, this may not have been an issue, but increasing the concentration of the acid and not the flow rate may have affected the efficiency of quenching. Increasing the concentration of acetic acid may not have been able to lower the pH enough to fully quench the reaction, due to the increased volume of deuterium used.

In summary, due to the increased volume to volume ratio of deuterium to acid, in conjunction with the larger chip, the uptake of this method was much higher, but did not negatively affect the ability to observe gradual uptake over time.

3.1.6 HDX Profile of Smaller Gauge Outer Capillary with Altered Notch Position

While there was a significant drop in uptake at the 10mm timepoint, the rest of the results when utilizing a smaller gauge capillary were comparable to the original setup. Data collected for this setup is presented in **Figure 3.11**. Gradual uptake over time could be observed, along with differential peptide

uptake. By altering the size of the inner diameter and the position of the notch in the reaction chamber, the reaction time was decreased by a significant amount. At 0mm, the reaction time would be 0.006s, and at 10mm, it would be 2.7s, which is just a few fractions of a second longer than the reaction time at 5mm for the original setup. However, the uptake profile of this experiment was similar to the ones of other experiments, despite the reduced reaction times. From an ease-of-use perspective. no significant technical issues were experienced.

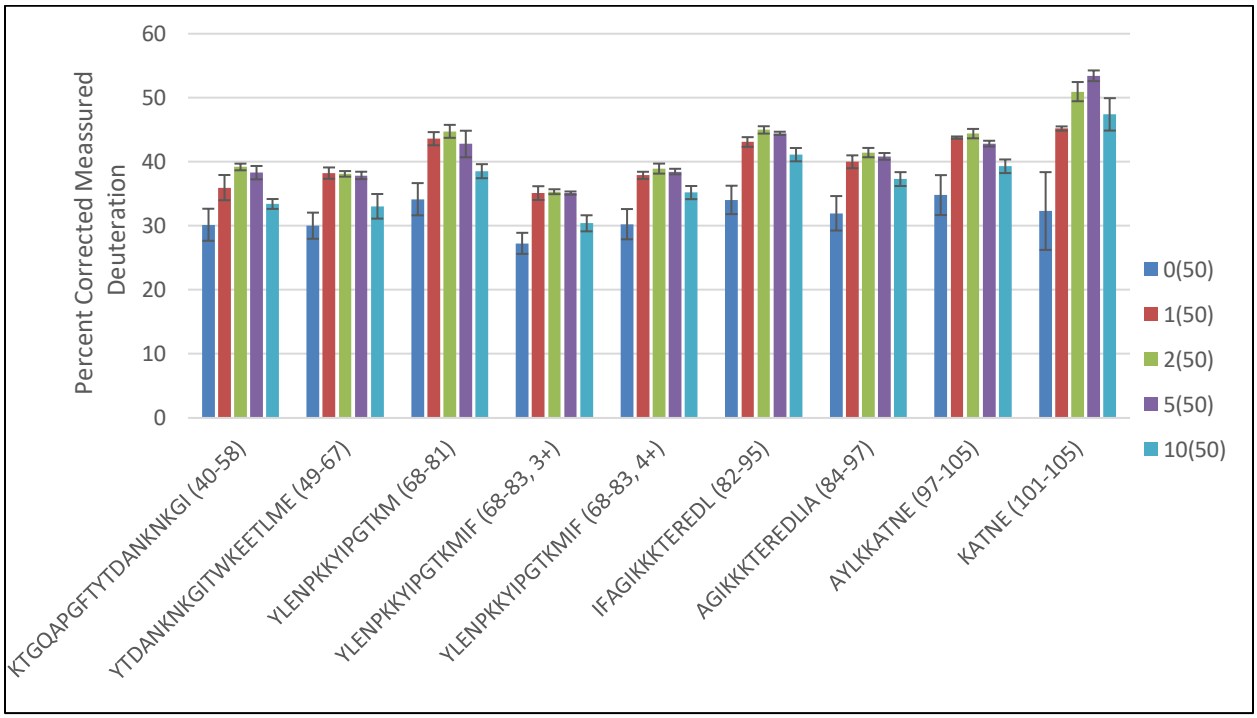

Figure 3.11. HDX analysis of cytochrome C utilizing smaller gauge outer capillary with altered notch position. Representative kinetic plots of % corrected deuterium uptake vs. time for 9 peptides from cytochrome C. Error bars represent standard deviation values of one replicate.

As can be seen in the peptide map in **Figure 3.12**, some peptides had higher uptake values than others, but all gradually increased over time. However, in the 10mm timepoint the uptake for most of the peptides decreased.

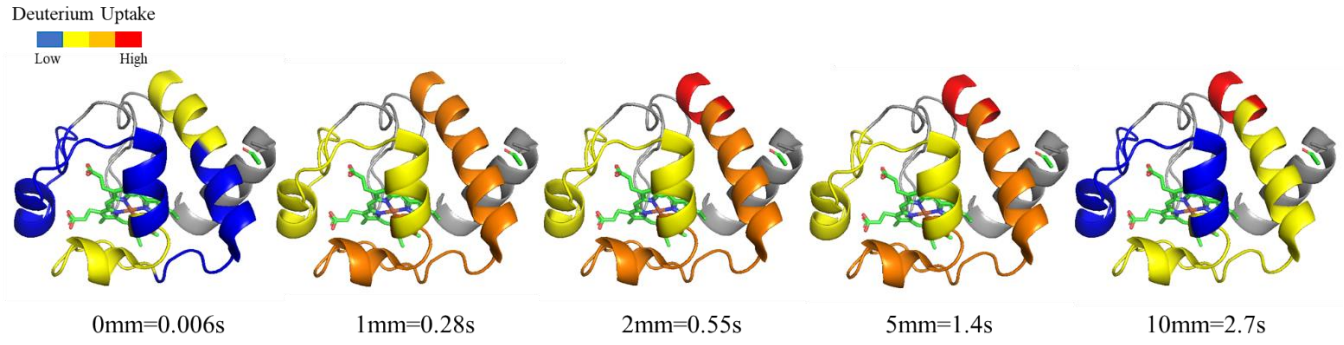

Figure 3.12. Deuterium uptake profiles of cytochrome C peptides mapped onto protein structure utilizing smaller gauge outer capillary with altered notch position. The measured profiles are coloured according to total deuterium uptake: red (46-53%), orange (40-46%), yellow (33-40%), and blue (27-33%) Regions for which no peptides were observed are coloured in grey.

The use of a 30-gauge outer metal capillary reduced the inner diameter from 0.178mm to only 0.1524mm, which is slightly larger than the outer diameter of the internal fused silica capillary (0.152mm). This greatly reduced the reaction time by limiting the volume for the HDX reaction before reaching the quenching mixing chamber. Increasing the distance of the notch increased the reaction time, but the alteration in the gauge of the capillary produces far more significant effects on the reaction time. Therefore, it would be expected that this method should produce lower uptake than the others due to the reduced reaction time. This was not seen, as the uptake was only slightly lower than what was seen in the original setup. This indicates that there was some issue in the reaction chamber, whereby the reaction time was not actually reduced.

There could be several issues that lead to this result, such as pressure issues, or the failure of the notch. As seen in the other setups with suspected pressure issues, there is a “flattened” look to the uptake, meaning that the protein and deuterium may be not flowing through the setup at the correct rate, in addition to the reaction being quenched inefficiently. Another possibility could be a failure in the notch, where there is not enough space for the protein to adequately flow out of the notch, due to the reduced inter-capillary space. This could result in the protein flowing extremely slowly and spending more time than intended exchanging with deuterium. Either of these failures could lead to the higher than expected and flat uptake trends that were seen.

3.1.7 Comparison of Methods Utilizing Cytochrome C

The general trends of each method used to analyze cytochrome C is presented in **Figure 3.13.** The method that produced the greatest uptake was increasing the flow rate of protein and deuterium, while the method producing the lowest overall uptake was using a smaller 30-gauge outer capillary with an altered notch position. Aside from the increased flow rate method, the uptake profiles of the other experiments are relatively flat, with uptake not changing much with the increasing timepoints.

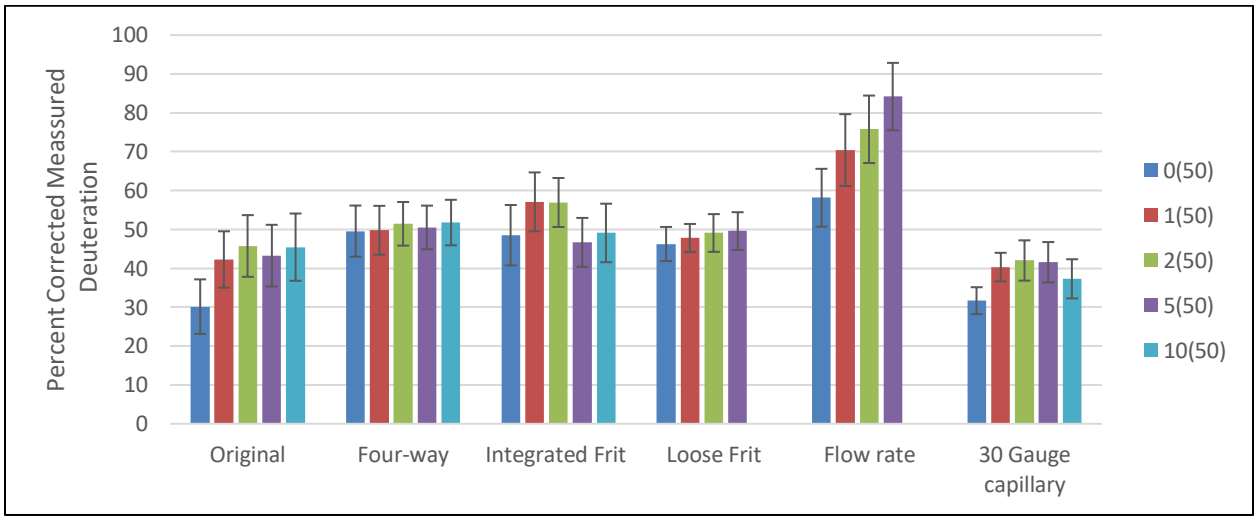

Figure 3.13. Summary of HDX analysis of cytochrome C comparing uptake across all methods. Representative kinetic plots of % corrected deuterium uptake vs. time for the 6 methods tested using cytochrome C. The % corrected deuterium uptake of the peptides in each method were averaged according to timepoint. Error bars represent standard deviation values of the all the peptides in the timepoint for each method.

What was desired from the implementation of the new methods was a clear gradual increase in uptake across the timepoints, as well as consistency between replicates. The flat uptake profiles of the four-way mixer, the integrated frit, and the loose frit do not produce defined uptake over time, and therefore were not improvements on the original setup.

While the flow rate modification had an improved the gradual uptake profile in comparison to the original setup, the greatly increased uptake presented issues as it could indicate that quenching did not occur correctly and resulted in longer than intended reaction times.

Finally, the 30-gauge capillary had a similar uptake profile to the original setup, but as the reaction times should have been much lower than the original setup, there may be issues with this setup that resulted in high deuterium uptake when the reaction time should be low.

When looking at the error between all the methods implemented, even though the original contains four replicates while the other methods either only are composed of one or two, the error is often comparable. This illustrates the low consistency of these methods.

In summary, while the original setup suffered from consistency issues, none of the newly implemented methods provided a conclusive improvement to both gradual uptake and repeatability using cytochrome C.

3.2 Modifications using Tau

3.2.1 HDX Profile of Original Microfluidic Setup Utilizing Pepsin

Using the original setup with pepsin as the proteolytic enzyme resulted in pronounced stepwise uptake in accordance with the timepoint, with distinct uptake profiles across the different peptides. The data for this setup is presented in **Figure 3.14.** As the timepoints increased, the uptake of all peptides also increased, with little to no instances of identical uptake from one timepoint to the next. Differential uptake between peptides could be observed, such as peptides 74-105 and 115-127 experiencing lower uptake, and peptides 2-8 and 310-315 experiencing higher uptake relative to one another. There were few technical difficulties when utilising this method, with minimal troubleshooting required.

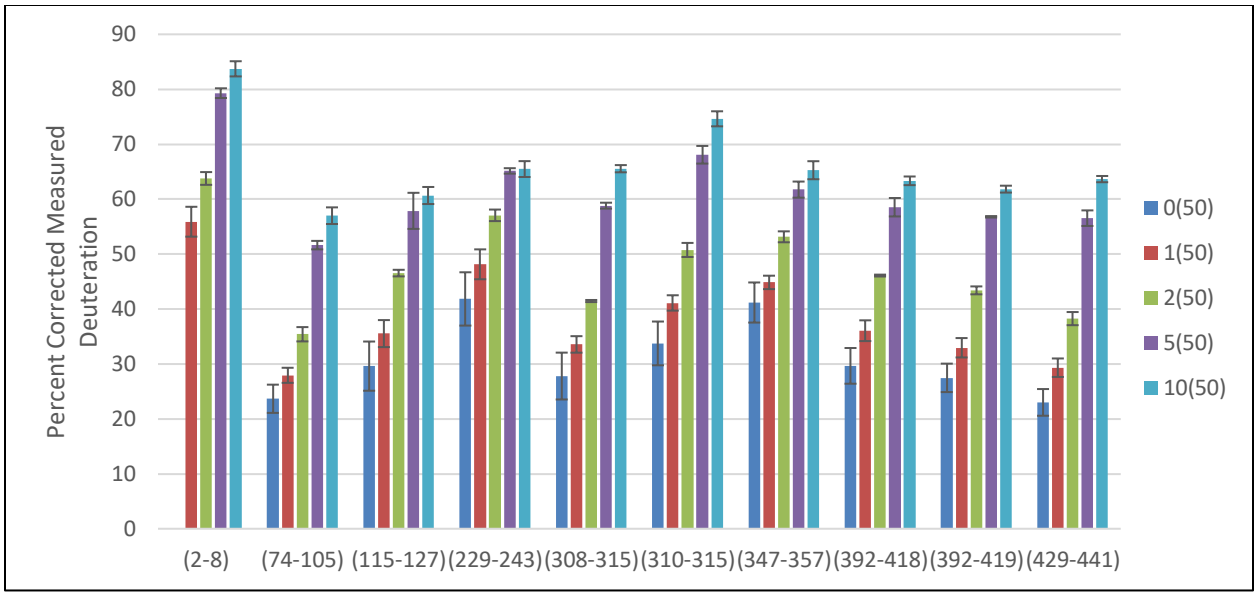

Figure 3.14. HDX analysis of Tau utilizing the original setup with pepsin. Representative kinetic plots of % corrected deuterium uptake vs. time for 10 peptides from tau. Error bars represent standard deviation values of one replicate.

As can be seen in the peptide map in **Figure 3.15**, peptides slowly uptake deuterium at different rates, with most of them reaching a high level by the last timepoint. Differential uptake was maintained, as all the peptides did not reach the same levels of deuteriation by the 10mm timepoint.

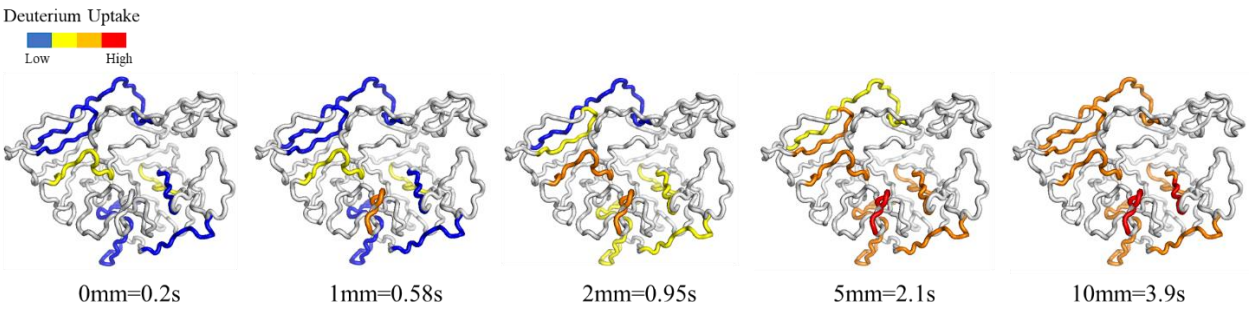

Figure 3.15. Deuterium uptake profiles of Tau peptides mapped onto protein structure utilizing original setup with pepsin. The measured profiles are coloured according to total deuterium uptake: red (68-83%), orange (53-68%), yellow (38-53%), and blue (23-38%) Regions for which no peptides were observed are coloured in grey.

The uptake for this method is what would be expected for HDX of a disordered protein, as there was significant uptake over time, with the increased reaction time producing peptides with higher uptake. The uptake profile is far better than what was seen using cytochrome C, as no plateauing of uptake past a

certain percentage uptake was seen. The more uniform uptake of deuterium with the increasing timepoints resulted in a better original setup as a baseline to contrast with other methods. Any changes in exchange from the stepwise uptake of this experiment would be more obvious than what would be seen in cytochrome C. This also highlights how TRESI may be better suited to studying disordered proteins rather than the more structured cytochrome C.

3.2.2 HDX Profile of Three Junction Mixing Chamber Utilizing Loose Frit with Pepsin

The method used was nearly identical with the loose frit method with cytochrome C in **Section 3.1.4.** This setup resulted in no real gradual uptake with increasing timepoints, with the 2mm timepoint equal to or lower than the uptake at the 0mm timepoint. The data from this method is presented in **Figure 3.16**. Peptide specific uptake is still distinct with the peptides displaying the same uptake trends as seen in the original setup. For example, the 74-105 peptide exhibiting the lowest uptake, and 2-8 the highest uptake. The loss of the 5mm and 10mm timepoints were not due to issues with the setup, but rather an experimental error discovered later. Aside from this, no major issues were experienced when obtaining the three timepoints before this error.

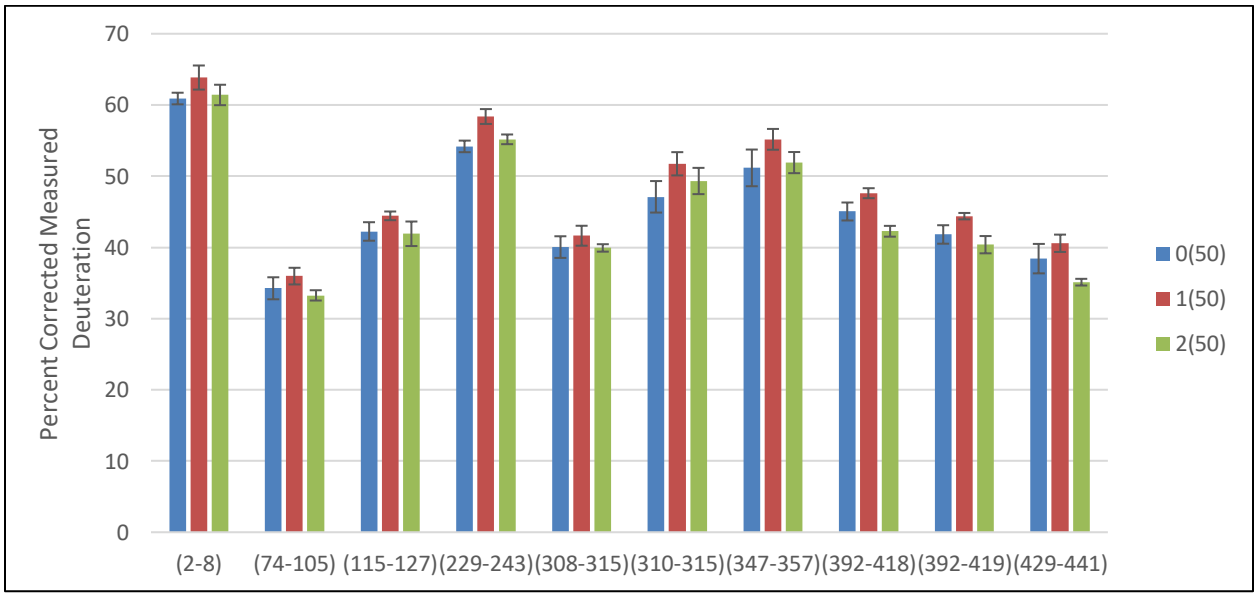

Figure 3.16. HDX analysis of Tau utilizing three junction mixing chamber with loose frit using pepsin. Representative kinetic plots of % corrected deuterium uptake vs. time for 10 peptides from tau. Error bars represent standard deviation values of one replicate.

As seen in the peptide map in **Figure 3.17**, some peptides increased in uptake during the 1mm timepoint, only to decrease in the 2mm timepoint.

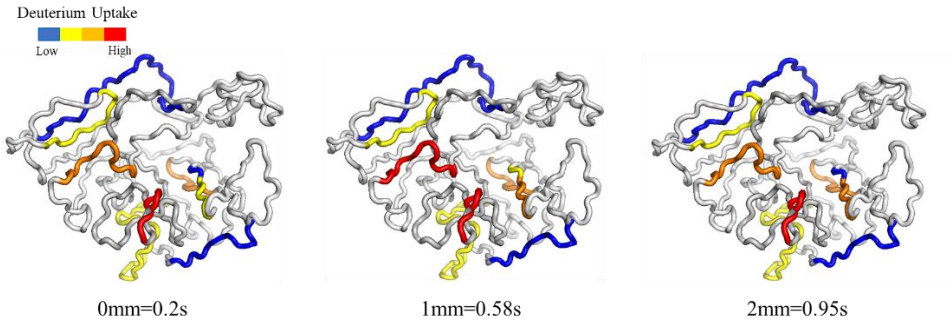

Figure 3.17. Deuterium uptake profiles of Tau peptides mapped onto protein structure utilizing three junction mixing chamber with loose frit using pepsin. The measured profiles are coloured according to total deuterium uptake: red (56-63%), orange (48-56%), yellow (40-48%), and blue (33-40%) Regions for which no peptides were observed are coloured in grey.

The HDX uptake shows a similar flattening of uptake over the timepoints which was seen in the cytochrome C results. This shows that the use of a loose frit in the mixing chamber produced flattening of uptake even with the disordered protein, which was previously seen to have better stepwise uptake than cytochrome C. This means that the flattening of uptake was unrelated to the protein cytochrome C, and pressure issues likely prevented the apparent reaction time from uniformly increasing with the timepoints, possibly due to inefficient quenching at all timepoints. In summary, these results indicate that this specific method consistently produces flat uptake that prevents change in uptake over time.

3.2.3. HDX Profile of Original Microfluidic Setup Utilizing Protease XIII

There were some issues with using pepsin for digestion, so another original setup was run using Protease XIII instead of pepsin. Switching the proteolytic enzyme to Protease XIII from pepsin still produced distinct stepwise uptake with increasing timepoints, while also exhibiting differential peptide uptake. The data from this method is presented in **Figure 3.16.** Since a different set of peptides were analyzed, the relative uptake profiles of the peptides could not be compared with the peptides analyzed in the pepsin experiment. However, the peptide 429-441 was present in both protease types. There is a large

jump in uptake from the 1mm to the 2mm timepoint, with the uptake increasing more gradually from the 2mm timepoint to the 10mm timepoint.

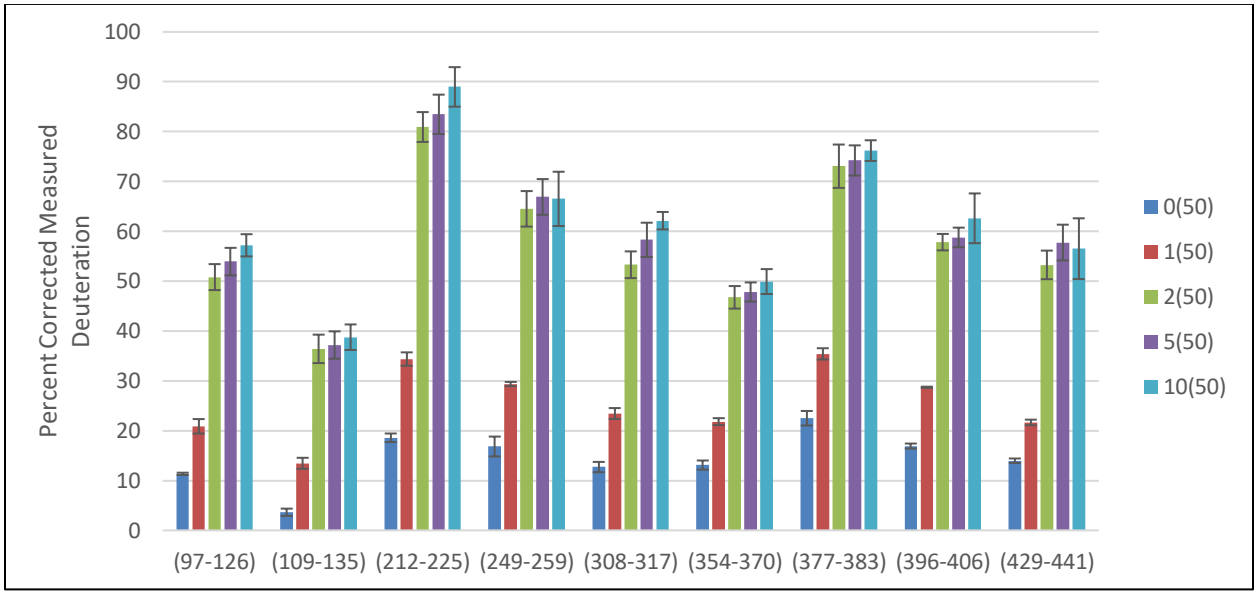

Figure 3.18 HDX analysis of Tau utilizing the original setup with Protease XIII. Representative kinetic plots of % corrected deuterium uptake vs. time for 9 peptides from tau. Error bars represent standard deviation values of one replicate.

The increase in uptake over time can be seen in the peptide map in **Figure 3.17**, where the peptides increase in uptake across the timepoints, with a large increase from the 1mm to the 2mm timepoint. The differential peptide uptake is also visible as the peptides increased in uptake at different rates, and not all peptides reached the same level of uptake by the final timepoint.

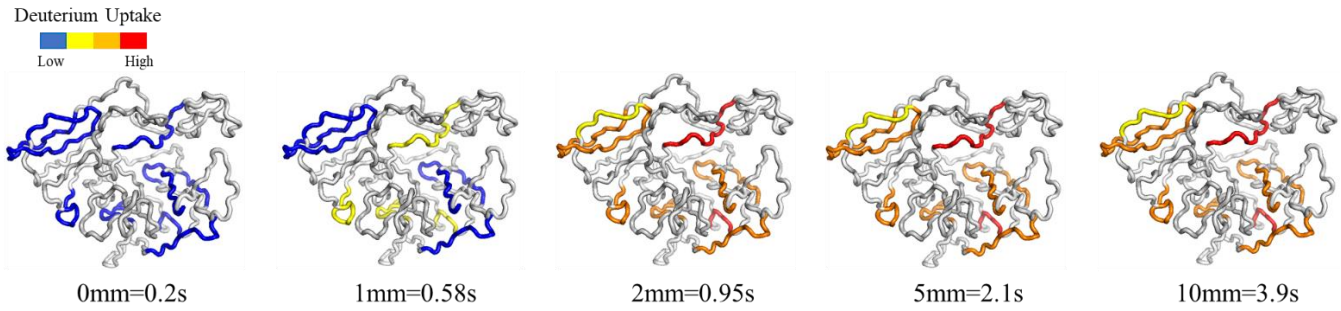

Figure 3.19 Deuterium uptake profiles of Tau peptides mapped onto protein structure utilizing the original setup with Protease XIII. The measured profiles are coloured according to total deuterium uptake: red (67-88%), orange (46-67%), yellow (24-46%), and blue (3-24%) Regions for which no peptides were observed are coloured in grey.

During the execution of this experiment there was a large number of air bubbles produced within the chip and replacing the acid line resolved the issue. The 0mm and 1mm timepoints were acquired before the bubbles became an issue, with the 2mm-10mm timepoints acquired with the bubbles filling the chip. However, once the bubble issue was resolved, the 2mm-10mm timepoints were re-acquired a second time but produced similar uptake to the first replicate. These two replicates of the 2mm-10mm timepoints were both included in the data. Other than the air bubbles, there was minimal troubleshooting, however multiple aspects of the setup needed to be replaced before the source of the bubble issue was discovered and resolved.

Similar to the original setup utilizing pepsin, the deuterium uptake of this original setup clearly increased with the longer reaction times, however the first two timepoints had a much lower uptake than expected. The uptake profile was similar to what was seen in the original setup utilizing pepsin, indicating that the original setup stays fairly consistent between proteases when analysing tau. In summary, this setup served as a good standard to contrast against the other methods implemented to determine how alterations to the microfluidic device affect the uptake profile of tau peptides produced by Protease XIII.

3.2.4. HDX Profile of FEP Tubing Utilizing Protease XIII

This setup resulted in little change in uptake over time across all the timepoints. Data for this experiment is presented in **Figure 3.20**. Significant differential peptide uptake could be observed, which were consistent with the trends seen in the other experiments using tau. However, aside from the 0mm timepoint, all other timepoints had similar uptake, with the increase in the reaction volume seemingly having no effect on deuterium uptake.

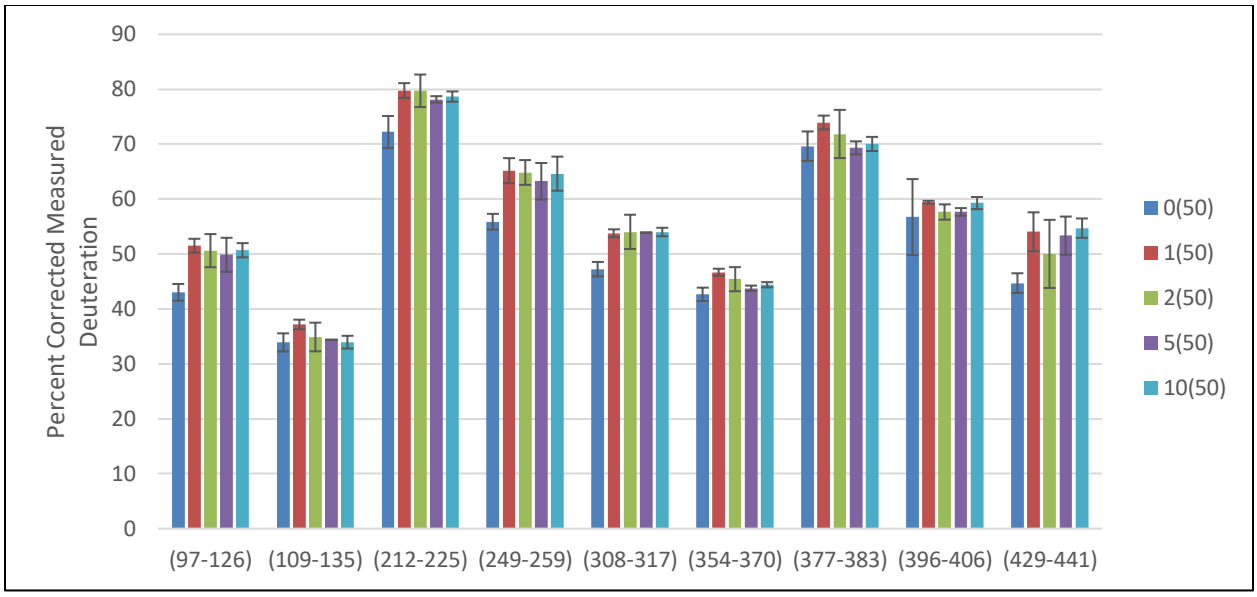

Figure 3.20. HDX analysis of Tau utilizing FEP tubing with Protease XIII. Representative kinetic plots of % corrected deuterium uptake vs. time for 9 peptides from tau. Error bars represent standard deviation values of one replicate.

The lack of change in uptake can be seen in the peptide map in **Figure 3.21**, where most peptides did not change in uptake over all the timepoints. The differential uptake is also prominent, as in the final timepoint there were peptides with low, medium, and high uptake.

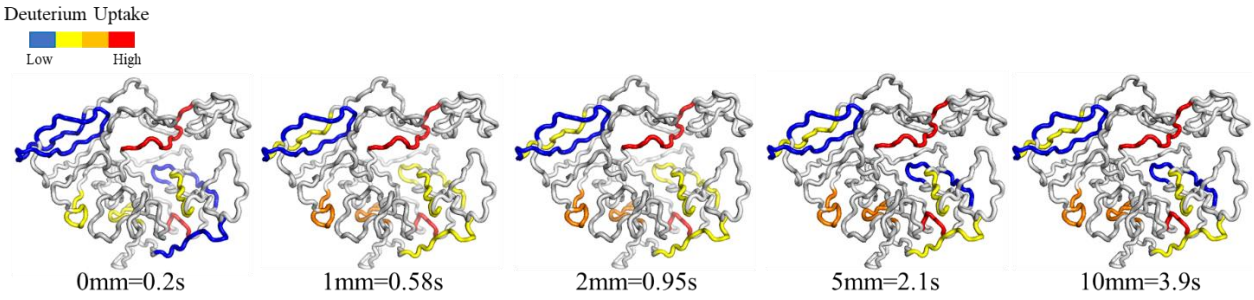

Figure 3.21. Deuterium uptake profiles of Tau peptides mapped onto protein structure utilizing FEP tubing with Protease XIII. The measured profiles are coloured according to total deuterium uptake: red (68-79%), orange (56-68%), yellow (45-56%), and blue (33-45%) Regions for which no peptides were observed are coloured in grey.

Little troubleshooting was required for this method, with the implementation of the FEP tubing not causing any major issues that could be seen on a mechanical level. The only issue that was

experienced was that due to the inflexibility of the tube it was more difficult to orient the chip so it lay flat, but this issue was eventually resolved.

Once again, the implementation of a new method other than the original setup produced a flattened uptake profile. This was not expected, as the use of the FEP tubing was intended to reduce pressure by increasing the inner diameter of the connection between the mixing chamber and the proteolytic chip. Pressure issues are what were suspected to be causing the flatting of uptake seen in many of the other methods, but this setup designed to reduce pressure did not improve uptake. The reason for this was believed to be that the pressure issues do not reside between the mixing chamber and the chip, and perhaps existed elsewhere in the setup. One final possible reason for the flat uptake profile could be that the increased diameter of the FEP tubing did reduce pressure, but low pressure in turn reduced how fast the protein was travelling through the final portion of the setup, allowing more time for acid back exchanging.

In summary, this setup still maintained the differential peptide uptake that was seen in all the tau experiments, and the relative amount of uptake matched what was observed in the original setup using Protease XIII. However, the implementation of this modification did not produce gradual uptake over time comparable to the original setup, and therefore was not an improvement to the setup.

3.2.5 HDX Profile of No Proteolytic Frit Utilizing Protease XIII

Removing the proteolytic chip frit did produce gradual uptake over time and resulted in differential uptake across the peptides. Data for this experiment is presented in **Figure 3.22**. There was gradual uptake from the 0mm timepoint until the 5mm timepoint, with a sudden drop in uptake in the 10mm timepoint. During the execution of the experiment there was a constant leak from behind the TRESI reaction chamber, and troubleshooting was unable to remove it.

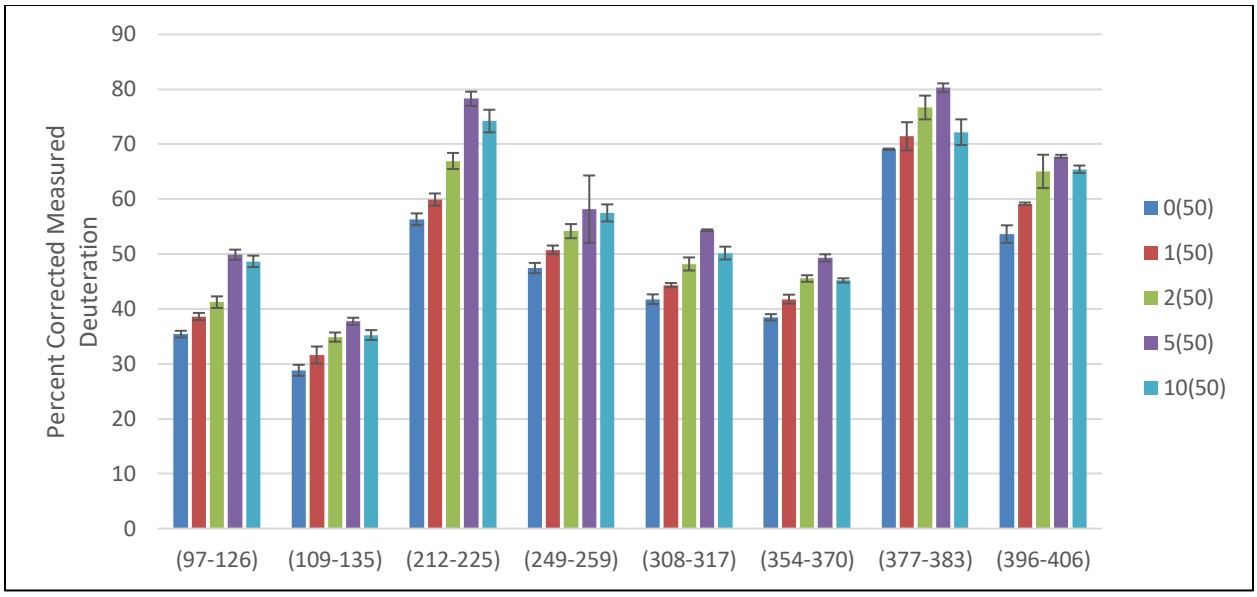

Figure 3.22. HDX analysis of Tau utilizing no proteolytic frit with Protease XIII. Representative kinetic plots of % corrected deuterium uptake vs. time for 8 peptides from tau. Error bars represent standard deviation values of one replicate.

The gradual uptake over time can be seen in the peptide map in **Figure 3.23**, where some peptides can be seen to increase in uptake. Other peptides remain at the same uptake level for the majority of the timepoints.

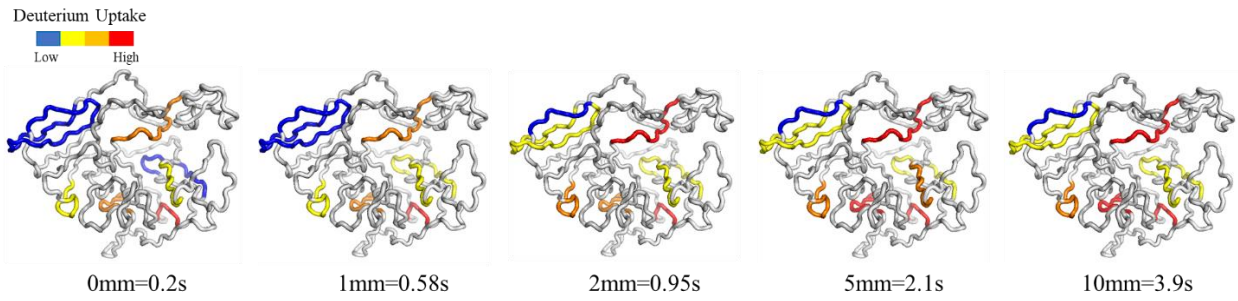

Figure 3.23. Deuterium uptake profiles of Tau peptides mapped onto protein structure utilizing no proteolytic frit with Protease XIII. The measured profiles are coloured according to total deuterium uptake: red (65-78%), orange (53-65%), yellow (41-53%), and blue (28-41%) Regions for which no peptides were observed are coloured in grey.

The removal of the frit was expected to reduce system reliability, as the frit was assumed to prevent clogging of the ESI nozzle and prevent pressure issues. However, the clogging and pressure issues that were expected when running tau without a frit were not observed. While the uptake was flatter

than the original setup, there was still distinct uptake over time. The data was expected to be much flatter than what was seen since the frit was supposed to reduce any high pressure issues. In addition, despite there being leaking issues, the troubleshooting required for this method was not significantly worse than the original setup. This indicates that while the frit may improve the gradual uptake, it may not be as necessary as it was believed to be. As only one replicate was performed, the consistency of this setup could not be determined.

In summary, as removing the frit did not appear to improve the quality of data, there would be no reason to remove it during experiments. The frit may prevent protease-linked beads from entering the ESI nozzle, and therefore its continued use could assist in preventing potential issues that may occur during an experiment.

3.3.6 Comparison of Methods Utilizing Tau

The general trends of each method used to analyse tau are presented in **Figure 3.13.** The methods that resulted in the greatest percent deuterium uptake were the original setups using pepsin and Protease XIII, with both methods also demonstrating the most pronounced stepwise gradual uptake. The loose frit and FEP tubing methods produced flat uptake profiles, showing little change in uptake over time. The method where the frit in the proteolytic chip was removed did not produce a flat uptake profile, however the uptake between timepoints were not as distinct as the original setups, in addition to the 10mm timepoint experiencing a drop in uptake. The error bars for all methods were large, due to the distinct differential uptake between peptides.

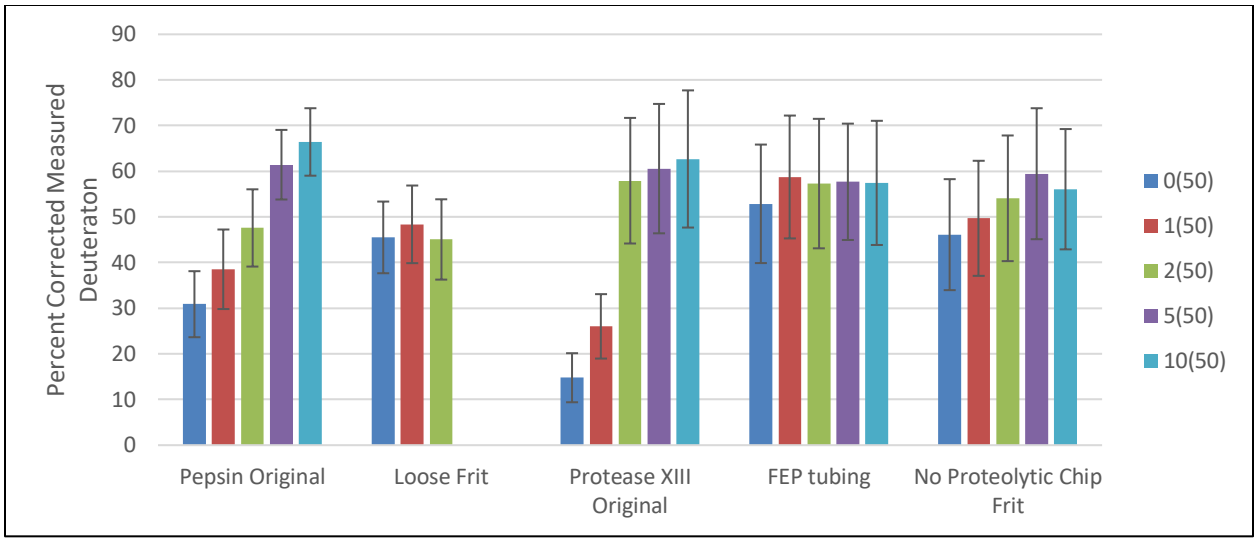

Figure 3.24. Summary of HDX analysis of Tau comparing uptake across all methods. Representative kinetic plots of % corrected deuterium uptake vs. time for the 5 methods tested using tau. The % corrected deuterium uptake of the peptides in each method were averaged according to timepoint. Error bars represent standard deviation values of the all the peptides in the timepoint for each method.

The very distinct stepwise uptake seen in the original setups provided a good baseline of what would be expected in a successful TRESI-HDX experiment. While the removal of the proteolytic chip frit produced reasonable gradual uptake comparable to the original setup, the other two methods implemented did not come close to the success of the original setups. This was not what was expected, as these two methods were intended to improve either the efficiency of quenching or alleviate pressure issues, but neither alteration conferred any improvement to the quality of the data. In addition, the setup where the frit was removed and was expected to produce clogging issues and increase the amount of troubleshooting required, instead resulted in better data than the other two methods that were intended to improve the setup. These unexpected results culminated in the inability to justify any changes to the original setup that could improve upon consistency between replicates or reduce mechanical issues.

Chapter 4: Conclusions and Future Work

4.1 Conclusions

Through modification and experimentation on the microfluidic TRESI HDX apparatus, insights were gained on which mechanical setups produced the most consistent results and were least prone to error. The setup currently in use with no modifications tended to result in the greatest consistency and produced the expected deuterium uptake profiles. Alterations made to this setup resulted in mechanical failures during execution and tended to produce lower quality data. These results highlight the sensitivity of this microfluidic setup to alterations, as small alterations often resulted in significant changes to the data acquired. Therefore, any changes made to the TRESI microfluidic setup between replicates should be closely monitored or avoided entirely when performing future analytical experiments.

During the study, the protein being initially being analysed, cytochrome C, was changed to tau. Due to the flat uptake profiles that were acquired from cytochrome C, it was difficult to evaluate the ability of the TRESI setup to produce gradual uptake with increasing reaction times with a structured protein. The deuterium uptake of cytochrome C tended to plateau after a certain percentage, with the final timepoints obtained producing similar results. With the disordered protein tau, uptake continued to increase until the final timepoints, allowing for greater scrutiny of how gradual uptake was affected with the alterations to the setup. The higher quality of gradual deuterium uptake seen when analyzing tau highlights how the TRESI method is best suited to the study of less structured proteins.

4.2 Future Work

As a conclusive modification that improved upon the original setup was not discovered, alternative modifications could continue to be tested to further investigate the microfluidic setup. One potential modification could be to reduce the gauge of the reaction chamber from 28 gauge and thereby increasing the size of the inner diameter. The main challenge that seemed to result in flat uptake profile were high pressure issues which could prevent the protein from exiting the silica capillary via the notch. The increased space between the fused silica capillary and the inside of the metal capillary could reduce

the operating pressure. This may have the drawback of reduced mixing efficiency of deuterium and protein, as it has been previously observed that reduced volume results in greater mixing. In addition, a larger gauge would result in larger reaction times, which is generally undesired.

One more significant alteration to the microfluidic setup could be the replacement of the syringe pumps that were used to force the solutions through the capillaries, with the use of an LC pump. Many mechanical issues were experienced while using the syringe pumps, in particular, high pressures would cause the pumps to stall. An LC pump can tolerate high pressures which would reduce mechanical failures while running experiments.

Finally, current experimentation is being conducted into automation of the pullback of the fused silica capillary to allow for continuous pullback instead of manually adjusting the distance. This is done using stacked syringe pumps, whereby one pump injects the protein into the solation, while the second pump pulls back the first pump, increasing the reaction volume in the reaction chamber by retracting the fused silica capillary. This could potentially increase consistency between experiments by removing the human error involved in measuring the distance of fused silica capillary pullback.

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