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In Situ and Multisubstrate Detection of Elastase Enzymatic Activity External to Microdialysis Sampling Probes Using LC−ESI-MS

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Extracellular proteases play significant roles in mammalian development and disease. Enzymatic activity external to a microdialysis sampling probe can be determined by infusing judicious choices of substrates followed by collecting and measuring the products. Porcine pancreatic elastase was used as a model enzyme with two substrates possessing different cleavage sites, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin (FL-substrate) and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (UV-substrate). These substrates were infused through the microdialysis sampling probe to a solution containing elastase. The resulting four products and the remaining two substrates were collected into the dialysate and were subsequently analyzed off-line using liquid chromatography−mass spectrometry (LC−MS) with electrospray ionization (ESI). All analytes were identified using extracted ion chromatograms of *m/z* 628 (FL-substrate), *m/z* 452 (UV-substrate), *m/z* 471 (*N*-methoxysuccinyl-Ala-Ala-Pro-Val, FL-NTP), *m/z* 332 (*N*-succinyl-Ala-Ala-Ala, UV-NTP), *m/z* 176 (7-amino-4-methylcoumarin, AMC), and *m/z* 139 (*p*-nitroaniline, *p*NA). FL-NTP and FL-substrate exhibited 10-fold higher ion production as compared to AMC with equimolar standards. Microdialysis sampling combined with LC−ESI-MS detection allowed for in situ determination of the enzymatic activity of a protease external to the microdialysis probe when using different peptide-based substrates.

Extracellular proteinases play essential roles in the degradation of extracellular proteins allowing for multicellular organs to develop and function normally.[1] Matrix metalloproteinases (MMPs), a family of zinc-dependent proteinases, have been recognized for their ability to cleave extracellular matrix (ECM) structural proteins including collagen, laminin, and fibronectin.[2] MMPs regulate cell behavior via cleavage of cell surface molecules and other pericellular nonmatrix proteins including proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor binding proteins, cell surface receptors, and cell−cell and cell−matrix adhesion molecules.[3] MMPs have known involvement with numerous disease states including arthritis, cancer, and cardiovascular disease. Elucidating the roles that MMPs play in vivo via chemical analysis is challenging since their endogenous substrates are typically insoluble proteins. The multiplicity of MMP substrates makes it difficult to effectively measure the activity of a single enzyme.[4] Additionally, once MMPs cleave their targeted substrate, the resulting product can be cleaved by additional MMPs or extracellular proteases. Many analytical methods have been described to assay the clinically relevant MMPs ex vivo from tissue biopsies.[5] Immunochemical methods give quantitative information of total enzyme concentrations, but they cannot discriminate between zymogens and active enzymes.[6] MMPs are synthesized as inactive zymogens (inactive proteins) and require enzymatic activation that is tightly regulated via tissue inhibitors of metalloproteinases (TIMPs). Zymography is a common method to separate and quantify the concentrations between the proenzyme and the active enzyme.[7] Although zymography allows visualization of enzyme activities, it is a tedious method and is difficult to quantify.[8] A variety of fluorescence and colorimetric assays have been applied to quantify the active enzyme based on changes in spectroscopic properties of the resulting products after substrate cleavage.[9−13]

Few methods are available to determine in vivo MMP activity. Assessing MMP enzymatic activity is important for identification and localization of activated MMPs during various disease states. Additionally, such assays would be useful in pharmaceutical studies designed to inhibit targeted MMPs. Considerable efforts have been made in the areas of fluorescent-based imaging

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techniques for in vivo assessment of MMP activity.[14−16] However, the target specificity for these approaches is presently limited by the number of fluorophores available that would provide nonoverlapping emission bands for the enzymatic products.

Microdialysis sampling is a well-established separation technique for continuous collection of analytes, such as neurotransmitters, drugs, and their metabolites, both in vivo and in vitro.[17,18] It is accomplished by using a probe consisting of a semipermeable hollow-fiber dialysis membrane affixed to inlet and outlet tubing. A perfusion solution is passed through the probe at microliter per minute flow rates and collected for analysis. Analytes that are smaller than the membrane pores can diffuse freely through the membrane and are carried to the outlet by the perfusion fluid. Larger analytes will be rejected by the membrane pores so that their recovery is negligible. Microdialysis sampling can also be used to deliver certain substances with a molecular weight smaller than the membrane molecular weight cutoff (MWCO) to the fluid outside the probe thus allowing localized metabolism to be monitored,[19] including MMP-2 and MMP-9 activity in breast cancer,[20] phenol metabolism from multiple sites in the liver,[21] and substance P metabolism at the blood−brain barrier.[22] During microdialysis sampling, sampling effectiveness is determined via the bidirectional extraction efficiency (EE), which can be calculated as shown in eq 1,

$$\mathrm{EE}\ (\%) = \frac{C_\mathrm{d} - C_\mathrm{i}}{C_\mathrm{e} - C_\mathrm{i}} \times 100 \qquad (1)$$

where C_d, C_e, and C_i stand for the analyte concentration in dialysate, external sample medium, and perfusion fluid, respectively.[23] This equation allows the calculation of extraction for a delivered substrate that is locally infused through the dialysis probe. Additionally, when the analyte concentration in the perfusion fluid is zero, the equation simplifies to the percentage of analyte concentration in the dialysate divided by the analyte concentration in the external sample medium and is usually termed relative recovery (RR). Here, loss of substrate from the probe will be termed $\mathrm{EE_{loss}}$ and recovery will be termed $\mathrm{EE_{rec}}$.

In this work, in vitro microdialysis sampling and liquid chromatography−electrospray ionization mass spectrometry (LC−ESI-MS) was applied to determine the activity of porcine elastase external to a microdialysis sampling probe. Elastase exhibits similar substrate specificity to neutrophil elastase (MMP-12) at a substantially reduced cost. Since many of the enzymatic substrates for different MMPs contain similar colorimetric or fluorescent products, we chose to focus on using the N-terminal peptide as an analytical target. Peptides are easily ionized using ESI conditions and the use of chromatographic separation prior to the detection allows quantitative measurements for each individual compound. Such an approach would ultimately allow multiplexed analysis of various MMP or protease activities at their site of action. An advantage for using LC−MS is that it allows simultaneous monitoring of multiple analytes in a single experiment as long as the substrate and product species exhibit mass to charge ratios (m/z) that can be spectroscopically resolved.[24,25] LC−MS analysis of peptide products for various in vitro studies of enzymatic activity and profiles have been reported.[26−29] Recently, an LC−MS approach has been described for peptide biomarker studies of MMP-13 activity in synovial fluid and urine.[30]

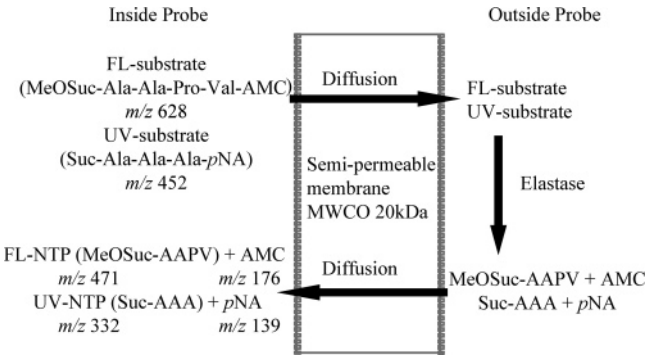

Figure 1. Microdialysis sampling schematic. FL-substrate and UV-substrate diffuse to the surrounding medium react with elastase forming products that diffuse back into the probe.

To determine the elastase enzymatic activity external to the microdialysis probe, a fluorogenic elastase substrate and a colorimetric elastase substrate with different N-terminal peptides were coperfused and delivered through the dialysis probe as shown in Figure 1. The organic and N-terminal peptide products were detected and quantified along with the substrates using LC−ESI-MS.

EXPERIMENTAL SECTION

Chemicals. *Fluorescent Series Chemicals.* Self-quenched fluorogenic elastase substrate V, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin (MeOSuc-AAPV-AMC, FL-substrate), was obtained from Calbiochem (San Diego, CA), *N*-methoxysuccinyl-Ala-Ala-Pro-Val (MeOSuc-AAPV, fluorescent substrate N-terminal peptide, FL-NTP) was purchased from Bachem (Bubendorf, Switzerland), and 7-amino-4-methylcoumarin (AMC) was purchased from Sigma (St. Louis, MO).

Colorimetric Series Chemicals. N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (Suc-AAA-*p*NA, UV-substrate) and *p*-nitroaniline (*p*NA) were both purchased from Sigma (St. Louis, MO).

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Porcine pancreatic elastase (type I, aqueous suspension) was purchased from Sigma (St. Louis, MO). One unit of elastase will hydrolyze 1.0 μmol of Suc-AAA-*p*NA per min, pH 8.0 at 25 °C. All other chemicals were reagent grade or better. HPLC-grade water was purchased from Fisher Scientific. Stock solutions (2 mM) of FL-substrate, FL-NTP, AMC, and UV-substrate were prepared in DMSO and stored at 4 °C. All other solutions were prepared with HPLC-grade water.

High-Performance Liquid Chromatography−Mass Spectrometry System (LC−MS). For separation and detection, an Agilent 1100 series high-performance liquid chromatograph (HPLC) with the MSD 1100 ion trap mass analyzer (Agilent Technologies, CA) operating in the ESI mode was used. All samples were injected using an autosampler with an injection volume of 2 μL. An Alltima 5 μm C18 column (150 mm × 4.6 mm i.d.) with guard column was employed to separate the substrates and products. A binary pump was used to deliver the solvents at 0.5 mL/min flow rate. Solvent A contained 0.2% formic acid in water, and solvent B contained 0.2% formic acid in acetonitrile. A linear gradient with solvent B changing from 25% (v/v) to 40% over a 20 min period was used to separate the analytes.

Mass spectrometric measurements were performed in the positive ion mode. Mass spectra were recorded over the range from m/z 100 to 700. The ESI source conditions were as follows: capillary voltage 4.5 kV, nebulizer gas (N_2) pressure 25.0 psi, dry gas (N_2) flow rate 10 L/min, dry temperature 350 °C.

Calibration Curves and Analyte Quantitation. Standard solutions of FL-substrate (0.5−50 μM), FL-NTP (1−20 μM), AMC (2−20 μM), and UV-substrate (0.5−50 μM) were prepared by appropriately diluting the corresponding 2 mM stock solution with water. Standards (2 μL) were analyzed by the LC−ESI-MS system in triplicate. Peak areas of extracted ion chromatograms (EIC) of m/z 628 (FL-substrate), 471 (FL-NTP), 176 (AMC), 452 (UV-substrate) versus concentration were used to create calibration curves.

Samples (2 μL) were analyzed in duplicate by the LC−ESI-MS system. Base peak chromatograms (BPC) of m/z 100−700 and EIC m/z 628 (FL-substrate), 471 (FL-NTP), 452 (UV-substrate), 332 (UV-substrate N-terminal peptide, UV-NTP), and 139 (*p*NA) were monitored. Each peak was characterized by their specific mass spectrum. Peak areas from the above EIC were used for quantitation.

Analyte Stability. The stability of FL-substrate, FL-NTP, and AMC were tested by storing 50 μM FL-substrate, 20 μM FL-NTP, and 20 μM AMC (diluted from 2 mM stock solution by water, respectively) in the dark at room temperature for various time periods. Samples were analyzed by LC−ESI-MS using the above conditions and quantified by using the calibration curves.

High-Performance Liquid Chromatography−Fluorescence system (LC−FL). In order to compare with the LC−MS detection, separation and quantitation were also performed on a LC−FL system, which includes a Shimadzu SIL-10ADvp autoinjector, LC-10ADvp pumps, SCL-10vp system controller, and RF-551 fluorescence HPLC detector. The same column, flow rate, injection volume, and gradient were used as in the LC−MS system. Fluorescence detection at an excitation (Ex) wavelength of 370 nm and emission (Em) wavelength of 460 nm was chosen for AMC detection.

Microdialysis Sampling. Microdialysis sampling was performed with a 1 mL Hamilton syringe (Hamilton Company, Reno, NV) with plastic tip and a CMA-102 syringe pump (CMA/Microdialysis, North Chelmsford, MA). CMA 20/04 microdialysis probes with a 10 mm 20 kDa MWCO polycarbonate/polyether (PC) membrane (CMA/Microdialysis, North Chelmsford, MA) were used in all experiments. All experiments were performed in quiescent solutions at 37 °C maintained by a sand bath (Fisher Scientific, U.S.A.).

The extraction efficiency for the FL-series substrate and products in the absence of enzyme was obtained at different flow rates. For AMC and FL-NTP, microdialysis was performed in recovery mode with water perfused through a probe to a solution containing 15 μM AMC or 15 μM FL-NTP, respectively. For the FL-substrate, microdialysis was performed in delivery mode with 15 μM FL-substrate perfused through a probe immersed into water. Dialysates (60 μL) were collected in triplicate at different flow rates (0.5, 1.0, 2.0, 3.0 μL/min) and analyzed by the LC−ESI-MS.

Next, enzyme was included in the microdialysis system. The solution external to the microdialysis probe contained 0.5 units/mL elastase in water. In the single-substrate microdialysis assay, the perfusion fluid passing through the dialysis probe contained 50 μM FL-substrate in HPLC-grade water. For the multisubstrate microdialysis assay, the perfusion fluid contained 50 μM FL-substrate and 50 μM UV-substrate in HPLC-grade water. The microdialysis sampling perfusion fluid flow rate was 1 μL/min. Dialysate collection was initiated 25 min post probe immersion into the enzyme solution to allow for product concentrations to reach detectable levels. Samples were collected for 60 min. Three replicate samples were collected during single-substrate experiments. Four replicate samples were collected during multisubstrate experiments. Statistical analysis required pooling the data due to differences in replicate sample numbers.

Due to the presence of enzyme outside the probe, the extraction efficiency of products may differ from the values obtained when there is no enzyme present. Standard addition experiments were performed to test the EE_{rec} of FL-NTP and AMC in the presence of enzyme. In this experiment, water was perfused to microdialysis probes at 1 μL/min flow rate. Elastase was added to the FL-substrate solution to achieve a concentration of 0.5 units/mL elastase in 25 μM FL-substrate, and the enzymatic reaction was initiated. This reaction mixture was vortexed and incubated at 37 °C for 15 min. Fluorescence at Ex 360 nm, Em 465 nm was monitored to ensure reaction completion. Then microdialysis probes were immersed in this reaction mixture, and 10 μL of dialysate was collected. Next, FL-NTP solution was added to the reaction mixture to achieve an increase of FL-NTP concentration of 20 μM. The reaction mixture was well-mixed, and 10 μL of dialysate was collected. Then AMC solution was added to the reaction mixture to achieve an increase of AMC concentration of 20 μM. The reaction mixture was well-mixed, and 10 μL of dialysate was collected. All dialysates were subsequently analyzed by LC−ESI-MS.

Kinetics. A Tecan SPECTRAFluor plate reader (Tecan Group Ltd., Männedorf, Switzerland) was used for all the kinetics measurements. The K_M, k_{cat}, and k_{cat}/K_M values for both substrates

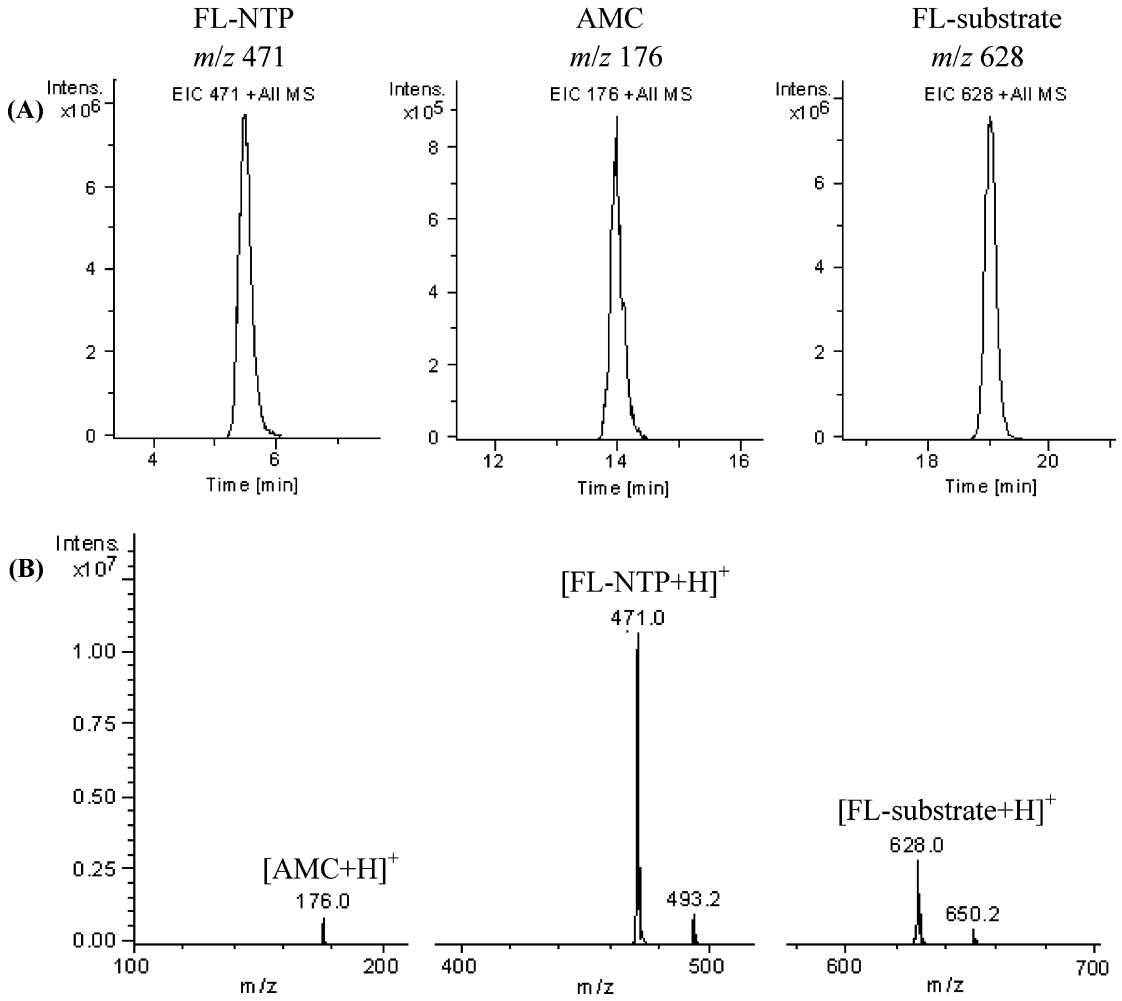

Figure 2. LC chromatograms and mass spectra for the FL-substrate and its products, FL-NTP and AMC. (A) Extracted ion chromatogram (EIC) of FL-NTP (*m*/*z* 471), AMC (*m*/*z* 176), and FL-substrate (*m*/*z* 628). (B) Mass spectra of FL-NTP, AMC, and FL-substrate in full scan mode from *m*/*z* 100 to 700. Peaks at *m*/*z* 493 and 650 are Na^+ adducts and were not quantified.

were determined by plotting $1/v$ versus $1/[S]$ in a Lineweaver−Burk plot.

Elastase kinetics for the UV-substrate was determined by adding 50, 63, 83, 100, 125, 167, 250, and 333 μM UV-substrate to 0.01 units/mL elastate. The reaction progress was monitored at 405 nm every 30 s for 8 min using a *p*NA calibration curve between 0 and 400 μM. At 405 nm, the product of the reaction, *p*NA, has significant molar absorbtivity and the UV-substrate exhibits no absorbance.

Kinetics of the FL-substrate in the presence of elastase were determined by adding 67, 75, 86, 100, 120, 150, and 200 μM FL-substrate to an enzyme concentration of 0.2 units/mL. The production of AMC from the cleavage reaction was measured using a 360 nm for excitation and 465 nm for emission every 30 s for 8 min. The calibration curve of AMC concentration ranging from 0 to 250 μM was obtained using fluorescence Ex 360 nm, Em 465 nm.

RESULTS AND DISCUSSION

Analyte Stability and Method Optimization. At room temperature, the FL-substrate was found to be stable for 24 h; the FL-NTP was stable for greater than 12 h and was reduced to 96% of its initial concentration after 24 h. AMC was found to be the least stable. It was stable for at least 10 h and was reduced to 90% of its initial concentration after 24 h at room temperature. Collection followed by detection of dialysates was often less than 24 h suggesting that no more than 10% variation in sample concentration would be expected under these conditions.

Figure 2 shows a typical LC chromatogram and mass spectra for the single-substrate dialysate analyzed by LC−ESI-MS. Separation was achieved on a C18 column with FL-NTP, AMC, and FL-substrate eluting at 5.4, 14, and 19 min, respectively. The retention behavior of the FL-substrate and products was consistent with a reversed-phase separation mechanism as the N-terminal peptide is more hydrophilic than AMC. ESI-MS spectra showed peaks of $[M + H]^+$ ions of FL-NTP (*m*/*z* 471), AMC (*m*/*z* 176), and FL-substrate (*m*/*z* 628). Sodium ion adducts, $[M + Na]^+$ were observed for FL-NTP (*m*/*z* 493) and FL-substrate (*m*/*z* 650) despite the addition of formic acid to suppress sodium adduct formation; however, their intensities are much lower compared to the ions of the protonated molecules. Only the EIC peak area of $[M + H]^+$ ions was used for quantitation.

Linear calibration curves were achieved for FL-substrate (0.5−50 μM), FL-NTP (1−20 μM), and AMC (2−20 μM) (figures not shown), based on the peak area from EIC of $[M + H]^+$ ions versus

Table 1. FL-Substrate and UV-Substrate Kinetic Parameters

	FL-substrate[a]	UV-substrate[b]
K_M (μM)	79.62	65.78
k_{cat} (min^{-1})	1.59	22.74
k_{cat}/K_M ($min^{-1}\,\mu M^{-1}$)	0.02	0.35

[a] FL-substrate kinetics were measured using 0.2 units/mL elastase. [b] UV-substrate kinetics were measured using 0.01 units/mL elastase.

concentration. The limit of quantitation (LOQ) was used as the lowest concentration standard for the calibration curve and was achieved at the concentration that yields a signal-to-noise ratio (S/N) of 10:1.

For equimolar concentrations of all three analytes, the signal of AMC was about 10 times lower and it exhibited poor S/N due to the low ionization efficiency compared to that of the peptides.[31,32] Several methods were applied to optimize AMC signal, including tuning the capillary voltage to a relatively high value of 4.5 kV,[31,32] using a narrow scan range (m/z 170 to ~190), or using selected-reaction monitoring (SRM) for m/z 176. A narrow scan range can relatively increase the sensitivity, but the S/N is poor; SRM can significantly increase S/N though it did not cause an obvious signal increase. Additionally, MS/MS detection (FL-NTP m/z 471 → m/z 215, AMC m/z 176 → m/z 117, FL-substrate m/z 628 → m/z 443) can reach significantly lower LOQ for all analytes: 100 nM for FL-NTP, 500 nM for AMC, and 50 nM for FL-substrate. Yet, the calibration curve for AMC using MS/MS did not exhibit a linear concentration relationship above a concentration of 5 μM, but was linear for the FL-substrate and FL-NTP up to 20 μM. Extracted ion chromatograms obtained from the mass spectra recorded over the range from m/z 100 to 700 were used for quantitation of dialysates.

The difference in ionization efficiency between the peptide product and AMC also suggests the advantage for using peptide substrates for the protease analysis due to the high ionization efficiency typically obtained for peptides. Although two different peptide products may exhibit differences in their ionization efficiency because of their different amino acid sequences, generally their ESI signal will be much higher than typically observed for organic products such as AMC that are used in conventional protease assays.[31,32]

Microdialysis Sampling Extraction Efficiency without Elastase. In vitro probe calibration for all three analytes without elastase was performed, and the determined EE values are shown in Table 2. At 1 μL/min, the EE_{loss} of FL-substrate in water was 48.5% ± 2.6% (n = 3) and the UV-substrate was 42.8 ± 2.1 (n = 3). The in vitro EE_{rec} of FL-NTP at 1 μL/min flow rate was 11.5 ± 0.2 (n = 3), which is much lower than the EE_{loss} for the FL-substrate. FL-NTP standard prepared in water instead of from the 2 mM DMSO standard and diluted to an external concentration of 15 μM gave an EE_{rec} of 17.5 ± 0.4 (n = 3), indicating that use of the DMSO solvent did not cause the significantly lower EE_{rec} for the FL-NTP compared to the FL-substrate. Microdialysis sampling was also performed in delivery mode where 15 μM FL-NTP was included in the perfusion fluid and perfused to water at 1 μL/min flow rate. In the delivery mode, the EE_{loss} of FL-NTP was 14.7 ± 3.6 (n = 3) indicating that FL-NTP mass transport characteristics to and from the microdialysis probe are similar as would be expected.[33]

Table 2. FL-Substrate, FL-NTP, and AMC Extraction Efficiencies[a]

flow rate (μL/min)	AMC EE_{rec}%	FL-NTP EE_{rec}%	FL-substrate EE_{loss}%
0.5	83.7 ± 1.3	18.3 ± 0.3	63.6 ± 5.5
1.0	76.3 ± 1.7	11.5 ± 0.2	48.5 ± 2.6
2.0	68.2 ± 8.3	8.7 ± 1.8	32.8 ± 3.9
3.0	56.0 ± 1.8	7.6 ± 0.2	23.0 ± 0.2

[a] EE_{rec}% denotes extraction efficiency in recovery mode. EE_{loss}% denotes extraction efficiency in delivery mode. All experiments were performed in quiescent solutions at 37 °C. Data represent mean ± SD, n = 3.

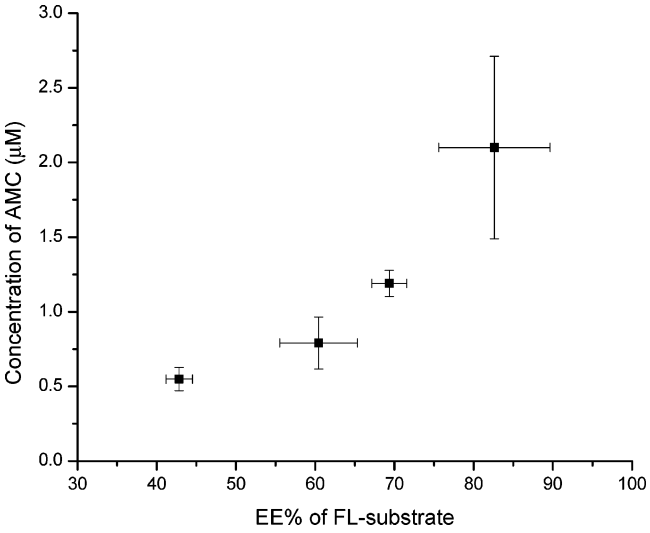

Figure 3. FL-substrate delivery and collected AMC concentrations as a function of perfusion fluid flow rate. The probes were perfused with 50 μM FL-substrate to a quiescent 0.5 units/mL elastase solution at 37 °C. Symbols and error bars denote mean ± SD, n = 3.

Single-Substrate Microdialysate Analysis in the Presence of Enzyme. The flow-rate-dependent FL-substrate EE_{loss} to a solution containing elastase and the collected AMC concentrations are determined by LC−FL and shown in Figure 3. As the perfusion flow rate increased, both the concentration of AMC collected into the probe and the FL-substrate EE_{loss} decreased. The time-dependent delivery of FL-substrate and collected FL-NTP concentrations are determined by LC−MS and shown in Figure 4. Through the duration of the collection time, the FL-substrate EE_{loss} remained constant and the recovered concentration of FL-NTP increased. These results are consistent with microdialysis sampling mass transport principles. It is well-established that mass transport rates in the dialysate are affected by the analyte aqueous solution diffusion coefficient and the volumetric flow rate (Q_d) of the microdialysis perfusion fluid; an increase in Q_d leads to a decrease in the microdialysis EE.[34] With a localized infusion

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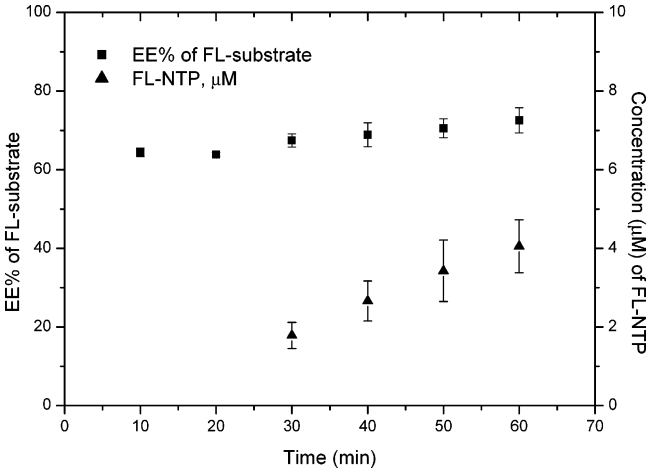

Figure 4. Time-dependent delivery of FL-substrate and FL-NTP recovery during single-substrate dialysis. FL-substrate (50 μM) was perfused at 1 μL/min to a quiescent solution containing 0.5 units/mL elastase at 37 °C. FL-NTP concentrations in the 10 and 20 min dialysate samples were below the LOQ. Symbols and error bars denote mean ± SD, $n = 3$.

Table 3. Estimated Extraction Efficiencies in Elastase[a]

	dialysate product concn (μM)	product concn (μM) after spike to external soln	EE_{enzyme} (%)
FLNTP	4.8 ± 0.7	9.3 ± 1.8	22.3
AMC	12.4 ± 2.4	22.3 ± 2.9	49.5

[a] Microdialysis samples (1 μL/min) were collected after 25 μM FL-substrate was incubated with 0.5 units/mL at 37 °C for 15 min. Then FL-NTP and AMC were spiked such that the external concentration was increased by 20 μM. EE_{enzyme} was calculated by dividing the difference of recovered product concentration by 20 μM. Data represent mean ± SD, $n = 3$ for 10 μL dialysates.

combined with product collection, the concentration of products collected would be expected to increase with an increasing infusion into a quiescent system. Additionally, the enzymatic reaction external to the probe causes the FL-substrate to reach an EE_{loss} steady state.

To determine approximate EE_{rec} values of the products in the presence of elastase (EE_{enzyme}) values were determined using a standard addition experiment. Table 3 shows that although AMC EE_{rec} values decreased under these experimental conditions from 76.3% (water) to 49.5% (enzyme solution), the FL-NTP EE_{rec} slightly increased in the presence of the enzyme to 22.3%. Although it is possible that AMC binds nonspecifically to the elastase enzyme causing a reduction in its EE_{rec}, it is not clear why the FL-NTP has an increased EE_{rec} value under these conditions.

In the single-substrate microdialysis experiment, FL-substrate (50 or 100 μM) was perfused through the probe immersed in a medium containing 0.5 units/mL elastase. The EE data for the FL-substrate and collected product concentrations are listed in Table 4. With a 50 μM FL-substrate infusion, the EE_{loss} was 66.6% ± 4.6% ($n = 4$) using the LC−MS detection method, which is comparable to 69.4% ± 2.2% ($n = 3$) using LC−fluorescence detection method ($p < 0.05$ level). The FL-substrate EE_{loss} is greater for probes immersed in an elastase solution as compared to solutions with no enzyme (48.5%) as would be expected since

Table 4. Single-Substrate versus Multisubstrate Comparison[a,b]

perfusate	EE_{enzyme} of FL-substrate (%)	delivered FL-substrate (pmol/min) outside probe	recovered FL-NTP concn (μM) in the dialysate	recovered FL-NTP (pmol/min) in the dialysate
FL-substrate 50 μM	66.5 ± 4.6*	33.3 ± 2.3*	4.3 ± 0.7*	4.3 ± 0.7*
FL-substrate 100 μM	62.1 ± 0.9*	62.1 ± 0.9	11.0 ± 0.5	11.0 ± 0.5
FL-substrate 50 mM and UV-substrate 50 μM	67.3 ± 3.6*	33.7 ± 1.8*	5.6 ± 0.1*	5.6 ± 0.1*

[a] An "*" denotes no statistical significance difference at the 95% confidence level between each designated value and the FL-substrate 50 μM value. [b] Microdialysis was performed at 1 μL/min with quiescent conditions at 37 °C. Sample collection (60 μL) was initiated 25 min post probe insertion. Data represent mean ± SD, $n = 3$ for single-substrate experiments and $n = 4$ for multisubstrate experiments.

the enzyme removes the substrate as it diffuses from the probe. When either 50 or 100 μM FL-substrate was perfused, the FL-substrate delivered outside the probe was 33.3 ± 2.3 and 62.1 ± 0.9 pmol/min. The FL-NTP collected in the dialysate was 4.3 ± 0.7 and 11.0 ± 0.5 pmol/min. Combining this information, it shows a higher percentage conversion of substrate to product outside the probe when 100 μM FL-substrate was perfused as compared to when 50 μM FL-substrate was perfused. This increase is expected considering the enzyme kinetics, that when more substrate is present, the reaction will proceed at a faster rate, and thus, within a certain collection time more substrate is converted to products and the conversion rate is higher.

Multisubstrate Microdialysate Analysis in the Presence of Elastase. On the basis of all the previous MS setup, a multisubstrate microdialysis assay scheme was developed to determine the potential for multiple substrate infusion followed by product detection. FL-substrate (50 μM) and UV-substrate (50 μM) were coperfused through the dialysis probe to a surrounding solution containing 0.5 units/mL elastase. Four different products, FL-NTP, AMC, UV-NTP, and *p*NA diffused back to the probe combined with the two substrates remaining in the dialysate. With the use of the same HPLC gradient, all six analytes can be resolved and quantified based on the EIC of their $[M + H]^+$ ions. Figure 5 shows the LC−ESI-MS chromatograms and mass spectra obtained during the multisubstrate infusion to elastase.

When a single enzyme acts on two different substrates, each substrate will work as a competitive inhibitor to the other.[35] The two substrates will compete for the binding site, and the enzyme exhibits kinetics that make it appear as if the K_M increases for each substrate. Comparing the kinetics data shown in Table 1, elastase has a weaker affinity to the FL-substrate (K_M 79.62 μM) than to the UV-substrate (K_M 65.78 μM), and the k_{cat} is slower for the FL-substrate. Considering the k_{cat}/K_M value, which allows the direct comparison of the effectiveness of the enzymatic conversion toward different substrates, it is much more effective for elastase to cleave the UV-substrate than to cleave the FL-substrate.

The FL-substrate and FL-NTP were quantified by comparing with the calibration curves. The EE_{loss} and product amounts

(35) Segel, I. H. *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*; John Wiley & Sons, Inc.: 1975.

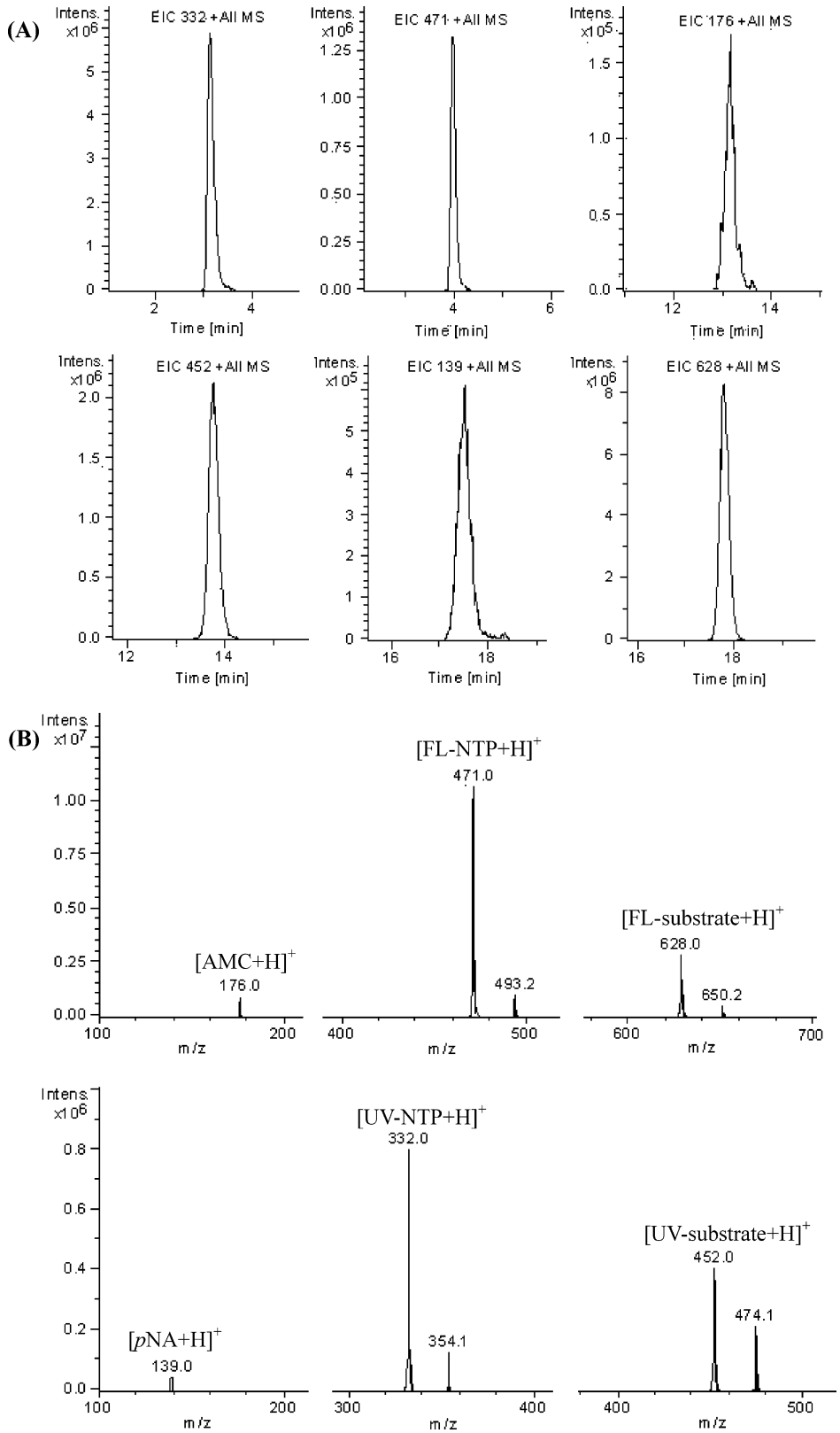

Figure 5. LC chromatograms and mass spectra of multisubstrate dialysate experiments. FL-substrate (50 μM) and UV-substrate (50 μM) were coperfused at 1.0 μL/min to a quiescent medium containing 0.5 units/mL elastase at 37 °C. (A) EIC of FL-NTP (*m*/*z* 471), AMC (*m*/*z* 139), FL-substrate (*m*/*z* 628), and UV-NTP (*m*/*z* 332), *p*NA (*m*/*z* 139), UV-substrate (*m*/*z* 452). (B) Mass spectra of FL-NTP, AMC, FL-substrate, and UV-NTP, *p*NA, UV-substrate in full scan mode from *m*/*z* 100 to 700. Peaks at *m*/*z* 354, 474, 493, and 650 are Na^+ adducts and were not quantified.

recovered between the multisubstrate versus the single-substrate dialysate are shown in Table 4. There is no significant difference ($p < 0.05$ level) between the EE_{loss} value of FL-substrate in the multisubstrate dialysate and in the single-substrate dialysate, when either 50 or 100 μM FL-substrate was perfused. This indicates that the EE value is not as sensitive to minor changes in kinetics

external to the probe. The recovered concentrations of FL-NTP during the multisubstrate versus the single-substrate infusion exhibited no significant differences ($p < 0.05$ level). Despite the higher k_{cat}/K_M value of UV-substrate as compared to the FL-substrate, there appeared to be no potential inhibition of the elastase catalytic activity toward the FL-substrate. This is most likely due to the higher enzyme concentrations used in these studies. Finally, the collected recovered concentrations of FL-NTP were doubled when 100 μM FL-substrate was perfused.

CONCLUSIONS

Enzymatic activity external to a microdialysis sampling probe can be detected by infusing proper enzymatic substrates through the dialysis probe followed by analysis of the products in the dialysate. In comparison to LC−FL detection, the use of LC−ESI-MS in enzymatic bioassay offers many distinct advantages. The main advantage with the LC−MS approach is that selected peptide substrates can be used allowing for a multiplexed approach toward determining enzymatic activity.

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