QUANTITATIVE DETECTION OF TRYPSIN ACTIVITY VIA SURFACE ENHANCED RAMAN SCATTERING

A Thesis

by

CHIN-AN LEE

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Hung-Jen Wu
Committee Members,	Katy C. Kao
	Victor M. Ugaz
Head of Department,	Muhammad N. Karim

August 2018

Major Subject: Chemical Engineering

ABSTRACT

Protease activities provide essential physiological functions in the human body, such as food absorption, and abnormal protease activities can lead to severe diseases. In order to diagnose and monitor these diseases, it is urgent and desirable to develop quantitative detection methods of proteolysis. Currently, the surface-enhanced Raman scattering (SERS) technique has received much attention due to its multiplexed analysis and signal enhancement. However, SERS quantitative analysis remains a challenge because of variable SERS enhancements. To overcome this barrier, we introduced an internal standard to minimize the signal variations. In this thesis, silver nanocubes (AgNCs) were used as SERS substrates. The short peptide (sequence CQSARW) was used to monitor the proteolysis of trypsin. Peptides and internal standards were chemisorbed on AgNC surfaces via metal-thiolate bonding. After Raman measurements, the degree of digestion was determined by comparing the normalized spectra of the peptide before and after trypsin digestion. To demonstrate the application of this technology in drug discovery, we evaluated the inhibition capability (IC_{50}) of two known trypsin inhibitors, Ovomucoid and Bowman-Birk inhibitor (BBI). It was found that the measured IC_{50} values of the trypsin inhibitors are at the same orders of magnitude as the literature values.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Wu, and my committee members, Dr. Kao and Prof. Ugaz, for their guidance and support throughout the course of this research.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, thanks to my mother and father for their encouragement and to my wife for her patience and love.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a thesis committee consisting of Professor Hung-Jen Wu and Katy C. Kao of the Department of Chemical Engineering and Professor Victor M. Ugaz of the Department of Biomedical Engineering. All work for the thesis was completed independently by the student.

Graduate study was supported by a fellowship from Texas A&M University and funds from the National Institutes of Health under award number R03AI113585.

NOMENCLATURE

SERS	Surface-enhanced Raman scattering
LSPR	Pressure localized surface plasmon resonance
AgNC	Silver nanocube
2-MES	2-mercaptoethanesulfonate
BBI	Bowman-Birk inhibitor
IC_{50}	Half maximal inhibitory concentration

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Proteases

Proteases are the enzymes which can catalyze the hydrolysis of peptide bond in the peptides or proteins. The reaction catalyzed by proteases is called proteolysis. The catalytic capability of proteases brings many functions in human biological systems. For example, dietary proteins are cleaved by proteases catalysis in the small intestine and digested to amino acids for cell absorption. Also, proteases mediate blood coagulation, immune function, maturation of prohormones, bone formation, programmed cell death. Additionally, they participate in many pathological processes and serve as biomarkers and targets in medical diagnostics and therapies, such as Alzheimer's disease and ovarian cancer.[1–5]

Generally, proteases are mainly classified into seven groups, including, serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases, metalloproteases, and asparagine peptide lyases. Because some types of proteases perform similar mechanisms, there are mainly two different mechanisms for all proteases (**Fig. 1**). First mechanism is that one specific amino acid of proteases serves as nucleophilic residue to attack the carbon of peptide bond of specific residue, and then break carbon-nitrogen bond to release one peptide chain. Next, water in the solution hydrolyzes the intermediate to detach original proteases in order to release the other peptide chain. Serine proteases, cysteine proteases, and threonine proteases apply to this mechanism. For serine proteases, serine serves as nucleophilic residue to catalyze. Also, cysteine and threonine are nucleophilic residues for cysteine proteases and threonine proteases,

individually. Second mechanism is that the active site of proteases to activate water to be nucleophile, and water attack carbon of peptide bond of specific residue. Finally, two peptide chains are formed by catalysis of proteases. Aspartic proteases, glutamic proteases, and metalloproteases apply to second mechanism.[2,6]

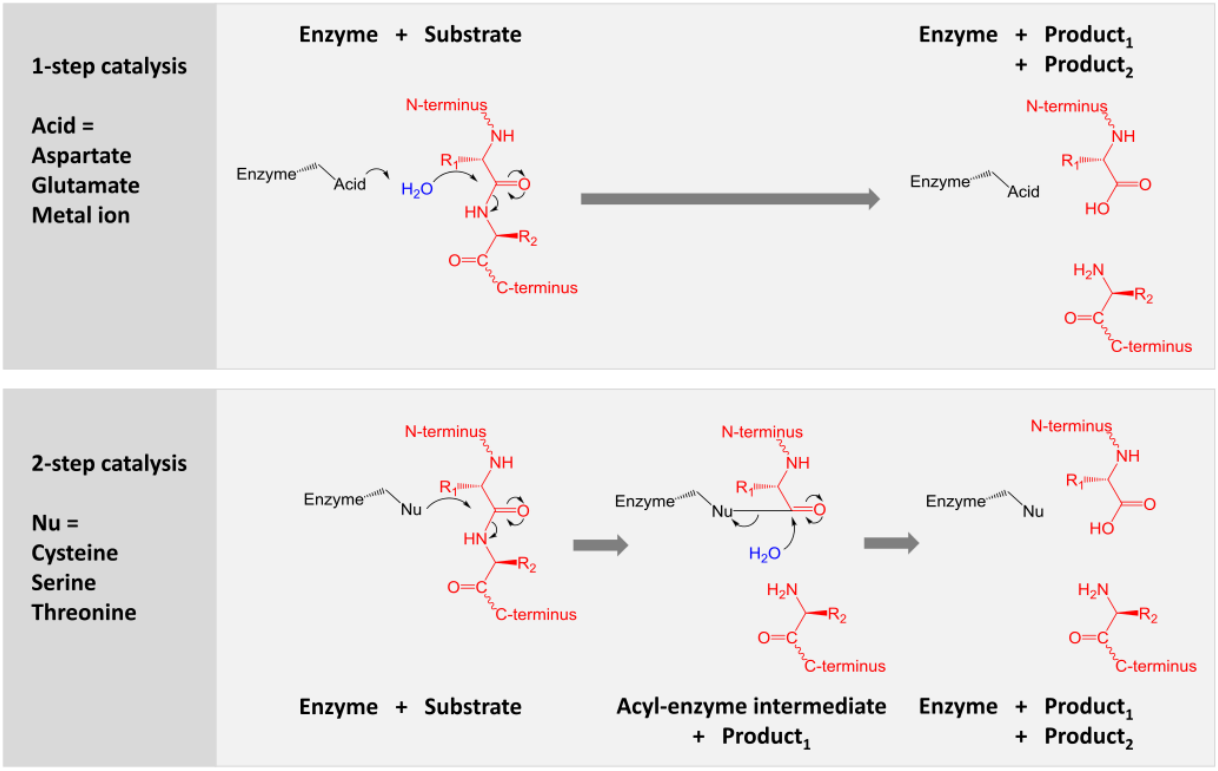

Figure 1: The mechanisms of hydrolysis catalyzed by different types of proteases.[2]

Reprinted from Wikipedia: Protease.

With many biological functions, proteases play a pivotal role in numerous biological reactions in organisms. When proteases malfunction, some biological functions are not able to execute normally, thereby causing some diseases, such as Alzheimer's disease (AD). It is well-known that human dementia results from Alzheimer's disease (AD) especially in the aging population. Although the entire mechanism remains unknown, the disease can be characterized in terms of accumulation of proteins and protein fragments in the brain, progressive neuronal loss. Recently, some research revealed disbalance of cysteine proteases are the key for treatments in AD and other neurodegenerative diseases since cysteine protease inhibitor protects neuronal cells from a wide range of attack, thereby causing cell death induced by oligomeric and fibrillar Aβ. It is worth to investigate the detailed mechanism of AD for therapeutic development. To do so, a reliable and high-throughput detection tool is required. In this thesis, we developed a new platform that can quickly determine proteases disbalance or activities.[4,5,7]

1.2 Literature Review: Existing Detection Methods of Proteolysis

A number of detection methods of proteolysis have been developed, including mainly colorimetry, electrochemistry, fluorescence and surface-enhanced Raman scattering (SERS). Each analytical method has its unique advantages and disadvantages, so we briefly explain these analytical methods in the following content.

(1) **Colorimetric assays** utilize the property of localized surface plasmon resonance (LSPR) of gold nanoparticles (AuNPs) for protease detection. The aggregation of nanoparticle alters the LSPR, leading to the solution color change. For example, Xue's group designed peptides with repeated arginine residues to induce the aggregation of gold nanoparticles; however, the nanoparticles aggregation would gradually collapse after peptide digestions catalyzed by proteases. Finally, the color change was observed from purple to red because the nanoparticles resuspended.[8] Moreover, Chen's group detected the color change by inducing the nanoparticles aggregation after proteolysis.[9] Overall, detection by naked eyes or by spectrometers for quantitative analysis is one advantage of colorimetric assays. Also, LSPR is relatively stable during detection compared to photo-bleaching phenomenon observed in fluorescent-based methods. However, the colloidal stability of nanoparticles is still difficult to control in a complex matrix. The fact that nanoparticles aggregations interfere reactions must be studied.[10,11]

(2) **Electrochemical assays** measure electrical signals produced from oxidation or reduction reactions.[12] In these assays (**Fig. 2**), peptides are usually labelled with redox reporters and immobilized on the electrodes. Then, peptide hydrolysis led to the detachment of redox reporters, thereby decreasing the electrochemical signal. Additionally, after hydrolysis redox reporters are designed to link to the cleaved peptide chain immobilized on the electrode; thus, the

increase of electrochemical signal would be observed.[11,13–15] In current developments, electrochemical assays show high sensitivity, and the sensors can be miniaturized for point-of-care applications. However, peptides with electrostatic residues may interfere electrochemical signals due to the variation of amino acids in the peptides, especially the chain length of the peptide. Therefore, calibrations for a variety of peptides must be carefully studied.[11]

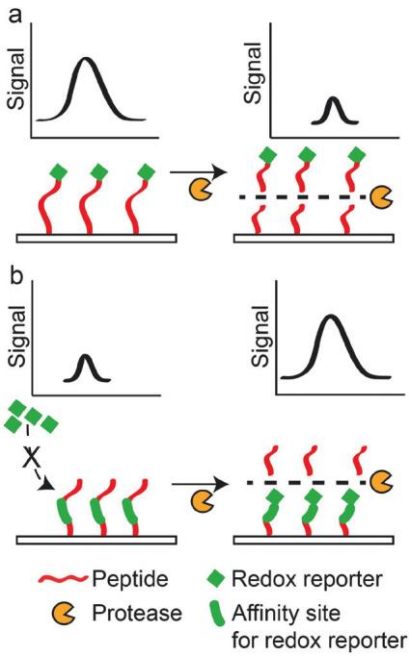

Figure 2: The mechanism of electrochemical assays. (a) Proteolysis of the peptide labelled with a redox reporter decreases the electrochemical signal. (b) Proteolysis of the peptide allows a redox reporter to link, thereby increasing the electrochemical signal.[11] Reprinted from "Recent developments in protease activity assays and sensors."

(3) **Fluorescence assays**: Proteases activities can be measured by detecting fluorescent signals of peptide substrates. So far, fluorescence resonance energy transfer (FRET) is widely used in protease detections. In FRET, a donor fluorophore in an excited electronic state transfer its excitation energy to a nearby acceptor chromophore by long-range dipole-dipole interactions.[16] The typical design of protease FRET detections is shown in **Fig. 3**. A short peptide consists of a FRET donor and a FRET quencher in very close proximity (1-10nm) so that acceptor can quench the emission of the fluorescence donor. Then, the donor and the quencher are separated after proteolysis, so the FRET phenomenon gradually decreases. In other words, the fluorescence signal would appear and increase. Traditionally, organic fluorescent dyes serve as FRET acceptors or donors; however, nanomaterials such as gold nanoparticles and quantum dots also are used as FRET acceptors or donors in order to increase efficiency.[17–19] Additionally, the more complexed fluorescent systems have been developed, such as BRET (Bioluminescence resonance energy transfer), due to the coupling between fluorescent proteins and enzymatic reactions with bioluminescence.[20]

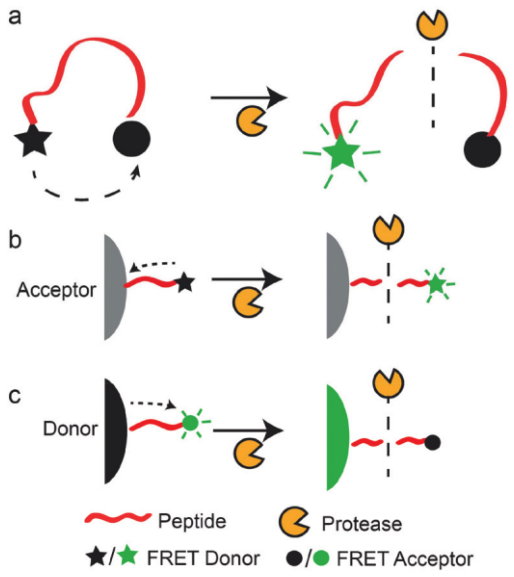

Figure 3: The mechanism of fluorescent assays. (a) Peptides are labelled with organic FRET donors and acceptors. (b) Peptides are labelled with organic FRET donors and nanomaterials FRET acceptors. (c) Peptides are labelled with nanomaterials FRET donors and organic FRET acceptors.[11] Reprinted from "Recent developments in protease activity assays and sensors."

Among the above-mentioned techniques, many efforts have focused on fluorescent assays due to its unique advantages. First, current fluorescence spectrometry can perform fluorescent tag of single molecule; thus, fluorescent assays possesses very high sensitivity. Second, fluorescent assays can be used in homogeneous or heterogeneous phases; however, colorimetric or

electrochemical assays are utilized in only heterogeneous phases due to the presence of nanoparticles or electrodes. Photo-bleaching phenomenon is still challenging for fluorescent detection. Also, there are some limitations on the peptide length because of FRET donors and acceptors in close proximity. Moreover, the label of FRET donors and acceptors is more complicated than other two techniques.[11,17,21,22] Herein, we used surface-enhanced Raman scattering (SERS) to detect protease activity in order to avoid some problems in above-mentioned methods and maintain high sensitivity for quantitative analysis.

1.3 Surface-enhanced Raman Scattering (SERS)

For the detection of protease activities, surface-enhanced Raman scattering (SERS) is a relatively emerging technique that has attracted much attention due to its multiplexed analysis and non-destructive detection. Basically, SERS is an extended technique of Raman spectroscopy. Therefore, we illustrate Raman spectroscopy and then interpret SERS principle and applications in the following content.

Raman spectroscopy is a scattering technique used to identify vibrational, rotational, and other low-frequency modes of molecules by measuring two different frequencies between excitation and emission. In principle (**Fig. 4**), when the monochromatic radiation light strikes at sample, the radiation would excite an electron to a higher virtual energy state, and then the electron immediately decay to a lower energy state; in the meantime, a scattering radiation would be emitted. After emission, most electrons would return to the original energy states, so the frequency of most scattered radiation is equal to the frequency of incident radiation. This phenomenon is

called Rayleigh scattering or elastic scattering. However, only about 1 part in 10 million of the incident photons would not follow elastic scattering since incident radiation interacts with the electron dipole of the molecule to change the original frequency of emission radiation. This situation is called Raman scattering or inelastic scattering. If the frequency of emission radiation is lower than the frequency of excitation, it is Stokes scattering; on the other hand, it is anti-Stokes scattering if the frequency of emission radiation is higher than the frequency of excitation.[23–26]

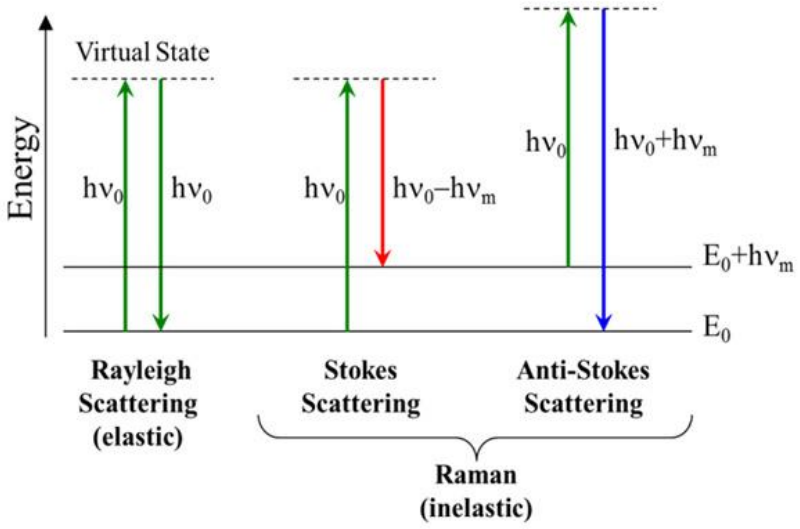

Figure 4: The mechanisms of Rayleigh, Stokes, and anti-Stokes scattering, respectively.[23] Reprinted from Raman Spectroscopy.

For inelastic scattering, due to obvious frequency change between excitation and emission, it represents the energy difference between initial and final energy states that corresponds to

rotational or vibrational modes of the molecule; thus, Raman spectroscopy provides information about rotational or vibrational modes of the molecule. The Raman spectrum of molecules obtained by Raman spectroscopy is like a structural fingerprint by which molecules can be identified. Overall, Raman spectroscopy has several advantages over the above-mentioned techniques. For example, organic and inorganic compound can be detected in the form of solids, liquids, polymers or vapors. Also, Raman is non-destructive detection and not interfered by water due to very low Raman signal for water. On the contrary, fluorescent sample or impurities may interfere with the Raman spectrum. Most importantly, the Raman effect is very weak due to approximately 1 of 10^7 photons by Raman scattering.[27] Overall, Raman spectroscopy is an advantageous technique; however, the weak Raman effect blocks the development of its applications. In order to improve this disadvantage, surface-enhanced Raman scattering (SERS) was invented to overcome this weakness.

Basically, the function of SERS is to enhance Raman scattering when analyte molecules are close to rough metal surfaces or nanostructures. The enhancement factor for Raman intensity can achieve up to 8 orders of magnitude. It means SERS not only has high sensitivity but also has a majority of capabilities Raman spectroscopy possesses. As a result, SERS is widely used for the technologies development of highly sensitive detection, especially in analytical chemistry and biomedical engineering.[28,29]

Regarding the mechanism of SERS, electromagnetic theory and chemical theory were proposed to reasonably explain the enhancement of Raman scattering. For electromagnetic theory, the localized surface plasmons are excited as incident radiation strikes the surface. When the frequency of localized surface plasmons resonates with the radiation, the electric field is maximized. Since plasmon oscillations must have the direction which is perpendicular to the

surface, the roughed surfaces or nanoparticles provide the area to produce plasmon oscillations with perpendicular directions. For chemical theory, when photons strikes metal surface, electrons in the metal are excited to cause charge transfer between the metal surface and chemisorbed molecules. The fact that the molecules may stay in the new excited-vibrational state renders Raman-scattered photons, leading to chemical enhancements.[28–31]

Additionally, SERS substrates are a parameter to control enhancement factor. For current SERS detection methods, silver (Ag) and gold (Au) are used as the SERS substrates since they have dielectric properties to sustain strong plasmon resonances in the visible range. Also, the enhancement of Raman scattering usually occurs in the presence of nanostructures, so silver or gold nanoparticles are enormously utilized in SERS detections.[30]

Overall, SERS possesses very low detection of limit due to high enhancement factor, and it applies to colloidal solution or solid film. Besides, multiplexed analysis is its unique advantage over other techniques. Since Raman spectrum provides information about many vibrational modes of many species, distinct vibrational signals can be identified as different species in complexed system. Compared to other techniques, SERS can detect and analyze more complicated system, such as bio-reactions with enzyme catalysis. With these merits, this project utilized SERS technique to detect protease activities.

1.4 SERS-based Protease Assays

Due to SERS unique advantages, prior studies have designed various types of SERS substrates to detect protease activities. These SERS platforms have successfully measured proteolysis for qualitative analysis. For example, gold nanoparticles (AuNPs) labeled with 4-mercaptobenzoic acid (4-MBA modified AuNPs) has been used as the SERS substrates (**Fig. 5**).[32] To detect serine proteolysis, the positive charged peptide with 9 arginine amino acids are cleaved into smaller arginine fragments by thrombin. In the absence of thrombin, SERS substrates would aggregate because the strong electrostatic attraction between peptide and negatively charged 4-MBA modified AuNPs. The aggregations of SERS substrates form electromagnetic "hot spots", so large electromagnetic coupling effect exist between AuNPs in the aggregate, leading to the enhancement of 4-MBA Raman signal. Contrarily, peptide chains are hydrolyzed into arginine fragments in the presence of thrombin. Because of weak electrostatic interaction between fragments and 4-MBA modified AuNPs, the degree of AuNP aggregation reduced, thereby minimizing enhancement factor. Thus, the SERS substrates can be used to detect proteolysis by measuring the intensity change of Raman signal.[32]

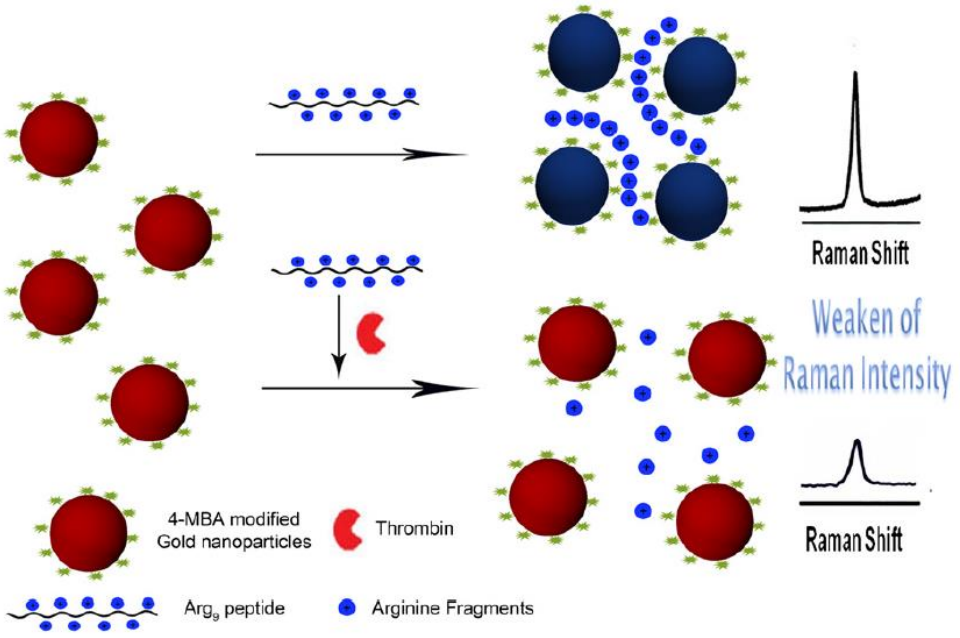

Figure 5: The mechanisms of SERS biosensor for thrombin based on enzymatic assays.[32] Reprinted from "A "turn-off" SERS-based detection platform for ultrasensitive detection of thrombin based on enzymatic assays."

Moreover, Hu's group proposed a new SERS platform using the opposite effect compared to the above SERS substrates (**Fig. 6**).[33] The designed peptide chain was divided into positive and negative charged fragments after trypsin digestion. When mixing peptide and 4-MBA modified AuNPs, peptide chain with cysteine amino acid can bind to AuNPs through Au-S bond. After digestion, cleaved peptide with cysteine would bind to 4-MBA modified AuNPs, inducing SERS substrates aggregate due to strong electrostatic interaction between positively charged peptide and negatively charged AuNPs. The aggregations increase the hot spots, improving the enhancement

of Raman signal. On the other hand, there is weak electrostatic interaction between SERS substrates labeled with intact peptides, so the reduction of hot spots lowers enhancement factors.[33]

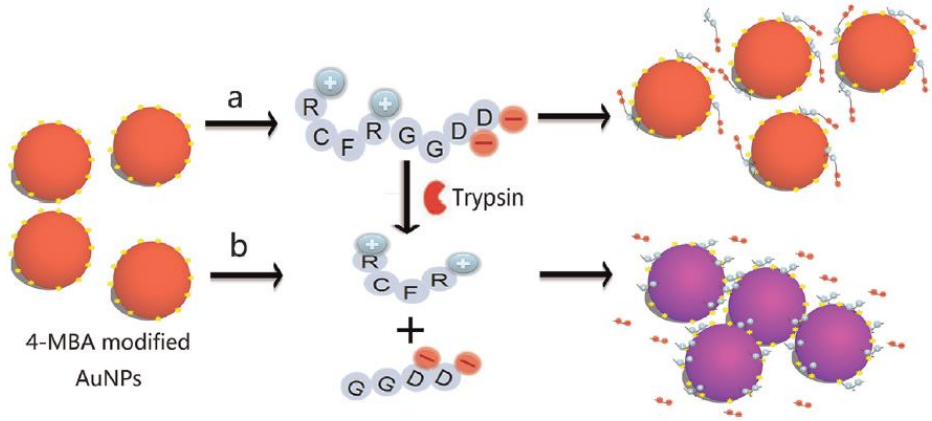

Figure 6: The mechanisms of SERS-based enzyme assay for trypsin.[33] Reprinted from "A simple and universal "turn-on" detection platform for proteases based on surface enhanced Raman scattering (SERS)."

As a whole, these SERS-based assays exhibited successfully the qualitative analysis of proteolysis detection. For medical applications, quantitative analysis is urgent desire to provide accurate information to diagnose. So far, SERS-based assays with quantitative detection are rare and uncertain since enhancement factors of SERS substrates vary with location. Therefore, it remains a great challenge to design stable SERS-based protease assays with convincing quantitative analysis.

1.5 SERS with Quantitative Analysis

The challenge of quantitative SERS analysis is that the local nanostructures and the coupling between SERS substrates severely influence the enhancement factors by several orders of magnitude. Even though the molecules are evenly distributed on the surface of metal nanoparticles, the intensities of SERS signal are not uniform. In this case, the SERS intensities are not proportional to the concentrations of target molecules. In other words, we cannot use SERS intensities to reflect concentration of target molecules. Thus, the SERS quantitative analysis was restricted.[34,35]

In order to satisfy quantitative analysis, internal standards were often introduced in SERS substrates. Briefly, internal standards are non-reactive reference molecules with high Raman responses in systems. As analyte molecules and internal standards are simultaneously adsorbed on the surface of metal nanoparticles, both molecules on the surface experience in the same environment. In other words, the SERS signals of both molecules have the same enhancement factors at each location even enhancement varies with location. Due to non-reactive property, the challenge of uneven enhancement can be overcome by normalizing SERS intensity of internal standards.[35] So far, with the addition of internal standards, our group successfully performed SERS quantitative detection for aldol condensation reaction (**Fig. 7**).[35] 4-mercaptophenol (MP) and 4-nitrothiophenol (NTP) are used as internal standard and reactant. Both reactants and internal standards were adsorbed on the particle surfaces via thiol-gold interactions. In colloidal solution, the aldehyde group on the nanoparticle reacted with acetone at alkaline condition via aldol condensation reaction. The relative intensities of the product are obtained by normalizing SERS

intensities of internal standards (**Fig. 8**). This quantitative analysis allows to monitor the kinetics of the chemical reactions and further explore the reaction mechanism.[35]

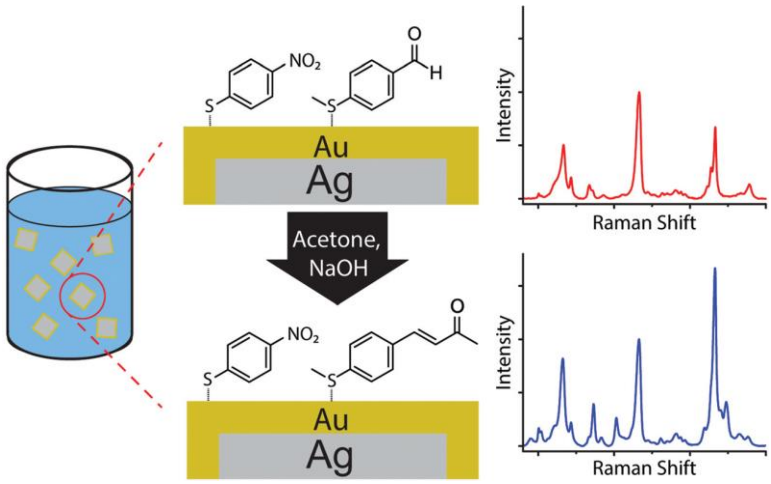

Figure 7: Schematic of the aldol condensation kinetic assay.[35] Reprinted from "Quantitative surface-enhanced Raman spectroscopy for kinetic analysis of aldol condensation using Ag–Au core–shell nanocubes."

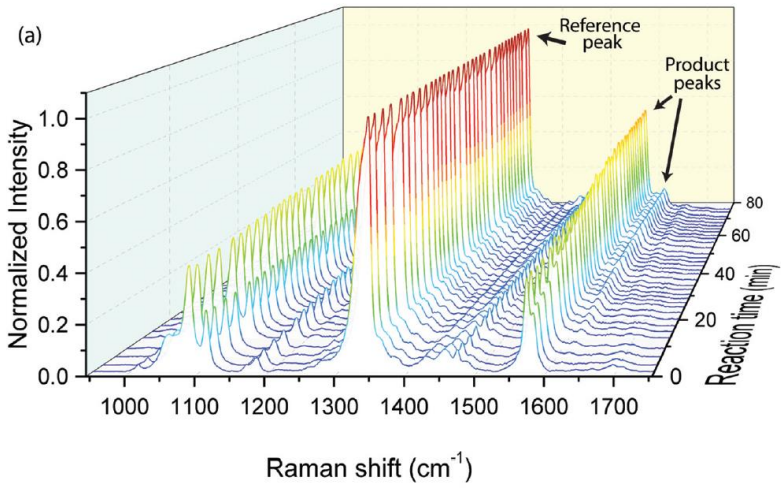

Figure 8: 3D SERS spectrum with time evolution during the aldol condensation reaction.[35] Reprinted from "Quantitative surface-enhanced Raman spectroscopy for kinetic analysis of aldol condensation using Ag–Au core–shell nanocubes."

Overall, our group fabricated successfully a SERS-based assay to satisfy quantitative requirement. Therefore, in this thesis, I adopted the same concept and replace aldol condensation reaction with proteolysis to achieve quantitative detection of protease activities.

1.6 Experimental Design

According to the above SERS-based assay from our group[35], my project also introduced the internal standards in the same manner to satisfy quantification. Here, I investigated the proteolysis reaction. For the proof-of-concept, I investigated the classic trypsin enzyme (23.8 kDa). Briefly, trypsin is a common serine protease and is produced in the pancreas. Its main function is to digest protein product into smaller peptide for absorption into the blood stream. Trypsin has histidine-57, aspartate-102 and serine-195 to form catalytic triad (**Fig. 9**). Initially, these three residues have electrostatic interactions to relay charge to increase nucleophilicity of the active site serine; thus, oxygen in the serine attacks the peptide bond to cause extra electrons on the carbonyl oxygen due to high nucleophilicity. Next, the extra electrons on the oxygen can reform a carbon-oxygen double bond so that carbon-nitrogen bond is broken to release first protein residue. Also, the nitrogen in the first protein residue receives a hydrogen from the histidine nitrogen. Then, the second protein residue is released by water attack in the same manner as serine. Besides, the function of aspartate residue located in the catalytic pocket is to attract and stabilize the positively charged residue, such as lysine and arginine. In other words, trypsin has digestion specificity at lysine and arginine.[36–38]

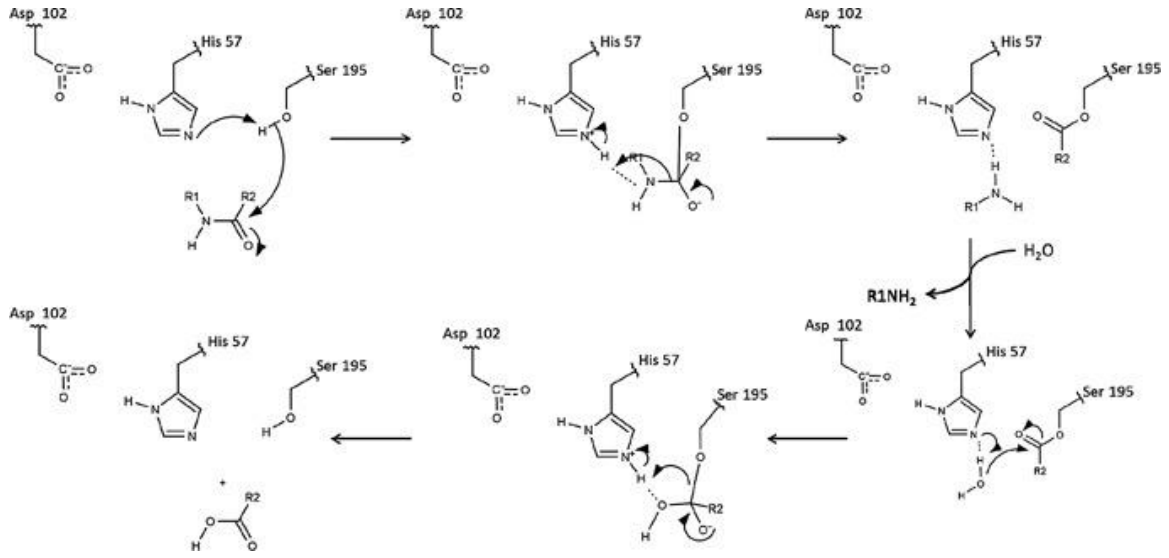

Figure 9: The mechanism of proteolysis by trypsin.[36] Reprinted from "Getting intimate with trypsin, the leading protease in proteomics."

In our experiment, target peptide sequence we tested is CQSARW (749.84 Da). With the catalysis of trypsin, the peptide chains are cleaved at the carboxyl side of arginine (R) and separated into a shorter peptide chain (sequence CQSAR) and a tryptophan (W) amino acid. For intact and cleaved peptide chains, the cysteine (C) provides a metal-thiolate linkage between silver nanocube (AgNC) and peptide. Additionally, only tryptophan (W) in the peptide sequence has strong SERS signal due to aromatic side chains. In this case, the cleaved peptide chains without tryptophan would have weak SERS signal; thus, the existence of tryptophan signal can represent intact peptide chains. In other words, the intensity change of tryptophan signal reflects the degree of proteolysis by trypsin. For quantitative analysis, the internal standard we used is 2-

mercaptoethanesulfonate (2-MES) in this assay since SERS signal of 2-MES does not overlap with the signal of target peptide.

After explaining individual conditions in our experiment, we illustrate all experimental procedures in detail from **Fig. 10**. First, intact peptide (CQSARW) is cleaved into shorter peptide (CQSAR) and tryptophan (W) in the presence of trypsin in aqueous solution. The peptide solution in initial and final reaction states mixed with internal standards is added on the solid film of silver nanocubes (AgNCs), respectively. After several washing and drying, these two SERS substrates that represent initial and final reaction states are detected by Raman spectroscopy. Due to the addition of internal standard, the reaction degree of proteolysis can be calculated by normalizing the signal of internal standard and comparing normalized intensity of target peptides. In this situation, the trypsin activities are monitored and determined in this SERS assay.

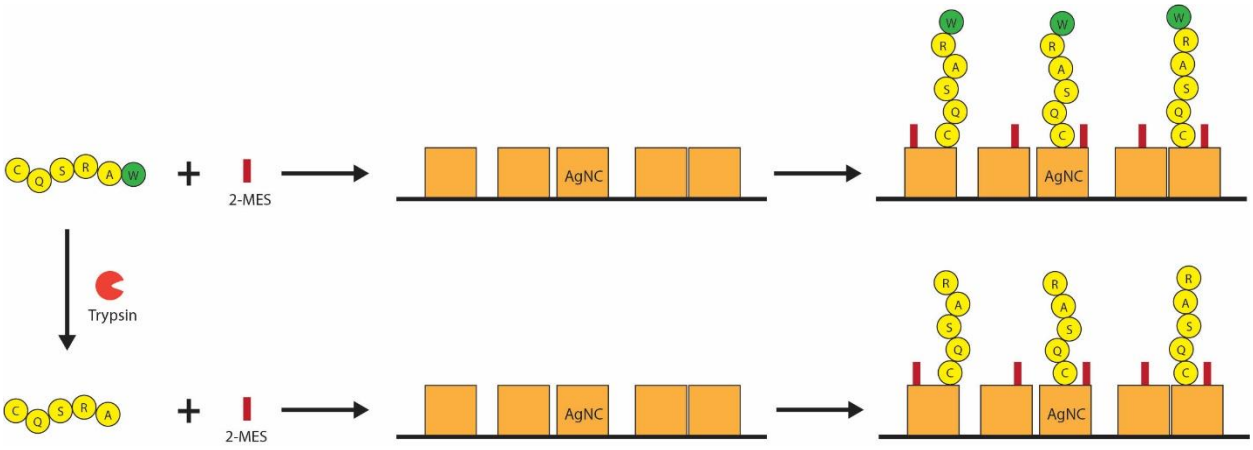

Figure 10: Schematic of SERS-based enzymes assay for trypsin.

CHAPTER II

EXPERIMENTAL SECTION

2.1 Materials

1,5-Pentanediol 98% (PD) were purchased from Acros Organics. Silicone oil, polyvinylpyrrolidone avg. MW 55000 (PVP), copper(II) chloride, Trypsin from bovine pancreas, trypsin inhibitor from chicken egg white (Type II-O), trypsin-chymotrypsin inhibitor from Glycine max (soybean), Tris buffer, and 2-mercaptoethanesulfonate (2-MES) were purchased from Sigma-Aldrich. Silver nitrate 99.9995% and sodium hydroxide pellets 98% were purchased from Alfa Aesar. L-Ascorbic acid 99.9% was purchased from Fisher Chemical. Peptide (CQSARW) 98.73% was purchased from NeoScientific.

2.2 Silver Nanocube (AgNC) Synthesis Procedure

We synthesized silver nanocubes (AgNCs) by following the polyol method.[39,40] First, silicone oil bath with magnetic stirring was prepared and fixed at 190 °C. 20 mL of 1,5-Pentanediol (PD) was heated in the silicone oil bath with temperature control by thermometer. As the temperature of PD solution increased to approximately 130 °C, 250 µL silver nitrate solution (0.2 g, in 10 mL PD containing ~0.02 mg $CuCl_2$ seeding agent) was added to initiate redox reaction. After 30 seconds, 500 µL PVP solution (0.2 g, in 10 mL PD) was injected to the reactor. After one minute, we repeated to inject 500 µL silver nitrate solution and 500 µL PVP solution every minute.

Once the solution became dark green, the solution was cooled down to stop reaction. Next, the nanocube solution was washed with 200 proof ethanol at least 4 times in order to remove the reactant and PD. Finally, the nanocube solution are purified by passing through 0.22 μm filter at least 5 times, and suspended in 50 mL ethanol.

2.3 Procedure for SERS-based Trypsin Assay

300μL AgNC in ethanol were washed with double deionized (DDI) water three times, and then suspend in 200μl DDI water. Also, a glass slide was covered with aluminum foil. Then, 15μL AgNC solution was added on aluminum foil at specific location as one spot, and a microscope glass slide usually was occupied by 12 spots for AgNC. After evaporating solution, AgNC would deposit on aluminum foil and form a circular pattern. The peptide digestion was performed in Tris buffer. 5μL of 0.5mg/ml peptide solution was mixed with 25μL of 1X Tris buffer. 5μL of 1mg/ml trypsin solution and 5μL DDI water were added to the peptide solution, and then incubated at room temperature (23°C) for 4 hours. For the control experiment, the trypsin solution was replaced with the DDI water. For trypsin inhibition experiments, trypsin inhibitor solutions were added to the peptide solutions to reach the desired inhibitor concentrations. 34μL of 20μM 2-MES dissolved in water was added to 18μL reacted solution at t=0hr and t=4hr. After digestion, pipetted 15μL of peptide solution on each printed SERS spot, and then dried the solution at 50°C. Then, pipetted 15μl DDI water on each printed spot three times to wash the salt and residual proteins. Finally, the SERS spectra of these printed spots were acquired using Raman microscope.

2.4 Instruments

Thermo Scientific DXR Raman microscope (Thermo Fisher Scientific, Inc.) with 780 nm diode laser excitation is used to measure Raman spectra. This Raman microscope is equipped with a Rayleigh rejection filter, a high-resolution (~2 cm^{-1}) diffraction grating, and a CCD detector. The laser focused on top surface of deposit AgNCs through 50X dark field objective. All spectra were measured at 5 mW laser power with an integration time of 5 seconds and 8 accumulations in the range of 300 cm^{-1} and 1874 cm^{-1}.

2.5 Spectral Data Analysis

The spectral data were pre-processed with background subtraction in the Raman in-built software, followed by baseline subtraction and spectral normalization in the Raman Processing software of MATLAB 2013b (MathWorks, Inc., Natick, MA). A spectrum peak at 1040 cm^{-1} represents the internal standard (2-MES), and a spectrum peak at 877 cm^{-1} represent the tryptophan (W) at the C-terminus. For quantification, the intensity of peptide at 877 cm^{-1} was normalized by the intensity of internal standard at 1040 cm^{-1}.

$$Normalized\ Intensity\ = \frac{I_{877cm^{-1}}}{I_{1040\ cm^{-1}}}$$

Also, "degree of digestion" was defined by dividing normalized intensity at t=0hr to normalized intensity t=4hr. Its physical meaning is 1 minus real degree of reaction, unreacted degree of proteolysis. As all peptides are completely cleaved, the degree of digestion is close to 0. When most peptides are intact without digestion, the degree of digestion remains about 1. Although degree of digestion should be equal to 1 minus the normalized intensity ratio in original definition, we still used "degree of digestion" to represent unreacted degree of proteolysis in the following content.

$$Degree\ of\ digestion\ = \frac{Normalized\ Intensity\ at\ t = 4\ hr}{Normalized\ Intensity\ at\ t = 0\ hr}$$

For degree of digestion at the concentration range of trypsin inhibitors, logistic with four parameters in the OriginPro 9.1 software (OriginLab Corp., Northampton, MA) was used to fit the curve. The equation of logistic with four parameters is shown as follows, including A1, A2, x0, and p fitted constants. Y is the response corresponding to the degree of digestion, and x is the concentration of trypsin inhibitors. A1 and A2 refer to the asymptote value on bottom and upper plateau, corresponding to the minimum and maximum response, respectively. p is the slope factor on linear region of the curve between bottom and upper plateau. x0 is the middle response between minimum and maximum response, corresponding to IC_{50}.[41]

$$Y\ =\ \mathrm{A2} + \frac{(A1 - A2)}{(1 + (x/x0)\hat{}p)}$$

CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Analytical Principle

We first analyzed the characteristic Raman peaks of peptide and 2-MES. Comparing the Raman spectra of peptide on AgNC (**Fig. 11B**) and bare AgNC (**Fig. 11D**), the peptide exhibited their characteristic bands at 757 cm^{-1}, 877 cm^{-1}, and 1010 cm^{-1} that are associated with the terminal amino acid, tryptophan (W). The bands at 756 cm^{-1} and 1010 cm^{-1} correspond to the ring breathing vibrations of indole ring, and the band at 875 cm^{-1} represents the H-scissoring on indole ring.[42] Also, the spectrum of internal standards on AgNC (**Fig. 11C**) showed the characteristic bands of 2-MES are located at 789 cm^{-1} and 1040 cm^{-1}, corresponding to C-S (sulfonic group) stretching vibration and symmetric n(SO_3^-) stretch, respectively.[43] In the following analysis, we investigated these characteristic SERS peaks of peptide and 2-MES.

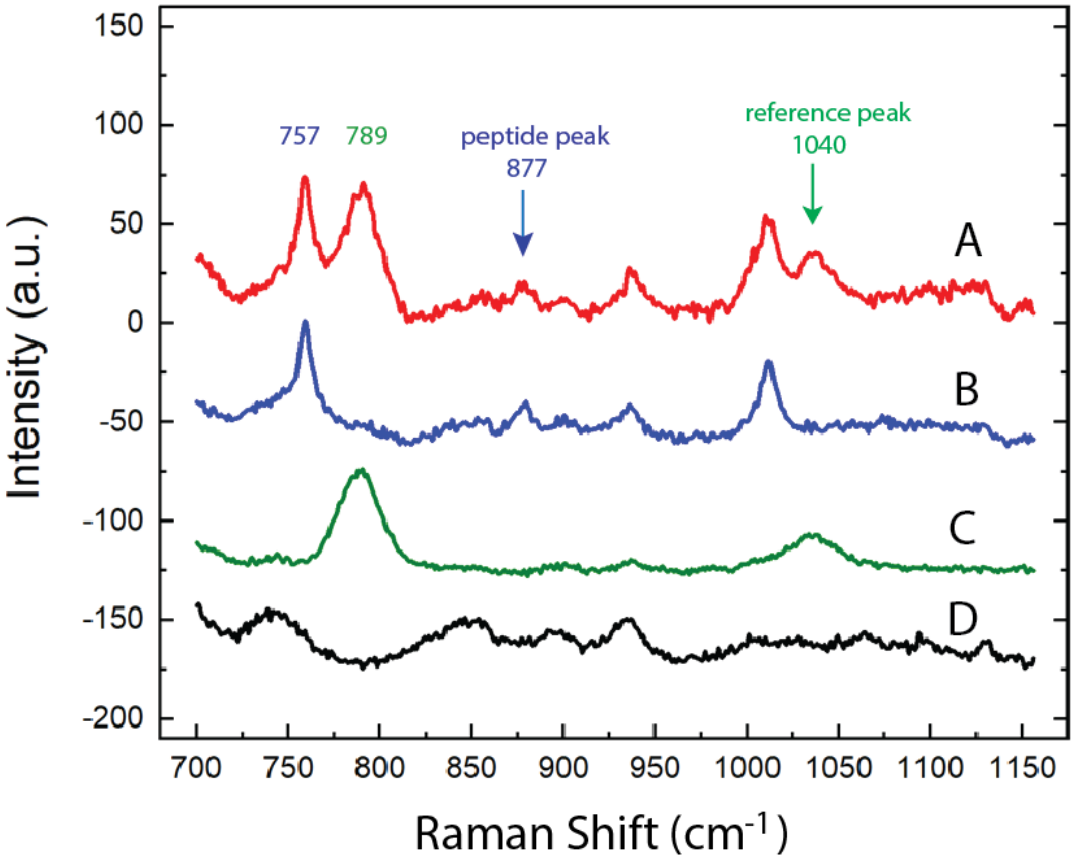

Figure 11: SERS spectra of (A) peptide and 2-MES on AgNC (B) peptide on AgNC (C) 2-MES on AgNC (D) bare AgNC. (B) tryptophan representing intact peptide has the characteristic bands at 757 $cm^{-1}$and 877 cm^{-1}. (C) internal standard (2-MES) has the characteristic bands at 789 $cm^{-1}$and 1040 cm^{-1}. The spectra data were pre-processed with background and baseline subtraction and were offset in y-axis for visualization.

According to **Fig. 11**, the Raman bands of the peptide at 757 cm^{-1} and 1010 cm^{-1} partially overlap with the background signals from the bare AgNC. However, there is no significant interference for the peptide band at 877 cm^{-1} compared with other spectra. Therefore, we chose the

band at 877 cm^{-1} to represent peptide signal in our assay. For 2-MES, both characteristic bands at 789 cm^{-1} and 1040 cm^{-1} could represent the signal of the internal standard. Because the band at 789 cm^{-1} exhibited the higher intensity, we first used this band as the reference to normalize the peptide signal. The normalized intensity of the peptide is defined as the ratio of the intensity at 877 cm^{-1} to the one at 789 cm^{-1}.

$$Normalized\ Intensity\ = \frac{I_{877 cm^{-1}}}{I_{789\ cm^{-1}}}$$

To quantify the degree of trypsin digestion, we compare the peptide intensities at t=0 hr to the peptide signals after 4 hr digestion.

$$Degree\ of\ digestion\ = \frac{Normalized\ Intensity\ at\ t = 4\ hr}{Normalized\ Intensity\ at\ t = 0\ hr}$$

To evaluate the reliability of our assay, the control experiments were performed in the absence of trypsin for 4 hours, and the degree of digestion should be approximately one. However, we observed significant data fluctuation when using the 789 cm^{-1} as the reference peak. (**Table 1**) In contrast, the variation of the degree of digestion could be minimized by using 1040 cm^{-1} as the reference. We observed the intensity variation of the peak at 789 cm^{-1} was about one order of

magnitude higher than the variation of the peak at 1040 cm^{-1}. In this case, the characteristic band at 789 cm^{-1} is more sensitive to the SERS environment. Therefore, the reference peak at 1040 cm^{-1} was applied to the following quantitative analysis. The normalized intensity of peptide is re-defined as the ratio of the intensity at 877 cm^{-1} to the one at 1040 cm^{-1}.

$$Normalized\ Intensity\ = \frac{I_{877cm^{-1}}}{I_{1040\ cm^{-1}}}$$

Table 1: The degree of digestion with standard errors for control experiments in the absence of trypsin based on two reference peaks (2-MES) and two peptide peaks. (n=6)

Degree of digestion		**Reference peak (cm^{-1})**	
		789	**1040**
Peptide peak (cm^{-1})	**757**	**2.154 ± 0.321**	**1.058 ± 0.054**
	877	**1.993 ± 0.456**	**0.964 ± 0.050**

3.2 SERS Analysis of Trypsin

With reliable analytical principle, we investigated proteolytic activities in the presence of trypsin. For proteolytic conditions, the common protocols of trypsin digestion in mass spectroscopy indicate that the ratio of trypsin and peptide is 1:20 to 1:100 (w/w); here we adopted 1:20 (w/w) for the ratio of trypsin and peptide in all experiments.[44]Also, the peptide digestion were performed for 4 hours in the addition of 0.25 mg/ml trypsin. Then, the peptide solution at t=0hr and t=4hr mixed with internal standards (2-MES) was added on the AgNC spots. Finally, the SERS substrates for t=0hr and t=4hr were measured by Raman spectroscopy.

Through the above-mentioned analytical principle, the raw SERS spectra were processed and shown in **Fig. 12**. After normalizing the Raman spectra by the peak at 1040 cm^{-1}, there are obvious intensity changed at 757 cm^{-1}, 877 cm^{-1}, and 1010 cm^{-1} after digestion (t=4hr). The decrease of the intensities demonstrated that the peptides were cleaved by trypsin. Meanwhile, it clearly explained the peak at 877 cm^{-1} is chosen to stand for the peptide due to partial overlap between the peaks at 757 $cm^{-1}$and 1010 cm^{-1} and the adjacent reference peaks at 789 cm^{-1} and 1040 cm^{-1} (**Fig. 11**). The normalized intensity at 877 cm^{-1} in initial reaction is approximately 0.5, and the normalized intensity is close to zero after 4 hours. By dividing these two normalized intensities, the degree of digestion (approximately 0.11) is used to quantify trypsin activities.

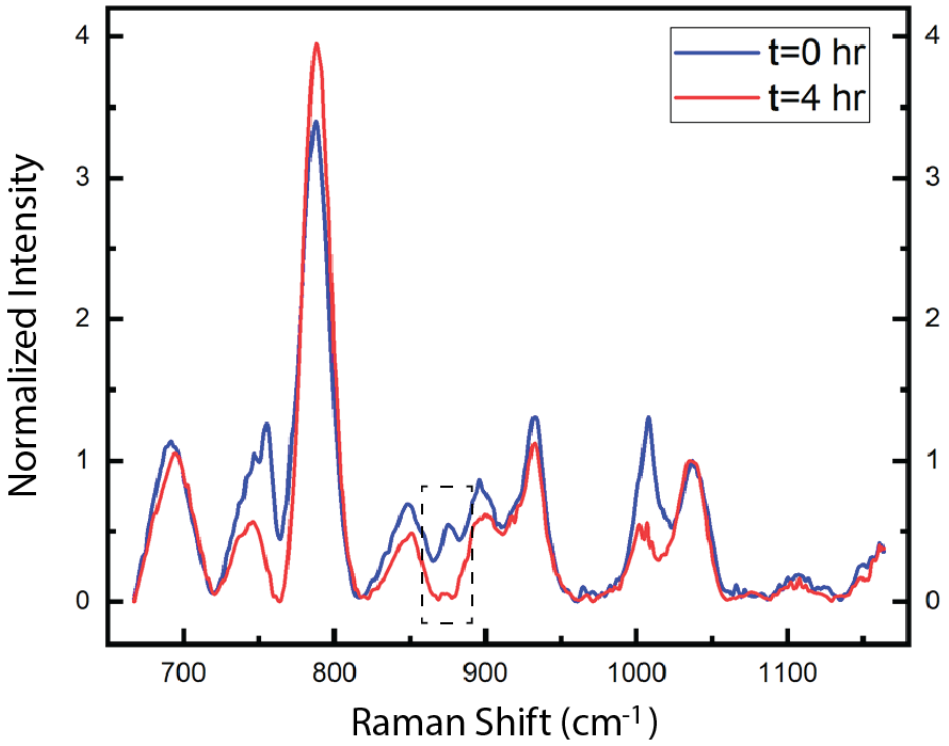

Figure 12: The SERS spectra of printed SERS substrates at t=0hr and t=4hr. Blue and red curves represent SERS spectrum at t=0hr and t=4hr, respectively. Black dashed box shows the change of normalized intensity of peptide between initial and final reaction states.

3.3 SERS Analysis of Trypsin Inhibitors

Protease inhibitors which block the function of proteases also play an essential role in the biology. Especially for HIV diseases, they can bind to viral HIV-1 proteases to prevent viral replication.[45,46] Herein, we investigated the performance of this SERS assay in the presence of trypsin inhibitors. The trypsin inhibitors used in our experiment are the trypsin inhibitors from chicken egg white type II-O (~28 kDa), Ovomucoid. At the fixed trypsin concentration (0.25 mg/ml), **Fig. 13** shows the normalized intensity ratios on the concentration range of trypsin inhibitors from 0.01 to 100μM and exhibits a reasonable sigmoidal trend. The degree of digestion at low concentrations of trypsin inhibitors is below 0.3, and the degree of digestion at high concentrations is approximately one. Also, there is a dramatic change between 4 to 20 μM trypsin inhibitors. This overall trend is the same as particular drug or other inhibitors used in biological processes. Usually, the half maximal inhibitory concentration (IC_{50}) is commonly used to explain the potency of inhibitors in a specific biological reaction. The definition of IC_{50} is the concentration of an inhibitor when its response is the middle of top and bottom plateaus. According to the literature[41,47], logistic with four parameters is a common equation used in pharmaceutical field to fit the data to obtain parameters including IC_{50}. The equation of logistic with four parameters is shown as follows, including A1, A2, x0, and p fitted constants. Y is the response corresponding to the degree of digestion, and x is the concentration of trypsin inhibitors. A1 and A2 refer to the asymptote value on bottom and upper plateau, corresponding to the minimum and maximum response, respectively. p is the slope factor on linear region of the curve between lower and upper plateau. x0 is the middle response between minimum and maximum response, corresponding to IC_{50}.

$$Y = \mathrm{A2} + \frac{(A1 - A2)}{(1 + (x/x0)\text{^}p)}$$

For Ovomucoid, we fitted the curve to acquire that minimum degree of digestion =0.241, maximum degree of digestion=1.03, IC_{50}=7.21 μM.. Furthermore, we tested the second trypsin inhibitor, trypsin-chymotrypsin inhibitor from Glycine max soybean (8 kDa), Bowman-Birk inhibitor (BBI). According to the **Fig. 13**, the protease activities of BBI show the similar sigmoidal curve from 6 to 400 μM trypsin inhibitors. In the same fitting manner, the degree of digestion on the bottom and upper plateau are 0.423 and 0.999 and the IC_{50} is 30.61 μM for Bowman-Birk inhibitor (BBI).

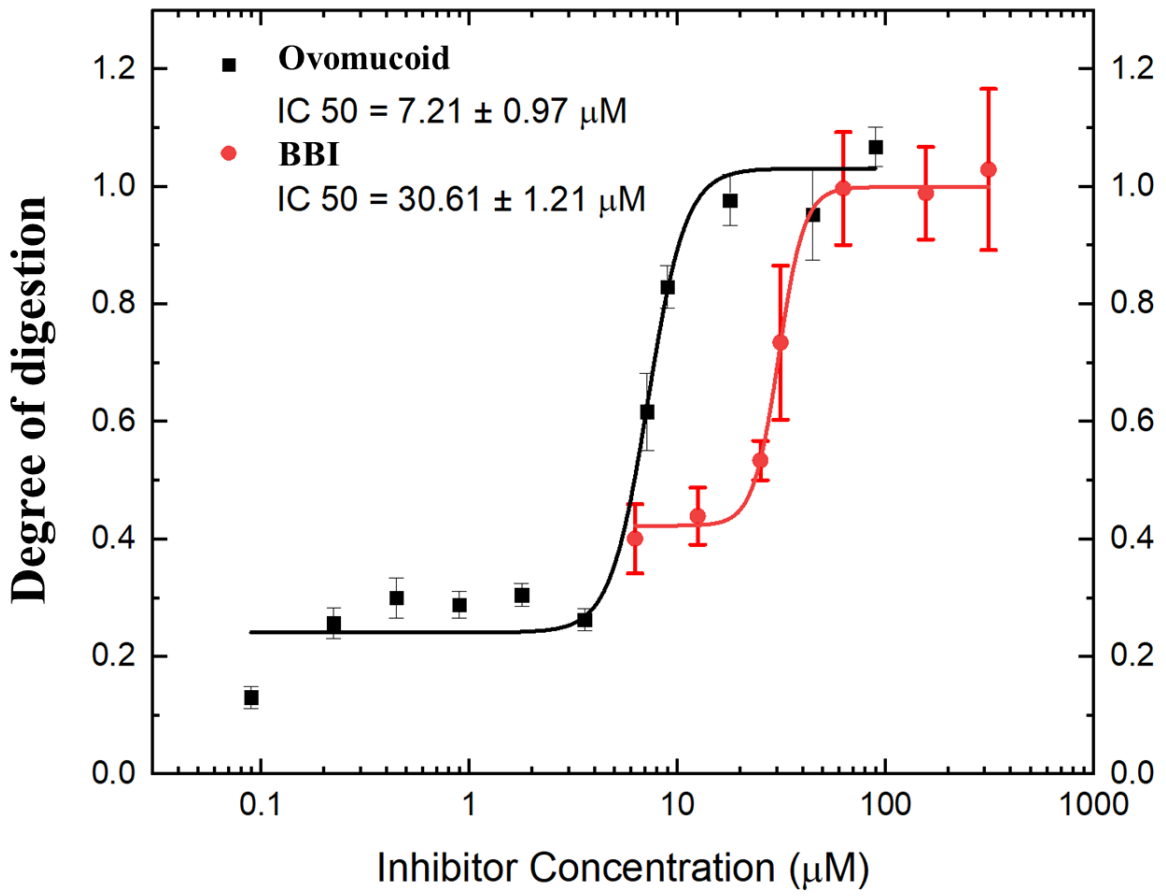

Figure 13: The degree of digestion with standard errors at the range of inhibitor concentrations. Black squares (n=12) and red circles (n=10) represent Ovomucoid and Bowman-Birk inhibitor (BBI), respectively.

3.4 Discussions

In our SERS assay, there is no process of trypsin removal before peptide solution was added on AgNCs spots. When added on AgNCs spots, the molecules in the solution including trypsin, peptide, and internal standards would diffuse on the AgNCs surface, parts of trypsin with positively charged amino acids were attracted on the AgNCs surface through electrostatic interactions. In this situation, trypsin probably would compete with the adsorption of peptide and internal standards to influence the detection results. Upon further considerations, we found that trypsin is a larger molecule (23.8 kDa) compared to peptides and internal standards, which leads to low diffusion rate before adsorption on the AgNCs surface. More importantly, the electrostatic interactions between AgNCs and trypsin are less than the Ag-S covalent bond for peptide and internal standard, so the adsorption of peptide or internal standard would replace original trypsin adsorption. Overall, based on these two points, the competitive adsorption of trypsin can be ignored in our SERS assay. Additionally, trypsin was removed with several washings on AgNCs spots before Raman measurements in order to avoid the interference of trypsin signal.

In biological systems, protease inhibitors also participate in the proteolysis to inhibit the protease activities in order to protect from viral replication. Therefore, we tested two trypsin inhibitors to demonstrate the availability of our SERS assay. For Ovomucoid, its function in 1 mg can inhibit approximately 1mg of trypsin with digestion of 10,000 Nα-Benzoyl-L-arginine ethyl ester (BAEE) unit for 1mg peptides.[48] From **Fig. 13**, Ovomucoid in our SERS assay exhibit inhibitory capabilities at the IC_{50} = 7.21 μM. According to the literatures[49,50], the IC_{50} of Ovomucoid for inhibition of sea urchin egg trypsin is 1.1 μM (**Table 2**). Thus, these similar IC_{50} values validated our SERS assay. On the other hand, Bowman-Birk inhibitor (BBI) we used in a

mg can inhibits ≥0.5 mg trypsin with activity of ~10,000 BAEE per mg peptides.[51] Our results showed Bowman-Birk inhibitor (BBI) in our SERS assay exhibit inhibitory capabilities at the IC_{50} = 30.61 μM. According to the prior research[49,50], the IC_{50} value of BBI is 6 μM for the inhibition of sea urchin egg trypsin (**Table 2**). Comparing our experimental data, it was found out both IC_{50} values exhibit the same orders of magnitude for two trypsin inhibitors. Overall, our SERS assay successfully demonstrates the feasibility in the detection of protease activities in terms of these two trypsin inhibitors.

In the literatures, the IC_{50} values for various trypsin inhibitors were obtained by monitoring hydrolysis of β-casein. The extent of hydrolysis is determined through measurements of the absorption at 280-290 nm in the clear filtrate due to acid precipitation of the unaltered casein. In their assay procedures, the mixture contained 0.5mM BAEE and 0.2M Tris buffer (pH=8), and one unit of trypsin enzyme can hydrolyze 1 μmol of BAEE per minute.

Table 2: The IC_{50} of various trypsin inhibitors for sea urchin egg trypsin.[49]

Trypsin inhibitors	**IC_{50} (μM)**
Chicken Ovomucoid	1.1
Bowman-Birk inhibitor	6
Soybean trypsin inhibitor	0.006

It is worth noting that all experiment data were collected from the interday experiments. The low interday variation demonstrates the reliability of our quantitative SERS analysis. Our SERS assay is convincing and reliable for proteolysis detection. With further modifications in the future, this SERS assay will be implemented for proteases activities in medical fields.

Although our SERS assay successfully demonstrates quantitative detection of proteolysis, current endpoint detection limits the applications on kinetic detection that may provide more information of the enzymatic mechanism. For example, the rate constant of aldol condensation reaction could be acquired via colloid SERS substrates.[35] In order to satisfy the transient detection, reaction phase must be changed from aqueous solution to colloidal solution because peptides chemisorbed adsorbed on AgNCs should continuously exhibit SERS signal. Thus, reactions must occur near the surfaces of AgNCs in colloidal solution. In this situation, the reaction near surface restricts the degree of freedom of reaction region and enzymes. In other words, adjacent peptides on AgNCs would not be cleaved simultaneously by proteases due to large molecule weights, leading to lower catalytic efficiency of proteases. Moreover, proteases sequences contain many amino acids with charges, so proteases adsorption on AgNCs probably causes unstable colloidal phase, thereby leading to AgNCs aggregation. These potential problems should be addressed in the future modification of our SERS assay, especially for kinetic detection.

CHAPTER IV

CONCLUSION

Due to high signal enhancement and multiplexed analysis, SERS overcame limitations on other techniques and is widely used to detect various processes or reactions in pratice. Herein, we utilized SERS to detect and investigate the protease activities that are related to some diseases, such as Alzheimer's disease. For the design of our SERS assay, trypsin was chosen to be our target protease and deposit AgNCs are our SERS substrates. Also, quantitative analysis for protease activities is required in our SERS assay for medical applications. The introduction of internal standards provides reference probes on AgNCs to satisfy quantitative detection. With the catalysis of trypsin in 4 hours, our SERS assay clearly observed the decrease of peptides signal according to SERS spectra. By normalizing the characteristic peak of internal standard 1040 cm^{-1} and comparing normalized peptide peak at 877 cm^{-1} between t=0 and t=4hr, the degree of digestion was approximate 0.11, which represents high degree of reaction. Furthermore, the two trypsin inhibitors, Ovomucoid and Bowman-Birk inhibitor (BBI), were also involved in the reaction to test the feasibility of our SERS assay. Ovomucoid and Bowman-Birk inhibitor (BBI) in our SERS assay exhibit reasonable inhibitory trend with the IC_{50} = 7.21 μM and 30.61 μM, respectively. Overall, we successfully invented a new SERS assay to detect protease activities with quantitative analysis.

REFERENCES

1. De Strooper, B. Proteases and Proteolysis in Alzheimer Disease: A Multifactorial View on the Disease Process. *Physiological Reviews* **90,** 465–494 (2010).
2. Protease - Wikipedia. Available at: https://en.wikipedia.org/wiki/Protease. (Accessed: 19th April 2018)
3. The function of Proteases-enzyme. Available at: https://www.sinobiological.com/the-function-of-proteases-enzyme.html. (Accessed: 19th April 2018)
4. Hasanbasic, S., Jahic, A., Karahmet, E., Sejranic, A. & Prnjavorac, and. The Role of Cysteine Protease in Alzheimer Disease. *Materia Socio Medica* **28,** 235 (2016).
5. Hirota, M., Ohmuraya, M. & Baba, H. The role of trypsin, trypsin inhibitor, and trypsin receptor in the onset and aggravation of pancreatitis. *Journal of Gastroenterology* **41,** 832–836 (2006).
6. Protease mechanisms | Learn Science at Scitable. Available at: https://www.nature.com/scitable/content/protease-mechanisms-14462487. (Accessed: 19th April 2018)
7. López-Otín, C. & Bond, J. S. Proteases: Multifunctional Enzymes in Life and Disease. *Journal of Biological Chemistry* **283,** 30433–30437 (2008).
8. Xue, W., Zhang, G. & Zhang, D. A sensitive colorimetric label-free assay for trypsin and inhibitor screening with gold nanoparticles. *The Analyst* **136,** 3136 (2011).
9. Chen, G. *et al.* A general colorimetric method for detecting protease activity based on peptide-induced gold nanoparticle aggregation. *RSC Adv.* **4,** 6560–6563 (2014).

10. Chen, C.-K., Huang, C.-C. & Chang, H.-T. Label-free colorimetric detection of picomolar thrombin in blood plasma using a gold nanoparticle-based assay. *Biosensors and Bioelectronics* **25,** 1922–1927 (2010).
11. Ong, I. L. H. & Yang, K.-L. Recent developments in protease activity assays and sensors. *The Analyst* **142,** 1867–1881 (2017).
12. Marcus, R. A. Chemical and Electrochemical Electron-Transfer Theory. *Annual Review of Physical Chemistry* **15,** 155–196 (1964).
13. Cao, Y., Yu, J., Bo, B., Shu, Y. & Li, G. A simple and general approach to assay protease activity with electrochemical technique. *Biosensors and Bioelectronics* **45,** 1–5 (2013).
14. Ji, J. *et al.* Electrochemical detection of the activities of thrombin and its inhibitor. *Electrochemistry Communications* **16,** 53–56 (2012).
15. Shin, D.-S. *et al.* Micropatterned Surfaces Functionalized with Electroactive Peptides for Detecting Protease Release from Cells. *Analytical Chemistry* **85,** 220–227 (2013).
16. Jares-Erijman, E. A. & Jovin, T. M. FRET imaging. *Nature Biotechnology* **21,** 1387–1395 (2003).
17. Matayoshi, E., Wang, G., Krafft, G. & Erickson, J. Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *Science* **247,** 954–958 (1990).
18. Huang, Y. *et al.* Carbon Nanotube-Enhanced Polarization of Fluorescent Peptides: A Novel Amplification Strategy for Homogeneous Detection of Proteases. *Chemistry - An Asian Journal* **9,** 87–92 (2014).
19. Dresselhaus, M. S. & Terrones, M. Carbon-Based Nanomaterials From a Historical Perspective. *Proceedings of the IEEE* **101,** 1522–1535 (2013).

20. Bacart, J., Corbel, C., Jockers, R., Bach, S. & Couturier, C. The BRET technology and its application to screening assays. *Biotechnology Journal* **3,** 311–324 (2008).
21. Zhao, Q., Li, X.-F. & Le, X. C. Aptamer Capturing of Enzymes on Magnetic Beads to Enhance Assay Specificity and Sensitivity. *Analytical Chemistry* **83,** 9234–9236 (2011).
22. Zhang, Y. *et al.* Histone controlled aggregation of tetraphenylethene probe: A new method for the detection of protease activity. *Sensors and Actuators B: Chemical* **257,** 1143–1149 (2018).
23. Raman Spectroscopy. Available at: http://www.personal.psu.edu/users/a/z/azn5189/assignment6.html. (Accessed: 20th April 2018)
24. Bumbrah, G. S. & Sharma, R. M. Raman spectroscopy – Basic principle, instrumentation and selected applications for the characterization of drugs of abuse. *Egyptian Journal of Forensic Sciences* **6,** 209–215 (2016).
25. Raman spectroscopy - Wikipedia. Available at: https://en.wikipedia.org/wiki/Raman_spectroscopy. (Accessed: 20th April 2018)
26. Raman Spectroscopy - A Tutorial | Kaiser Optical Systems, Inc. Available at: http://www.kosi.com/na_en/products/raman-spectroscopy/raman-technical-resources/raman-tutorial.php. (Accessed: 20th April 2018)
27. Advantages and disadvantages of Raman Spectroscopy - Romanian Database of Raman Spectroscopy. Available at: http://www.rdrs.ro/blog/articles/advantages-disadvantages-raman-spectroscopy/. (Accessed: 20th April 2018)

28. Yuen, C., Zheng, W. & Huang, Z. Surface-enhanced RAMAN scattering: Principles, Nanostructures, Fabrications, and Biomedical Applications. *Journal of Innovative Optical Health Sciences* **01,** 267–284 (2008).
29. Surface-enhanced Raman spectroscopy - Wikipedia. Available at: https://en.wikipedia.org/wiki/Surface-enhanced_Raman_spectroscopy. (Accessed: 20th April 2018)
30. Le Ru, E. C. & Etchegoin, P. G. *Principles of surface-enhanced Raman spectroscopy: and related plasmonic effects*. (Elsevier, 2009).
31. Moskovits, M. Surface-enhanced spectroscopy. *Rev. Mod. Phys.* **57,** 46 (1985).
32. Wu, Z., Liu, Y., Zhou, X., Shen, A. & Hu, J. A "turn-off" SERS-based detection platform for ultrasensitive detection of thrombin based on enzymatic assays. *Biosensors and Bioelectronics* **44,** 10–15 (2013).
33. Wu, Z. *et al.* A simple and universal "turn-on" detection platform for proteases based on surface enhanced Raman scattering (SERS). *Biosensors and Bioelectronics* **65,** 375–381 (2015).
34. Shen, W. *et al.* Reliable Quantitative SERS Analysis Facilitated by Core-Shell Nanoparticles with Embedded Internal Standards. *Angewandte Chemie International Edition* **54,** 7308–7312 (2015).
35. Weatherston, J. D., Worstell, N. C. & Wu, H.-J. Quantitative surface-enhanced Raman spectroscopy for kinetic analysis of aldol condensation using Ag–Au core–shell nanocubes. *The Analyst* **141,** 6051–6060 (2016).
36. Vandermarliere E, Mueller M, Martens L. Getting intimate with trypsin, the leading protease in proteomics. *Mass Spectrom Rev.* **32**, 453-465 (2013).

37. Trypsin - Wikipedia. Available at: https://en.wikipedia.org/wiki/Trypsin. (Accessed: 23rd April 2018)
38. Vandermarliere, E., Mueller, M. & Martens, L. *Getting intimate with trypsin, the leading protease in proteomics*. **32,** (2013).
39. Wu, H.-J. *et al.* Membrane-protein binding measured with solution-phase plasmonic nanocube sensors. *Nature Methods* **9,** 1189–1191 (2012).
40. Tao, A., Sinsermsuksakul, P. & Yang, P. Polyhedral Silver Nanocrystals with Distinct Scattering Signatures. *Angewandte Chemie International Edition* **45,** 4597–4601 (2006).
41. Sebaugh, J. L. Guidelines for accurate EC50/IC50 estimation. *Pharmaceutical Statistics* **10,** 128–134 (2011).
42. Zhu, G., Zhu, X., Fan, Q. & Wan, X. Raman spectra of amino acids and their aqueous solutions. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **78,** 1187–1195 (2011).
43. Zheng, X. *et al.* Determination of the sodium 2-mercaptoethanesulfonate based on surface-enhanced Raman scattering. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **81,** 578–582 (2011).
44. Trypsin Digestion Protocols | Proteomics & Mass Spectrometry |SUNY Upstate Medical University. Available at: http://www.upstate.edu/proteomics/prep/trypsin.php. (Accessed: 27th April 2018)
45. Ghosh, A. K., Osswald, H. L. & Prato, G. Recent Progress in the Development of HIV-1 Protease Inhibitors for the Treatment of HIV/AIDS. *Journal of Medicinal Chemistry* **59,** 5172–5208 (2016).

46. Protease inhibitor (pharmacology) - Wikipedia. Available at: https://en.wikipedia.org/wiki/Protease_inhibitor_(pharmacology). (Accessed: 29th April 2018)
47. IC50 - Wikipedia. Available at: https://en.wikipedia.org/wiki/IC50. (Accessed: 27th April 2018)
48. Trypsin inhibitor from chicken egg white Type II-O, Partially purified ovomucoid, containing ovoinhibitor | Sigma-Aldrich. Available at: https://www.sigmaaldrich.com/catalog/product/SIGMA/T9253?lang=en&region=US. (Accessed: 1st May 2018)
49. Alliegro, M. C. & Schuel, H. Characterization of soybean trypsin inhibitor sensitive protease from unfertilized sea urchin eggs. *Biochemistry* **24,** 3926–3931 (1985).
50. Sato, D. & Kato, T. Novel fluorescent substrates for detection of trypsin activity and inhibitor screening by self-quenching. *Bioorganic & Medicinal Chemistry Letters* **26,** 5736–5740 (2016).
51. Trypsin-chymotrypsin inhibitor from Glycine max (soybean) lyophilized powder | Sigma-Aldrich. Available at: https://www.sigmaaldrich.com/catalog/product/SIGMA/T9777?lang=en&region=US. (Accessed: 1st May 2018)