

Development of microchip isoelectric focusing and scanning laser
induced fluorescence detection for simultaneous enzyme
activity

by

Shu Jia

B.S., Sun Yat-sen University 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

Doctor of Philosophy

Department of Chemistry

College of Arts and Sciences

Kansas State University

Manhattan, Kansas

2021

Abstract

Matrix Metalloproteinases (MMPs) are a group of extracellular matrix proteinases expressed in normal and pathological processes, such as immune response and carcinogenesis. Dysregulation of MMP activities is commonly found in multiple pathways in cancer development, including degradation of extracellular matrix, reduction of cell-cell/cell-matrix adhesion, and facilitation of tumor cells metastatic migration.¹ Quantification of MMP activities has been utilized in cancer early detection, cancer staging, and immunotherapies.^{2,3} Currently, the ability to measure MMP activities with an affordable point of care device is still lacking. This project aims at addressing the need by developing a microfluidic isoelectric focusing device with modified magnetic nanoparticles to measure MMP activities in blood serum samples. Microfluidic isoelectric focusing devices were developed to separate fluorescently labeled peptide substrates based on isoelectric point difference. Sample handling, sample treatment, analyte separation, and fluorescence detection were integrated into the microfluidic IEF device. Magnetic nanoparticles with MMP substrates were digested by MMPs in the sample to cleave peptides. MMP activities were quantified by the ratio of cleaved and uncleaved substrates. This thesis/presentation will review the

development of the novel automated microfluidic IEF device and its application in enzyme activity assays.

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Table of contents

List of Figures	x
List of Tables	xiii
Acknowledgment.....	xiv
Chapter 1 Introduction	1
1.1 Analytical Separations	1
1.2 Electrophoresis and isoelectric focusing.....	5
1.3 Enzyme activity assay	14
1.4 Conclusion	24
Chapter 2 Cancer early detection and biomarkers	26
2.1 Introduction.....	26
2.2 Detection methods and cancer biomarkers	28
2.3 Matrix metalloproteinases.....	30
2.4 Conclusions.....	33
Chapter 3 Integrated microfluidic isoelectric focusing and scanning laser-induced fluorescence detection for peptide separation....	34
3.1 Introduction.....	34
3.2 Materials and methods	37
3.3 Results and discussion	45
3.4 Conclusions.....	56

Chapter 4 Point of care matrix metalloproteinase activity assay for cancer early detection using microchip isoelectric focusing and bioengineered magnetic nanoparticles.....	57
4.1 Introduction.....	57
4.2 Materials and methods	60
4.3 Results and discussion	67
4.4 Conclusions.....	79
Chapter 5 Conclusion and future outlook	80
References	84

List of Figures

Fig. 1 Baseline separation $R_s > 1.5$ (A) partial separation $R_s \sim 1.2$ (B) and poor separation $R_s \sim 1$ (C)	4
Fig. 2 Electroosmotic flow.....	7
Fig. 3 carrier ampholyte example	10
Fig. 4 isoelectric focusing processes.....	10
Fig. 5 charge of pI 8.2 peptide in pH gradient	12
Fig. 6 Product concentration over time in condition $[S] \gg K_M$	17
Fig. 7 Theoretical relation between substrate concentration and reaction rate.....	18
Fig. 8 FRET mechanism	22
Fig. 9 Sensitivity of matrix proteinases on consensus sequence substrates ³³ (highest signal is scaled to 100% for each enzyme).....	24
Fig. 10 5-year survival rate by stage at diagnosis (100% relative to in-situ).....	27
Fig. 11 Expression, activation and inhibition of MMPs	32
Fig. 12 single point LIF (A) and whole column imaging (B).....	36
Fig. 13 Soft lithography photomask.....	39
Fig. 14 Demonstration of IEF setup (A) and schematic of fluorescence detection (B)....	41
Fig. 15 Repetitive scanning of pre-marked chip	45
Fig. 16 Universal joint (A) and scanning of pre-marked chip with universal joints (B) ..	46
Fig. 17 LIF setup sensitivity with Rhodamine B	47
Fig. 18 Glass chip profiler results	48
Fig. 19 (RhB)-GCEEH substrate LCMS	49
Fig. 20 (RhB)-GCEHH substrate LCMS	49

Fig. 21 (RhB)-GCYYKK substrate LCMS.....	50
Fig. 22 Gel IEF of (1) GCEE (2) GCEEH (3) GCEHH (4) pI 4.4 marker	51
Fig. 23 0.02 mg/mL RhB-GCEE and RhB-GCEEH IEF CA3-10.....	52
Fig. 24 0.02 mg/mL RhB-GCEE and RhB-GCEEH IEF CA3-6.....	52
Fig. 25 0.035 mg/mL RhB-GCEHH IEF CA 3-6	53
Fig. 26 0.035 mg/mL RhB-GCEHH IEF CA 3-10	54
Fig. 27 RhB-GCYYKK IEF chromatogram CA 3-10	54
Fig. 28 pH switching of fluorescent(left) and non-fluorescent(right) Rhodamine B amide	55
Fig. 29 Iron/iron oxide magnetic nanoplatfrom working mechanism	59
Fig. 30 Microchip isoelectric focusing procedures.....	62
Fig. 31 Microchip magnetic rotation mixer	63
Fig. 32 Mixing of magnetic nanoparticles with a rotating magnet	67
Fig. 33 LCMS of MMP-7 substrates total ion chromatogram (above) MS at retention time 3.86-3.99 (below).....	68
Fig. 34 IEF of intact (pI=3.65) and cleaved (pI 4.20) MMP-9 substrate.....	68
Fig. 35 LCMS of MMP-9 substrates total ion chromatogram (above) MS at retention time 4.30-4.43 (below).....	69
Fig. 36 Gel IEF of intact MMP-7 (channel 2) and MMP-9 (channel 3, 4).....	70
Fig. 37 0.05mg/mL substrate digested by 100nM MMP-7 enzyme	71
Fig. 38 0.005mg/mL substrate digested by 100nM MMP-7 enzyme	72
Fig. 39 Product signal/internal standard over time	72
Fig. 40 substrate concentration over time	73

Fig. 41 Peak area ratio of cleaved/(substrate +cleaved)	74
Fig. 42 IEF of RhB-GCEEH (above) with 0.01% THPP (below)	75
Fig. 43 Free MMP-9 substrate (above) and reduced MMP-9 nanoparticles (below)	76
Fig. 44 MS of retention time 4.34min free MMP9substrate(above) and reduced nanoparticles(below).....	77
Fig. 45 HPLC of cleaved substrate, uncleaved substrate and nanoparticle reduction product after 4 hours enzyme digestion	78

List of Tables

Table. 1 List of common tumor biomarkers	29
Table. 2 pI standards labeled with Rhodamine B	44
Table. 3 MMP consensus sequence and isoelectric points	65

Acknowledgment

I would like to thank Kansas State Chemistry Department for offering me the opportunity to join the graduate program here. Thanks to the financial support, I am able to carry out fantastic research with many talented minds. I am sure the time I spent in Manhattan will be unforgettable throughout my life.

I would like to thank my advisor, Dr. Christopher. T. Culbertson for the guidance over these years. Chris is a great analytical chemistry scientist, and working under him was an honorable experience. Chris gave me the opportunity to master various analytical techniques and the ability to develop devices of medical importance.

I would also like to thank Dr. Stefan Bossmann and Bossmann group members, Dr. Madulali Kalubowilage, Dr. Obdulia Covarrubias, Dr. Jose Covarrubias, Sumia Ehsan, for their wits and effort in the synthesis of the biomarker platforms in my isoelectric focusing project.

I would like to thank Culbertson group members Dr. Kathleen Sellens, Dr. Jalal Sadeghi and Jeremiah Shipman for their direct support in my research projects. In addition, technicians Tod Ron, Jim from the chemistry department and Tim from the electronic design lab have assisted in tinkering of the electronic device used in my projects. I would also like to thank other Culbertson group members, Dr. Jay Sibbits, Abby Kreznor, Kushan Kompalage, for the companion and exchange of ideas during my graduate studies.

I would like to thank Karen Li and my parent Shi Zhang and Xiaoli Jia, for their family support during my study. Their love is what supported me through challenging times.

Chapter 1 Introduction

This introductory chapter discusses the fundamental technical background upon which this dissertation is based. General analytical separation techniques and basic chromatography terms are covered in **1.1 Analytical Separations**. Biological analyte separation is a rapidly growing branch in separation science. The advantages and disadvantages of **Electrophoresis and Isoelectric focusing** are discussed in section 1.2. How to apply these tools to overcome challenges faced currently with **enzyme activity assays** are explained in section 1.3.

1.1 Analytical Separations

Separation techniques are commonly used to isolate or purify one or a group of chemicals from a mixture. As a branch of separation science, analytical separations focus on the identification or quantification of interested chemicals(analytes) in mixtures that the direct measurement without separation cannot be readily achieved due to the intrinsically complicated sample matrix. The ability to analyze different samples requires not only the use of separation devices but also detectors that are able to measure analytes to give quantitative information. A variety of common analytical separation methods are gas chromatography(GC), high-performance liquid chromatography(HPLC), thin-layer chromatography(TLC), electrophoresis, and capillary electrophoresis(CE). Among these separation methods, chromatography is a prevalent method for separating complex

samples by passing them through a column with the medium that interacts with analytes and causes them to migrate at different rates. Separation methods are often evaluated using chromatography terms: 1) the ability to resolve closely resembled analytes, 2) the ability to retain separation efficiency in the presence of a complicated sample matrix, and 3) the time and the cost of the assay.

Chemical industries, such as oil refineries and pharmaceuticals, have establishments devoted to characterizing and measuring the chemical composition in their raw materials and intermediates. This process is called quality control (QC). The process of quantification of active contents in the final products is called quality assurance (QA). A typical QC/QA lab often performs separations routinely on a large scale. Companies with QC/QA facilities are willing to purchase expensive and bulky instrument options in exchange for reliability and sample processing speed.

Another branch of analytical separations is to provide ‘personalized’ information on samples that are unique and difficult to obtain, such as medical diagnosis and forensic science. Facilities that provide these services are often smaller in scale and maintain a smaller team compared to QC/QA labs. As such, analytical methods used by these labs are less price-sensitive and cost per sample could be an obstacle when carried out on a larger scale.

Both industrial and academic chromatography applications need to be evaluated in a standardized way. **Separation efficiency** is often evaluated using theoretical plate heights and plate numbers. Plate number depends on column length, making comparisons between different methods complicated. When comparing various chromatographic

methods, plate height (H) is used to describe unit length efficiency to prevent confusion over different column lengths.

$$H = \frac{\sigma^2}{L} \quad (1.1.1)$$

where L is the length of analyte movement(column length), σ^2 is the standard deviation representing the distribution of analytes in the separation zone and H is the length of a single plate, representing the separation method's efficiency.

Plate number is defined as

$$N = \left(\frac{t_R}{\sigma}\right)^2 = 16 \left(\frac{t_R}{w}\right)^2 \quad (1.1.2)$$

where t_R is the retention time of the analyte peak, w is the base peak width (4σ) of the analyte peak.

High plate numbers are always favorable to separation efficiency. Typical HPLC methods offer approximately 10,000 plates, UPLC ~100,000 plates, and capillary electrophoresis even greater than 100,000 plates.

While plate number represents separation efficiency, the separation efficiency (plate number) is limited by column length and other factors determining theoretical plate heights. Challenging separation tasks, such as the separation of chiral molecules, shall focus on the selectivity of separation rather than separation efficiency. Selectivity is a parameter that measures how well one analyte is separated from another.

Separation efficiency and selectivity are the two most common approaches to evaluate the goodness of the chromatographic method. Plate number is a popular way to represent

the efficiency of a chromatographic method in a normalized way to be able to compare different types of separation methods.

The selective separation between two analytes is evaluated by **resolution** (R_s):

$$R_s = \frac{d}{w} \quad (1.1.3)$$

where d is the distance between peak A and peak B. w is the base peak width.

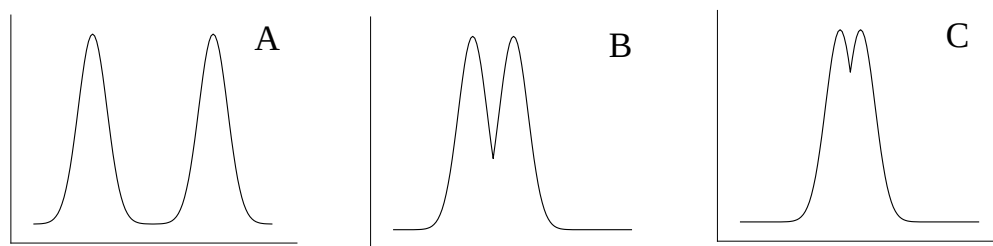


Fig. 1 Baseline separation $R_s > 1.5$ (A) partial separation $R_s \sim 1.2$ (B) and poor separation $R_s \sim 1$ (C)

A resolution of at least 1.5 is necessary for the baseline separation of two analytes.

If the separation efficiency (plate height H) remains constant, the separation distance is proportional to the column length,

$$d \propto L \quad (1.1.4)$$

Combining (1.1.1) and (1.1.4), we can get resolution R_s

$$R_s \propto \frac{L}{\sqrt{L}} = \sqrt{L} \quad (1.1.5)$$

As long as two analytes have different retention factors, it will be possible to separate two analytes with a long enough column. However, in practice, longer column lengths come at the cost of longer elution times, more solvent consumption and stronger band

broadening, which may reduce analyte signals lower than the detection limit of the instrument.

As for analytes of different peak widths, resolution R_s is calculated using the peak widths of each analyte,

$$R_s = \frac{d}{[(w_h)_a + (w_h)_b]/2} \quad (1.1.6)$$

$(w_h)_a$ and $(w_h)_b$ are the base width of peak A and peak B, respectively.

The final measure of column efficiency is the **peak capacity**. In definition, peak capacity represents the number of peaks that can be resolved ($R_s=1$) in a given time. In practice, peak capacity can be estimated by the average peak width of analytes in the separation:

$$\text{Peak capacity} = 1 + \frac{t_g}{\frac{1}{n} \sum_1^n w_p} \quad (1.1.7)$$

The larger the peak capacity, the more analytes could be potentially resolved with the separation method.

1.2 Electrophoresis and isoelectric focusing

Electrophoresis is not a chromatographic method, but both techniques show similarities in separation and are both commonly used for analytical purposes. Electrophoresis is a technique that mobilizes dissolved charged molecules in an electric field and causes the molecules to move towards electrodes of opposite charge. Suspended ions have counter charges surrounding the charged ions to form an electrical double layer. When a uniform electric field is applied, the ions are exposed to an electrostatic force to move them

towards the electrode, corresponding to the charge opposite of the ions. The ions also receive viscous stress from the interaction with counter charge in the electrical double layer and frictional force from the movement in the opposite direction of electrostatic movement. This phenomenon causes ions to move at various velocities. Separation occurs when charged molecules migrate at different velocities.

The force on the ion in an electric field can be expressed as:

$$F_E = \frac{V_{ion}}{E} = \frac{V_{ion}}{\left(\frac{V}{L}\right)} \quad (1.2.1)$$

where V_{ion} is the velocity of analyte ion and E is the electric field strength in volts per centimeter (V/L).

If the analyte ion is assumed to be spherical and follows Stoke's law, the Force on the ion can be expressed as:

$$F_{ion-drag} = \frac{z}{6\pi r\eta} \quad (1.2.2)$$

where z is the number of charges, r is the hydrodynamic radius of the ion, η is the viscosity of the solvent.

When the 2 forces on the ion are equal and opposite, the ion moves at a constant velocity that can be calculated by combining (1.2.1) and (1.2.2), and rearranging to solve for velocity:

$$V_{ion} = \frac{zV}{6\pi r\eta L} \quad (1.2.3)$$

Under a certain electric field (V/L), analytes migrate at different velocities based on the charge to size (hydrated ionic radius) ratio (z/r).

Electroosmosis is another common electrokinetic phenomenon accompanying electrophoresis. The electrical double layer at the interface between the channel wall and the electrolyte offers charged ions that can be mobilized under an electric field (Fig. 2). The movement of these charged ions and the shear viscosity of the solution can force the bulk liquid to flow through the channel. The combination of electroosmotic and electrophoretic movement determines the actual velocity of analyte ions, and hence the separation results.

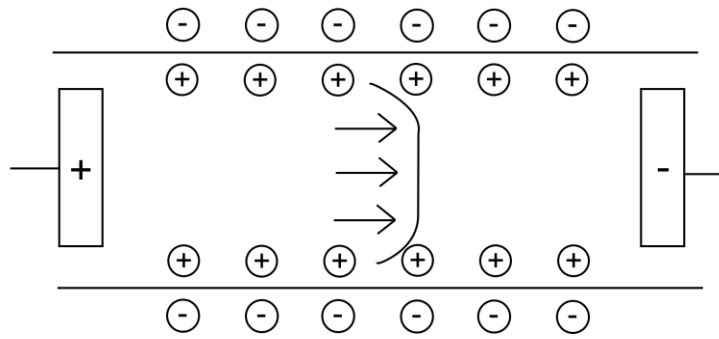


Fig. 2 Electroosmotic flow

Unlike electrophoretic movements, electroosmotic flows are only influenced by the property of the bulk electrolyte. Electroosmotic flow rate V_{eo} can be defined:

$$V_{eo} = u_{eo}E \quad (1.2.4)$$

where E is the electric field strength and u_{eo} is the electroosmotic mobility:

$$u_{eo} = \frac{K\epsilon_0\zeta}{\mu} \quad (1.2.5)$$

where ζ is the zeta potential of the electrical double layer and μ is the dynamic viscosity of the solution and ϵ_0 is the permeability of free space and K is the dielectric constant.

Although most electrophoretic separations involve both electrophoretic and electroosmotic movements, in some fields, such as isoelectric focusing, electroosmosis needs to be manipulated in favor/disfavor of electrophoresis. Since the electric field strength is crucial to both movements, the control of electroosmosis is accomplished via changing the viscosity of the electrolyte or zeta potential at the channel wall (1.2.5). In practice, thickening reagents can be added to increase solution viscosity, and coating reagents can be used to modify interfacial zeta potential.

Electrophoresis was first introduced in 1937 Tiselius's work⁴ about the first laboratory apparatus to electrophoretically separate blood serum proteins. Later in the 60s, Hjerten developed a series of apparatus to achieve open tubular electrophoresis with similarity to modern capillary electrophoresis (CE).⁵ The largest advancement of CE over traditional gel electrophoresis is the high surface-to-volume ratio of the capillary and the resulting extraordinary heat dissipation ability, which allows the deployment of higher electric field strength with higher resolution and shorter separation time. In addition to open tubular CE, micellar electrokinetic chromatography allows the separation of uncharged analytes by adding high concentration surfactant to form pseudostationary micelles around neutral molecules. Also, packed or coated capillary columns have been combined into CE, providing a hybrid separation mode that is effectively a combination of HPLC and CE.

As compared to conventional electrophoresis, isoelectric focusing (IEF) achieves analyte separation based on the different isoelectric points (pI) of zwitterionic molecules. When zwitterionic molecules are exposed to pH's lower than their pI, they will be positively charged, and at pH's higher than their pIs, they will be negatively charged. The pI value

of a zwitterionic molecule is determined by the quantity and the types of acidic/ basic groups.

For a simple zwitterionic molecule with only one acid and one base group, such as a single amino acid, the pI can be calculated as the mean of the pKas of both groups:

$$pI = \frac{pK_a(acid) + pK_a(base)}{2} \quad (1.2.4)$$

For zwitterionic molecules with multiple acidic groups and basic groups, the following equations (1.2.5) and (1.2.6) can be derived from Henderson-Hasselbach equation⁶:

$$\text{Number of positive charges} = \sum_{i=1}^n \frac{-1}{1 + 10^{pKa(acid\ i) - pH}} \quad (1.2.5)$$

$$\text{Number of negative charges} = \sum_{i=1}^n \frac{1}{1 + 10^{pH - pKa(base\ i)}} \quad (1.2.6)$$

The pI value can be solved, with the known pKa of all acidic and basic groups, when the number of positive charges equals the number of negative charges.

For more complicated molecules, such as peptides and proteins, the pKa of carboxyl groups and amine groups can also be influenced by the structure and the sequence.

Theoretical calculation methods have been developed to include the influence from adjacent amino acids, side chain ionizable groups of neighboring amino acids, and sequence of amino acids⁷. The increasing calculation ability of computers has enabled more complicated and accurate predictions of pIs.

For IEF, a stable pH gradient is established across the sample region using carrier ampholytes, which are a series of aliphatic polymers with polyamino-polycarboxylic

acids (Fig. 3)⁸. The carrier ampholytes are ‘sorted out’ under an electric field based on their pIs and this establishes a stable pH gradient from anode to cathode.

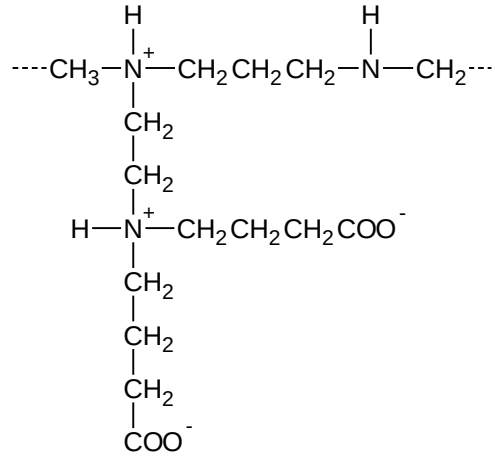


Fig. 3 carrier ampholyte example

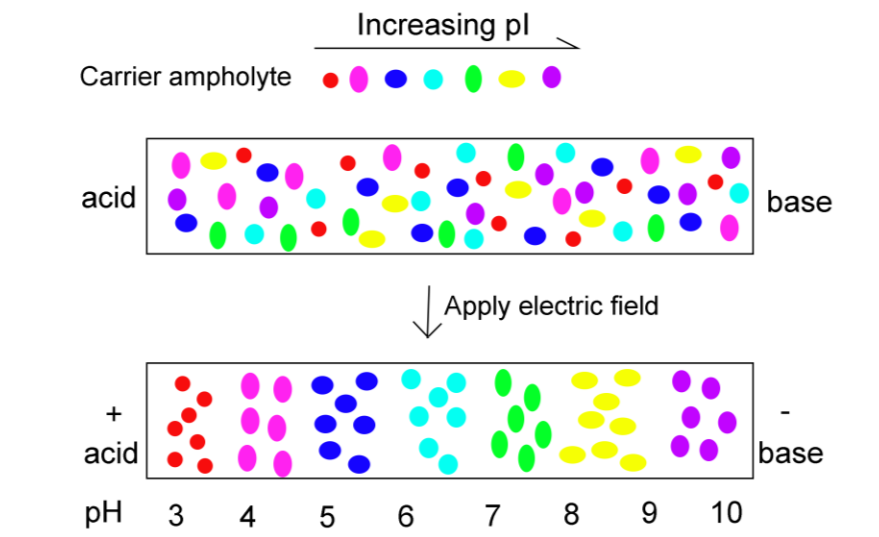


Fig. 4 isoelectric focusing processes

Svensson in 1961 showed an equilibrium analyzing the concentration of an ampholyte between electric mass transfer and diffusion⁹:

$$\frac{C_{ui}}{q\kappa} = D \frac{dC}{dx} \quad (1.2.7)$$

where C is the concentration of the ampholyte, u is the electric mobility of the ampholyte, i is the electric current, q is the cross-sectional area, κ is the local conductivity at the point of interest, D is the diffusion coefficient and x is the distance of the interested point from anode to cathode.

Svensson also showed the distribution of ampholyte followed bell-shaped Gaussian distribution:

$$x_1 = \sqrt{\frac{D}{pE}} \quad (1.2.8)$$

where E is the electric field, and

$$p = -\frac{du}{d(pH)} \frac{d(pH)}{dx} \quad (1.2.9)$$

Since the electric field shall be kept relatively stable across the length of the separation channel (column) (1.2.7) during the focusing, multiple ampholytes shall be used across the pH range, which display zero mobility around their pIs. Also, the ampholytes shall be soluble in water, which makes them easily ionized for focusing movements. The key to IEF is the generation of a stable pH gradient. To achieve this, a special pH buffer consisting of ‘carrier ampholytes’ is used. Carrier ampholytes are a mixture of soluble polymers containing multiple carboxylic groups and amino groups. These polymers display a variety of isoelectric points (pIs) across a range of pH values. The anode will be filled with an acidic buffer (anolyte) and the cathode with a basic buffer (catholyte) that determines the lower and upper limit of the pH gradient. When electric field is applied, the components of the carrier ampholyte will each migrate to its specific pI and participate in pH buffering locally. With hundreds of buffering components scattered

across the separation channel, the pH in the channel forms a gradient that increases progressively from the acidic anode to the basic cathode.¹⁰

When the sample proteins/peptides are introduced to the formed pH gradient, the protein/peptide will carry positive or negative net charges based on the local pH (Fig. 5)¹¹. An electric field is applied to mobilize the positively charged molecules towards the negatively charged electrode and negatively charged molecules towards the positive electrode. Eventually, proteins/peptides can be focused to their isoelectric points where the proteins/peptides carry zero charges and thus have zero mobility in the electric field.

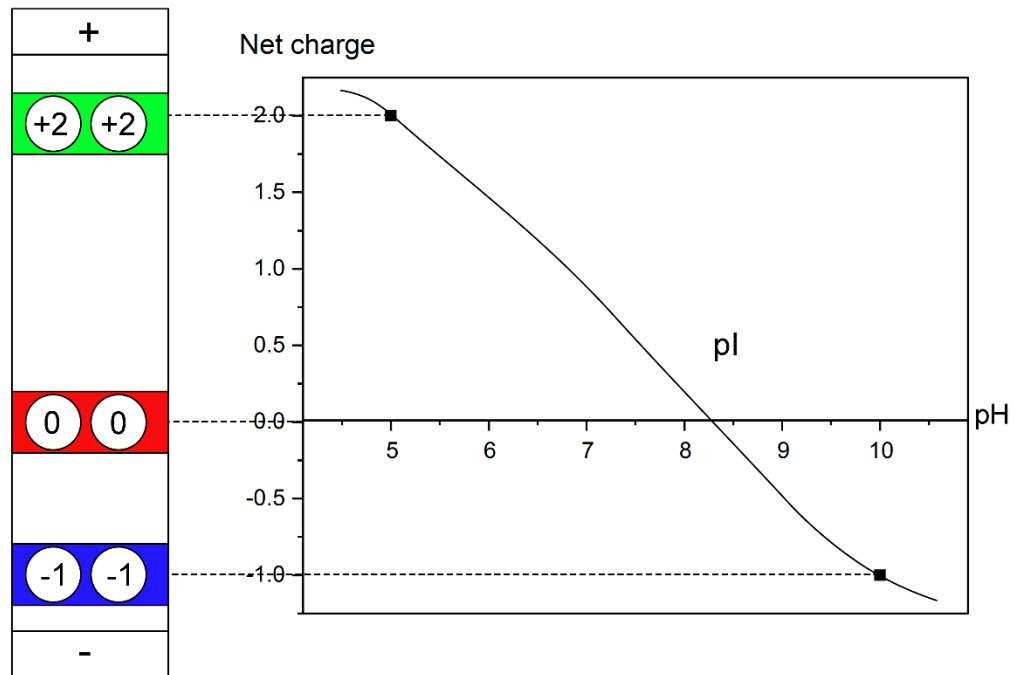


Fig. 5 charge of pI 8.2 peptide in pH gradient

One of the biggest challenges in IEF is electroosmotic flow (EOF) suppression. EOF is the bulk flow generated by the migration of ions in the electrical double layer between fluid and channel wall. EOF is harmful in IEF as the separation resolution weakens when

the focused analytes sustain driving force from EOF. Many IEF applications attempt to address EOF using a covalent coating in capillary IEF¹² and dynamic coating in microchip IEF¹³.

Several strategies based on gel IEF were used to further improve cIEF and mIEF. High local concentrations of proteins during focusing can lead to analyte precipitation. The precipitation can be alleviated by adding non-ionic detergents to increase protein solubility at their pIs.¹⁴ Cathodic drift is another phenomenon affecting separation resolution and reproducibility. To address this, anticonvective reagents such as methylcellulose can be added to the carrier ampholyte solution.

Capillary isoelectric focusing (cIEF) represents one of the highest resolving power separation techniques. Compared to classic gel electrophoresis, cIEF allows for higher resolution, shorter analysis time, and higher sensitivity if coupled with fluorescence detection. The biggest structural difference between gel IEF and cIEF is the use of fused silica capillary in cIEF, which offers great heat dissipation, making higher electric field strength possible. For intact protein screening, a minimum pI difference of 0.004 has been resolved while an average resolution supported a peak capacity of over 800.¹⁵ With proper coating on the capillary inner wall, peptide samples could be concentrated about 500-fold during focusing.¹⁶ Similar to gel IEF, the separation power of single dimension IEF could be further improved when coupled to another separation method, for example, capillary electrophoresis or mass spectrometry. cIEF-ESI-MS was used to analyze the tryptic digestion product of Bovine Serum A. 24 peptide fragments were identified with a limit of detection of Bovine Serum A at 7 nM.¹⁷

Microchip IEF (mIEF) is a competing technique for capillary IEF. mIEF has the following advantages: 1) instrument miniaturization; 2) short analysis times; 3) the ability to couple to separation and detection methods. One group reported a miniature of cIEF using a thermoplastic microfluidic chip.¹⁸ For a mere 4.7 cm long separation channel, a theoretical plate number of 1.5×10^5 plates could be achieved within several minutes, the plate number of which was comparable to cIEF and much higher than most traditional column-based chromatography. Full mIEF setup including the power supply and detection system can be packed inside the size of a shoe box. Besides miniaturized sizes, due to the short channel length and high electric field, focusing time of mIEF is about several minutes, which is much shorter than a common twenty minutes for the cIEF method. 2D separation with mIEF as the first dimension is a popular area. Isoelectric point-based separation can provide some qualitative information on the separated proteins. Preconcentration of proteins often reached 100-fold or more. Proteins collected from mIEF are highly concentrated, allowing space for dilution benefiting techniques like Mass Spectrometry.¹⁹ Facile separation and fractionation are also compatible with on-chip capillary electrophoresis, off-chip gel electrophoresis and chromatography.

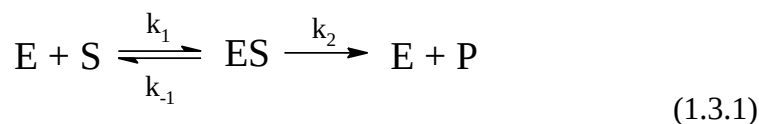
1.3 Enzyme activity assay

1.3.1 enzymes and substrates

Enzymes are bioactive macromolecules catalyzing specific biochemical reactions. In a relatively stable biological system where various delicate balances are equilibrated, and

biochemical reaction rates are often tightly controlled. Throughout the evolution of living organisms, these enzymes played essential roles in the kinetics of many biological processes. Studying the kinetic properties of these enzymes helps us understand the workflow of our body, allows us to determine when a system is no longer functioning properly and provides clues for the development of drugs that modulate critical biochemical reactions.

In the aspect of catalysis, enzymes are no different compared to other chemical catalysts. In the simplified model of an enzyme-catalyzed reaction (2-1), hypothetically, an enzyme molecule (E) first reacts with the reactant, or substrates (S) in enzyme reaction terminology, to form an enzyme-substrate complex (ES), then the complex further reacts to yield the final product (P) and releases the enzyme (E). As the quantity of enzyme remains unchanged, the enzyme serves as catalyst.



Similar to inorganic and organic catalysts, enzymes offer an alternative reaction path by forming low energy barrier intermediate products. In the discussed enzyme reaction model, the reaction rate constant (K) is determined by coefficient (A), the temperature of the reaction (T) and the activation energy (E_A):

$$K = Ae^{-E_A/(RT)} \quad (1.3.2)$$

Enzyme activity is defined as the catalytic ability of the enzyme. Enzyme activity is a comprehensive result of reaction rate coefficients and enzyme concentrations. Enzyme catalytic abilities are often described by using substrate concentration change per unit

time under catalysis. Although some techniques are able to determine the concentrations of enzymes, for example Mass Spectrometry and Gel Electrophoresis, these concentrations are not directly related to activity due to the existence of inactive state enzymes, such as pseudoenzymes²⁰, and other activity affecting factors, such as temperature, pH or the presence and concentration of coenzymes and cofactors. Activity is defined as the catalytic ability of active enzymes. The quantification of enzyme activity is based on the quantitative information of the reaction from substrate to product. Compared to enzyme concentrations, activities focus on the actual catalytic ability rather than the absolute quantity of the enzymes in a given sample.

However, due to the spectral overlapping of labeling components, spectrochemical detection can only monitor one enzyme reaction at a time.

In the simplified model (1.3.1), the initial reaction rate (V_0) follows the Michaelis-Menten equation:

$$V_0 = \frac{k_2[E][S]}{K_M + [S]}, \quad (K_M = \frac{k_{-1} + k_2}{k_1}) \quad (1.3.3)$$

Enzyme activity $[E]$ can be determined by building a simple linear correlation with initial reaction rate. At the beginning of a reaction when substrate concentration $[S]$ term $\frac{[S]}{K_M + [S]}$ remains largely constant, (1.3.3) can be interpreted as $v_0 = k[E]$. Unknown enzyme activity $[E]$ can be extrapolated from a linear calibration curve of v_0 against $[E]$.

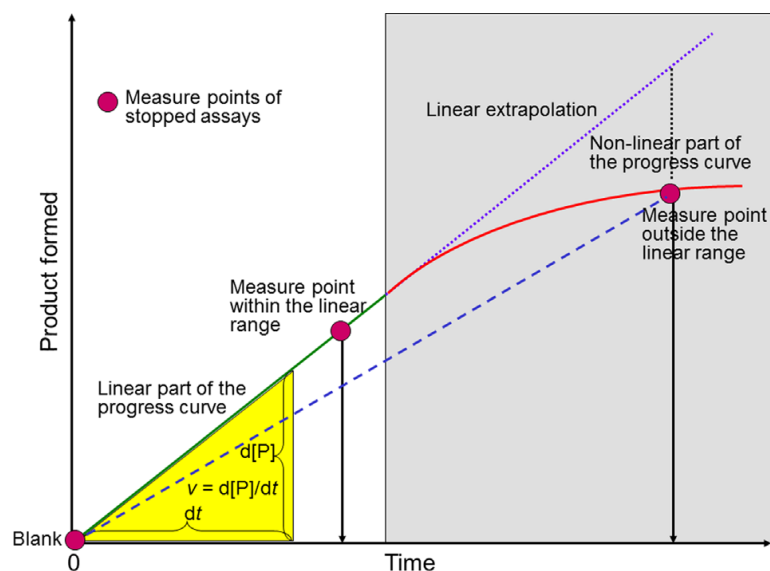


Fig. 6 Product concentration over time in condition $[S] \gg K_M$. ²¹

Practical analysis of enzyme activities involves two important factors. The first one is **the control of enzyme reaction time**. On one hand, the final concentration of reaction products must be higher than the detection limit within a given reaction time. Longer reaction times allow more reaction to be catalyzed per unit concentration enzyme and therefore increase activity determination sensitivity. On the other hand, however, reaction rates measured at long reaction times deviate from the constant **initial reaction rate** zone (green line in Fig. 6), which is the key area where to perform enzyme activity determination. The initial reaction rate, according to the Michaelis-Menten equation (2-3a), is proportional to enzyme activity $[E]$. With short reaction time (green line in Fig. 6), the reaction rate approximates initial reaction rate and product concentration follows a linear trend over time. The initial reaction rate can be calculated simply by dividing the product concentration by the reaction time. At long reaction times, reaction rate is affected by a decrease in the concentration of substrate and, therefore, deviates from initial reaction rate (red line in Fig. 6). To summarize, the reaction time has to be 1) long

enough to build up product concentration into the range of quantification and 2) short enough to keep reaction rate constant 3) can be interpreted as initial reaction rate.

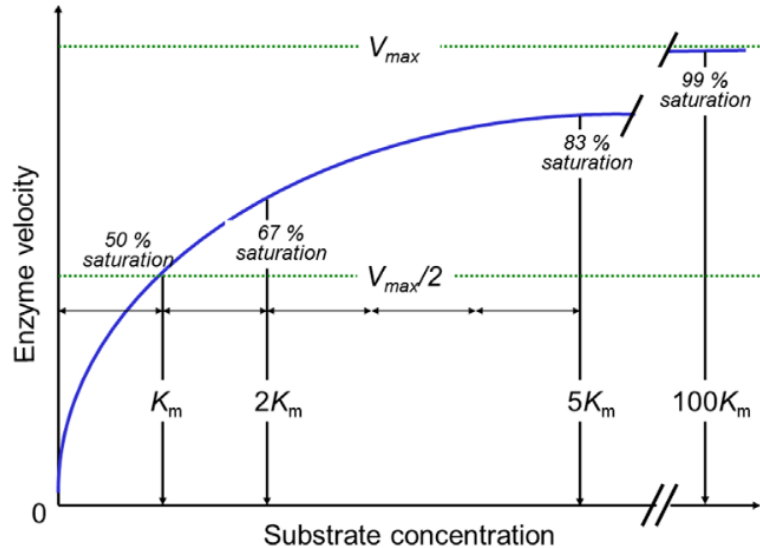


Fig. 7 Theoretical relation between substrate concentration and reaction rate²¹

The second consideration is **the factors that influence the measurement of initial reaction rate, especially substrate concentrations**. Briefly mentioned in the first problem, the **initial reaction rate** is calculated by dividing the product concentration by reaction time inside the constant reaction rate area at the beginning of the enzyme reaction (green line in Fig. 5). As such, a constantly changing reaction rate can lead to an erroneous result in determining the initial reaction rate. As shown in (Fig. 7), reaction rates are more stable at high substrate concentrations rather than at low substrate concentrations. If low substrate concentrations are used in the analysis, the reaction rate would drastically change and deviate from **initial reaction rate** (go into red line in Fig. 6). Another benefit from using an excessive amount of substrate is that the initial reaction rate can be simplified to $v_0 = k_2[E]$, which is totally substrate concentration-independent.

In summary, high substrate concentrations are beneficial for the initial reaction rate measurement and activity determination.

To conclude, the two major methods for enzyme activity determinations are: continuous monitoring and stop reaction measurement. A common method is to utilize the constant reaction rate region where enzyme activity is proportional to the initial reaction rate. In continuous monitoring like fluorescence or absorbance monitoring, substrates are kept at relatively high concentrations to allow one to extrapolate the linear region. In stop reaction measurements, such as Mass Spectroscopy and Gel Electrophoresis, substrates are added at high concentrations and the reaction time is kept as short as possible.

1.3.2 Proteinase activity assay methods

Briefly discussed in the previous section, enzyme activities are measured via the quantification of the consumption of substrates or the production of reacted products. Any detection methods that are capable of the quantification of substrates or products could be categorized as ‘enzyme activity assays’. Depending on how the data is collected, enzyme assays can be split into continuous assays or non-continuous assays. Continuous assays allow continuous activity monitoring as the enzyme reaction progresses, while non-continuous assays involve stopping the reaction and measuring the sample. Continuous monitoring methods are often optical detection methods like colorimetry, fluorescence, chemiluminescence. These detection methods offers real-time responses from the enzymatic reaction and, therefore, can provide kinetic information within the sample²². Non-continuous monitoring methods are single-point detection methods that

might be irreversibly destructive to enzyme activities. Common non-continuous monitoring techniques are separation-based chromatography or gel electrophoresis.

Among all types of enzymes, proteinases are enzymes that catalyze the hydrolysis of peptides or proteins and break them into smaller peptides. For example, some proteinases like Matrix Metalloproteinases (MMPs) can break down extracellular matrix proteins for other physiological processes. Two common methods to measure proteinase activities are zymography and fluorescence resonance energy transfer (FRET) assays.

In **zymography**, protein substrates are digested with proteinases for a certain reaction time, after which the concentration of the substrates or the digested products are analyzed with separation techniques like HPLC or gel electrophoresis. To measure proteinase activities with gel electrophoresis, the gel is cast with mixtures of digestible substrates. The proteinases from tested samples are separated electrophoretically, and then the separated proteinases are allowed to incubate and react with the local substrates. After the catalyzed reaction is completed, all remaining substrates are stained for optical detection or imaging. This method relies heavily on the separation quality of gel electrophoresis and can only offer semi-quantitative information on proteinase activities. Some works have demonstrated the separation of the latent forms and the active forms of MMP-2 and MMP-9 and showed quantification of the reacted gelatin in the gel.^{23,24} Zymography enables semi-quantitative activity measurement of multiple MMPs, which is beneficial to studies of complex samples containing multiple active proteinases. Detection limits of MMP-2 and MMP-9 can reach 5 pg per 10 μ L total sample ($\sim 10^{-9}$) with the inclusion of several simple pre-purification steps.²⁵ Conversely, zymography can not fully mimic the activation/inhibition conditions in a sample since the tested MMPs are separated from

their activators and inhibitors during gel electrophoresis. This results in the tested sample being in an activation/inhibition-free zone and hence greatly limits the selection of samples, thus yielding little information of actual MMP activities in many situations. Zymography has been mainly used to study the activation and inhibition interaction under theoretical conditions rather than in real samples.

The second common method that assays MMP activity is **fluorescence resonance energy transfer (FRET)**. This method involves engineering a digestible peptide that is chemically attached with both donor fluorophores and acceptor quenchers (Fig. 8). On such fluorescent peptides, the donor and the acceptor are incorporated on opposite sides of the scissile bond at a short distance from each other, allowing high efficiency energy transfer. When excited, donor fluorophores emit undetectable virtual photons that are instantly absorbed by the acceptor, leading to fluorescence quenching. Due to this mechanism, a successful FRET pair requires the emission spectrum of donor fluorophore to overlap with the absorption spectrum of acceptor fluorophore.²⁶ Upon any cleavage of peptide bonds connecting the donor with the acceptor, the physical distance between the donor and the acceptor increases and quenching effect no longer exists.

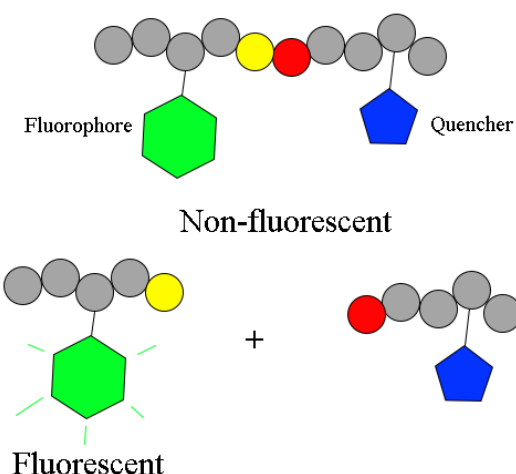


Fig. 8 FRET mechanism

The specificity of a FRET substrate is crucial if the activity of a single type of MMP is desired. Unlike serine proteases which have clear recognition mechanisms, the MMP family displays low specificity towards various peptide sequences²⁷. Measuring the degradation of one fluorescent substrate can only profile the ‘total MMP activity’ instead of the activity contributed by a single type of MMP. Many attempts have been made to understand the recognition mechanism and apply that to design high selectivity substrates. One early work, which is commercially available today, reported a substrate with a high k_{cat}/k_m value (selectivity) of $619,000 \text{ M}^{-1}\text{s}^{-1}$ for MMP-2, 3-fold higher than that of MMP-9, 15-fold higher than MMP-3 and 30-fold higher than MMP-1.²⁸ Another work reported that a large set of substrate sequences were tested and selected for MMP-16 and MMP-17 transmutation specificity comparisons. The optimal substrate sequence was picked from about 10,000 cleavage measurements and achieved over 50-fold reaction rate coefficient (k) difference.²⁹ Broadband specificity over the whole MMP family has always been a challenging topic. One group set up a small library of substrates for 6 MMPs and analyzed the amino acid preference at different positions of the

substrate.³⁰ Based on this result, substrate peptides were synthesized carrying the consensus motif of a specific MMP and tested for their actual specificity. Among six predicted consensus peptides, MMP-2 consensus sequence had a k_{cat}/k_m value for MMP-2 8-fold higher than the highest k_{cat}/k_m of other MMPs.

Besides low specificity of the assays, only one type of assay can be used in one sample due to the distribution of emission spectrum and limited spectral space. The use of multiple assays often leads to emission spectra overlapping and requires complicated experimental efforts to compensate for the mixed fluorescence signal. As only one assay can be used and the assays show low specificity, the applications of fluorescence-based assays are limited to samples with simple compositions of MMPs. As a separation-based method, zymography, which can determine activities of multiple MMPs in a single experiment, while fluorescence-based assays require a vast number of parallel experiments with different substrates that are specific to the tested MMP type. As such, fluorescence-based assays often find application in activity measurements of a single MMP type that is most meaningful in carcinogenesis, for example MMP-2³¹ and MMP-9³².

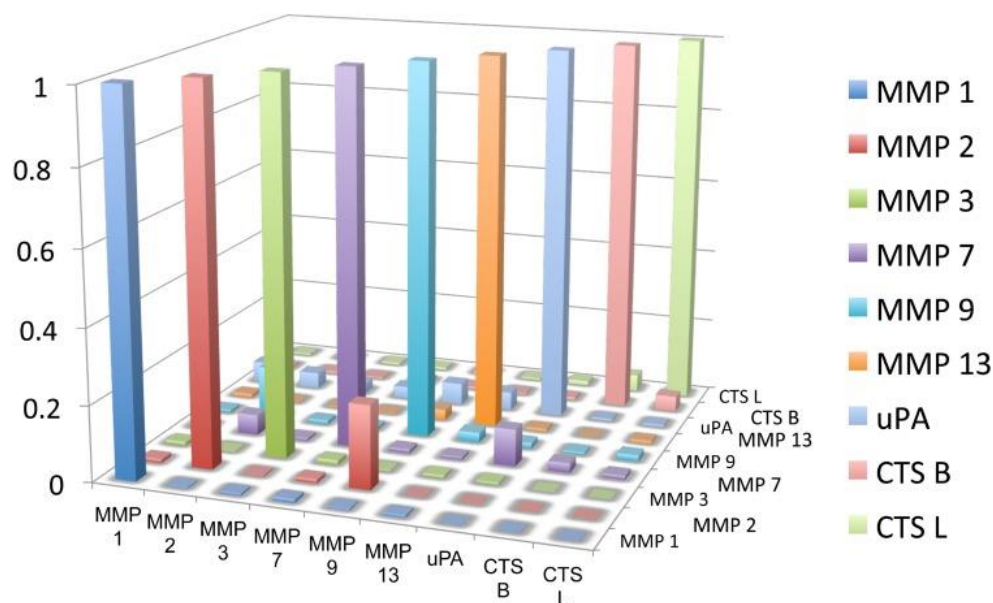


Fig. 9 Sensitivity of matrix proteinases on consensus sequence substrates³³ (highest signal is scaled to 100% for each enzyme)

Since MMPs are low specificity proteinases, to reduce the obscureness, high specificity peptide substrates need to be used. Bossmann et al. have designed and synthesized a series of peptide substrates linked to nanoparticles that are of high specificity for specific MMPs.³³ The increases of fluorescence signal were measured after coincubation of one type of MMP and one type of substrate(Fig. 9). MMP 1, MMP 3, MMP 7, MMP 9 and MMP 13 substrates showed high selective response towards their targeted enzyme. These high specificity substrates allow interpretation of individual MMP activities in mixed MMP samples.

1.4 Conclusion

In subsequent chapters, I will present the development of microfluidic devices for early cancer detection. Chapter 2 discusses the merit of cancer early detection and explores potential techniques that can improve diagnostic accuracy and reduce testing expenses.

Chapter 3 reviews the advancement of microfluidic devices and their application in cancer early detection. Chapter 4 presents the development of microfluidic isoelectric focusing and integrated scanning laser-induced fluorescence detector capable of peptide quantification. Chapter 5 describes the application of microfluidic isoelectric focusing device in enzyme activities assays. Chapter 6 discusses the future outlook for the microfluidic isoelectric focusing project.

Chapter 2 Cancer early detection and biomarkers

2.1 Introduction

Despite decades of effort dedicated to cancer diagnosis and treatment, by 2020 cancer contributed the second highest most mortality with COVID-19 ranked the third³⁴.

Cancers refer to a series of diseases that involve the mutation of normal cells, their malignant growth, spreading to other organs and there forming secondary tumors.

Although various mutations are happening in our body every day, some studies have found groups of genes that give an edge to the proliferation of cancer cells over normal cells^{35,36}. Compared to normal cells, cancer cells display six different characteristics: limitless proliferation potential, evading programmed cell death(apoptosis), self-sufficient growth signals, insensitivity against anti-growth signals, promoted angiogenesis, invasive spreading and metastasis³⁷.

The metastatic nature of cancer cells is one of the main reasons why some types of cancer can be difficult to treat. When cancerous mutations occur at primary sites that are insensitive to pain or pressure, cancer cells can grow and metastasize asymptotically until patients show symptoms at a later stage. For late-stage cancers, not only the primary tumor needs treatment, but tumors from metastasized sites also need to be eliminated. Once cancer cells metastasize, medical providers face increased difficulty to surgically remove all the tumors, which can also be highly risky for the elderly and those with weaker recovery abilities^{38,39}. Moreover, secondary tumors can be too small for detection

and untreated tumor sites may cause recurrence. Treatments are more efficient at earlier stages when metastasis is limited (Fig. 10)⁴⁰.

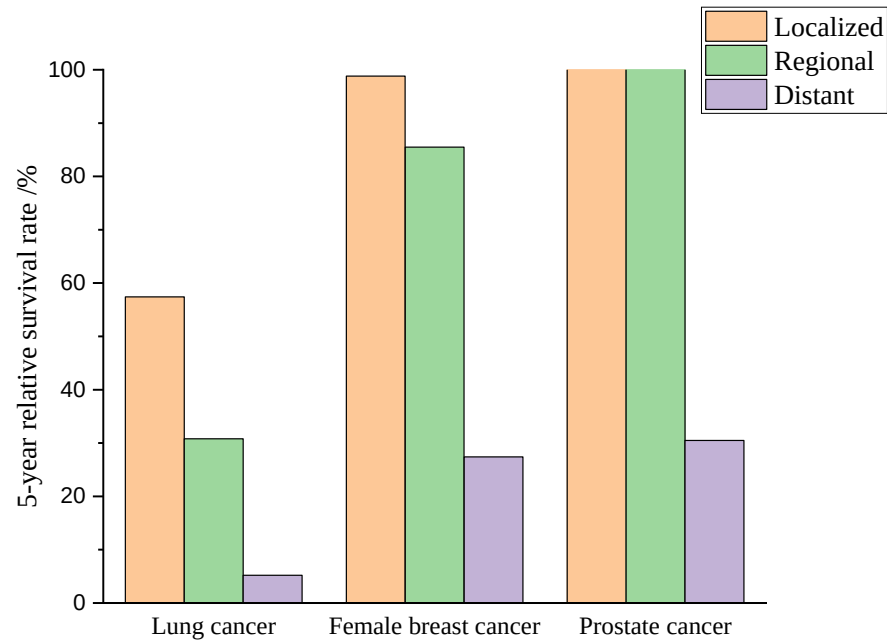


Fig. 10 5-year survival rate by stage at diagnosis (100% relative to in-situ)⁴⁰

Depending on the progress of metastasis to nearby tissues or distant organs, all types of cancer can also be categorized into four stages: in-situ, localized, regional and distant metastasis. In-situ stage includes the appearance of mutated cancerous tissue without any spreading. Localized stage includes the formation of a primary tumor without any spreading to nearby tissues. Regional stage includes the formation of primary tumor and the spreading to nearby tissues or organs. Distant stage includes the tumor and its spreading to distant tissues or organs.

2.2 Detection methods and cancer biomarkers

To improve the overall survival rate, a successful early diagnostic method must be sensitive, reliable, low-cost and relatively harmless to patients. Due to increasing demands of early detection, government and companies are making heavy investments in research projects to introduce new diagnostic tools that can detect asymptomatic cancers in early stages. For early detection purposes, one important factor society values is the accessibility of a diagnostic method to high-risk individuals. Expensive test fees and aggressive sampling methods, such as the surgical removal of tissues for examination, would inevitably translate into low demands.

Two major routes to achieve early detection are imaging-based tests and chemical signal based assays. Chemical-based diagnostic tools focus on the detection of biomarkers released during tumor development and metastasis, while imaging methods focus on the discovery of tumorous sites in the body. Both routes have witnessed great commercial applications over the past decades and people can even find some of these tests in a regular annual body checkup. Typical commercial imaging tools include standard X-ray scans, X-ray computer tomography (CT) scans, magnetic resonance imaging (MRI) scans and ultrasounds. CT scans, for example, measure the X-ray attenuation in the tissues as the X-rays pass through the body to generate images based on different tissue densities, which can be used to distinguish malignancy from normal tissues. Tumors ranging from millimeter to centimeter sizes can be imaged using X-ray CT scans^{41,42}. While imaging techniques like CT or MRI can be powerful early detection tools, these tests require expensive instrumentation, causing costly test fees, like hundreds of dollars for a single

scan on a certain organ⁴³. Therefore, lower cost chemical assays are under surging demands in the cancer early detection market.

Chemical assays depend on the quantification or semi-quantification of biomarkers to specific malignant processes. These biomarkers are **mutated genes** or **proteins** secreted by cancer cells or other normal cells in response to tumor development. Although most biomarkers are concentrated at the tumor sites, some are found leaked into the circulatory system, allowing diagnostic methods based on blood or other body fluids samples. Cancer cells can also be used as biomarkers as circulating cancer cells have the ability to metastasize in distant organs via blood circulation.

Table. 1 List of common tumor biomarkers

Biomarker	Cancer type	Sample	Refence
Alpha-fetoprotein (AFP)	Liver and germ cell cancer	Blood	44
CD117	gastrointestinal stromal tumors, acute myeloid leukemia	Blood	45,46
CA-125	Ovarian	Blood	47
CD19	B-cell lymphomas and leukemias	Blood	48
Chromogranin A	Neuroendocrine tumors	Blood	49
5-HIAA	Neuroendocrine Tumor	Urine	50
Neuron-specific enolase (NSE)	Small cell lung cancer	Blood	51

Genetic biomarker tests screen for inheritable or altered genes to evaluate a person's susceptibility to cancer. Inheritable cancers contribute 3-10% of all cancers. Test results can provide guidance to conduct more specific tests or to adjust daily habits for reduced cancer risks. Gene biomarkers are often analyzed using real-time polymerase chain reactions (RT-PCR)⁵³, fluorescence in-situ hybridization (FISH)⁵⁴ and DNA sequencing⁵⁵. Genetic testing only shows specific genes risky for tumor development but not necessarily detect an actual malignancy.

2.3 Matrix metalloproteinases

Matrix Metalloproteinases (MMPs) are a family of extracellular enzymes that target extracellular matrix components (ECM) and regulate tissue degradation in tissue remodeling, wound healing, and immune response. Elevated expressions of MMPs are also found in cancer development, especially at metastasis stages.⁵⁶ A better understanding of MMP activities in a specific organ or tissue will allow detection of early-stage cancer and evaluation of tumor malignancy.

Twenty-three different types of MMPs have been discovered in humans. MMP enzymes are initially secreted as inactive proenzymes and later activated via the cleavage of stabilizing bonds. The family of MMPs is not only categorized by their proteolytic ability in ECM but also by their similar structures. Despite minor structural differences, like

domains for transmembrane anchors, most intact MMPs share several conserved structural components: signal peptide, propeptide domain, catalytic domain and hemopexin-like domain. Signal peptides, commonly found in secretory proteins, contribute to protein recognition for various secretory pathways and are removed after protein translocation.⁵⁷ The propeptide domain in the proMMP suppresses proteolytic activities via a 'Cysteine Switch'. The cysteine residue in the propeptide interacts with Zn^{2+} ions in the catalytic domain and forms a stabilizing conformation that blocks the active site. During activation, the propeptide domain containing the 'Cysteine Switch' is disrupted by external proteinases or autocleavage, resulting in an increase in MMP activity.⁵⁸ The catalytic domain consists of a Zn^{2+} ion, a Ca^{2+} ion and substrate binding sites. Upon activation, the Zn^{2+} ion is released from the 'Cysteine Switch' and bound with one water molecule. The active site water molecule attacks the scissile bond of polarized carbonyl group on the peptide substrate for catalysis.⁵⁹ The hemopexin-like domain is related to substrate recognition, especially for collagen degradation.⁶⁰ MMPs missing this recognition domain display activity towards a broad range of substrates and lack the catalytic ability to degrade triple helical collagen.⁶¹

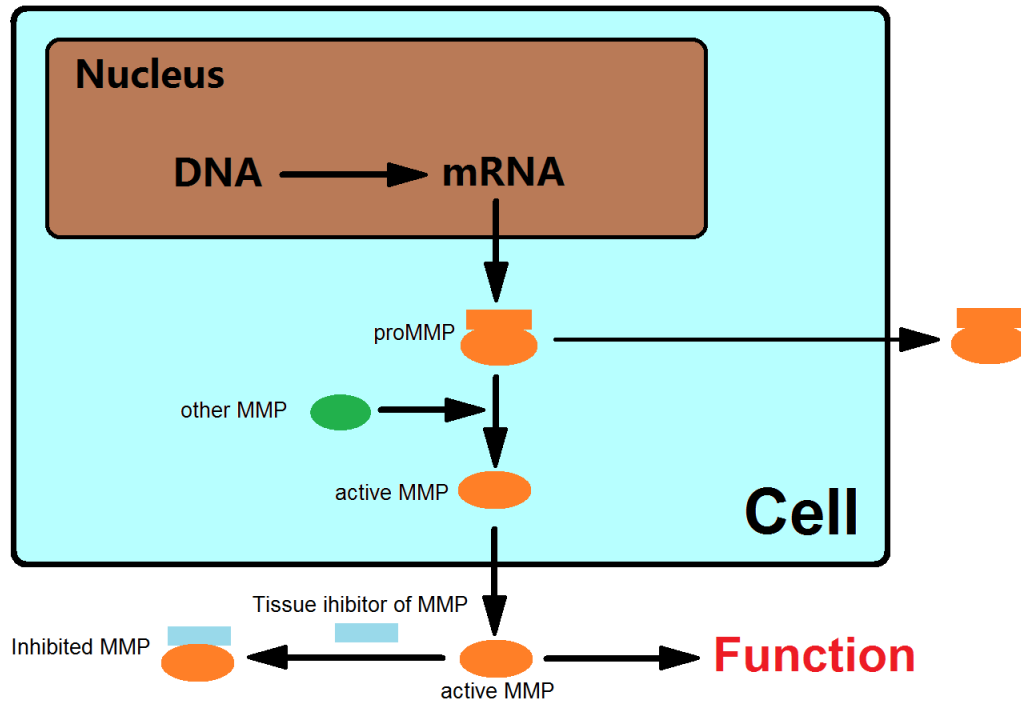


Fig. 11 Expression, activation and inhibition of MMPs

In carcinogenesis, MMP activities have been reported to be closely related to metastasis, which helps to spread malignant cells to distant organs and induce new tumors. The expression of MMPs is upregulated not only in tumor cells but surrounding immune cells (i.e., macrophages) and stromal cells. These overexpressed MMPs assist metastasis in several ways: 1) degradation of physical tissue barriers limiting tumor growth; 2) cleavage of malignant cell apoptosis signal receptors; 3) facilitation of tumor supporting angiogenesis; 4) reduction of cell adhering components and facilitation of tumor cell invasion.

Although MMPs are widely distributed in diverse tissues, their matrix-digesting catalytic abilities are carefully regulated via transcription, expression and extracellular inhibition. Understanding the actual activities of MMPs can provide insight into the development of pathologic tissue matrix degradation.

2.4 Conclusions

Detecting cancers early allows treatment before they have the chance to grow and spread. Current cancer screening methods include regular physical check-ups, CT scans and chemical biomarkers assays on high risk populations. Biomarkers are used to screen healthy populations for signs of malignancy, determine types of malignancy, assist prognosis and guide further treatment plans. Matrix metalloproteinases are interesting cancer biomarkers that have not been studied intensively compared to other biomarkers. New detection and separation methods are needed before MMPs can be analyzed at clinical level.

Chapter 3 Integrated microfluidic isoelectric focusing and scanning laser-induced fluorescence detection for peptide separation

3.1 Introduction

Isoelectric focusing (IEF) is a powerful separation technique for protein or peptide analysis. Although the first IEF instrument was based on open tubular design⁴, IEF matured as a separation tool when performed on a gel similar to gel electrophoresis^{62,63}. One important distinction between IEF and gel electrophoresis is that IEF separates analytes based on isoelectric points rather than mass to charge ratio as in gel electrophoresis. IEF not only offers a different separation mechanism but it can be coupled with gel electrophoresis to perform multi-dimension separation for complicated protein mixture samples^{64,65}. Proteins or peptides containing multiple amino acids often display different isoelectric points due to the difference in the numbers of amino acids and charges. This is especially useful for the separation of small peptides/proteins whose low molecular weights cause excessive migration and therefore poor separation resolution in gel electrophoresis. Separation resolution of IEF is mainly determined by the pH gradient. Two common pH gradient generating reagents are carrier ampholytes and immobilized pH gradient gels^{66,67}. While immobilized pH gradient are limited to gel

IEF⁶⁸, carrier ampholytes can be used in both gel and free flow devices in the capillary and microfluidic formats.^{69,70}

In free flow devices, electroosmotic flow is detrimental to the focusing of peptides and proteins. When an electric field is applied, electroosmotic flow (EOF) is generated and analytes are slowly driven by the EOF towards the anode instead of being focused at a fixed position. EOF suppression methods in free flow device have been studied intensively, especially in the area of applying surface modifications that reduce wall surface charges (ζ potential), which is proportional to EOF^{71,72}. To reduce the ζ potential, polymeric detergents can be coated onto the channel walls to suppress EOF. Both permanent covalent bonding coatings and temporary adsorption based coatings can provide significant EOF suppression^{73,74}. But temporary coatings excel at stability and reproducibility. One popular temporary coating is linear polyacrylamide (LPA) which not only suppresses EOF but also reduces protein adsorption onto channel walls⁷⁵. Other polymer coatings such as polyethylene oxide (PEO) have been investigated for improved EOF suppression, pH stability and coating lifetime^{76,77}. Methylcellulose in particular offers stability over a pH range of 2-12 and displays resilience over 135 IEF runs⁷⁸. In addition to lower surface charge at the channel wall, methylcellulose at high concentrations increases sample viscosity, which further suppresses EOF and also suppresses passive convection of focused proteins.

Microfluidic devices have many amazing applications in analytical separations in that they can integrate fluidic control, mixing, reaction and detection into one device⁷⁹.

Detection components in microfluidic devices often fall into three categories: 1) Optical detection, 2) electrochemical detection, 3) mass spectrometry⁸⁰. While mass spectrometry

requires bulky instrumentation, optical and electrochemical detections can be miniaturized and integrated into a portable sized workstation while retaining low below detection limits. Even detection at the single-molecule level has been demonstrated using laser-induced fluorescence (LIF) detection in integrated microfluidic devices⁸¹⁻⁸³. LIF detectors can be built with low-cost mini-sized optics commercially available due to the CD and DVD reader industry, which makes LIF the ideal detector for our IEF project. Since analytes in IEF are focused to different positions based on their isoelectric points in the pH gradient, LIF detection could be applied using two basic design concepts: single point LIF (Fig. 12 A) or whole column imaging (Fig. 12 B)^{84,85}. Although whole column imaging enables signal collection over the whole column, single point detection offers higher detection sensitivities. Single point LIF design was selected and we built a mobilization platform to allow scanning of the microfluidic chip.

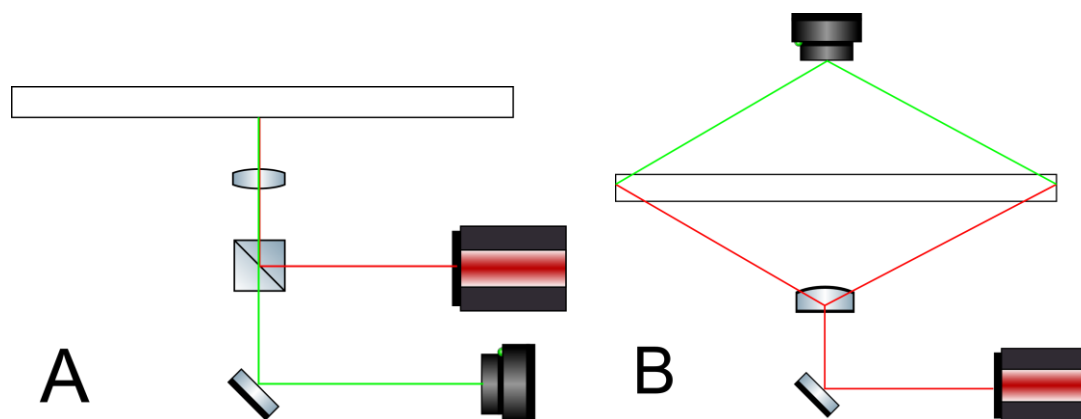


Fig. 12 single point LIF (A) and whole column imaging (B)

In this chapter, the development of microchip isoelectric focusing devices with laser-induced fluorescence detection is reported. The microchip is fabricated with

polydimethylsiloxane via soft lithography and with glass that is etched to create patterned channels.

3.2 Materials and methods

3.2.1 Reagents and materials

Isoelectric focusing reagents: Arginine, methylcellulose viscosity 1500cp were purchased from Sigma-Aldrich (St. Louis, MO, USA), Brij 35 was purchased from ThermoFisher Scientific, Servalytes 3-6, 3-10 were purchased from Biophoretics (Sparks, NV, USA), deionized water was collected from a Barnstead E-pure water purification system (Lake Balboa, CA, USA).

PDMS was cast using a Dow Corning Sylgard 184 Silicone elastomer kit clear that was purchased from Fisher Scientific, SU-2010 photoresist was purchased from MicroChem Corp, 1-methoxy-2-propanol acetate (Sigma Aldrich), isopropyl alcohol (Fisher Scientific), AZ photoresist P4620 (Integrated micro materials, TX, USA), AZ developer (Integrated micro materials, TX, USA), plain glass slides(Fisher Scientific), Buffered hydrofluoric acid (Transene Company, MA, USA), calcium gluconate gel(Calgonate, FL, USA), 100mm diameter silicon wafers (University Wafers Inc, MA, USA), laser printed photomask was ordered and printed by Fineline Imaging (CO, USA).

IEF setup components: stepper motor 28BYJ-48, ULN2003 stepper motor driver, 5V DC power supply, breadboard were included in an Arduino kit Elegoo UNO R3 starter kit, stepper motor driving rod setup, flexible shaft universal joint, photomultiplier tube (PMT) TypeH10722-20 (Hamamatsu Corp, NJ), high voltage power supply Ultravolt

HV-RACK-4-250-00400, NI USB6002 data acquisition device (National Instruments), Labview control program.

3D printer: Makergear M2 and PLA printing filament Ingeo biopolymer were purchased from MakerGear (Beachwood, OH, USA)

Gel electrophoresis: Gel tank Xcell Surelock Mini cell, Novex pH 3-10 IEF gel and buffer were purchased from Sigma Aldrich. Fluorescence pictures of focused samples were taken with a Samsung Galaxy A31 cellphone.

3.2.2 Microchip fabrication

3.2.2.1 PDMS microchip fabrication:

PDMS chips were fabricated with patterned channels in the PDMS layer which is coupled to a plain glass slide as backing. The PDMS layer was fabricated via standard soft lithography methods. In short, a master mold was created using photoresist epoxy SU-8 2010 on a bare silicon wafer. 1mL of SU-8 2010 was applied to a clean silicon wafer and spin-coated at 2000 rpm for 32 s. The coated wafer was pre-baked at 95°C for 3 min. After the wafer cooled down to room temperature, the patterned photomask film (Fig .13) was placed directly on top of the coated wafer and a glass block was placed over the photomask to ensure the photomask was in close contact with the SU-8 surface. The whole device was exposed to UV light for a dosage intensity of 150 mJ/cm². The exposed SU-8 wafer was post-baked at 95°C for four min. After the wafer cooled down, the SU-8 wafer was developed with 1-methoxy-2-propanol acetate to remove unwanted SU-8. The SU-8 master mold was ready after rinsing with isopropyl alcohol and dried.

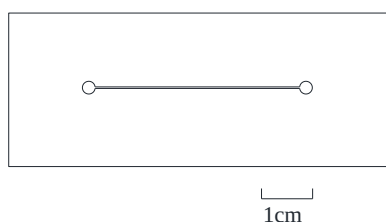


Fig. 13 Soft lithography photomask

The PDMS chips were prepared using the Dow Corning Sylgard 184 Silicone elastomer kit. Briefly, the PDMS base and curing reagent were mixed at a ratio of 10:1. The mixing process might generate some air bubbles and they were removed by placing the mixture under vacuum for 40 min. The mixture was then poured onto the SU-8 master mold and incubated at 65°C for 90 min. Punctures and cuts were made to the PDMS chip to create solution reservoirs connecting the microfluidic channels. The PDMS chip was reversibly sealed onto a clean glass slide for further experiments.

3.2.2.2 Glass microchip fabrication

The fabrication of glass microchips was similar to PDMS microchip except that the pattern was etched into the glass chip instead of the PDMS polymer. The fabrication of the glass chip followed an HF etching protocol. In short, glass substrates were pre-rinsed with deionized water, acetone, and then isopropyl alcohol. Clean substrates were baked at 150°C for 5 min and let cool to room temperature. Scotch tape was used to fully cover one side of the glass. Ready a hotplate at 65°C before proceeding to coating the glass substrate.

The glass substrate was set onto the spincoater, with the tape-covered side facing down. Approximately 1mL of AZ p4620 photoresist was applied to the middle of the the glass substrate. After the photoresist was spun at 2000 rpm for 17 s, the glass substrate was taken out and the Scotch Tape was carefully removed without touching the coated side. With the coated side facing up, the substrate was set on hot plate at 65°C for 2 min, ramp to 95°C stay for 2 min and then to 120 °C for 4 min. The substrate was let cool in a dark environment to allow rehydration for 1 hr. The AZ side was covered with a photomask and exposed for total energy 800mJ/cm². The exposed substrate was cooled for 10 min and then developed in 1:4 AZ developer. The solution was gently shaken and all unexposed photoresist was removed within 4 min. The substrate was rinsed with deionized water and dried. A post-bake for the glass substrate was carried out at 120 °C for 10 min.

Before HF etching, caution should be taken to wear suitable PPE and ready first-aid medicines as the HF is a highly toxic and volatile reagent. The magnetic stirrer was set to an appropriate speed to allow thorough mixing in HF solution. The AZ coated substrate was placed in a Teflon holder and lowered into HF to etch for 30 min. Etched glass substrates was taken out carefully and rinsed with water thoroughly. HF-free glass was developed in acetone for 2 min to remove the AZ coating. The final glass substrate was rinsed with deionized water and blown dry for characterization and further IEF experiments.

3.2.3 IEF setup development

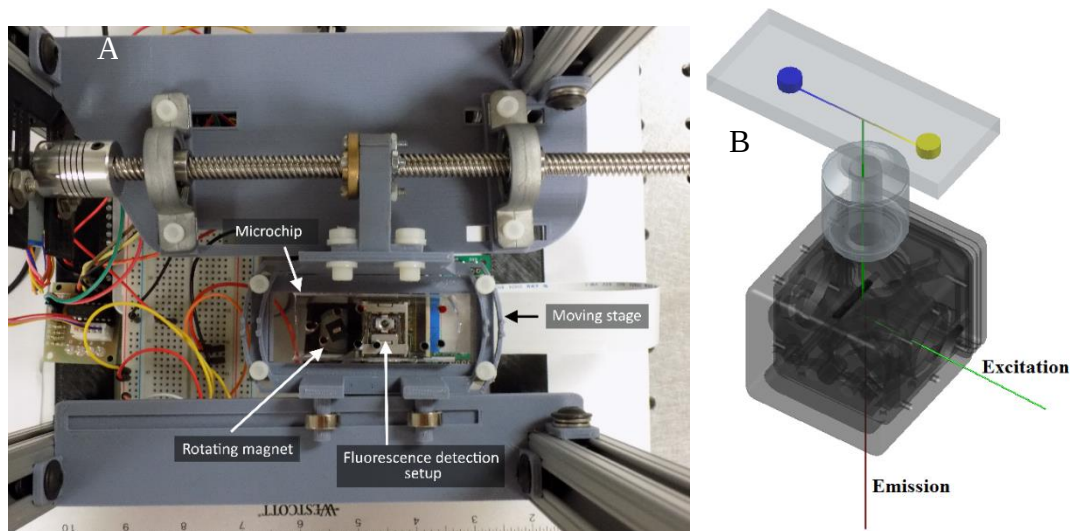


Fig. 14 Demonstration of IEF setup (A) and schematic of fluorescence detection (B)

Linear motion was crucial to scanning LIF detection. A 3D-printed microchip holder (Fig. 14 A) was designed to carry the IEF microchip along a linear motion trail to travel above the LIF detection spot. The chip holder was designed using AutoCAD and printed on 3D printer MakerGear with PLA filaments. The chip holder was linked to a threaded driving rod which was directly anchored on the shaft of a stepper motor, converting the stepper motor rotational movement to linear motion of the chip holder and IEF microchip. The 3D-printed platform was calibrated with a bubble-liquid level was used to ensure that the linear motion platform was installed horizontally with no height difference across all four corners. Height differences would negatively impact the reproducibility of IEF experiments as the microchip had to be maintained on the proper focal plane of the microscope objective for the LIF detection system. The reproducibility was tested with a dummy chip with marked targets that represent focused peptides substrates at fixed positions.

The optical detection system was a standard laser-induced fluorescence setup consisting of an excitation 532 nm green laser, a long working distance microscope objective, a

dichroic lens, a reflective mirror and a mini photomultiplier tube detector(PMT) (Fig.14 B). This system can precisely excite a spot less than 30 μm in diameter and measure the fluorescence from it. An alternative fluorescence detection method is whole column imaging. But whole column imaging has a LOD that is 1-3 orders of magnitude poorer and requires the use of a large-sized light source. A DVD pickup head that has a structure similar to a laser-induced fluorescence setup can be incorporated into the device as a miniature version of the same system. These several dollar DVD pickup heads provide laser diodes at both 405 nm and 633 nm, as well as a series of miniaturized mirrors and a focusing objective. The detector of the DVD pickup head can be replaced by a micro PMT for higher sensitivity and better reproducibility. A dark box was set up enclosing the whole device to prevent stray light from the environment.

The stepper motor, PMT detector and high voltage power supply were controlled via customized LabView programs.

In all, the combination of microchip IEF and scanning fluorescence detection offers unprecedented advantages in sensitivity and flexibility. IEF provides one-dimensional separation that the fluorescence signals from multiple MMP substrates can be resolved and detected individually. As a result, the complicated composition of MMPs in a biological sample can be detected simultaneously. On the other hand, fluorescence detection and the focusing effect of IEF will put detection sensitivity at a similar, if not better, level as regular fluorescence assays. As low concentration substrates are detectable, MMP reaction times can be decreased, increasing sample throughput.

3.2.4 pI standards characterization

Fluorescently tagged pI standards were characterized with LCMS. The fluorescent peptides were diluted in deionized water to 0.1mg/mL for injection. The characterization was performed on a Waters Acquity UPLC-MS. The running conditions included: 5 μ L injection volume, solvent: (A) water + 0.1% formic acid (B) acetonitrile + 0.1% formic acid, at constant flow rate 0.3 mL/min; gradients starting at 5% B at 0min, ramped to 20% B at 1 min, 45% at 2.5 min, 80% at 4 min and retained until 5.50 min, then to 5% B; column temperature at 40°C, column: ACQUITY UPLC peptide CSH C18 column (Waters), 2.1 mm x 150 mm, 130 Angstrom, 1.7 micrometer. MS electrospray ionization conditions included: 3.50 kV capillary voltage, 22 V cone voltage, 3 V extractor voltage, 150°C source temperature, 400°C desolvation temperature, 600 L/hr desolvation gas flow, 20 L/hr cone gas flow.

Gel electrophoresis was performed on three pI standards GCEE(theoretical pI 3.46), GCEEH(theoretical pI 4.39), GCEHH(theoretical pI 5.67) using NovexTM pH 3-10 IEF gel. The pI standards were diluted to 0.1mg/mL for sample loading. Gel electrophoresis experiment was performed following the protocol provided by Novex (https://assets.thermofisher.com/TFSAssets/LSG/manuals/IEFgel_card.pdf).

3.2.5 pI standards IEF

To understand the physical position of the pH gradient in the micro channel, pI standards were synthesized by our collaborators in the Bossmann group. The pI standards were fluorescently tagged small peptides whose isoelectric points could be predicted by

the composition of amino acids. The synthesis process, in short, followed a classic solid phase peptide synthesis method³³. When the peptide synthesis was complete, the fluorophore Rhodamine B was attached via a protocol similar to adding new amino acids to peptides by coupling the carboxylic end to peptide amine end with HBTU in DMF/DIEA solvents^{86,87}.

Besides synthesized pI standards, a natural fluorescent protein Allophycocyanine with a theoretical pI at 5.09 and λ_{ex} 650 nm λ_{em} 660 nm was also used as pI standard with the red laser detection setup.

Table. 2 pI standards labeled with Rhodamine B

Peptide sequence	Theoretical pI*	Molecular weight /Da
(RhB)-GCEE	3.46	860.94
(RhB)-GCEEH	4.39	998.08
(RhB)-GCEHH	5.67	1006.11
(RhB)-GCYYKK	8.79	1185.41

*Theoretical isoelectric points were calculated with peptide sequences in [Protein Isoelectric Point Calculator](#).

The synthesized peptides were dissolved in deionized water at 1 mg/mL as stock solutions. The synthesized peptides were characterized with HPLC and LCMS to confirm the purity.

A typical IEF sample consists of 5 μ L peptide analyte solution, 5 μ L 1.1% Brij 35 with 500 mM Arginine, 2.5 μ L 40% carrier ampholyte pH 3-10, 10 μ L 2% methylcellulose in water, and 27.5 μ L deionized water. The IEF sample was vortexed until the solution was

mixed well and no visible layers could be seen. The IEF sample was then ready to be loaded into the microfluidic device.

To prepare the microchip for IEF experiments, the microchip was first rinsed with methanol and then deionized water to remove any potential contaminants in the chip. The microchip was then filled with 0.4% methylcellulose in water and let sit for 10 min. After the methylcellulose coating was completed, the microchip was filled with IEF samples prepared as above.

Sample loaded microchip was connected with electrodes from the high voltage power supply. PMT power was turned on and amplification voltage was set to 0.45 V.

3.3 Results and discussion

3.3.1 IEF setup development

The reproducibility of the start point and end point was examined by running a microchip with pre-marked target, which left a distinctive fluorescence pattern(Fig. 15).

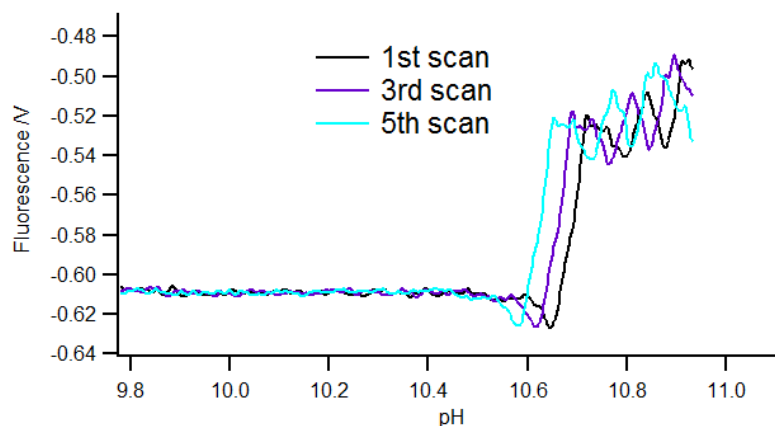


Fig. 15 Repetitive scanning of pre-marked chip

When scanning the chip back and forth multiple times, systematic drift was observed at both the start point(low pH) and end point(high pH). This drifting could cause false reporting of isoelectric point position. By analyzing the mechanical connection between the chip holder and stepper motor, the cause of the drifting was determined to be the direct mechanical connection between stepper motor and the driving rod. We proposed to exchange the mechanical connection for soft connection using a universal joint(Fig.16 A).

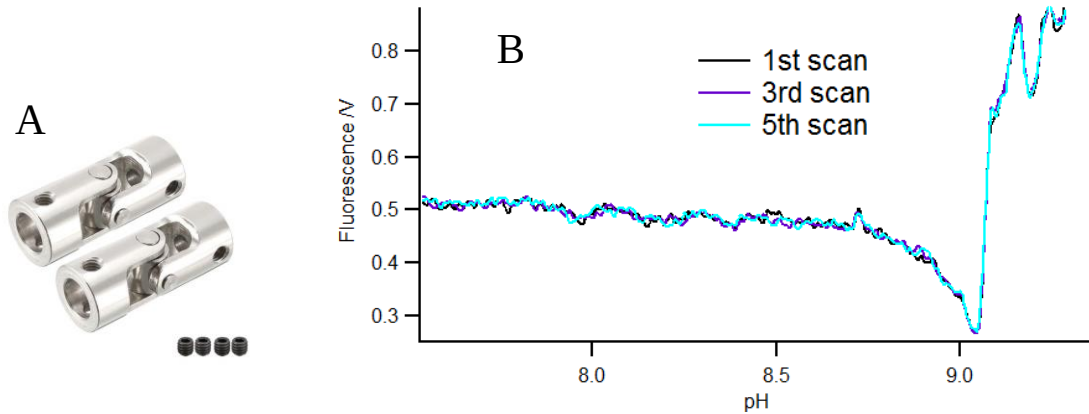


Fig. 16 Universal joint (A) and scanning of pre-marked chip with universal joints (B)

Reproducible fluorescence signal positions were observed over five rounds of scanning. The drifting issue was resolved using flexible connection with the universal joint(Fig. 16 B).

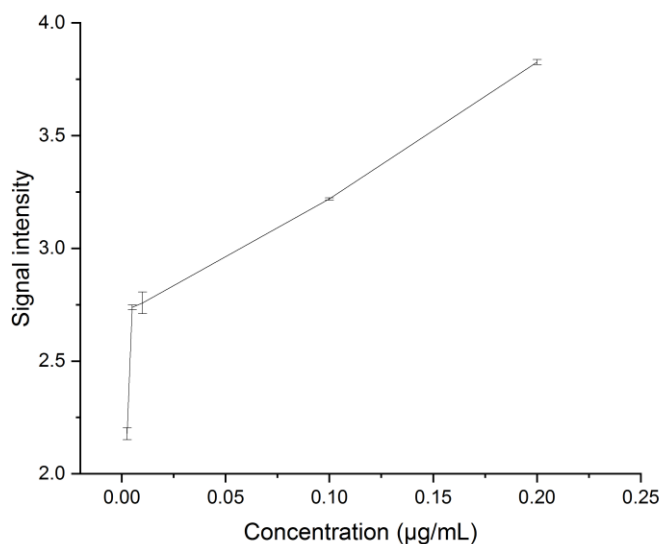


Fig. 17 LIF setup sensitivity with Rhodamine B

The fluorescence detection capability of the LIF setup was tested using Rhodamine B isothiocyanate aqueous solution (Fig. 17). The detection limit was 0.0103 µg/mL (18.6nM).

3.3.2 Microchip fabrication

Initially, the microchip was fabricated using soft lithography with PDMS chips coupled to plain glass slides. But PDMS chips were found to have serious staining issues as focused fluorescent peptides formed high local concentration bands, part of which was absorbed onto the hydrophobic PDMS chip. PDMS chips were replaced by glass chips that have separation channels etched into the glass to decrease surface contamination.

The HF etching of glass was highly time-dependent due to actual HF concentration being unknown. For chemical safety considerations, the attempt to test HF concentration

was given up. Instead, glass chips were etched and pulled out every 10 minutes to measure etching results. Eventually, glass chips were etched to approximately 30 μm in depth after 30 min of etching.

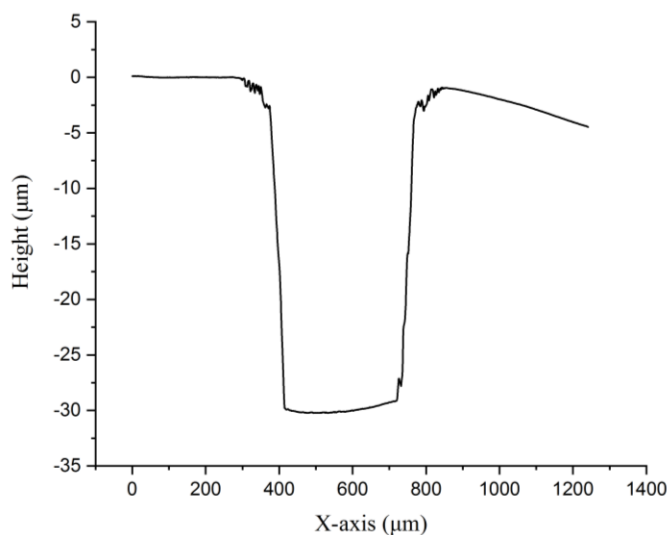


Fig. 18 Glass chip profiler results

Glass microchip was measured with channel width was 442 μm and channel depth 30.1 μm . HF etching. While the width of etched channel (442 μm) was slightly larger than the channel width on the photomask (300 μm), the long etching times created horizontal etching which increased the actual channel width.

3.3.3 pI standards characterization

Fluorescently tagged peptide substrates were characterized on LCMS to assess purity before they could be used as standards. Peptide purity can be enhanced using prep-LC if needed.

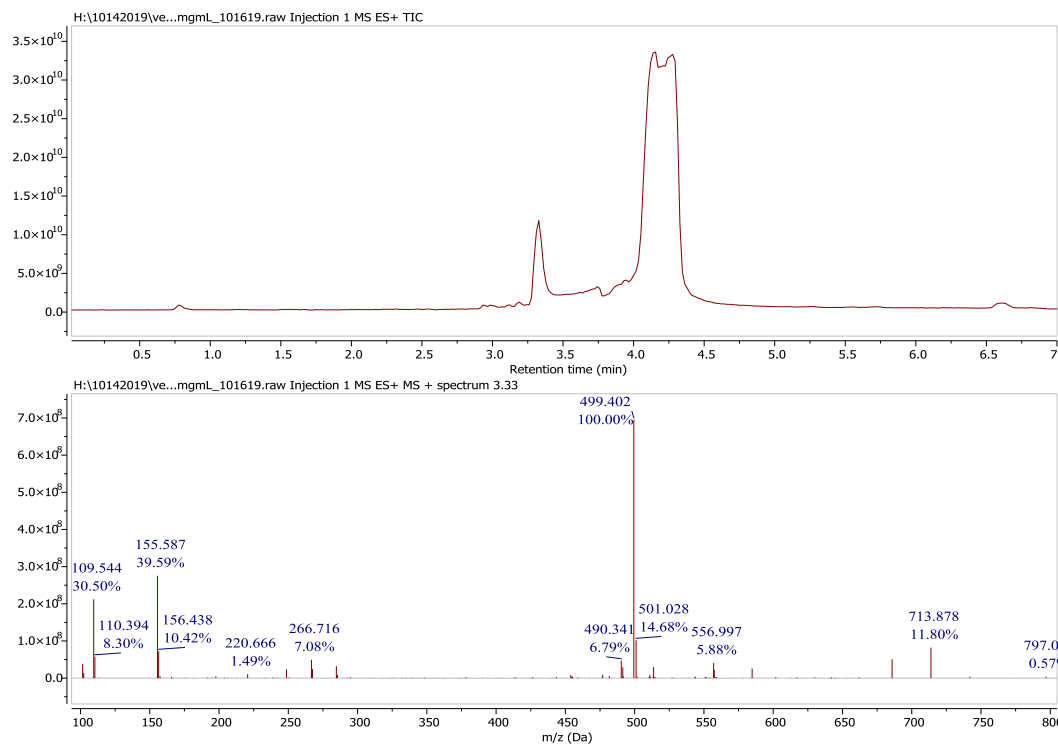


Fig. 19 (RhB)-GCEEH substrate LCMS

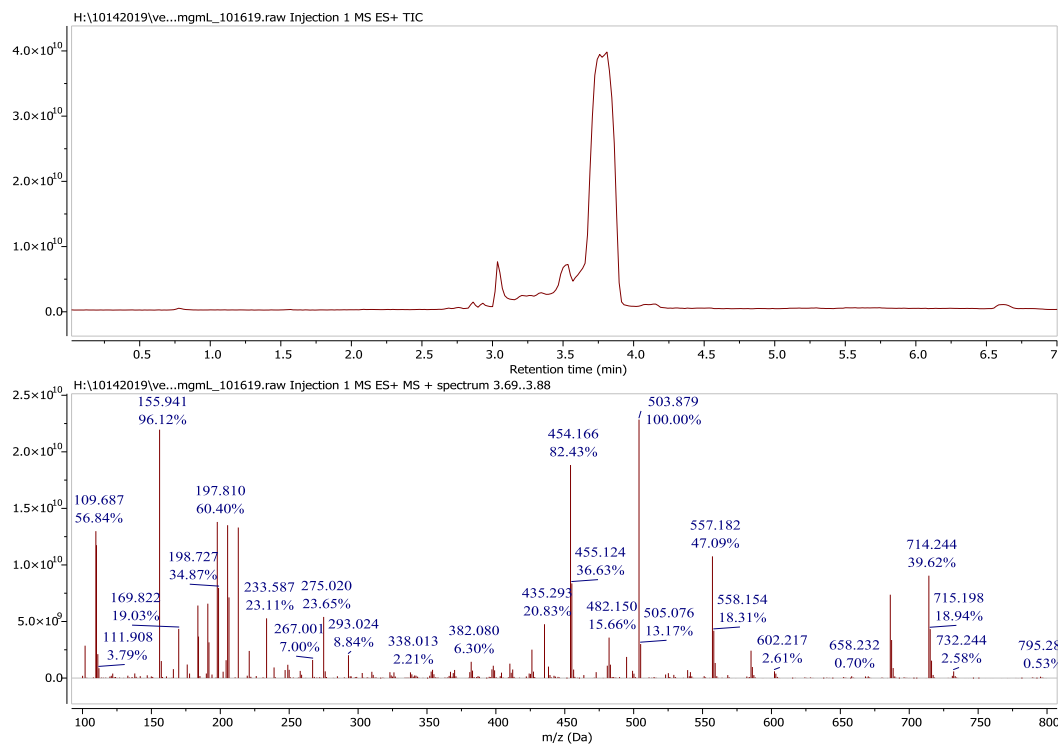


Fig. 20 (RhB)-GCEEH substrate LCMS

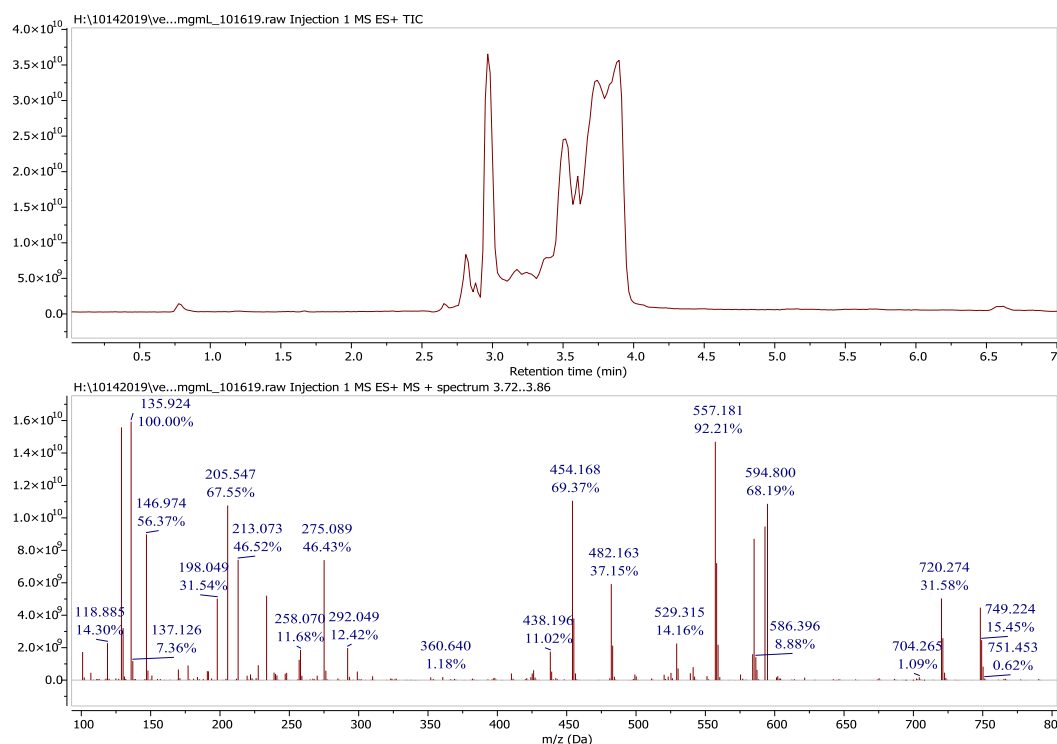


Fig. 21 (RhB)-GCYYKK substrate LCMS

LCMS results of RhB-GCEEH (Fig. 19) reported $[M+2H^+]^{2+}$ peak ($m/z = 499.04$) at retention time 4.22 min and purity calculated from peak area was 90.2%. LCMS results of RhB-GCEEH (Fig. 20) showed $[M+2H^+]^{2+}$ peak ($m/z = 504.05$) at retention time 3.81 min and purity was 86.0%. LCMS results of RhB-GCYYKK (Fig. 21) reported $[M+2H^+]^{2+}$ peak ($m/z = 593.70$) at retention time 3.78 min and purity was 68.8%. Besides RhB-GCYYKK, other pI markers were relatively pure and could be used as pI standards.

Gel electrophoresis was performed on pI markers to compare their pI value with theoretical pIs. A fluorescence photo of the gel isoelectric focusing of synthesized pI standard RhB-GCEE, RhB-GCEEH, RhB-GCEEH and pI 4.4 commercial marker are shown (Fig. 22).

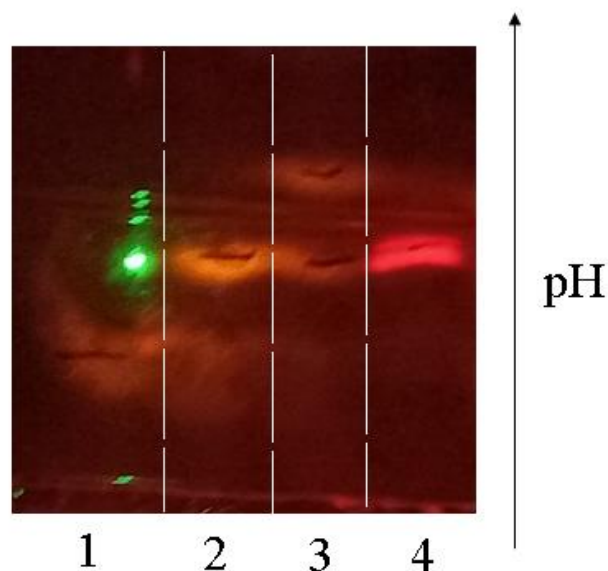


Fig. 22 Gel IEF of (1) GCEE (2) GCEEH (3) GCEHH (4) pI 4.4 marker

Compared to the pI 4.4 standard in channel 4, GCEE (channel 1) with theoretical pI 3.46, GCEEH (channel 2) with theoretical pI 4.39, GCEHH (channel 1) with theoretical pI 5.67 were focused to positions that matched their theoretical pIs. GCEEH, in particular, was focused in close proximity to the pI 4.4 standard. The isoelectric points of the synthesized pI standards were relatively close to theoretical pI. If more precise measurements of pI are needed, additional pI standards covering pH 3-6 could be used to create a calibration curve.

Another interesting finding was that the theoretical pIs of synthesized standards were highly close to theoretical value as the theoretical pI values were calculated solely based on the contribution from single amino acids without considering the influence from Rhodamine B attachment.

3.3.3 pI standards IEF

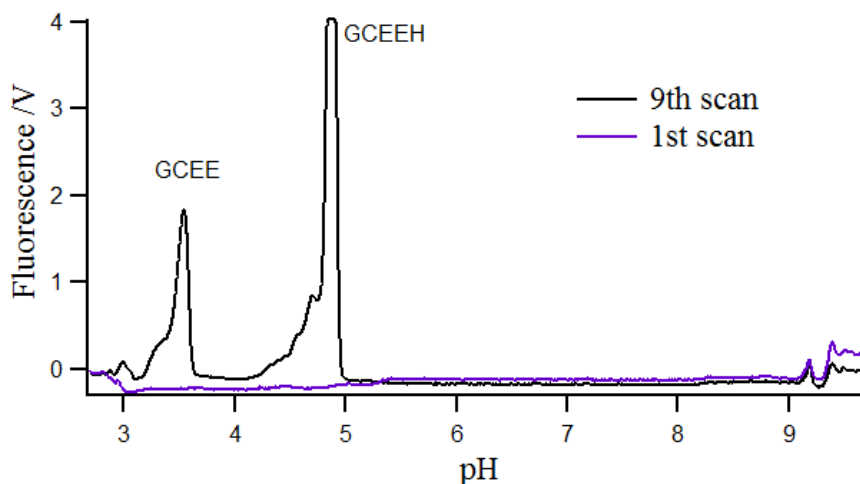


Fig. 23 0.02 mg/mL RhB-GCEE and RhB-GCEEH IEF CA3-10

Using carrier ampholyte pH 3-10 (Fig. 23), the GCEE isoelectric point was 3.54 compared to theoretical pI 3.46 while pI of GCEEH was 4.84 compared to theoretical pI 4.39. As the pH scale was set based on the physical position of the scanning stage, the actual pH gradient might not align with the physical position. Based on the theoretical pI of peptide standards, the pH gradient can be calibrated.

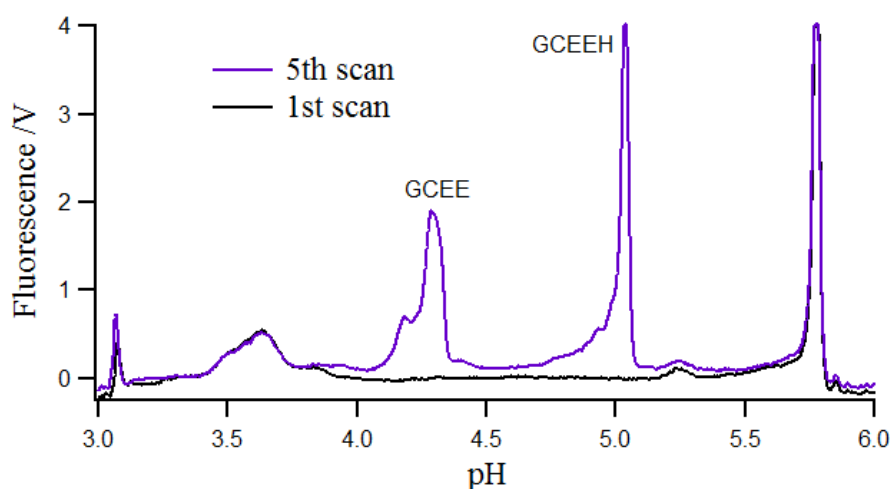


Fig. 24 0.02 mg/mL RhB-GCEE and RhB-GCEEH IEF CA3-6

RhB-GCEE and RhB-GCEEH markers were separated with carrier ampholyte range pH 3-10 and pH 3-6. With carrier ampholyte range pH 3-6 (Fig. 24). The peak to the high pH

side was an irrelevant contaminant adsorbed on PDMS slide at fixed positions. pI of GCEE was 4.29 compared to theoretical pI 3.46 while pI of GCEEH was 5.03 compared to theoretical pI 4.39. The pI positions of both peptide standards further deviated from theoretical pI but could still be used as calibration standards.

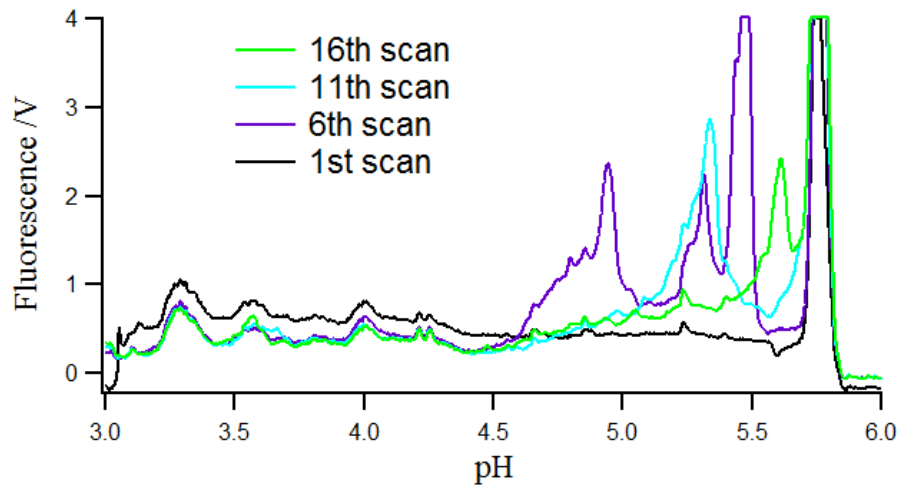


Fig. 25 0.035 mg/mL RhB-GCEEH IEF CA 3-6

During the focusing, RhB-GCEEH theoretical pI 5.67 was slowly focused outside to the high pH end of the separation channel, indicating the actual high end of the pH gradient inside separation channel was less than pH 6. RhB-GCEEH could not be used as pI standard in pH 3-6 range.

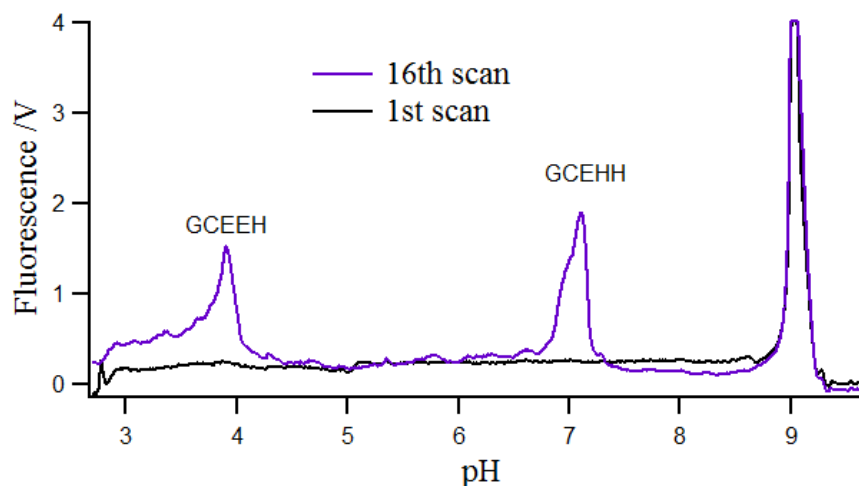


Fig. 26 0.035 mg/mL RhB-GCEEH IEF CA 3-10

With carrier ampholyte pH 3-10, RhB-GCEEH was successfully focused in the separation channel. RhB-GCEEH showed pI 3.90 compared to theoretical pI 4.39 and RhB-GCEEH displayed a pI of 7.08 compared to the theoretical pI 5.67.

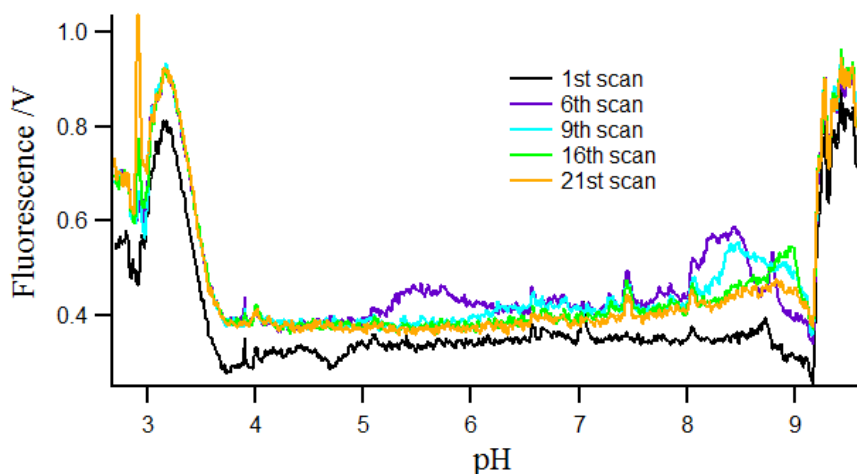


Fig. 27 RhB-GCYYKK IEF chromatogram CA 3-10

RhB-GCYYKK theoretical pI at 8.79 was focused outside of the separation channel for carrier ampholyte 3-10, possibly indicating the pH gradient in the channel was less than pH 10. Compared to GCEEH (theoretical pI 5.67) that was focused to pH 7.08 in carrier ampholyte 3-10, GCYYKK with a higher theoretical pI (8.79) than GCEEH (5.67) could

have been focused outside the separation channel. If the GCYYKK peptide were focused to high pH end outside the detection window, a peak would show up at the low pH end and migrate towards the high pH end over time and eventually disappear from the high pH end. But from the 1st scan to the 21st scan, no apparent peak was found, which indicated no peptide migration towards the high pH end. This abnormal result could not be explained by GCYYKK focused at high pH end outside separation channel.

Another possible explanation was that the peptide was not generating adequate fluorescence response under current experimental conditions. After a literature search, a theory was found to back this idea. When fluorophore Rhodamine B was covalently bonded to peptides, the fluorescence quantum yield of the formed amide was sensitive to pH (Fig. 28)⁸⁸.

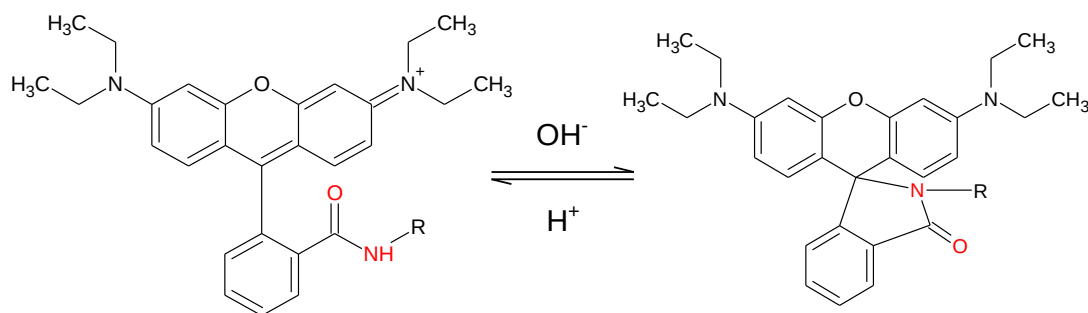


Fig. 28 pH switching of fluorescent(left) and non-fluorescent(right) Rhodamine B amide

When GCYYKK was focused to its pI 8.79, the fluorescence of Rhodamine B tag would be suppressed, which resulted in low fluorescence signal. While fluorescence tag Rhodamine B was initially selected for its simplicity in coupling chemistry similar to amino acid addition in solid phase peptide synthesis, its fluorescence quantum yield could be influenced by pH. Alexa fluor could be a potential alternative to Rhodamine B in 3 to 10 pH gradients⁸⁹.

3.4 Conclusions

A point-of-care microfluidic isoelectric focusing device was successfully developed. The device was capable of fluorescence detection over a 5cm microchannel by scanning laser-induced fluorescence detector across the channel with sharp detection sensitivity and the ability to report the position of fluorescence signal. To evaluate the efficiency of the microchip IEF device, peptide isoelectric point markers were designed and successfully synthesized. Peptide isoelectric point calibration markers showed a strong correlation between the designed isoelectric point and the actual isoelectric point. The separation of peptide markers on IEF devices was successful with the three low pI markers. The high pI marker was not detected because the fluorophores undergo a structural conversion at high pH, which decreases the fluorescence quantum yield. In the future, a pH stable fluorophore throughout the expected IEF pH range will be investigated.

Chapter 4 Point of care matrix metalloproteinase activity assay for cancer early detection using microchip isoelectric focusing and bioengineered magnetic nanoparticles

4.1 Introduction

Currently there is no commercially available matrix metalloproteinase (MMP) activity assays at the clinical level. Available analytical techniques in the research community include activity-based gel zymography, fluorescence resonance energy transfer and protein-concentration-based enzyme linked immunosorbent assays(ELISA)^{24,90,91}. Briefly discussed in chapter 2.3 Matrix Metalloproteinases, protein-concentration-based techniques measure the absolute concentration of a certain form of MMPs (pro-enzyme or active enzyme), while activity-based techniques measure the catalytic capability of MMPs in a given sample matrix environment. Among these techniques, **ELISA** was the most accessible technique at clinical level to measure antibodies and protein biomarkers^{92,93}. The ELISA of active enzyme MMPs, however, is not generally used since the function of active enzymes is determined by many factors rather than simply the concentration. Activity-based **gel zymography** is a technique similar to gel electrophoresis except that the analytes in gel zymography are pre-loaded enzyme substrates rather than the enzyme itself. The disadvantages of gel zymography are also

similar to gel electrophoresis for being labor intensive, time consuming, and semi-quantitative. Activity-based **FRET** assays are potentially the best match to measure MMP activities at clinical level. FRET substrates are peptide substrates attached with a fluorophore and a quencher which suppresses fluorescence if the quencher is in close proximity of the fluorophore. When the peptide substrates are hydrolyzed by MMPs, the fluorophore is released from the quencher and fluorescence will no longer be suppressed. Fluorescence signal increment can be used to quantify hydrolyzed substrates and allow one to determine the activity of MMPs⁹⁴. The fluorescence can be monitored continuously by a fluorimeter or a 96-well fluorescence plate reader to detect nanomolar level proteinases⁹⁵. However, FRET is still lacking at clinical level due to complicated sample pre-treatment and the inability to process multiple MMPs in parallel in a single well.

With optimal MMP substrates, the determination of MMP activities is still a challenge at the clinical level. A method that can readily deliver substrates into the MMP containing the sample and recover the remaining substrates after reaction is needed for the development of a sample-in answer-out integrated device.

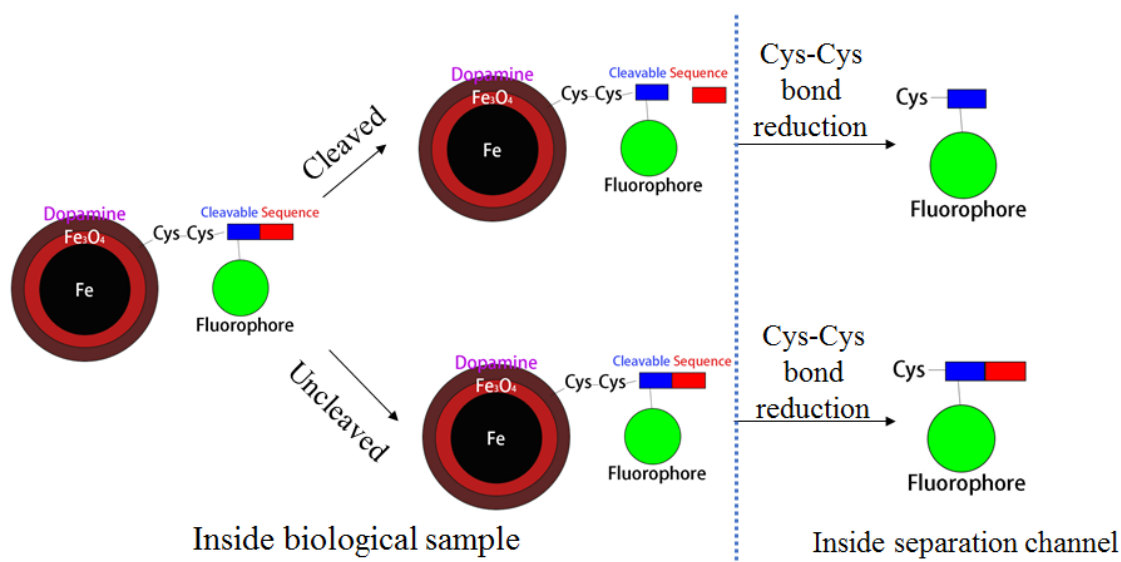


Fig. 29 Iron/iron oxide magnetic nanopatform working mechanism

The basic detection mechanism of MMP substrates is similar to zymography or any other separation-based techniques. Cleaved MMP substrates have different lengths, amino acid compositions and pIs compared to uncleaved substrates (right of dashed line Fig. 29). With isoelectric focusing separation, based on the pI differences, the cleaved and uncleaved substrates can be separated, focused and enriched at their pIs.

The magnetic nanoparticles carrying MMP substrates are first mixed with biological samples. A certain period of incubation is allowed for the MMPs to react with substrates. As the reaction progresses, some of the substrates are cleaved. The reaction is then quenched by moving the nanoparticle out of the MMP containing sample. In the separation channel where IEF will be performed, a reduction reagent is added in order to reduce and break the cysteine-cysteine bond and release the reacted/unreacted substrates from the nanopatform. An electric field is then applied to form a pH gradient, which separates free substrate peptides based on their pIs. Fluorescence emission along the pH

gradient is measured. Finally, fluorescence intensity is reported along with the isoelectric point position in the pH gradient.

Synthesis of the magnetic nanoplatform was done in collaboration with the Bossmann group of Kansas State University. In short, consensus peptide sequences are designed based on the cleavage motif of amino acid at the cleavage site³⁰. Dopamine coated magnetic nanoparticles synthesis are described in another work of Bossmann's group for in vitro MMP activity measurement³³. Cysteine-cysteine disulfide bond formation strategies are reviewed elsewhere.⁹⁶

4.2 Materials and methods

MMP digestion: MMP-7 (catalytic domain) enzyme and MMP-9 (catalytic domain) enzyme were purchased from Enzo life science(Farmingdale, NY, USA), Tris-HCl was purchased from Sigma Aldrich, NaCl and CaCl₂ were purchased from Fisher scientific, Reducing reagent 80% Tris (3-hydroxypropyl) phosphine (THPP) was purchased from Sigma Aldrich.

Isoelectric focusing reagents: Arginine, methylcellulose viscosity 1500cp are purchased from Sigma-Aldrich (St. Louis, MO, USA), Brij 35 was purchased from ThermoFisher Scientific, Servalytes 3-10 were purchased from Biophoretics (Sparks, NV, USA), deionized water is collected from Barnstead E-pure water purification system (Lake Balboa, CA, USA).

4.2.1 IEF setup improvement

The workflow of the IEF microchip is illustrated in (Fig. 30). Liquid samples were collected for immediate analysis or storage at low temperatures. 1) Approximately 30 μL sample was added to the sample reservoir, where it was mixed with the synthesized magnetic nanoparticles. With the aid of a rotating permanent magnet underneath the microchip, the nanoparticles were continuously mixed with the biosample, allowing MMP enzymatic reaction to take place. 2) After a reaction time of less than 10 min, a portion of the substrates on nanoparticles were cleaved under the catalysis of MMP enzymes. The unreacted substrates remained full length on the nanoparticle. 3) All the nanoparticles were magnetically transported from the sample reservoir into the wash channel. The enzyme sample was replaced by wash buffer quenching reaction.

4) The nanoparticles were transported into the IEF separation channel. Buffer in the separation channel contained carrier ampholyte for pH gradient generation, surfactants for increasing peptide substrate solubility, and a reducing reagent for cleaving the substrates off the nanoparticles. After the detachment of substrates, the magnetic nanoparticles were removed from the separation channel. 5) An electric field was applied between anode and cathode to generate a pH gradient and focus the peptides to their pIs. During the focusing process, the cleaved peptide substrates are focused into small bands of tens of micrometers. Peptide substrates focused into small bands were measured with a scanning LIF detector.

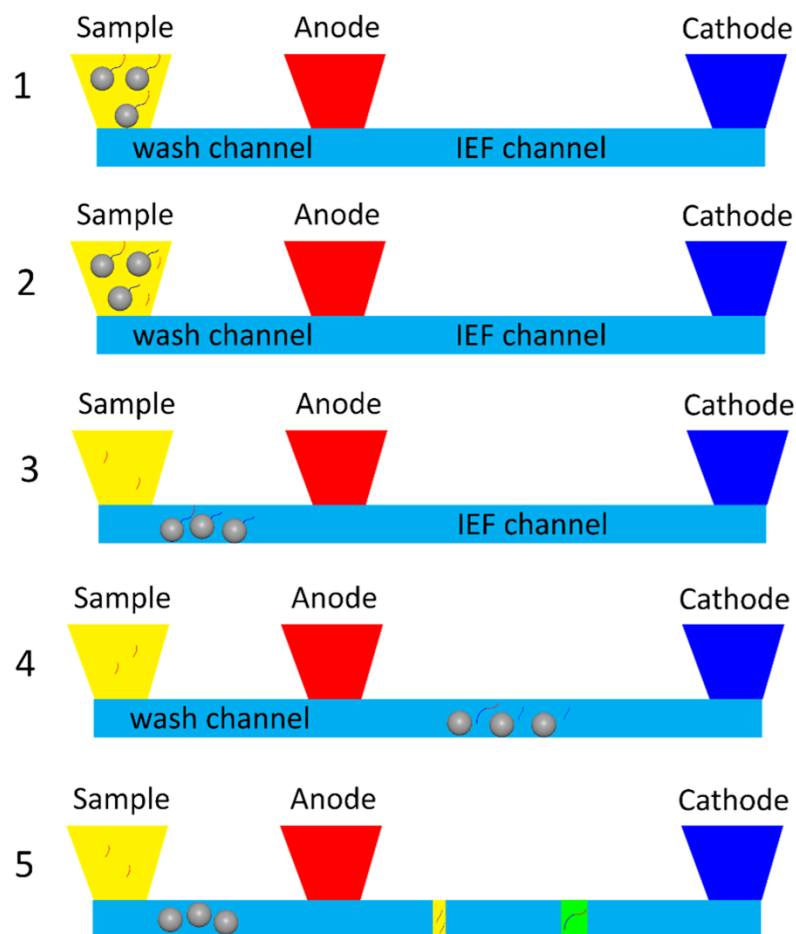


Fig. 30 Microchip isoelectric focusing procedures

Microfluidic chips will have channels with a cross-section at micrometer scales. This mini size can make sample and reagent consumption as low as several microliters. The microchip was fabricated with polydimethylsiloxane (PDMS) using standard soft lithography methods. The microchip was placed inside a holder made by 3D printing. Connected to a stepper motor, the holder and the microchip move linearly to expose different parts of the channel to the optical detection system. The stepper motor is controlled by Arduino or LabVIEW program, which controls the microchip in scan rate, direction and distances. The goal of this motion system is to enable scanning optical detection and magnetic nanoparticle manipulation.

4.2.2 IEF setup magnetic mixing

The manipulation of magnetic nanoparticles needs a permanent magnet to provide attraction and create mixing in the sample. In order to manipulate magnetic nanoparticles onchip, a custom device was designed to use external magnetic field to move or mix magnetic nanoparticles in the solution.

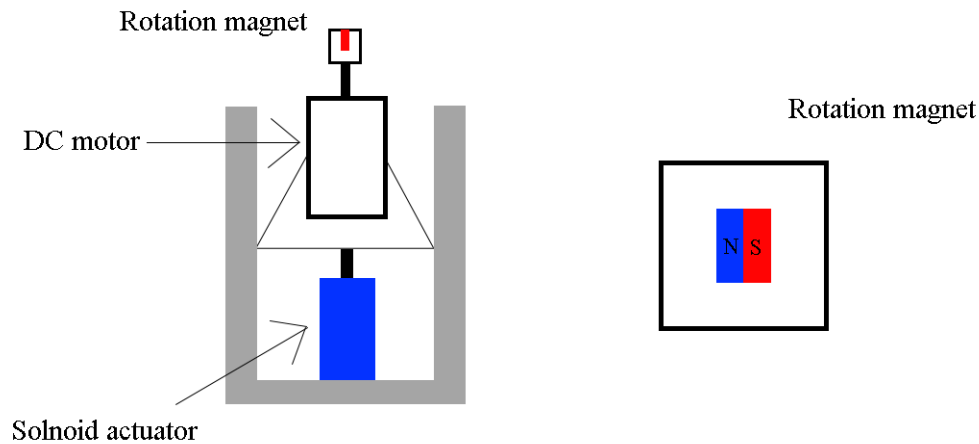


Fig. 31 Microchip magnetic rotation mixer

A Neodymium magnet was embedded into a 3D printed chip holder attached to a DC motor, with the magnet's north pole and south pole facing horizontally. When rotating the magnet, magnetic nanoparticles, similar to tiny magnets, would be attracted following the changing magnetic field, creating a vortex. Besides the vortex-like mixing capability, a rotating magnet also attracts magnetic nanoparticles, making positional manipulation of nanoparticles possible. As the permanent magnet moves along the channel, magnetic nanoparticles are attracted to the position in the channel right above the permanent magnet.

4.2.3 IEF chip fabrication

The fabrication of IEF glass chip is based on hydrofluoric acid and extreme caution should be used to avoid direct contact with the hydrofluoric acid. The first step was to create a mask on glass chips for selective hydrofluoric acid etching. Designs with desired channel patterns and geometry were plotted on computer using AutoCAD(Autodesk Inc.). The designs were sent to a laser printing facility(Fine Line Imaging) to create a photoplotter photomask. 1 mL of room temperature AZ photoresist P4620 was applied to a clean, dry glass chip and spin coated at 2000 rpm for 30 s. The coated glass chip was heated: at 65°C for 2 min, raised and maintained at 95°C for 2 min and finally raised and maintained at 120°C for 4 min. Let the glass chip cool and rehydrate in air for 1 hour in a dark environment. The glass chip then received a UV exposure at a dosage of 800mJ/cm². After the exposure, the glass chip is let cool for four min and developed with AZ developer for 4 min. The developed glass chip was rinsed with water and dried with nitrogen gas and heated at 120°C for 10 min.

The second step was to etch the masked glass chip with hydrofluoric acid. The masked glass chip was placed in a Teflon holder and submerged into 32% HF/NH₄F buffer with sufficient magnetic mixing. The glass chip was exposed for 30 min. The reacted glass chip was taken out with caution and rinsed with deionized water five times. The rinsed glass chip is briefly blown dried and transferred to a petri dish filled with acetone. The petri dish was gently shaken to make sure all the AZ coating was stripped off the glass chip. The clean glass chip was then rinsed with isopropyl alcohol and deionized water to

remove leftover acetone. The dimensions of the etched surface were measured using a surface profiler.

4.2.4 MMP substrate design and characterization

Cleavable MMP-7 and MMP-9 peptide sequences were designed based on the cleavage motif by MMP-7 and MMP-9 enzymes^{30,33}. The synthesis of MMP substrates was similar to that of the peptide pI markers in chapter 3. Besides the consensus sequences for MMP cleavage recognition, cysteine was added in order to provide thiol groups to form disulfide bonds with magnetic nanoparticles.

Table. 3 MMP consensus sequence and isoelectric points

Protease	Sequence	pI	Molecular weight
MMP-7	RhB-GCDGPQA↓IAGQHG	4.78	1635.79
Cleaved MMP-7	RhB-GCDGPQA	3.80	1072.18
MMP-9	RhB-GCDDHAAG↓LLGLDG	3.67	1738.91
Cleaved MMP-9	RhB-GCDDHAAG	4.20	1170.24

↓ represents cleavage site. Underlying sequence is the consensus sequence.

Synthesized MMP-7 and MMP-9 substrates were purified with dialysis and lyophilization. The purified products were characterized on LCMS and gel electrophoresis to assess purity and isoelectric point.

4.2.5 MMP substrate digestion

MMP substrates were dissolved in digestion buffer (50mM Tris-HCl pH 7.5, 10mM CaCl₂, 150mM NaCl, 0.1% Brij 35) to 1mg/mL as stock solution. MMP enzymes were added to nanomolar concentrations depending on digestion rate. Internal standard Rhodamine 6G was spiked to 1 μ M in the digestion buffer. The substrate-enzyme mixtures were incubated at 37°C to allow digestion. For kinetics studies, a fraction of samples were collected and placed at -20°C to stop the reaction. The collected digested sample could be directly injected into LCMS or spiked into IEF samples.

4.2.6 Nanoparticle disulfide bond reduction

To release the MMP substrates from the nanoparticles, the disulfide bond between nanoparticles and substrates had to be broken by reduction reactions. Tris (3-hydroxypropyl)phosphine (THPP) was selected as the reducing reagent due to its stability in air. 1mL reducing buffer was prepared with 50 mM Tris-HCl and 0.1% THPP. Substrate-loaded magnetic nanoparticles were rinsed with DI water three times. When rinsing magnetic nanoparticles, a permanent Neodymium magnet was used to attract and hold nanoparticles. 1 mg clean magnetic nanoparticles were mixed with the reducing buffer and incubated at 37°C for half an hour. The reduction product was centrifuged at 3000 g for 5min. Supernatant was taken while the magnetic nanoparticles were held down using Neodymium magnets. 800 μ L supernatants were collected analyzed by LCMS or IEF.

4.2.7 Nanoparticles digestion

1.0 mg of nanoparticles were suspended in deionized water in an Eppendorf tube. By using a Neodymium magnet to hold the nanoparticles, the nanoparticles were rinsed three times with digestion buffer (50mM Tris-HCl pH 7.5, 10mM CaCl₂, 150mM NaCl, 0.1% Brij 35).

4.3 Results and discussion

4.3.1 Magnetic nanoparticles onchip mixing

To demonstrate mixing capability, 35 nm diameter bare magnetic nanoparticles from Bossmann group were used (Fig. 32).



Fig. 32 Mixing of magnetic nanoparticles with a rotating magnet

Due to the vortex-like movement pattern, magnetic nanoparticles were confined inside a three-dimensional space rather than stuck on the glass slide. The movement and mixing were demonstrated. It is worth noting that the nanoparticles could be moved inside higher viscosity running buffers containing 2.5% methylcellulose. Additional experiments were done to evaluate the efficiency of this mixing process.

4.3.2 MMP substrate characterization

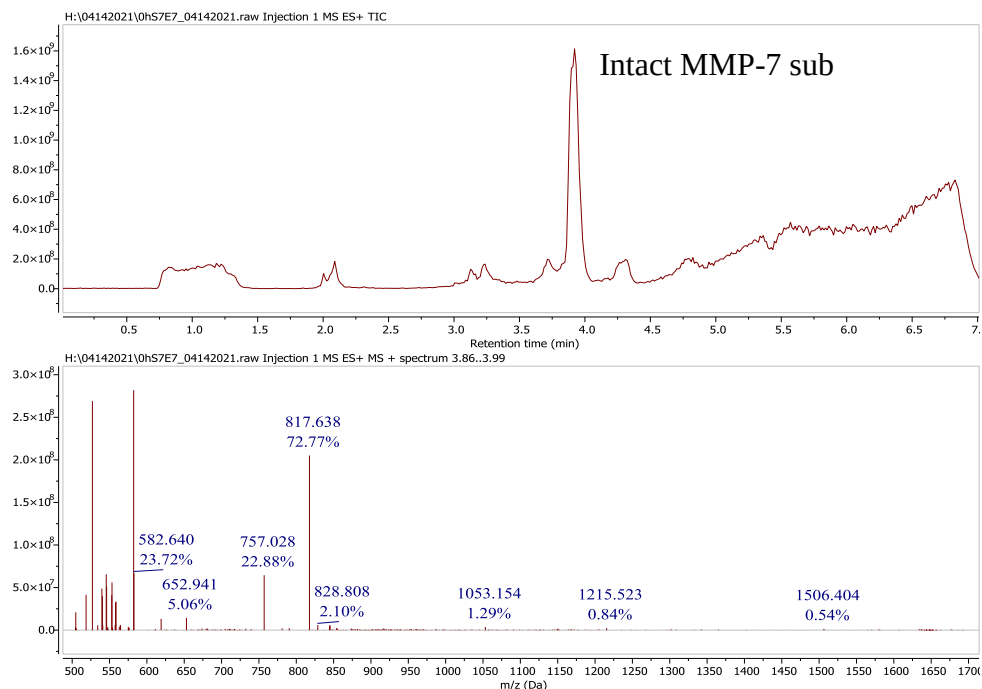


Fig. 33 LCMS of MMP-7 substrates total ion chromatogram (above) MS at retention time 3.86-3.99 (below)

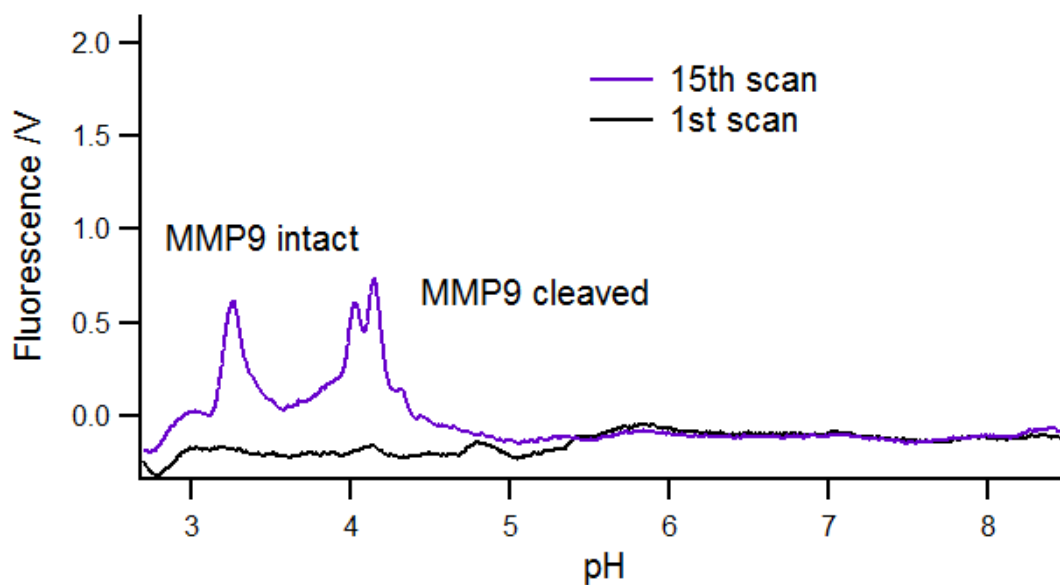


Fig. 34 IEF of intact (pI=3.65) and cleaved (pI 4.20) MMP-9 substrate

MMP-9 intact and cleaved substrates were focused to their respected isoelectric point.

MMP-9 intact substrate was focused to 3.24 compared to theoretical pI 3.65, while

MMP-9 cleaved substrate was focused to 4.15 compared to theoretical pI 4.20. It is worth

noting that MMP-9 cleaved substrates seemed to represent a split double peak which remained unresolved after eight scans.

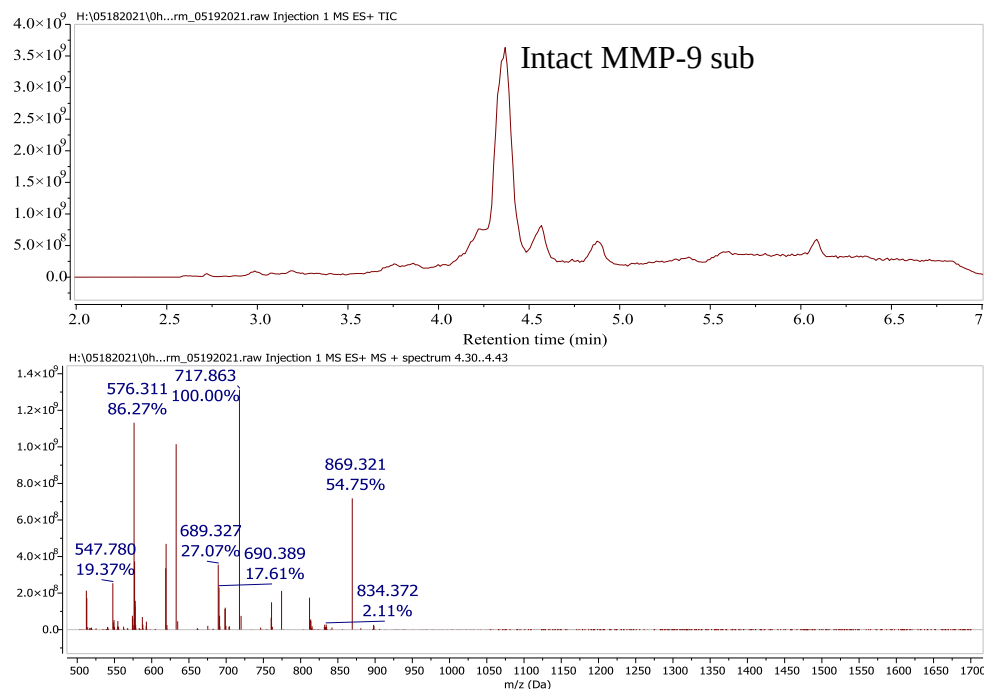


Fig. 35 LCMS of MMP-9 substrates total ion chromatogram (above) MS at retention time 4.30-4.43 (below)

MMP-7 substrate sample showed m/z 817.638 at major peak (retention time 3.90 min), which matched the $[M+2H^+]^{2+}$ peak of intact MMP-7 substrate (M.W. 1635.79). MMP-9 substrate sample showed m/z 869.321 at major peak (retention time 4.38 min), which matched the $[M+2H^+]^{2+}$ peak of intact MMP-9 substrate (M.W. 1738.91). Both substrates were successfully synthesized with 90%+ purity. Further purification was not necessary for the initial experiments performed and reported here.

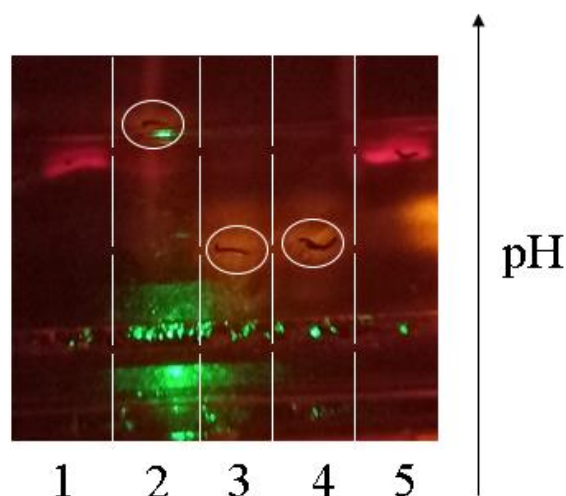


Fig. 36 Gel IEF of intact MMP-7 (channel 2) and MMP-9 (channel 3, 4)
(Fluorescence bands are highlighted in white circles for contrast)

Compared to the pI 4.4 marker in channel 1 and channel 5, MMP-7 substrate with theoretical pI 4.78 and MMP-9 substrate with theoretical pI 3.67 were focused to the proximity of their theoretical pI. Additional calibration samples of pI standards covering pH 3-6 would be useful for more accurate determination of the isoelectric points.

4.3.3 Free MMP substrate digestion

0.05mg/mL MMP-7 substrate was digested by 100nM MMP7 enzyme. Digestion samples were collected at 0 hours (sampling at enzyme addition), 2 hours, 4 hours, 6 hours and 24 hours after enzyme addition (Fig. 37). Collected samples were analyzed on HPLC with absorbance detection at 532 nm. 532 nm detection wavelength was selected to mimic the absorbance from excitation 532 nm green laser on IEF setup.

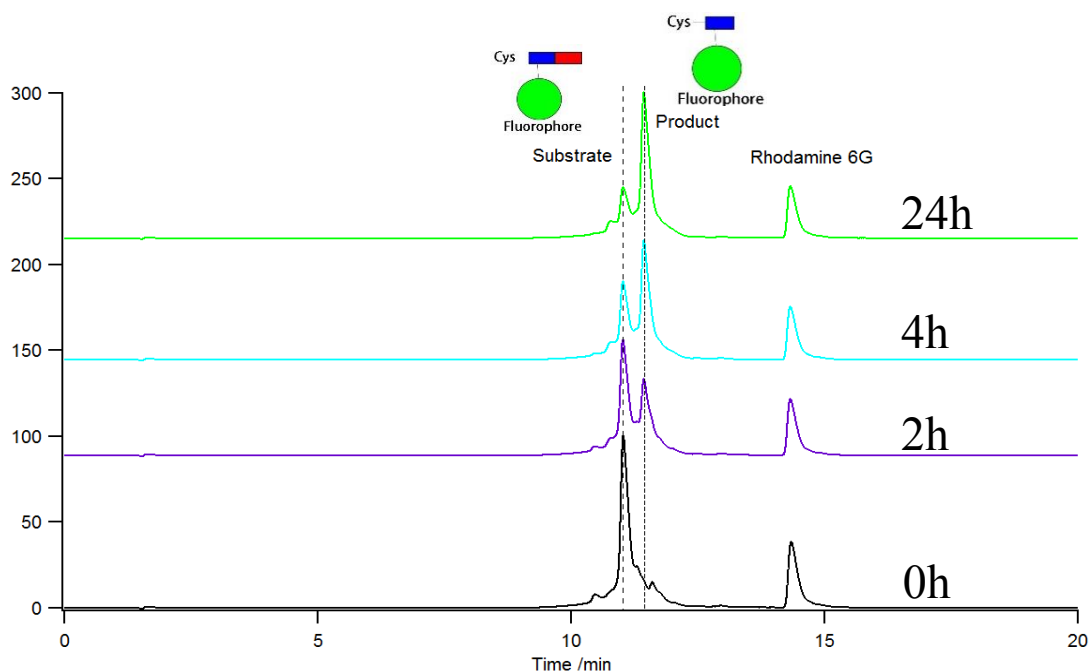


Fig. 37 0.05mg/mL substrate digested by 100nM MMP-7 enzyme

As digestion progressed, intact MMP-7 substrates were hydrolyzed and cleaved substrates were generated. Retention time of intact MMP-7 substrate was 10.90 min and cleaved product was 11.25 min. The intact substrates and cleaved products were not baseline resolved and this would lead to inaccuracy when integrating the peaks. To improve the separation resolution, the solvent gradient program could be modified to allow better separation between intact and cleaved peptides.

Lower concentration 0.005 mg/mL MMP-7 substrate was digested by same concentration 100nM MMP-7 enzyme (Fig. 38).

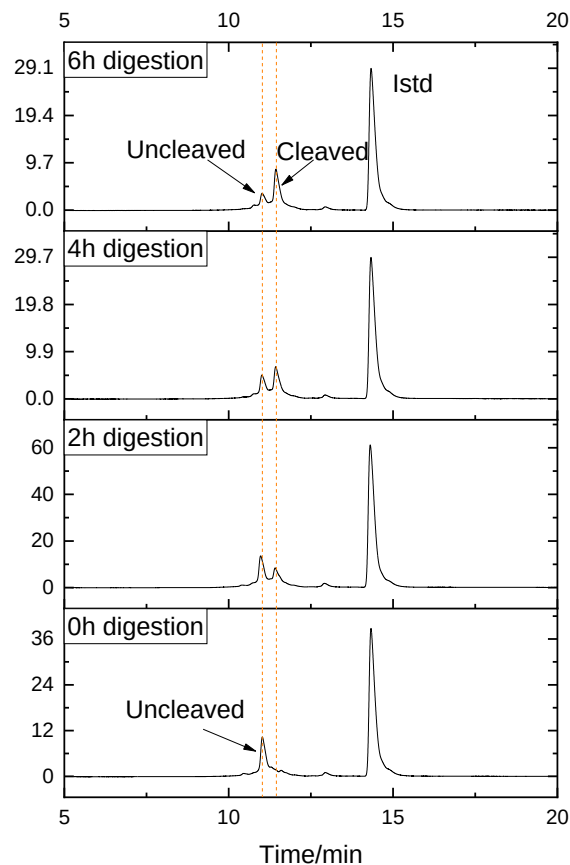


Fig. 38 0.005mg/mL substrate digested by 100nM MMP-7 enzyme

The reaction rate was determined by the signal decrease of intact peptides or the signal increase of cleaved product (Fig. 38). The signals were proportional to the concentration.

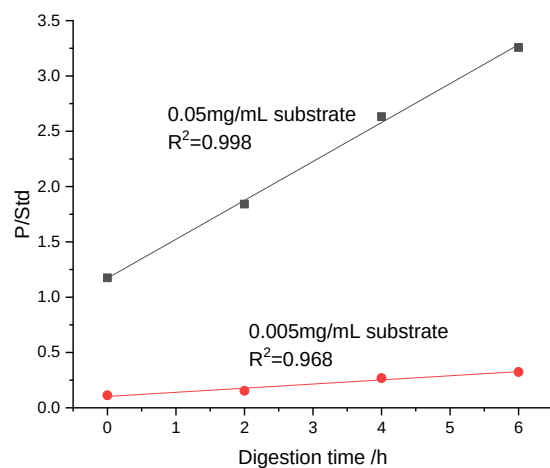


Fig. 39 Product signal/internal standard over time

The cleavage product concentration increased linearly with time. This indicated that the digestion rate remained stable within 6 hrs and the initial reaction rate V_0 could be determined simply by calculating the reaction at any point within those 6 hrs. As discussed in chapter 1, enzyme activity assays, according to Michaelis Menten equation, for initial reaction rate V_0 :

$$V_0 = \frac{k_2[E][S]}{K_M + [S]}, (K_M = \frac{k_{-1} + k_2}{k_1}) \quad (1.3.3)$$

When substrate concentration is kept constant, the initial reaction rate V_0 is proportional to enzyme activity $[E]$. Michaelis Menten equation could be rewritten into:

$$V_0 = k[E] \quad (4.1)$$

To measure unknown enzyme activities $[E]$, calibration samples were digested with known enzyme activity $[E]$, from which constant 'k' was extrapolated. Then unknown enzyme activity could be calculated by measuring initial reaction rate V_0 and 'k' constant.

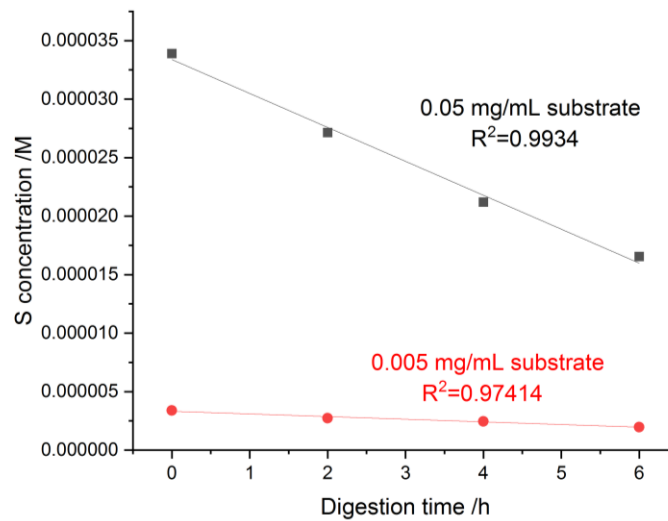


Fig. 40 substrate concentration over time

Substrate consumption also followed a linear trend with respect to digestion time(Fig. 40). The concentration decrease of MMP-7 substrate could also be used for activity determination.

Another interesting finding is that the digestion experiment did not necessarily require an internal standard. Based on the ratio between the cleaved product peak area and total peak area of (substrate and product), a linear relationship still existed (Fig. 41).

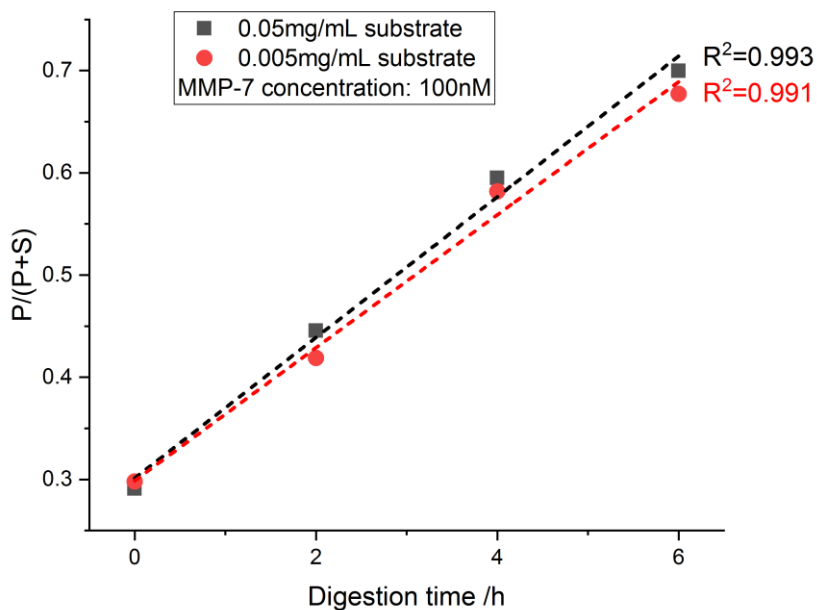


Fig. 41 Peak area ratio of cleaved/(substrate +cleaved)

4.3.3 Nanoparticle reduction

A reducing reagent was used to reduce the disulfide bonds between substrates and nanoparticles. Firstly, the influence of reducing reagent was studied because IEF involves high electric field strength that might reinforce redox reactions in the sample. Redox reactions occurring during IEF could increase current and induce electroosmotic flow that

compromises IEF separation. THPP was chosen as the reducing reagent as it was a water soluble, air stable, biologically pH stable reagent that was capable of rapid reduction of disulfide bonds.

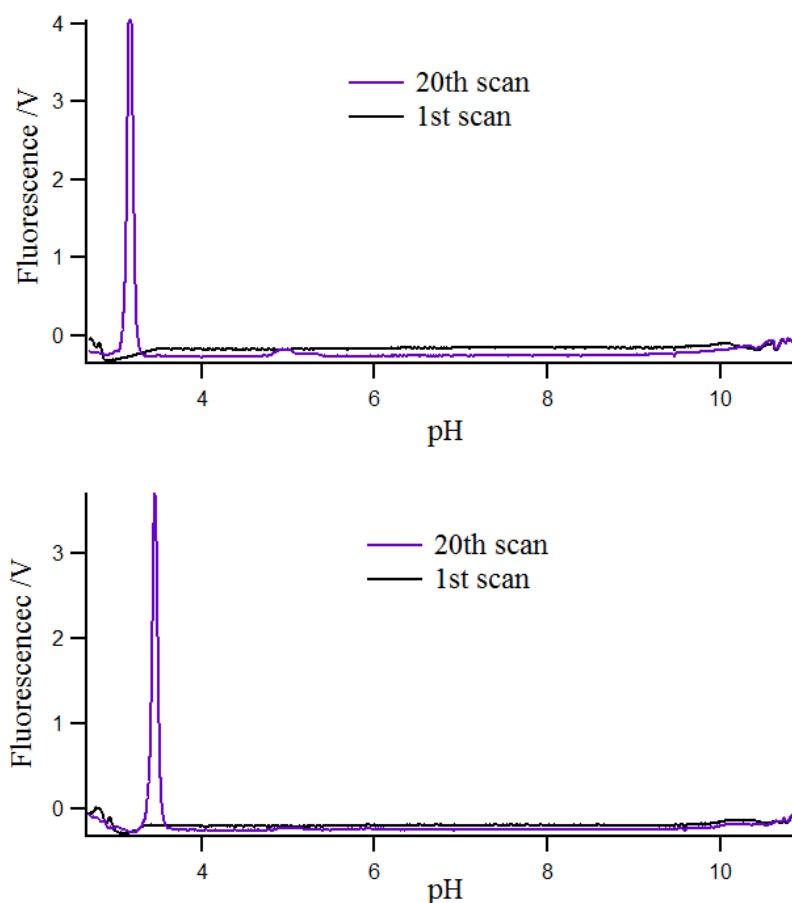


Fig. 42 IEF of RhB-GCEEH (above) with 0.01% THPP (below)

IEF of RhB-GCEEH was not affected by 0.01% THPP reducing reagent (Fig. 42).

Since the **reducing environment did not interfere with the isoelectric focusing** separation, the sample buffer onchip could use a mixture of IEF buffers and reduction buffers.

Nanoparticles reduction was performed on MMP-9 substrate-loaded nanoparticles. Reduction products were expected to be simply the free substrates. MMP-9 particles were rinsed with water three times and then placed in 0.1% THPP in digestion buffer (50 mM Tris-HCl pH 7.5, 10 mM CaCl₂, 150 mM NaCl, 0.1% Brij 35). The MMP-9 nanoparticles were vortexed and then placed on a shaker and shook for 30 min. The reduced MMP-9 nanoparticles were held by a Neodymium magnet while supernatants were collected. Collected supernatants were analyzed on LCMS.

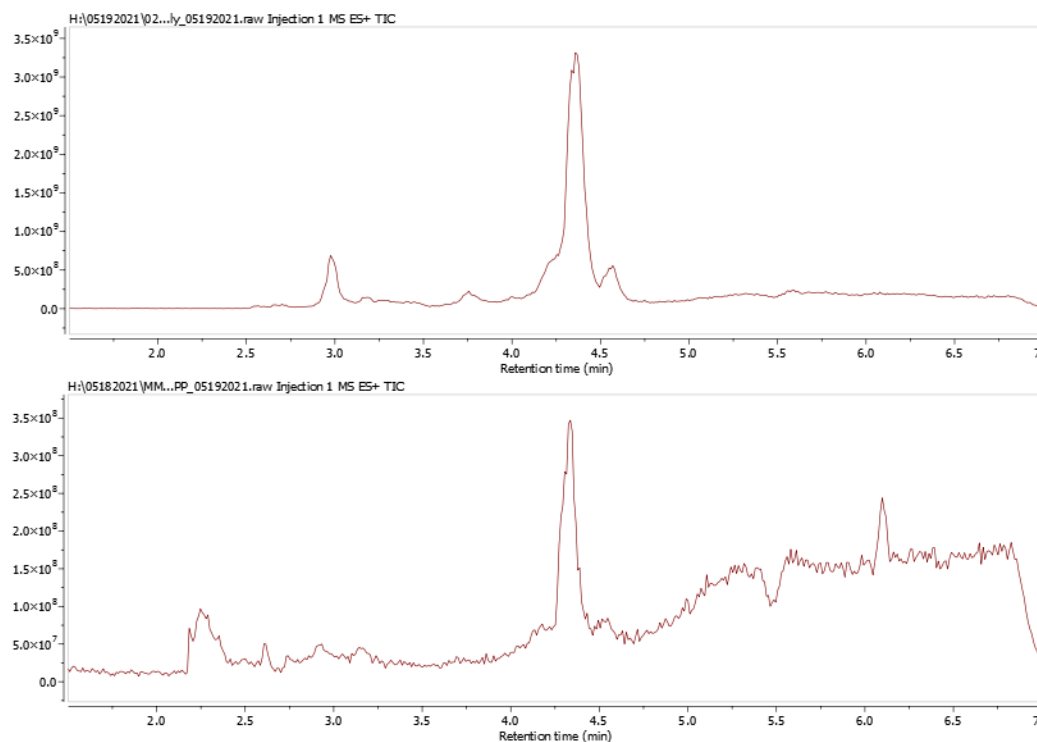


Fig. 43 Free MMP-9 substrate (above) and reduced MMP-9 nanoparticles (below)

A major peak found at retention 4.34 min for both free substrate and reduction products from the MMP-9 nanoparticles is shown in (Fig. 43). This might indicate the successful reduction of the disulfide linkage on MMP-9 nanoparticles and the release of MMP-9

substrates. This was confirmed by comparing the MS of free substrates to that of reduction products at retention time 4.34 min.

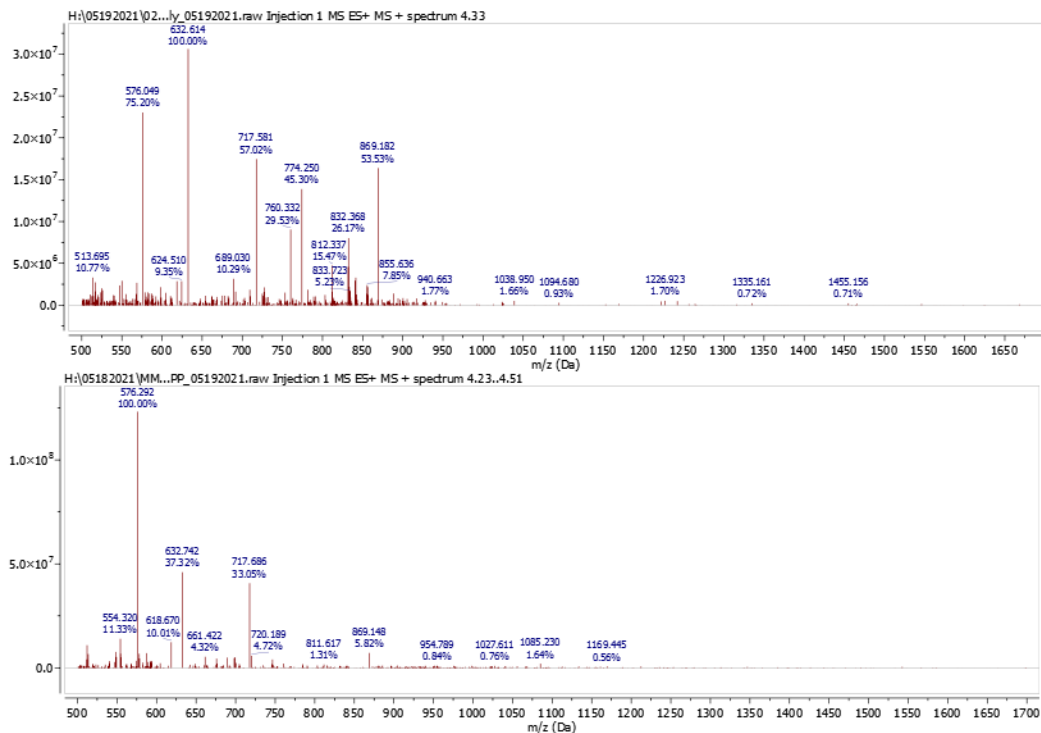


Fig. 44 MS of retention time 4.34min free MMP9substrate(above) and reduced nanoparticles(below)

Although the MS pattern was slightly different, characteristic mass peaks were found at m/z 869.1, which was the $[M+2H^+]^{2+}$ peak of MMP-9 substrates. Fragment mass peaks at m/z 576.2, 632.6, 717.6 were all found in both samples. The nanoparticle reduction products were confirmed to be MMP-9 substrates. The reduction of magnetic nanoparticles using THPP was successful and the THPP did not interfere with further IEF analysis of the reduced substrates.

4.3.3 Nanoparticle digestion

MMP-7 nanoparticles were first digested by 100nM MMP-7 enzymes in digestion buffer (50 mM Tris-HCl pH 7.5, 10 mM CaCl₂, 150 mM NaCl, 0.1% Brij 35). Then the digested nanoparticles were reduced with 0.1% THPP in digestion buffer. The reduction product was analyzed on HPLC.

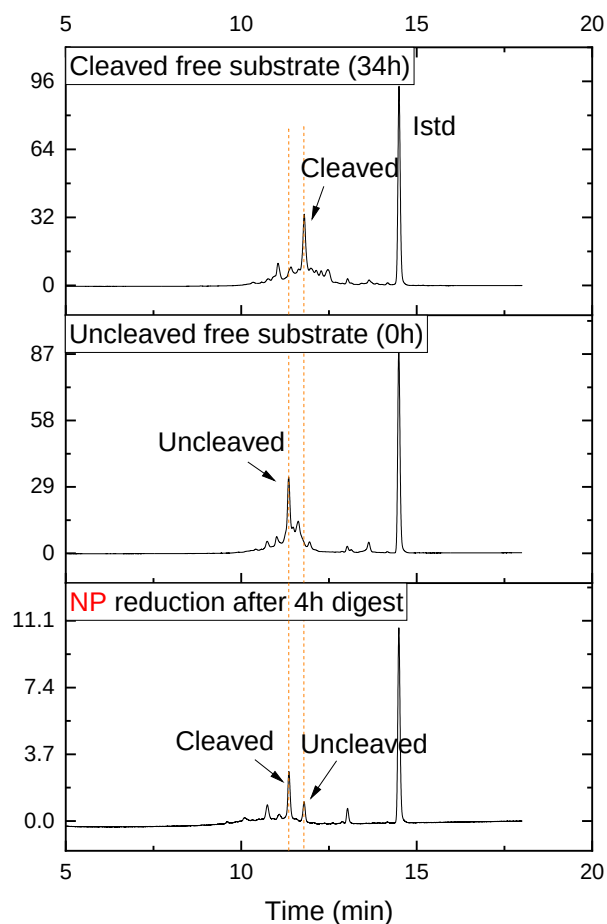


Fig. 45 HPLC of cleaved substrate, uncleaved substrate and nanoparticle reduction product after 4 hours enzyme digestion

The reduction product of the nanoparticles digested for 4 hrs showed both cleaved and uncleaved substrates. The digestion and reduction process was carried on magnetic particles to acquire kinetic information for enzyme activity determination.

4.4 Conclusions

MMP-7 and MMP-9 substrates were designed to measure digestion catalyzed by MMP-7 and MMP-9 enzymes. MMP-7 substrates were successfully digested. The ratio of digested and intact substrates was used to quantify enzyme activity. MMP substrates were loaded onto iron core magnetic nanoparticles via disulfide bonds. Digestion of substrates loaded onto nanoparticles was successful and the digested substrates could be reduced off the nanoparticles by THPP reduction. Potential future work would be the application of these nanoparticles on microchip IEF device.

Chapter 5 Conclusion and future outlook

This dissertation discussed some of the technical challenges we faced in cancer early detection. Some diagnostic techniques and cancer biomarkers were introduced in chapter 2, and questions were raised about a unique group of biomarker-Matrix Metalloproteinases. The dysregulation of these proteinase enzymes is a clear pathological sign of malignancy although their upregulation can also be seen in much earlier stages of cancer development.. However, current detection techniques are lacking in being able to measure the activities of multiple Matrix Metalloproteinases quickly and cheaply.

Isoelectric focusing is capable of accurate enzyme activity measurements and can be easily integrated into microfluidic devices. Initially, we proposed to design a mini-sized scanning laser-induced fluorescence detector to measure fluorescence signals from isoelectric focusing. Prototype devices were built using a commercially available DVD pickup head but we later switched to a photomultiplier tube detector single-point detection setup to improve sensitivity and reproducibility. To build a compact instrument, a scanning stage, isoelectric focusing power supply, and Labview program were developed to carry out microchip isoelectric focusing. Finally, to evaluate the performance of the microchip isoelectric focusing device, we designed and synthesized several isoelectric point markers using fluorescently tagged peptides. Although the IEF separation of pI standards was successful, we learned that the pH stability of the fluorescence tag was crucial to the detection of pI markers at high pH. Initial attempts at integrating red excitable and pH stable Cy5 dyes was unsuccessful, so we turned to

commercially available Rhodamine B to demonstrate proof of concept of the system. Rhodamine B, however, does not fluoresce at high pH's. pH-stable fluorescent dyes, such as Alexa fluor, could be a better alternative compared to rhodamine dyes for peptide labeling but they are much more expensive.

The second step was to apply the microchip IEF device to the separation MMP containing samples. First, we designed peptide MMP substrates and labelled them with Rhodamine B for fluorescence detection similar to the development of the pI standards. The synthesized MMP substrates were characterized and purified. The MMP substrates were successfully digested and the ratio of cleaved/uncleaved substrates was used to quantify enzyme activity. Then MMP substrates were attached to magnetic nanoparticles via a disulfide bond to allow enzyme digestions on the nanoparticles and magnetic retrieval of the reacted nanoparticles. After collecting magnetic nanoparticles, the substrates were exposed to a reducing environment which cleaved the intact and cleaved peptides reduced off the nanoparticles.

In the future, there are several improvements we can make to expand the application of microchip IEF in MMP activity determination. First, pH-stable fluorescent dyes could be used to broaden the available pH range to at least pH 10. Currently, fluorescent tag Rhodamine B limits the detectable pH range to pH 3-7. Above pH 7 Rhodamine B tagged substrates undergo conformation change which significantly reduces the fluorescence quantum yield and thus the detection sensitivity. This limits the use of high pI substrates on the device and, therefore, compromises peak capacity. Instead of Rhodamine B, labeling the substrates with a pH stable fluorescent dye will enable detection at pH range, allowing the use of standards or substrates with high pIs. Under current separation

resolution, opening up the high pH range can offer 50% more peak capacity and more substrates pairs can be simultaneously used within one IEF separation. The determination of multiple substrates pairs in one IEF separation would provide a bigger picture of individual MMP activities and may offer more accurate information for cancer typing or advanced health advice.

Secondly, the current separation resolution of the IEF microchip can be further improved by optimizing the detection area of the optical detection setup. With the current laser-induced fluorescence detection setup, excitation and emission were collected at the focal distance over a circle of $\sim 250\text{ }\mu\text{m}$ in diameter. As the fluorescence signals are collected over the whole detection spot, signals from different positions in the separation channel are averaged to display one fluorescence readout on the IEF chromatogram. The larger the detection circle is, the worse the averaging effect is, which leads to excessive band broadening. Reducing the detection spot size can help decrease the band broadening effect and improve the overall separation resolution. Higher magnification optics could be used to limit the detection spot to a smaller size. The current 20X objective on the setup can be exchanged to 40X or higher. Improving separation resolution could increase potential peak capacity as the MMP substrates could be engineered to have pIs closer to each other.

Third, several minor adjustments can be made to improve user experience of the IEF microchip. The current microchip is a PDMS-glass sandwich device, which can be beneficial to prototyping by the facile integration with different PDMS chip designs. However, PDMS chips may suffer from fluorescence dye staining as the focused fluorescent substrates form high local concentrations. When the microchip channel

design is determined, PDMS-glass chips could be replaced by all glass chips that are more resistant to stainings. Another potential improvement is the onchip heating function. While magnetic nanoparticles could be mixed with real samples, for example, serum, the MMP activities are also influenced by the sample temperature. To mimic the MMP activities in native environments, onchip temperature control would be a beneficial feature. Onchip heating is a well-researched field in microfluidics. ITO glass heating or onchip heating filaments could be potential options.

Besides the improvement mentioned above, the final step is to integrate the magnetic nanoparticles loaded with multiple substrates onto an IEF microchip. With the integrated device, real samples could be added to the microchip and handled with automatic sample preparation and IEF detection. The IEF device will not only fill the gap between separation-based assays and fluorescence-based assays but provide unprecedented sensitivity and high sample throughput. This would allow the research community to better understand MMP activities in blood or body fluid during tumor development. The cost-effective sample in-answer out device would provide clinical tools to improve early cancer detections as well as the overall survival rate of cancer patients.

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