An outer membrane protease based approach for detection of E. coli in water and food

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**AN OUTER MEMBRANE PROTEASE BASED APPROACH
FOR DETECTION OF *E. COLI* IN WATER AND FOOD**

GAURAV SINSINBAR

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2019

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FOR DETECTION OF *E. COLI* IN WATER AND FOOD**

GAURAV SINSINBAR

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

5th Aug 2019

Date

A handwritten signature in cursive script, reading 'Gaurav', with a long horizontal stroke extending to the right.

Gaurav Sinsinbar

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

5th Aug 2019

Date

Prof Bo Liedberg

Authorship Attribution Statement

This thesis contains material from one paper published and 2 submitted or under preparation in the following peer-reviewed journal(s) in which I am listed as an author.

1. Chapter 5 was published as - S. E. Wood, G. Sinsinbar, S. Gudlur, M. Nallani, C. F. Huang, B. Liedberg, M. Mrksich, *Angew. Chemie - Int. Ed.* **2017**, 56, 16531–16535.

The contributions of the co-authors are as follows:

- M. Nallani, B. Liedberg, and M. Mrksich conceived the project. B. Liedberg and M. Mrksich jointly supervised the entire project.
- I expressed and purified all the proteases required for this study and generated all data relevant to protease characterization, including fluorescence and MALDI-TOF, but excluding SAMDI-MS data.
- Based on what was already known about the proteases' substrate specificity, I designed the peptide libraries and the design for extending the peptide sequence for SAMDI-MS screening.
- S. Wood synthesised the peptide arrays and generated all SAMDI-MS data. C. F. Huang assisted in synthesis of the peptide arrays.
- I, S. Gudlur, and S. Wood analyzed and interpreted the SAMDI-MS results.
- S. Wood and S. Gudlur prepared the manuscript, and I helped revise the manuscript with final revisions from B. Liedberg, and M. Mrksich.

2. Chapter 6

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- B. Liedberg conceived the project and jointly supervised the entire project.

- S. Wood was involved in optimizing the substrate and SAMDI-MS experiments.
 - H. Yildiz, P. Alagappan and G. Ammanath helped with the polythiophene (PTAA).
 - I performed the experimental study and design of the OmpT-Pep-PT assay followed by benchmarking of the same with inputs from S. Gudlur.
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- M. Nallani, and B. Liedberg conceived the project and jointly supervised the entire project.
- K. J. Metcalf performed the LC-MS experiments.
- I performed the expression, purification and other experimental work with discussion and suggestion on experiments from S. Gudlur.
- I and S. Gudlur analyzed and interpreted the data and prepared the manuscript with final revisions from M. Mrksich, M. Nallani, and B. Liedberg.

5th Aug 2019

Date

Gaurav Sinsinbar

Abstract

Bacterial pathogens such as *E. coli* and *Salmonella* are major contributors to food and waterborne diarrhoeal illness throughout the world. According to a 2015 WHO report, nearly 0.42 million people worldwide die each year because of the consumption of contaminated food or water. The spread of foodborne infection outbreaks can be restricted, preventing the numbers of deaths caused, by early detection of these bacterial pathogens in contaminated food and water. However, most commercially available bacterial detection kits rely on lab-based approaches, which are time-consuming, labour-intensive, or require dedicated instruments with a trained user. This thesis describes the development of a simple and rapid colourimetric assay for detecting *E. coli* in water and food. This is the first reported *E. coli* detection method that relies on activity of an outer membrane protease (OmpT) while employing unlabelled peptides.

OmpT, a protease present on the outer membrane of *E. coli*, acts as a defense mechanism for *E. coli* against human immune response and cleaves cationic antimicrobial peptides (e.g. LL37) at dibasic site. As reported in the literature, obtaining a fully functional wild-type OmpT via *in vitro* recombinant protein expression is extremely difficult due to its propensity to undergo autoproteolysis. This is because, OmpT carries di-basic sites within its own sequence making it prone to autoproteolysis. However, the findings from the current study suggest that during *in vitro* protein expression, the properly folded protein does not undergo autoproteolysis, but it is only the misfolded protein that is prone to autoproteolysis. Hence, efforts to improve protein refolding can significantly limit *in vitro* autoproteolysis of wild-type OmpT. This study as well as previous investigations suggest that lipopolysaccharide (LPS) is crucial for protease activity. LPS activates OmpT and is reported to bind to a predicted-binding site composed of five amino acids. However, mutating these five residues did not diminish OmpT activity, suggesting that the predicted LPS binding residues on OmpT are not critical for OmpT activity or that LPS might have more than one binding site on OmpT.

OmpT's substrate preference was investigated by designing, synthesizing and screening peptide libraries to identify the most optimal residues at the catalytic site. A high throughput mass spectrometry-based technique, SAMDI-MS, was employed to screen

rationally designed peptide arrays composed of tetrapeptide sequences whose P2, P1, P1' and P2' positions were varied from a starting peptide sequence, ARRA. The data obtained from screening such a peptide array suggested an absolute requirement of dibasic amino acids at P1 and P1' position and between lysine and arginine, OmpT preferred arginines at both P1 and P1' positions. This first array was useful in determining the preferred residues at each of the four positions. However, it is recognized that there is often an interdependence of amino acids at each position in the peptide sequence, and therefore arrays having two variable positions would be expected to reveal still more active substrates. So, a larger peptide array was generated by simultaneously varying the amino acids at P2 and P2' and whose screening led to the identification of a tetrapeptide sequence, FRRV with high catalytic activity for OmpT. Further optimizations were performed by incorporating FRRV into a fragment of the bacteria's natural substrate, yielding a peptide sequence for which OmpT exhibited a catalytic efficiency of $6.1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, a 400-fold improvement in catalytic efficiency over the natural sequence. Thus, *E. coli* expressing OmpT on its surface can efficiently cleave this optimized 15-mer peptide into two shorter fragments of seven and eight residues.

By combining the (1) proteolytic activity of OmpT, (2) an optimized peptide substrate specific for OmpT, and (3) an anionic polythiophene (PT) as a reporter molecule, an assay was designed and developed to detect *E. coli* in water samples. Mixing the unlabelled peptide substrate and PT in an aqueous buffered solution resulted in a significant increase in PT's fluorescence intensity with a concomitant colour change from orange to yellow that is detectable via naked eye. However, exposing the unlabelled peptide substrate to *E. coli* (or OmpT) prior to mixing it with PT resulted in no colour change, thus facilitating the detection of *E. coli*. Circular dichroism data confirmed that this change in the optical properties of PT coincides with a conformational change in the secondary structure of both the PT and the peptide. Unlike the intact peptide, the cleaved peptide fragments were unable to bring about a conformational change in the PT. Besides relying on the proteolytic activity of surface OmpT on *E. coli*, the assay also relies on the ability of PT to distinguish between the intact and cleaved peptides. Using this assay, a detection limit of as little as 1 CFU/mL of *E. coli* (K12 and J96 strains) was achieved within 6 and 8 h, respectively. With an assay time that is ~70 % lower when compared to the standard 24 h for most

commercially sold home water testing kits that are based on β -galactosidase activity, this OmpT-Pep-PT assay is clearly a significant improvement towards a faster, easier, and more sensitive *E. coli* detection method.

Since OmpT belongs to the Omptin family of proteases that include membrane proteases from *Salmonella*, *Y. pestis*, and *Shigella* which share structural similarities with OmpT, a similar approach to assay development is underway to identify unique recognition sequences for these Gram-negative bacteria. The work described in this thesis provides a useful platform towards the development of a multiplex assay capable of identifying more than one bacteria in the same test.

Lay Summary

Bacterial pathogens such as *E. coli* and *Salmonella* are major contributors to food and waterborne diarrhoeal illness throughout the world. According to a 2015 WHO report, nearly 0.42 million people worldwide die each year because of the consumption of contaminated food or water. The spread of foodborne infection outbreaks can be restricted, preventing the numbers of deaths caused, by early detection of these bacterial pathogens in contaminated food and water. However, most commercially available bacterial detection kits rely on lab-based approaches, are time-consuming, labour-intensive, or require dedicated instruments with a trained user. This thesis describes the development of a simple and rapid colourimetric assay for detecting *E. coli* in water. As far as one can tell from the literature, this is the first reported functional assay that employs enzymes present on the surface of these bacterial pathogens.

Gram-negative bacteria such as *E. coli* and *Salmonella* have an inner and outer membrane surrounding the cell. A family of proteases (enzymes that cleave peptides or proteins) called OmpTins, reside on the outer membrane of such microbes and defend them from antimicrobial peptides secreted by human host cells. They do this by cleaving the antimicrobial peptides at specific locations and rendering them ineffective. OmpT is one such ompT protease present on the outer membrane of *E. coli*. For studying the activity of OmpT, it was produced in pure form using recombinant protein expression technology. During this process, OmpT showed a self-destruction property, which has been previously reported. However, the findings in this thesis suggests that only the fraction of OmpT that is improperly folded undergoes self-destruction, while the properly folded protein is resistant to self-destruction. The outer membrane of Gram-negative bacteria is abundantly covered with lipopolysaccharide (LPS), a negatively charged molecule that is also an activator of OmpT's protease activity. Previously, it was reported that LPS activates OmpT by binding to a specific site on OmpT and it is predicted that this site is most likely composed of five specific amino acids. However, in this study, it was shown that mutating these five amino acids did not affect OmpT activity, leading to the conclusion that the predicted LPS binding amino acids on OmpT are not critical for OmpT activity. It is possible that LPS might have more than one binding site on OmpT.

OmpT is known to cleave a short peptide composed of four amino acids in a defined order: Alanine-Arginine-Arginine-Alanine (ARRA). A library of different peptide substrates was designed and screened with OmpT to check how efficiently each peptide was cleaved into smaller fragments. For this, a technique called SAMDI-MS was used which can screen extensive peptide libraries for any mass-based change. From this screening, Phenylalanine-Arginine-Arginine-Valine (FRRV) was identified to be the best peptide sequence. Subsequently, a longer peptide was designed by substituting FRRV into a fragment of a natural substrate. This designed longer peptides substrate, LL37_{FRRV} was degraded by OmpT 400 times better as compared to the natural substrate used. LL37_{FRRV} was used in developing a simple method for detecting *E. coli* with a colour readout.

The detection method is based on a simple thought process that every organism has a preference for certain type of food(s) which can be used to identify them. Consider a situation where it becomes necessary to identify an animal without having the option to see, hear, or smell it. One way to go about identifying this animal would be to offer it a choice of bamboo and meat as its lunchtime meal. If the animal consumes bamboo leaving behind the meat, one would guess the animal to be a panda. However, if it ate the meat, there would be many options and more trials would be necessary before it is possible to establish the identity of this animal. Similarly, by designing a peptide substrate that is specifically degraded by a particular enzyme, it can be used for its detection. Hence, the longer peptide which is degraded efficiently by OmpT present on *E. coli* was used to develop a colour based sensor specific for *E. coli*.

By combining (1) OmpT, (2) LL37_{FRRV}, and (3) a light emitting polymer, a method was designed and developed to detect *E. coli* in water samples. The light emitting polymer when mixed with LL37_{FRRV} gives a bright orange colour when viewed under UV light. However, no bright orange colour was observed if LL37_{FRRV} was first exposed to water contaminated with *E. coli* before mixing it with the light emitting polymer. The LL37_{FRRV} peptide is positively charged and interacts with the negatively charged light emitting polymer via electrostatic attractions leading to changes in colour and such samples display a very bright orange colour under UV light. Interestingly, the longer peptide once degraded by OmpT present on *E. coli* does not show this colour change or bright orange colour under UV. The presence or absence of colour change is

correlated to the presence or absence of OmpT, and by extension, *E. coli*. Using this method, as little as 1 *E. coli*/ mL of water can be detected in as less as 8 hrs. This method is easy to use, fast, and cheap when compared to commercially available home *E. coli* detections kits. Like OmpT on *E. coli*, PgtE is present on *Salmonella*, and similar omptin proteases are present on other bacterial pathogens like *Y. pestis*, and *Shigella*. The proteases are structurally similar but have different substrate specificity. By designing substrates that are specific to each of these proteases, it is possible to develop a multiplex assay capable of identifying more than one bacterial pathogen at a time.

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Abbreviations

WHO	World Health Organization
OmpT	Outer membrane protease in <i>E. coli</i>
OMP	Outer membrane protein
LL37	A cationic antimicrobial peptide
LPS	Lipopolysaccharides
SAMDI-MS	Self-Assembled Monolayer Desorption Ionization-Mass Spectrometry
CFU	Colony Forming Unit
PT	Polythiophene
CAGR	Compound Annual Growth Rate
PCR	Polymerase Chain Reaction
QCM	Quartz crystal microbalance
cAMPs	cationic Anti-Microbial Peptides
HTS	High Throughput Screening
CP	Conducting Polymer
LOD	Limit of Detection
ELISA	Enzyme-linked immunosorbent assay
LAMP	Loop-mediated isothermal amplification
IMS	Immunomagnetic separation
SPR	Surface Plasmon Resonance
LFI	Lateral Flow Immunoassay
FRET	Fluorescence Resonance Energy Transfer
QDs	Quantum Dots
GO	Graphene Oxide
CNTs	Carbon Nanotubes
OMPs	Outer Membrane Proteins
RMSD	Root Mean Square Deviation
AUC	Area Under Curve
ICD	Induced Circular Dichroism
MW	Molecular Weight
BCA	Bicinchoninic acid assay
LC-MS	Liquid Chromatography-Mass Spectrometry

MALDI	Matrix-assisted laser desorption/ionization
FAM	Fluorescein Isothiocyanate
PTAA	Polythiophene Acetic Acid
PTFE	Polytetrafluoroethylene
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide gel electrophoresis
DTT	Dithiothreitol
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride

Chapter 1

Introduction

Every year, millions of people are affected by foodborne infections (FBIs). According to the most recent WHO report, FBIs are the reason for 0.42 million deaths globally. Among all different causative agents, E. coli and Salmonella are the two leading causes of FBIs. Consumption of water and food contaminated with such bacterial pathogens can cause diarrheal infections and in extreme cases, may result in death. Early detection of disease outbreak and the implementation of rigorous safety standards can help limit the spread of FBIs. Current bacterial detection methods – those commercially available in the market and the ones under development, have various limitations such as, being time-consuming, costly, labour-intensive, and require a trained user. Hence, the development of fast, cheap, sensitive, and fieldable sensing platforms are the need of the hour for controlling these outbreaks. This thesis focuses on understanding the substrate preferences of proteases present on the outer membrane of E. coli and Salmonella, and on developing a colourimetric assay that relies on the protease's activity for detecting the bacteria in contaminated water and food. This chapter briefly describes the current bacterial detection methods and their limitations, which sets up the foundation of the problem statement the thesis is trying to solve. It further outlines the strategy adopted to develop the assay, objectives and scope of the thesis, and an overview of each chapter in the thesis. The chapter concludes with the significant findings of the research work.

1.1 Background

Pathogenic bacteria pose a significant threat to human health. According to a report published by the World Health Organization (WHO) in 2015, food and water-borne illnesses affect one in 10 individuals around the world causatively contributing to mortality and morbidity, and impeding socio-economic development.¹ According to the WHO, these are mostly unreported, undiagnosed, and untreated class of diseases that take a substantial toll on societies. Among all causative agents spanning microbial, parasitic, and chemical origins, *E. coli* and *Salmonella* are the most prevalent, causing ~120,000 deaths each year globally. They spread via contaminated water, uncooked food such as vegetables, fruits, meat, eggs, or milk, and cause infections characterised by diarrhoea, fever, abdominal pain, nausea, leading, in extreme cases, to severe dehydration. Thus, early detection of bacterial contamination in food and water supplies is critical for containing, controlling, and preventing *E. coli* and other bacterial outbreaks.

According to a market intelligence report published in 2019 by MarketsandMarkets™, titled ‘Food Safety Testing Market by Target, Technology, Food Tested, and Region-Global Forecast to 2023’, the global food safety testing market is expected to reach \$24.6 billion by 2023, growing at a Compound Annual Growth Rate (CAGR) of 7.7 % from 2018-2023 (Figure 1.1 A).² The market for bacterial testing of water has also grown exponentially in the last few years. The market size is projected to reach \$ 1.59 billion by 2022, growing at a CAGR of around 7.0% from 2017 to 2022 (Figure 1.1 B).³ 3M Company, Thermo Fisher, Neogen Corporation, Pathogen Detection Systems Inc are some of the global companies that currently manufacture and supply commercial kits for testing water and food for bacterial contamination. Even though there is a substantial market for water and food testing kits, the bottleneck remains that the kits are not fast, cheap, easy to use etc.

The current gold standard (i.e. the culture method) for detecting bacterial pathogens require pre-processing (i.e. homogenisation, enrichment, and separation of pathogens) of water and food samples before the samples can be grown in a particular growth medium that can vary depending on the type of bacteria being detected. Only after growing sufficient bacterial colonies, which can take anywhere from 12 h to 1 week,

can a final biochemical assay be performed on them to identify and quantify the level of bacterial contamination in the source sample (Figure 1.2).^{4,5}

Figure 1.1: Market analysis and global forecast: A) Food safety B) Microbiological testing of water. CAGR refers to a compound annual growth rate. Source: a report published by MarketsandMarkets™ (2019).^{2,3}

The overall bacterial detection process can take up to seven days, which not only makes it time-consuming and labour-intensive, but also requires trained personnel. While this approach is acceptable for certain situations, it becomes meaningless for home-kits or for quick screening in the food industry, where it is not feasible to wait for several days to confirm if the food or water for consumption or packaging and shipment is contaminated. Although various rapid detection methods based on nucleic acid and immunoassays have been developed to reduce the assay time to as little as ~24 h, they are still laboratory-based techniques requiring specialised instruments and trained personnel.^{4,6}

Besides being time-consuming, the current gold standard for bacterial detection requires different strain-specific media for their growth and different biochemical assays for their detection, thereby increasing the overall cost as well.⁷ Commercially available nucleotide-based bacterial detection kits from Thermo Fisher Scientific, Hygiene, Neogen *etc.*, rely on polymerase chain reaction (PCR) and generally require 8-24 h, which involves pre-culturing of the samples. PCR based assays are based on nucleotide amplification that require specialised lab-based equipment, reagents,

temperature control, and a trained user to perform the assay and interpret the results thereby increases the overall cost of the assay.

Figure 1.2: Comparison of detection time for the gold standard and advanced detection methods for bacterial pathogen detection.

Similarly, bacterial detection kits based on immunoassays such as the kits from FOODCHECK, Solus scientific, Biocontrol Systems *etc.*, require 8-24 h to obtain the results. Furthermore, these assays are not cost effective due to the use of ELISA based kits which require refrigeration during shipping and storage besides the need for sophisticated instrumentation to read output signals (e.g. Spectrophotometers for UV/vis or fluorescence). Chromogenic media-based assays for bacterial detection, such as those from Bio-Rad, also possess an overall assay time of up to 48 h. Although such bacterial detection kits for household use are commercially available, they are time-consuming and non-specific. For instance, AqualVial™, a water testing kit sold by AquaBSafe (Ontario, Canada) for *E. coli* detection is time-consuming and requires an incubation time of around 24 h before the results can be reported. Given the limitations of current approaches described above, the development of fast, cheap, sensitive, and easy to use assays are the need of the hour for controlling outbreaks caused by contaminated water and food. An alternative to such expensive and time-consuming detection methods, is an easy-to-use polymer-based fluorescence biosensor. Polymers such as anionic poly(2-(2-(4-methylthiophen-3-yloxy) ethyl)malonate acid), poly(para-phenyleneethynylene), poly(3-alkoxy-4-methylthiophene) exhibit strong fluorescence properties and have been employed in various detection method.⁸⁻¹⁰ These approaches

are simple to use since they only require the mixing of the polymer and test sample. Any change in the fluorescence intensity of these polymers upon binding to the bacterial surface is a good indicator of bacterial contamination. Although such methods have been reported previously, they are either based on nonspecific electrostatic interactions between polymer chains and bacterial surface or involve the use peptides that bind differentially to the components of bacterial surfaces resulting in poor limits of detection.^{9,10} However, by combining the advantage of ease-of-use offered by the polymer-based sensing approach with the advantage of lower limit of detection and specificity offered by enzymatic reactions, it is possible to develop an assay that is sensitive, selective, easy to use, and cost-effective.

1.2 Problem Statement/Hypothesis

Bacterial contamination of water and food are a leading cause of death in developing and underdeveloped nations. There is a compelling need to develop fast and easy to use assays that can detect the presence of pathogenic bacteria in water and food. Such assays will help in early detection of disease outbreaks and limit the spread of pathogenic infections. *E. coli*, being one of the major causes of these outbreaks, is an essential target for the development of such sensing platforms/assays. Hence, the hypothesis/problem being addressed in the thesis are:

- I. Understanding and limiting the autoprolysis of OmpT in order to obtain OmpT protease in sufficient quantity for its characterisation, substrate optimisation and assay development.
- II. Optimisation of a protease substrate to improve protease's catalytic efficiency by a factor of 10 or more by using a high throughput screen technique, to improve the sensitivity and assay time for detection.
- III. Development of a fast, cheap, sensitive, and easy to use assay for detecting 1 CFU/mL of *E. coli* and similar bacterial pathogens in water and food within 10 h in order to control and prevent the spread of disease outbreaks spread via contaminated water and food.

1.3 Assay Development Strategy

For developing an assay to detect bacterial pathogens in water and food, different sensing strategies and platforms can be employed. The sensing strategies can be

directed at the detection of bacterial growth, viability, presence/absence of cellular components, nucleic acid composition, cell surface and cytoplasmic receptors, or enzymatic activity. On the other hand, a variety of sensing platforms have been previously developed and employed, including optical sensors based on: i) absorbance and fluorescence of dyes, gold nanoparticles, and quantum dots, ii) electrochemical sensors based on graphene oxide and carbon nanotubes, iii) piezoelectric biosensors based on Quartz crystal microbalance (QCM) iv) Magnetoelastic biosensors, and v) Field-effect transistor (FET)-based devices.^{4,6} Depending on the choice of the platform, different levels of sensitivity for real-time bacterial detection have been reported previously.^{4,6}

During the development phase of an assay, it is essential to first identify an appropriate target and understand it. Most bacterial detection assays generally target protein receptors present on the pathogen's cell surface or specific nucleic acid sequences within the cell. The former has an advantage because the number of preprocessing steps can be minimised by not having to lyse the cells. On the bacterial membrane, both receptors and enzymes are present which carry out a plethora of different functions. When targeting receptors or other proteins on the membrane surface, an immunological assay is usually employed wherein antibodies bind to the target protein. Although this approach has been proven to have good reliability and specificity, it does suffer from insufficient sensitivity. The ratio of target and sensing biomolecule is usually 1:1, and unless the particular receptor is over-expressed or the primary signal is amplified via additional steps, its detection is limited to the total amount of pathogen present in the sample, which can be extremely low if early detection is mandated.⁴ On the other hand, detection based on enzymatic activity can lead to better sensitivities since they are not limited by a 1:1 stoichiometry of target to signal. Presence of low amounts of the enzyme can be detected if the substrate is efficiently transformed (digested or modified) by the enzyme. Acetylcholinesterase (AChE) is an example of an enzyme that has high catalytic efficiency where a single enzyme hydrolyses 2×10^7 molecules of acetylcholine per minute.¹¹ Hence, given sufficient time, even low concentrations of the enzyme is capable of converting enough substrate into a product to generate a sufficiently strong signal that can be easily detected, leading to improved detection limits. Several enzymes have been targeted for developing fast and sensitive assays, but almost all of them target enzymes inside *E. coli* and none on the bacterial surface.

Targeting enzymes on the bacterial surface gives an advantage of avoiding labour-intensive steps involved in DNA/RNA isolation for nucleic acids assays and circumvents stability and storage issues commonly faced with antibodies in immunoassays. One such enzyme that is present on *E. coli* surface and which can be targeted for assay development, is OmpT. It is a protease that is known to cleave short peptides at dibasic site and belongs to the omptin family of proteases. Omptin is a family of proteases present on the outer membrane of bacterial pathogens (Table 1.1).^{12–14} These proteases cleave cationic anti-microbial peptides (cAMPs) that are secreted by the human body as a defence mechanism against *E. coli* and other gram-negative bacteria.^{15,16} cAMPs are generally positively charged and exhibit different polypeptide secondary structures like α -helical, β -sheet, or an extended conformation.^{17,18}

Table 1.1: Members of the Omptin family of proteases.

Protein	Host organism	Identity to OmpT (%)	Size of mature protein (kDa)
OmpT	<i>Escherichia coli</i> K12	100	33.4
OmpP	<i>Escherichia coli</i> K12	73	33.1
SopA	<i>Shigella flexneri</i>	60	33.4
PgtE/PgtE2	<i>Salmonella enterica</i>	50	32.8
Pla	<i>Yersinia pestis</i>	50	32.6
PlaA	<i>Erwinia pyrifoliae</i>	47	32.3
Q8ZGQ6	<i>Yersinia pestis</i>	40	32.4

Note: Adapted from Hritonenko, V. *et al.*, 2007.¹²

The omptin family of proteases include OmpT on *E. coli* (causative agent of water & food born infections and urinary tract infections), PgtE on *Salmonella enterica* (causative agent for water and food born infections and typhoid fever)¹⁹, SopA of *Shigella flexneri* (causative agent for bacillary dysentery), and Pla of *Yersinia pestis* (causative agent for plague).¹² Among these, OmpT is the most well-characterised protease, and its crystallographic structure is known as well (Figure 1.3 A).²⁰ For structural studies and high throughput screening of peptide substrate library, OmpT is required in relatively large quantities. It is not feasible to purify milligrams of OmpT with high yield from the outer membrane of *E. coli*. However, OmpT can be obtained in relatively large quantities by recombinantly overexpressing it in *E. coli* as inclusion

bodies (IBs). The IBs can then be unfolded and refolded in the presence of detergent or phospholipids to obtain an active OmpT (Figure 1.3 B). Unfortunately, OmpT recombinantly overexpressed *in vitro* is known to undergo autoproteolysis and results in an inactive protease. OmpT carries three di-basic sites in its own protein sequence (Refer to section 2.3.2 of chapter 2 for more details) and thus becomes prone to autoproteolysis.¹⁴ This thesis focusses on understanding and limiting the cause of autoproteolysis during *in vitro* refolding, in order to obtain OmpT protease in sufficient quantity for its characterisation and screening experiments.

Figure 1.3: A) Crystal structure of OmpT²⁰ and illustrating LPS docked to the protein. B) Illustration depicting OmpT incorporated in a detergent micelle.

This thesis attempts to gain a better understanding of OmpT and PgtE substrate preference in order to develop it into a possible target for *E. coli* and *Salmonella* detection. In this particular case, the substrate will be a polypeptide. From previous studies on OmpT, it is known that the protease prefers to cleave short peptides at dibasic residues (Figure 1.4).

Figure 1.4: Illustration of OmpT cleaving at dibasic sites. The P1 and P1' represent the enzymes cleavage site as the Schechter and Berger nomenclature of the substrate specificity of the protease. P2 and P2' represent the amino acids in the vicinity of the cleavage site.

Among the 20 naturally occurring amino acids, these basic residues include lysine (K), arginine (R). Hence, any short peptide carrying an -RR-, -RK-, -KR-, and -KK- in its sequence is likely to be cleaved by OmpT. However, the efficiency with which such peptides are cleaved by OmpT will depend on two fundamental factors that are governed by the Michaelis Menten equation, K_m and k_{cat} which define the binding affinity and the turnover number of an enzyme, respectively. A small K_m indicates high binding affinity since it means the reaction can reach half the maximum velocity (V_{max}) at low substrate concentration. Whereas k_{cat} measures the number of substrate molecules "turned over" by enzyme per second. Hence k_{cat}/K_m , also referred to as catalytic efficiency, gives a measure of enzyme efficiency with a unit of $1/(\text{Molarity} \cdot \text{second})$ or $\text{L}/(\text{mol} \cdot \text{s})$. The enzyme efficiency can be increased by increasing k_{cat} (high turnover) and by decreasing the value of K_m (binding affinity). The turnover number depends on the cleavage site on the substrate while the binding affinity depends on the amino acids in the vicinity of the cleavage site. One approach to identify optimal substrates is to first design a peptide library where the amino acids at the cleavage site are varied. Screening such a peptide library for OmpT activity will help identify peptide sequences for which OmpT displays a high turnover rate. After identifying and fixing the optimal amino acids at the cleavage site, the amino acids on either side of the cleavage site can be varied to further improve the peptide's binding affinity for OmpT. In this manner, it is possible to design, screen, and identify the most optimal peptide for which OmpT exhibits the highest catalytic efficiency. However, screening of large peptide libraries designed based on prior knowledge of substrate specificity is difficult and expensive with traditional methods of screening such as SPOT technique, ELISA-based methods, and other currently used High Throughput Screening (HTS) techniques.²¹ The SPOT method allows simultaneous synthesis of multiple peptides on cellulose sheets, using fluorenyl-methoxy-carbonyl (Fmoc) chemistry. The peptides can then be cleaved from the cellulose surface or used directly on the surface for assay.²² SPOT, ELISA-based, and other HTS approaches rely on the use of fluorescent- or radio-labelling of the substrates that are being screened. This labelling can affect the activity and may not reflect the true affinity or catalytic efficiency of the proteases towards the labelled substrates.^{14,23,24}

Self-Assembled Monolayer Desorption Ionization-Mass Spectrometry (SAMDI-MS) is a label-free HTS technology. SAMDI-MS combines matrix-assisted laser desorption

ionisation time-of-flight (MALDI-TOF) mass spectrometry with Self-Assembled Monolayers (SAMs) of alkane thiolates on gold. Substrates for enzyme reactions, including peptides, proteins, carbohydrates, metabolites and others are immobilised on these SAMs and analysed using MALDI-TOF. (Refer to section 2.6 of chapter 2 for a more detailed description of the technique). Using SAMDI-MS, the Mrksich lab in Northwestern University (Evanston, IL) have performed HTS of peptide arrays and developed quantitative assays in a label-free approach.^{21,25–28} SAMDI-MS has been extensively used for assays of various enzymes like kinases, proteases, glycosyltransferases, methyltransferases, lysine deacetylases, and nucleic acid ligases.^{25,29,30} SAMDI-MS has been used to screen substrates for enzymes using two approaches: (i) Pulldown approach, which is an indirect approach and (ii) On-surface approach, which is a direct approach (Figure 1.5).

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Figure 1.5: Alternative strategies for identification of peptide hits using SAMDI-MS. (1) Pull down Approach: Pre-incubation of the protease with the peptides from a generated library in microtiter wells. The cleaved and un-cleaved peptide fragments from each individual well is transferred to the metal array plate for SAMDI-MS analysis. (2) On-surface Approach: Immobilization of the peptides from the library on the metal array plate. The array plate is then treated with the enzyme followed by SAMDI-MS analysis.

In the pull-down approach, the enzyme and substrates react in microwell plates and later immobilised on the SAMs. Whereas, in the on-surface approach, the substrate is immobilised on SAMs and then treated with the enzyme. These SAMs are then analysed using a MALDI instrument where irradiation of the spot with the laser results in cleavage of the gold-thiolate bond and release of the intact alkanethiolate which then accelerates to the detector and has its mass determined. In this way, if a molecule has been modified, the mass of the alkanethiolate increases (or decreases) by a specific

amount, and that change is readily monitored using the MALDI instrument. Enzyme activity is quantified using the intensity (I) of the peak for product and substrate in MALDI ($\text{Activity} = I_{\text{product}} / (I_{\text{substrate}} + I_{\text{product}})$).^{31–34} By employing SAMDI-MS, it is possible to screen peptide libraries to identify the optimal residues at the cleavage site and the residues in the immediate vicinity of the cleavage site.

After identifying the most optimal peptide for OmpT, this peptide can be combined with a reporter molecule to develop a colourimetric/fluorimetric assay for *E. coli* detection. As previously described in section 1.1, polymer-based sensors offer the advantage of being easy-to-use since it involves a simple mixing of polymer and test sample. Conducting polymers (CP) such as polythiophenes and polypyrrole are examples of such polymer-based sensors that have found applications in a variety of chemical and biosensors in the recent past.^{35,36} The optical properties of polythiophenes are tuneable by modifying the side chain with different functional groups. These functional groups can interact with DNA, proteins, small bio-analytes, metal ions, and surfactants. Such interactions can affect the conformation of the polythiophene backbone and lead to changes in their structural, electronic, and optical properties.^{36,37}

Figure 1.6: Chemical structure of polythiophene and polypyrrole. The position marked as * in polythiophene represents the position where the side chain can be modified.

CP based sensors are very sensitive to even minor conformational changes because of response amplification by a collective system response. CPs can exhibit a million-fold increase or decrease in fluorescence intensity because of two factors – delocalisation of the electronic excited state (exciton) and ultrafast exciton mobility along the conjugated polymer chain.³⁸ These properties of CPs can make them very valuable for the development of optical biosensors in the future.

In this thesis, the polythiophenes (PT) were used as the reporter molecule. The physical and chemical properties of polythiophenes can be modified by the introduction of suitable side chains in the 3 position (Figure 1.6 position marked as * in polythiophene). A conformational change in the peptide substrate interacting with a polythiophene can force the polymer backbone to adopt different conformations leading to a change in absorption and emission properties of the polythiophene. This property can be used to develop a novel sensing platform based on the conformational change in peptides. The side chain in PT presents a carboxylic group that can interact with positively charged amino acids of the peptide. The interaction between the optimized peptide substrate for OmpT and PT is likely to bring about a significant change in fluorescence that can be exploited to determine the presence or absence of *E. coli* in a water or food sample.

1.4 Objectives and Scope

I. Obtained OmpT protease in sufficient quantity for its characterisation and used in screening experiments.

a. Recombinantly expressed and purified wild-type and mutant OmpT in E. coli.

Used the protocol established by Kramer *et al.*, 2000¹⁴ as a guide, wild-type OmpT and OmpT with one or more amino acids mutated were recombinantly expressed as inclusion bodies (IBs) in *E. coli*.

b. Explored and tried to limit the cause of autoproteolysis during in vitro refolding, a phenomenon that is absent during in vivo expression.

Wild-type OmpT, when expressed in vitro, undergoes autoproteolysis. Refolding conditions and concentrations of LPS during OmpT refolding were investigated in an effort to identify and limit autoproteolysis.

II. Optimised peptide substrates for OmpT

a. Designed, synthesised, and screened small peptide array to optimise the cleavage site of the substrate

In order to understand the amino acid preference of OmpT at different positions (P2, P1, P1' and P2' - Figure 1.4), all 4 positions in a previously known substrate, ARRA, were modified with 19 different amino acids one at a time. The peptide library of 76

peptides (XRRA, AXRA, ARXA and ARRX; where X is any of 19 amino acids except cysteine) were screened using SAMDI-MS.

- b. Designed, synthesised, and screened larger peptide array to optimise amino acids in the immediate vicinity of the cleavage site.*

Based on the results from screening the 76-peptide library for OmpT catalytic activity, amino acids at the cleavage site was fixed and the amino acids in next neighbour were varied simultaneously (**X** P1-P1' **Y**; where X and Y are any of 19 amino acids except cysteine). The larger library of 361 peptides was screened for OmpT catalytic activity using SAMDI-MS.

- c. Improved peptide binding affinity by extending the peptide length*

The optimised tetrapeptide from 1.4 ii b was substituted into the peptide sequence of a natural OmpT substrate such as LL37 to improve the binding affinity of the peptide substrate. The OmpTs catalytic activity against this peptide was compared with native LL37 peptide fragment and ARRA substituted LL37 fragments. Kinetic analysis for all 3 peptides was performed to obtain the k_{cat} and K_m values for these peptide substrates.

- d. Compared and investigated changes in peptide substrate preferences of wild-type and mutant OmpT*

Wild-type OmpT and OmpT with two residues mutated (to prevent autoproteolysis) were used to screen the 361-peptide library to examine if there is any change in the substrate preference for the two proteases.

III. Developed a polythiophene-based colourimetric assay that relies on OmpT activity for detecting pathogenic bacteria like E. coli in water.

- a. Evaluated the effect of peptide-polythiophene interaction on the polythiophene fluorescence, absorbance and secondary structure.*

Changes in optical properties (absorbance and fluorescence) of polythiophenes were investigated in the presence and absence of various peptide substrates for OmpT. The polythiophene and peptide pair that exhibit the maximum change in the optical property upon interaction were used for further experiments.

b. Evaluated assay with wild-type OmpT in detergent micelles

The assay was evaluated with recombinantly expressed OmpT reconstituted into detergent micelles. Fluorescence, absorbance and CD measurements were collected to characterise the assay.

c. Tested the assay with water contaminated with intact E. coli.

The assay was tested with live bacteria spiked in water and food samples to determine the limit of detection and assay time. Four different strains of *E. coli* were used to evaluate the assay.

In summary, an enzyme-based functional assay was developed based on the 3 pillars :

i) Expression, purification and characterization of the OmpT protease; ii) Synthesis of a substrate peptide library and screening it using SAMDI-MS to identify a substrate that showed high catalytic efficiency; and iii) Employed polythiophene as reporter for colorimetric/fluorimetric detection of *E. coli* in water. The assay will be referred as OmpT-Peptide-Polythiophene (OmpT-Pep-PT) assay.

1.5 Dissertation Overview

The thesis addresses the need to develop a protease-based assay for the detection of bacterial pathogens (*i.e. E. coli, Salmonella*) in water and food, and help in preventing the spread of bacterial foodborne infections. This thesis is comprised of 7 chapters:

Chapter 1 provides an overview of the prevalence of water and foodborne infections, current detection techniques for bacterial contamination in food and water, and some of the short-comings of the current detection techniques. This chapter also provides an outline of the strategy adopted to develop a new protease-based assay for *E. coli* detection, which is the focus of this thesis. The chapter ends with the list of objectives, scope, and original findings of this thesis.

This thesis describes a rational design approach to optimising substrates for outer membrane proteases and the identification of a substrate for which OmpT exhibited a 400-fold improvement in catalytic efficiency. These findings were validated when the substrate was employed in developing a colourimetric assay for E. coli detection, whose assay time is significantly shorter than most available

commercial detection kits.

Chapter 2 reviews the background literature concerning the global burden of water and foodborne infections and the limitations of current detection methods. This chapter further covers the previous work done on OmpT proteases, including the structural and catalytic properties of OmpT proteases. It also highlights the background literature on the purification of recombinantly expressed OmpT, design of peptide library for OmpT, and the techniques employed in screening the peptide libraries. Further, the scope of SAMDI-MS, a high throughput screening approach, for screening arrays is reviewed. The chapter ends with a brief review of polythiophenes as a reporter molecule for various sensing applications and their structural properties. This chapter gives an awareness of historical and contemporary literature and how it can be used to fill the gaps and develop on the knowledge.

Chapter 3 discusses the rationale and the methodologies of the experimental procedures that have been employed for protease expression and purification, peptide substrate design, and screening them using SAMDI-MS. It further explains the rationale and methodologies used for the assay development based on an optimised substrate for proteases and polythiophene as the reporter molecules.

Chapter 4 elaborates the results for expression, refolding, purification, and characterisation of OmpT. The chapter also covers the finding on LPS binding sites and autoproteolysis of OmpT. The results for the activity assay of OmpT are also discussed in the latter part of the chapter.

Chapter 5 elaborates on peptide library design and the findings from the SAMDI-MS based screening for the optimisation of amino acids at the cleavage site and in the immediate vicinity of the cleavage site. The chapter further covers the results of improvement in peptide substrate binding affinity by extending the peptide length. This chapter then elaborates on the results of comparative kinetic analysis of the three peptide substrates: LL37(substituted with optimised tetrapeptide), LL37 (ARRA substituted) and LL37 (native). The results of 361 peptide library screening with wild-type and mutant OmpT are discussed to investigate the effect of a mutation on substrate preference.

Chapter 6 is subdivided into 3 parts. The part A elaborates on the findings and results

of fluorescence and absorbance characterisation of peptide-polythiophene interaction studies. The chapter further explains how the electrostatic interaction between peptide and polythiophene brings about changes in their secondary structure, which is supported by circular dichroism data. The chapter further covers the results of the peptide-polythiophene based assay with wild-type OmpT and how the polythiophene can distinguish between cleaved and intact peptide. The chapter concludes with the results of *E. coli* detection in water using the assay. The part B of the chapter further focusses on improving assay sensitivity and scope by screening different anionic polythiophenes. The part C of the chapter covers the testing of food samples for *E. coli* contamination using the OmpT polythiophene assay.

Chapter 7 summarises the findings and results from chapter 4, 5, and 6, and how these results can contribute to future studies. This chapter also outlines the future work for developing the lab-based assay into a more user-friendly household assay. This chapter concludes by addressing the extent of the verification of the hypothesis and how far the objectives of the research were met.

1.6 Findings and Outcomes/Originality

This research led to several novel outcomes:

1. The refolding studies on OmpT clearly suggests that properly folded OmpT is resistant to autoprolysis and it is only the unfolded/improperly folded OmpT that undergoes autoprolysis.
2. The kinetic studies on the various mutant OmpTs indicate that the predicted LPS binding site is not critical for OmpT activity.
3. Screening of peptide libraries using SAMDI-MS led to the identification of a peptide substrate sequence for which OmpT exhibited a 400-fold improvement in catalytic efficiency.
4. Wild-type and mutant OmpT displayed significant differences in their substrate specificities, demonstrating that even modest mutants may not be suitable substitutes for the native enzyme.
5. A simple yet sensitive colourimetric/fluorimetric assay, OmpT-Pep-PT assay, to

detect very low levels of *E. coli* in water by targeting the bacteria's surface protease activity was developed (Figure 1.7). Using this assay *E. coli* K12 and J96 wild-type strains could be detected at as low as 1 CFU/mL in water within a total assay time of 6 and 8 h, respectively.

Figure 1.7: Schematic of the OmpT-Pep-PT assay for the detection of gram-negative bacteria i.e. *E. coli* and *Salmonella*. Red line corresponds to a peptide, which is cleaved by OmpT into small fragments. Orange line corresponds to the polythiophene. Intact peptide interacting with polythiophene results in structural change and increased fluorescence, while interaction with cleaved fragments doesn't bring about any change.

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Chapter 2

Literature Review

*This chapter provides a comprehensive review of the historical and contemporary work related to this thesis. It first overviews the background information related to the global burden of water and foodborne infections, and briefly highlights the major contributors to these infections like *E. coli* and *Salmonella*. It then presents a detailed description of the current detection methods for bacterial pathogens along with their limitations. The current detection methods cover the gold standards used for bacterial pathogen detection as well as the culture independent nucleotide, immuno, biosensor, or colourimetric based detection methods. This chapter further gives a brief overview of protease-based detection methods before describing the three pillars of the OmpT-Pep-PT assay: 1) Protease (OmpT in *E. coli*), 2) Substrate optimisation (peptide), 3) Reporter molecules (polythiophene). This brief overview starts with the description of the pathogenesis and structural aspects of OmpT proteases, and covers the expression, purification, and reconstitution of OmpT. Autoproteolysis and the importance of LPS for OmpT activity are also discussed after that. Peptide substrate design and various screening technique, with a focus on SAMDI-MS, are covered in the second part while the third part focusses on assay development. The chapter also briefly covers the previously reported polythiophene-based sensing methods and concludes with the scope of the thesis.*

2.1. Pathogenic bacteria - a leading cause of water and foodborne illness and a global burden

“Pathogens” are biological agents like bacteria, viruses, protozoan, viroid, or fungus that can cause infections. Pathogenic bacteria are widespread and are easily found in soil, animals and humans, food, and water. Hence, the infections they cause can spread quickly via inhalation, ingestion, abrasion to the skin, contaminated blood, or the bite of an insect. Foodborne diseases are infections of the gastrointestinal tract caused by harmful bacteria, parasites, viruses, or chemicals present in contaminated food or beverages. Improper handling and storage of foods, inadequate cooking or reheating, cross-contamination between raw and cooked foods or freshly produced food, and poor hygiene are some of the ways in which such infections are acquired. Various microbial products, also called virulence factors, help the pathogens survive and spread the disease. Virulence factors include toxins, molecules involved in adhesion, invasion, and evasion of the host’s immune system.

According to the most recent World Health Organization (WHO) report on foodborne illnesses, published in 2015, food and water-borne illnesses affect one in 10 individuals around the world causatively contributing to mortality and morbidity, and impeding socio-economic development.¹ The WHO estimates that 600 million people fall ill after consuming contaminated water-food and 420,000 of whom die each year (Figure 2.1).

Figure 2.1: Statistics on food and water-borne illnesses based on the 2015 WHO report.

Children below the age of 5 are worst hit with 140,000 deaths each year, which is 33% of the total deaths caused.¹ Diarrheal infections account for 550 million people falling ill and 230,000 deaths each year. These numbers make diarrhoeal infections the most common water-food borne illnesses caused by consuming contaminated water and

food.¹ This mostly unreported, undiagnosed, and untreated class of diseases, the WHO estimate found, takes a substantial toll on societies.

Figure 2.2 : Ranking of foodborne hazards, based on Disability-Adjusted Life Years at the global level, with 95% uncertainty intervals, in 2010. White dots indicate the median burden, black boxes the inter-quartile range (50% UI), black lines the 5 and 95 percentiles (90% UI) and grey lines the 2.5 and 97.5 percentiles (95% UI). Note that the y-axis is on a logarithmic scale. Abbreviations: EPEC = Enteropathogenic *Escherichia coli*; ETEC = Enterotoxigenic *E. coli*; STEC = Shiga toxin-producing *E. coli*.¹

Among all causative agents spanning microbial, parasitic, and chemical origins, *E. coli* and *Salmonella* are two of the most common ones, causing ~120,000 deaths each year throughout the world. These diarrhoeal infections have already become a big socio-economic burden in resource-limited nations where there is a shortage of clean drinking water and food. Even developed nations like the USA are not spared from these water and foodborne infections.^{2,3} According to a report published by the Centers for Disease Control and Prevention (CDC), approximately 48 million people in the USA fall ill, 128,000 people are hospitalized, and 3000 deaths are reported each year due to water and foodborne infections.⁴

2.2. Current bacterial detection methods

Present-day methods to detect pathogens in water, food, and biological samples are based on culturing, immunological, or nucleic acid-based techniques. Reliable and easy to use detection methods are not only critical for controlling pathogens during an outbreak, but also for assessing the microbial contamination in food, water, and medications. Nearly 38% of publications related to pathogen detection are related to food industry security (38%) followed by clinical diagnosis (18%) and water-environment quality (16%) control.⁵ It was also reported that the majority of detection methods deal with detecting *Salmonella* (33%), *E. coli* (27%), and *Listeria* (14%).⁵ Conventional methods used for detecting pathogenic bacteria in food and water samples rely on culturing the microorganisms on specific media and agar plates followed by different biochemical identifications, thereby increasing the overall cost and time. Only after the pathogen is grown in enough quantity (enough number of colonies), can biochemical tests be performed on them to identify the pathogen. Such methods are labour intensive, and for certain bacteria, it can take up to 5 days to culture them. Current rapid detection methods such as those based on nucleic acids, biosensors, and immunological approaches can reduce the time from a week to a few days. Immunological assays are based on antigen and antibody interaction, coupled with other methods to detect or quantify the interaction. Enzyme-linked immunosorbent assay (ELISA) and enzyme-linked fluorescent immunoassay have been extensively used for the detection of pathogenic bacteria including *E. coli* and *Salmonella typhi*.⁶⁻⁸ The limit of detection (LOD) of these immunoassays is usually 10^4 to 10^5 CFU per mL of water sample or gram of food samples. Different bio-sensing techniques based on optical, impedance, fluorescence, and electrochemical transduction principles have been developed to detect *Listeria monocytogenes*, *Salmonella enteritidis*, *E. coli* etc.⁹⁻¹⁴ Nucleic acid-based detection techniques such as real-time polymerase chain reaction (PCR)¹⁵⁻¹⁷, multiplex PCR¹⁸⁻²⁰ and loop-mediated isothermal amplification (LAMP)²¹⁻²³ methods have been developed to detect *E. coli*, *Kingella kingae*, *Staphylococcus aureus*, *Shigella*, *Salmonella*, *Vibrio cholerae*, *Yersinia pestis* etc. PCR based assays have a LOD of 10^3 to 10^4 CFU/mL. In order to obtain high yield and good quality DNA, a certain minimum number of cells in the sample is required. Most immunological and genetic-based methods are sensitive and specific but require technical expertise, laborious procedures, and expensive instrumentation that make them inappropriate for

on-site testing. Various bacterial detection kits based on such approaches are currently available in the market.

A significant challenge in testing food sample comes from the interference caused by the complex food matrix itself.²⁴ The food matrix is composed of various components such as inorganic particles, biochemical components, microflora, fats, carbohydrates etc. and these can interfere with antibody binding and nucleic acid amplification thereby affecting the results.²⁴ In order to avoid a preculturing step and also to reduce the effect of the food matrix, various non-culture methods have been developed. These methods not only separate the bacterial pathogens from the food matrix but also help in concentrating the pathogens to a detectable limit. Centrifugation and filtration are the most widely used physical separation methods because of ease of use.²⁵

Table 2.1: Advantages and limitations of rapid detection methods.

Detection method		Advantages	Limitations
Nucleic acid-based	Simple PCR	High sensitivity, High specificity, Automated and Reliable results	Affected by PCR inhibitors, Requires DNA purification, Difficult to distinguish between viable and non-viable cells
	Multiplex PCR	High sensitivity, High specificity, Detection of multiple pathogens, Automated, Reliable results, High sensitivity, High specificity, Detection of multiple pathogens, Automated and Reliable results	Affected by PCR inhibitors, Difficult to distinguish between viable and non-viable cells and Primer design is crucial
	Real-time PCR	High sensitivity, High specificity, Rapid cycling, Reproducible, does not require post-amplification products processing and Real-time monitoring PCR amplification products	High cost, Difficult for multiplex real-time PCR assay, Affected by PCR inhibitors, Difficult to distinguish between viable and non-viable cells, requires trained personnel, Cross contamination may occur
	NASBA	Sensitive, Specific, Low cost, does not require thermal cycling system and Able to detect viable microorganisms	Requires viable microorganisms and Difficulties in handling RNA
	LAMP	High sensitive, High specificity, Low cost, Easy to operate and Does not require thermal cycling system	Primer design is complicated and Insufficient to detect unknown or unsequenced targets

	Oligonucleotide DNA microarray	High sensitivity, High specificity, High throughput, enables detection of multiple pathogens, allows detection of specific serotype and Labour-saving	High cost, Difficult to distinguish between viable and non-viable cells, requires trained personnel, requires oligonucleotide probes and labelling of target genes
Biosensor-based	Optical biosensors	High sensitivity, Enables real-time or near real-time detection and Label-free detection system	High cost
	Electrochemical biosensors	Can handle large numbers of samples, Automated and Label-free detection	Low specificity, not suitable for analysing samples with low amount of microorganisms, Analysis may be interfered by food matrices and Many washing steps
	Mass-based biosensors	Cost effective, Easy to operate, Label-free detection and Real-time detection	Low specificity, Low sensitivity, Long incubation time of bacteria, many washing and drying steps and Regeneration of crystal surface may be problematic
Immunological-based	ELISA	Specific, can be automated so that it is more time efficient and labour-saving, Allows the detection of bacterial toxins and Can handle large numbers of samples	Low sensitivity, False negative results, may result in cross-reactivity with closely related antigens, Pre-enrichment is required in order to produce the cell surface antigens, requires trained personnel and Requires labelling of antibodies or antigens
	Lateral flow Immunoassay	Low cost, Reliable, Easy to operate, Sensitive, Specific and Allow the detection of bacterial toxins	Requires labelling of antibodies or antigens

Note: Adapted from Law, J. W.F.*et al* (2014).²⁶

With these pretreatment steps, 500-1000 fold increase in concentrations of microorganisms has been achieved.²⁴ Antibodies, aptamers, bacteriophage proteins and other binding molecules have been used on magnetic beads for immunomagnetic separation (IMS) and concentration of pathogen. IMS that is based on biological interactions has been widely used for sample preparation and cleanup before final sample analysis.^{27,28} Although these pretreatment steps improve the detection limits, they require a trained user to perform the same. All the three methods of detections mentioned above have their own advantages and disadvantages some of which are summarized in Table 2.1. One of the major problem that most of the approaches summarized in Table 2.1, is the lack of long-term stability of kits/assays when applied to detect bacteria in real environments outside the lab, they required storage at 4 °C which is difficulty to achieve.

2.2.1. Nucleotide-based detection methods

Commercially available nucleotide-based bacterial detection kits from Thermo Fisher Scientific, Hygiene, and Neogen utilise polymerase chain reaction (PCR) principle and generally require 8-24 h including preculturing of the samples. PCR is a lab-based technique used for making copies of DNA with the help of an enzyme called DNA polymerase and short nucleotide sequences called primers. The primers are short nucleotide sequences, complementary to the DNA which is to be copied. PCR amplifies the DNA sequence exponentially, making millions of copies of the DNA sequence in the reaction mixture, but it requires a repeated cycle of heating and cooling. PCR based approaches are the most widely used methods for bacterial pathogen detection in water and food. There are some commercial kits available based on the methods mentioned above.

The iQ-Check® *E. coli* O157:H7 PCR Detection Kit from Bio-Rad detects 1-10 CFU in 12-24 hours with preculturing of the sample in buffered peptone water (Figure 2.3). A few examples of qPCR-based kits are *E. coli* O157 BAX® PCR (DuPont Qualicon), ADIAFOOD rapid pathogen detection system for *E. coli* O157 and *E. coli* O157:H7 (AES Chemunex), and ADIAFOOD *Campylobacter* JCL qPCR commercial kit (AES Chemunex) for the detection of *E. coli* and *Campylobacter*. PCR not only increases the cost but also requires trained personnel to perform the assay. A summary of nucleic acid-based commercial detection kits that have been reported previously is compiled in Table 2.2 (for detailed information refer to Appendix 8.1). Other than the commercially available kits, nucleic acid-based bacterial detection methods reported in literature can be broadly divided into two different approaches: one which requires amplification of nucleic acids and the second approach which requires hybridisation of nucleic acids. A summary of nucleic acid-based detection methods that have been previously reported is compiled in Table 2.3.

Figure 2.3: Illustration of the protocol used for iQ-Check® *E. coli* O157:H7 PCR Detection Kit.²⁹

Table 2.2: Nucleotide based commercial kits targeting mostly *E. coli* in food samples (i.e. raw meat, green vegetables, milk, fruits) with storage at 2-8 °C.

Name	Pre-treatment and culture enrichment	Limit of detection	Time
MicroSEQ™ <i>E. coli</i> O157: H7 Detection Kit Thermofisher	Enrichment and DNA extraction	1-3 CFU/25 g sample	8-18 h
BAX® System PCR Assay for <i>E. coli</i> O157: H7 MP - Hygiena	8-24 h	10 ⁴ CFU/mL, after enrichment	3.5 h PCR
BAX® System Real-Time PCR Assay for <i>E. coli</i> O157: H7 Hygiena	8-10 h	10 ⁴ CFU/mL, after enrichment	1 h PCR
iQ-Check® <i>E. coli</i> O157:H7 PCR Detection Kit Bio-Rad	Media enrichment	1 – 10 CFU/25 g sample	12 h
3M™ Molecular Detection Assay <i>E. coli</i> O157 - 3M	Enrichment required	1-5 CFU	~ 1-2 h PCR
Foodproof® <i>E. coli</i> O157 Detection Kit - Biotecon Diagnostic	18-24 h	10 ³ - 10 ⁴ CFU/mL in enrichment cultures	~1.5 h PCR
GENE-UP® Biomérieux-industry	8-24 h		

<i>E. coli</i> O157 Food Safety Kit - Instant Labs	Enrichment 10 h, Extract DNA	1 CFU/mL	~ 2 h PCR
ANSR® for <i>E. coli</i> O157: H7 - Neogen		1 CFU/analytical unit 10 ⁴ CFU/mL post-enrichment	12-24 h
GeneDisc® Technologies - <i>E. coli</i> O157(:H7) - Pall Corporation	Enrichment 8 h and DNA extraction	1 CFU/25 g of sample 1 CFU/375 g raw beef	~2 h
Assurance GDS® for <i>E. coli</i> O157: H7 - BioControl	Enrichment and IMS		8-10 h
Mericon VTEC stx1/2 Kit - Qiagen	Enrichment culture and DNA isolations		18-27 h PCR 73 min

Multiplex PCR (mPCR) uses multiple sets of primers and can detect multiple targets in a single reaction. mPCR requires fewer efforts for the same detection results. Although mPCR is a robust and specific assay, it requires careful design of primers with similar annealing temperatures. Other limitations of the techniques are primer concentrations that need to be carefully validated, optimisation of buffer concentrations, proper cycling temperatures etc. Quantitative real-time PCR (qPCR) monitors the amplification of a targeted DNA molecule during the PCR in real time. With qPCR, it is possible to simultaneously amplify and detect the target DNA using fluorescently labelled molecules. The increase in amplified DNA concentration is directly proportional to fluorescence intensity. Hence, a change or increase in fluorescence of the sample indicates the presence of the target DNA. Quantitative reverse transcription PCR (RT-qPCR) uses RNA as starting material. It can detect and measure the quantity of targeted-amplified DNA. Since RNA degrades faster outside cells, RT-qPCR has an advantage that it mainly amplifies RNA from live cells.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid detection method that does not rely on PCR and is a single tube technique for nucleotide amplification. Unlike PCR based approaches, LAMP based nucleotide amplifications require isothermal temperature (59-65 °C). LAMP requires four primers, and the amplification can be detected by measuring fluorescence that can be visualised with the naked eye by using a SYBR green dye or gel electrophoresis. Thi Ngoc Diep Trinh and Nae Yoon Lee developed a microdevice to perform LAMP based detection of pathogens (*E.*

coli O157:H7, *Salmonella* spp., *S. aureus*, and *Cochlodinium polykrikoides*) in a single assay (Figure 2.4).³⁰ A polycarbonate microdevice integrated all the functions on one device: on paper DNA extraction, LAMP based DNA amplification, and detection of DNA on-chip. The microdevice showed high selectivity and LOD of approximately 170 CFU/mL for milk samples artificially spiked with *Salmonella*.³⁰ Nucleic acid sequence-based amplification (NASBA) is another non-PCR based method and is a primer-dependent technology that is used for continuous amplification of nucleic acid at an isothermal temperature in a single reaction.

Figure 2.4: Schematic illustrations demonstrating: (a) operation of the microdevice, (b) extraction process using heat-based lysis and polydopamine-coated paper, and (c) detection mechanism using fisetin. Reproduced with permission (Appendix - Permissions).³⁰

DNA microarrays are chips which have 25 to 80 bp long single-stranded DNA oligonucleotide probes attached to it. These probes are complementary to a gene or

genomic markers of various pathogenic microorganisms. DNA microarray-based assays reported detecting targets in the range of 10^6 to 10^8 CFU/mL, making them inappropriate under tested conditions.^{31,32} Microfluidic chips which require nano/picoliter of samples flowing through micron-sized channels in a device are also referred to as Lab on chip (LOC), and are portable diagnostic chips. These chips have an advantage that steps such as sample preparation, enrichment, labelling, signal amplification, and detection can be done in channels and wells on the chip. However, the DNA needs to be extracted from food samples before insertion into LOC.³³ LOC has been reported to detect approx. 10^4 genomic copies in 1 μ L of purified DNA in 14 min.³⁴

Table 2.3: Previously reported nucleotide-based assays targeting mostly *E. coli* and *Salmonella* in food samples (raw meat, green vegetables, milk products, fruits, and water).

Method	Pre-treatment approach	Limit of detection	Time
PCR ³⁵	Magnetic nanoparticles	8-13 CFU/mL	
PCR ³⁶	Centrifugation and bacterial immobilisation	10^3 CFU/g of ground beef	
PCR ³⁷	1) Filtration, centrifugation, and lysis using enzymatic digestion and successive freeze/thaw cycles. 2) DNA extraction by phenol/chloroform extraction	10^3 CFU/g of ground beef	12 h
Multiplex PCR ³⁸	8 h enrichment/preculture	1 CFU/mL milk or chicken meat homogenate	18 h
Multiplex PCR ³⁹	Immunomagnetic beads	10 - 10^4 CFU/mL	
Quantitative PCR ⁴⁰	Two step filtrations	7.5×10^2 CFU/100 mL	3 h
Quantitative PCR ⁴¹	None	10^4 CFU/mL 10^3 CFU/mL 10^5 CFU/g	
Quantitative PCR ⁴²	None	10^3 - 10^8 CFU/mL 10^4 - 10^8 CFU/mL	
Quantitative PCR ⁴³	None	10 CFU /reaction	6 h
Multiplex quantitative PCR ⁴⁴		10^3 CFU/g	
Multiplex quantitative PCR ⁴⁵	Immunomagnetic separation	10^2 in pure culture 10^3 spiked food	5 h
Quantitative reverse transcription RT-PCR ⁴⁶	Two step centrifugation and zirconium hydroxide for bacterial immobilization	10^2 CFU/mL (WM) 10 CF/mL (IC)	
Loop mediated isothermal amplification ⁴⁷	6-8 h enrichment 1 CFU/25 g of sample	10^5 - 10^6 CFU/25 g	-

Loop mediated isothermal amplification ⁴⁸	Propidium mono azide for LAMP	3.4 -34 CFU/g pure culture 6.1×10 ³ -10 ⁴ CFU/g spiked food samples	3 h
Loop mediated isothermal amplification ⁴⁹	None	547 CFU/mL	1 h
Nucleic acid sequence-based amplification ⁵⁰	Heat shock to cells (5-40 min)	40 CFU/mL	4 h
Nucleic acid sequence-based amplification ⁵¹	None	1-10 CFU/mL	3-4 h
Microfluidic lab on a chip ⁵²	DNA extraction	3.72 × 10 ⁴ DNA copies 3.58 × 10 ⁴ DNA copies 1.79 × 10 ⁴ DNA copies	14 min for PCR

2.2.2. Immuno-based methods

There are several commercially available bacterial detection kits based on immunoassay, such as the kits from FOODCHECK, Solus scientific, Biocontrol Systems, Reveal® test kits (Neogen) and VIP® GOLD™ (BioControl Systems) for *Listeria*, *Salmonella* and *Escherichia coli* O157 and DuPont™ Lateral Flow System (DuPont Qualicon) for *Listeria* etc., but they require 8-24 h to obtain the results. Some SPR-based optical biosensors for detecting *E. coli* and *Salmonella* are commercially available, such as SPREETA biosensor and BIACORE 3000 biosensor, having a limit of detection of 10²-10³ CFU/mL and 10⁵ cells/mL, respectively. A summary of Immuno-based (ELISA, LFI) commercial kits has been compiled in Table 2.4 (for detailed information refer to Appendix 8.1). Furthermore, these assays are not cost effective due to use of ELISA kits, BIACORE chips which require refrigeration during shipping and storage, besides the need for sophisticated instrumentation to read output signals (e.g. plate readers, BIACORE instrument).

Antibody-antigen interaction based bacterial detection methods are mainly grouped into two broad categories: Enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassays (LFA). ELISA based assays are used for the detection of an antigen in solutions using an antibody linked to an enzyme (Figure 2.5). The antigens are attached to a surface either through charged interactions or using a second antibody against the antigen. Enzyme-linked antibody specifically binds to the ligand attached to the surface. With the addition of an enzyme's substrate, a fluorogenic or chromogenic signal is produced by the enzyme. The rate of signal generation is proportional to the amount of antigen bound. Although LFA is also based on antibodies, it is a

paper/polymer membrane-based detection method, unlike ELISA. LFA is based on a series of capillary beds prepared from porous paper, microstructured polymer, or sintered polymers (Figure 2.5 B). The liquid samples are loaded onto an absorption pad, and the samples move across to the conjugation pad because of capillary forces. The analyte binds to the antibody functionalized particles in the conjugation pad. The analyte and functionalised particle complex move across the membrane and get captured by the antibodies at the test line producing a signal readout. The second line present on the LFA is a control line to show that the reaction conditions and assay worked fine. It captures the functionalised particles which pass through the test line.

Table 2.4: Immuno-based (ELISA, LFI) commercial kits targeting mostly *E. coli* in food samples (*i.e.* raw meat, green vegetable, milk, fruits) with storage conditions at 2-8 °C.

Name	Pre-treatment	Limit of detection	Time
Singlepath® & Duopath® Lateral Flow Tests for Immunological Detection - Merck	NA	NA	NA
FOODCHEK™ – <i>E. coli</i> O157 TEST KIT FOODCHEK™	7 h of enrichment, filtration, and temperature- controlled incubation	1CFU/375 g beef, 0.05 CFU/ 100 cm ²	~ 10-12 h *FOODCHEK™ reader required
Solus <i>E. coli</i> O157 ELISA Solus scientific	Enrichment	10 ⁴ - 10 ⁵ CFU/mL	18-22 h (2 h for ELISA)
Reveal® for <i>E. coli</i> O157:H7 - Neogen	Enrichment - 8 to 20 h culture enrichment	1 CFU/25 g, 1 CFU/375 g	15 min + enrichment
VIDAS® UP ECPT - Biomérieux-industry	Enrichment - 7 to 24 h culture enrichment for raw meat; 15-24 h other matrices		50 min for assay + enrichment
VIP® Gold – EHEC - Biocontrol–MiliiporeSigma	Enrichment 8 h minimum	NA	NA
Lateral Flow System for <i>E. coli</i> O157 - Hygiena	8 h for 25 g samples or 10 -15 h for 375 g samples	1-4 CFU/25g sample	10-15 min after enrichment

RapidChek® <i>E. coli</i> O157 - Romerlabs	NA	NA	8-18 h including enrichment
TRANSIA® AG - Biocontrolsys	Enrichment culture	NA	Beef in 10 h and 20 h for matrix

Figure 2.5: Illustration of Immunoassays A) ELISA B) Lateral flow assays.⁵³

A summary of ELISA and LFI based detection approaches have been compiled in Table 2.5. In the recent past, ELISA based approaches have focused on increasing antibody density, multiplex detection, and using fluorescent and chemiluminescent labels to increase the sensitivity and reduce the detection limits. Nanomaterials like Gold nanoparticles and single-walled carbon nanotubes are used for functionalization of antibodies with much higher antibody density. LFA provides a rapid and straightforward form of the detection assays. The is performed on different types of membrane strips, and the signal is represented by colour change produced by gold

nanoparticles or enzymatic reactions. LFAs are low cost, simple to use and are suitable for in-field screening of large samples giving qualitative data even though they have a higher false positive with food samples.⁵⁴

Table 2.5: Previously reported immuno-based assays targeting mostly *E. coli* and *Salmonella* in food samples (i.e. raw meat, green vegetable, milk products, fruits, water).

Method	Pre-treatment	Limit of detection	Time
Polyacrylonitrile fibre-based sandwich enzyme-linked immunosorbent assay (ELISA) ⁵⁵	None	10^6 - 10^7 CFU/mL	2 h
Single-walled carbon nanotubes enhanced direct and sandwich ELISA ⁵⁶	None	Fat-free milk: 10^3 (direct) and 10^4 (sandwich) CFU/mL; 9% milk: 10^4 CFU/mL	Direct – overnight, Sandwich – 3 h
Gold nanoparticle (AuNP) network ELISA ⁷	Immunomagnetic separation (IMS)	<i>E. coli</i> O157:H7: 3 CFU/mL in milk and beef; <i>S. Typhimurium</i> : 15 CFU/mL in juice	~ 2h
AuNP-enhanced ELISA ⁵⁷	Immunomagnetic separation (IMS)	6.8×10^2 CFU/mL in vegetable and milk, and 6.8×10^3 CFU/mL in ground beef	3 h
Sandwich chemiluminescent immunoassay ⁵⁸	NA	10^5 – 10^6 CFU/mL for the <i>E. coli</i> O157:H7 and 10^6 – 10^7 CFU/mL for <i>Salmonella</i>	4 h
Sandwich fluorescent immunoassay ⁵⁹	NA	<10 CFU/mL	2 h
Enzyme-linked antibody aptamer sandwich assay ⁶⁰	Aptamer-modified magnetic particles	10^3 CFU/mL	3 h
Multiplex LFI ⁶¹	Immunomagnetic separation and cell lysis	<i>E. coli</i> BL21: 10^2 CFU/mL in milk and juice and 10^6 CFU/mL in lettuce; <i>E. coli</i> O157:H7: 10 CFU/mL in milk and juice and 10^5 CFU/mL in lettuce	30 min

2.2.3. Biosensors based methods

Biosensors have been reported to achieve better sensitivity than previously reported culture-dependent and independent methods. Based on their transduction mechanism, biosensors can be categorised into optical biosensors, electrochemical biosensors, piezoelectric biosensors, magnetoelastic biosensors etc. A collection of these biosensors that are capable of detecting *E. coli* is presented in Table 2.6.

Optical biosensors are based on properties of light absorbance, chemiluminescence, fluorescence, light polarization and rotation, surface plasmon resonance (SPR) and total internal reflection. Also, nanomaterials like nanoparticles, nanofibers, and quantum dots have been extensively explored for bacterial biosensing because of their unique optical properties. SPR based platforms like BIACORE gives an advantage of real-time monitoring and label-free detection, but on the downside, the cost is very high. Bacterial pathogen sensing with a lower limit of 10^3 CFU/mL has been reported for SPR. Fibre-optic biosensors use optical fibre, which gives an advantage of various surface modifications and real-time monitoring. Fluorescence resonance energy transfer (FRET) immuno-sensor using optical fibre have been developed to detect *S. Typhimurium* with a limit of detection of 10^5 CFU/mL.⁶² Optical sensors based on readouts from colourimeter, spectrophotometer, light scattering measurement devices have been developed. These sensors might have a higher sensitivity, but the cost and technical know-how of these systems become a negative point for their use in the field.

Electrochemical sensors can be classified based on the type of signal readout into amperometric, potentiometric, conductometric, and impedimetric sensors. The electrochemical sensors can be applied to analytes present in any state of matter, including those in food, where the analytes are present mostly in liquid samples. Electrochemical sensors give an added advantage that they are not affected by the turbidity of the samples. Nanomaterials (graphene, GNPs etc.), enzymatic reactions etc. have been used in conjugations with electrochemical sensors to achieve LOD of approx. 10^4 CFU/mL.⁶³

Piezoelectric quartz crystal microbalance (QCM) has been extensively researched for its application in bacterial pathogen detection. QCM like SPR based BIACORE system gives an advantage of real-time monitoring and label-free detection. QCM also has multiple options of biocompatible electrodes for ligand immobilisation. QCM measures mass change in the nanogram levels, which is not enough for sensitive detection; hence, QCM is used along with GNPs or INPs for mass amplification. Whole cell imprinting on polymeric structures is reported and show potential for improving sensitivity.^{64,65}

Table 2.6: Previously reported different biosensor-based assays targeting mostly *E. coli* and *Salmonella* in food samples (i.e. raw meat, green vegetable, milk products, fruits, water).

Method	Pre-treatment	Limit of detection	Time
SPR immunosensor ⁶⁶	None	10 ² -10 ³ CFU/mL	30 min after sample injection
Sandwich SPR immunosensor (enzymatic signal enhancement) ⁶⁷	Centrifugation	10 ³ -10 ⁴ CFU/mL	2.5 h
SPR immunosensor ⁶⁸	Homogenized and filtered	10 ⁶ CFU/mL	~20 min
Sandwich multi-channel SPR immunosensor ⁶⁹	Heat-kill and ultrasonication of cells, and pH adjustment of apple juice pH 7.4	3.4 × 10 ³ to 1.2 × 10 ⁵ CFU/mL	-
SPR biosensor ⁷⁰	Homogenised and filtered	3.0 × 10 ⁴ CFU/mL in cucumber and 3.0 × 10 ⁵ CFU/mL in beef sample	-
Colorimetric biosensor ⁷¹	None	10 ⁸ CFU/mL	
Light scattering immunosensor ⁷²	Homogenised and filtered	10 CFU	<6 mins
FRET-based optical fiber biosensor ⁷³		10 ⁵ CFU/mL	5 min
Fluorescence nucleic acid biosensor ¹¹	Centrifugation and lysis of cells	1.5 × 10 ² CFU/mL	5.5 h
Amperometric immuno sensing strip ⁷⁴	Centrifugation, Lipid and protein removal	5 × 10 ³ CFU/mL	1 h
Voltammetric sandwich immunosensor ⁷⁵	Immunomagnetic separations and pH adjustment	5 × 10 ³ CFU/mL	80 min
Voltammetric sandwich immunosensor ⁶³	None	400 and 800 CFU/mL	-
Voltammetric sandwich immunosensor ⁷⁶	Immunomagnetic separation	10 ³ CFU/mL	1 h
Potentiometric aptamer biosensor ⁷⁷	Filtration and washing	6 CFU/mL in milk and 26 CFU/mL in apple juice	
Impedance immunosensor ⁷⁸	Immunomagnetic separations	7.4 × 10 ⁴ Culture media and 8.0 × 10 ⁵ CFU/mL in Grounded beef	35 min
Capacitive immunosensor ⁷⁹	Filtration	10 ³ , 10 ⁴ and 10 ³	1 h
Impedimetric immunosensor ⁸⁰	None	5 × 10 ³ CFU/mL	
Impedimetric immunosensor ⁸¹	none	83.7 CFU/mL	

Impedimetric immunosensor ⁸²	Homogenised and filtered	1.5×10^2 CFU/mL	
QCM DNA biosensor ⁸³	Centrifugation, DNA extraction and PCR amplification	5.3×10^3 CFU/ml	
MIP based QCM and SPR ⁶⁵		10^8 CFU/mL	
Cantilever immunosensor ⁸⁴	None	10 CFU/mL	10 min
Magnetoelastic (ME) phage-based biosensor ⁸⁵⁻⁸⁷	None	5×10^5 CFU/mL	~30 min
Hydrogel based colorimetric assay ³⁴	None	4×10^6 - 4×10^5 CFU/ml in 5 min and 4×10^4 -400 CFU/ml in 60 min	
Polymer and peptide ⁸⁸	None	$\sim 10^6$ CFU/ml	

Magnetoelastic biosensors use amorphous ferromagnetic materials for sensing. These materials under external time-varying magnetic field show a magnetoelastic resonance which could be measured using pickup coils. The resonance frequency changes when a target comes in contact with the sensor surface and this change in resonance frequency helps in detecting the presence of the pathogen in the sample. Magnetoelastic biosensors have shown a LOD of 5×10^2 CFU/mL with samples spiked on the surface.⁸⁵ From the information presented so far, it is evident that several methods to detect bacterial pathogens in water and food have been developed which have improved sensitivity and reduced the time required for results. However, the cost of using these methods because of reagents and instruments involved is quite high. Nucleotide-based assays are fast, specific, and reproducible but have high instrumental and reagent cost at the same time require a trained user to perform the reactions. ELISA and LFA have major concerns with sensitivity and stability of antibodies on the assay platform. Biosensors are rapid, sensitive, and low-cost devices, but much work needs to be done on their reliability, the stability of materials used, and require field testing.

All these immunological and nucleic acid based methods require technical expertise, laborious procedures, and expensive instrumentation that make them inappropriate for on-site testing. Hence, there is a need to upgrade existing detection methods and make them rapid and easy to use. Such detection techniques will considerably reduce the spread of infections and control outbreaks. It is required that one targets the bacterial surface and the approach should be designed to offer minimum interference from the

matrix of the sample. Hence, instead of using immunological assays or nucleotide-based approaches, it is better to target membrane enzymes for a more sensitive response with less sample processing.

2.2.4. Chromogenic

Chromogenic media-based assays for bacterial detection, such as those from Bio-Rad, also possess an overall assay time of up to 48 h. These chromogenic assays are mostly based on β -galactosidase enzyme, gold nanoparticle aggregation, sorbitol degradation etc. Although some bacterial detection kits for household use are commercially available, they are time-consuming and non-specific.

Table 2.7: Other commercially available colourimetric kits targeting mostly *E. coli* in food samples (i.e. raw meat, green vegetable, milk, fruits) with storage conditions at 2-8 °C.

Method	Name	Enrichment	Limit of detection	Time
DIP sticks	<i>E. coli</i> O157 Coli-Strip Corisbio	Broth enrichment	10 ⁴ CFU/mL	15 min + enrichment
Plating - Chromogenic media	RAPID <i>E. coli</i> O157:H7 Medium Bio-rad			48 h
Liquid Crystal Biosensor	<i>E. coli</i> O157 Antibody Kit - Crystal Diagnostics	Enrichment 10-15 h, IMS		
Latex agglutination	Pro-Lab Diagnostics™ Prolex™ - Blue <i>E. coli</i> O157 Latex Test Reagent Kits - Fisher scientific		One colony is enough	
Latex agglutination	DRYSPOT <i>E. coli</i> O157 TEST KIT - Thermo Scientific	Plating required		
Colourimetric assay	AquaVial <i>E. coli</i> and Coliform Water Test Kit - Aquabsafe	None	1 CFU/ mL	24 h at 37 °C
Colourimetric assay	WaterSafe Bacteria Test - Watersafe	None		48 h
Colourimetric assay	MicroSnap™ <i>E. coli</i> Test Kit - Hygiena	None	<10 CFU in 8 h	8 h
Colourimetric assay	Total Coliform/ <i>E. coli</i> Indicator Bacteria Test Kit - LaMotte Company	None	1-10 CFU/100 mL	24-48 h

Colourimetric assay	Drinking Water Test Kit (<i>E. coli</i> / Coliform Test) - Rakiro	None	1 CFU/100 mL	18-24 h
Colourimetric assay	Compartment Bag Test (CBT) Quantitative <i>E. coli</i> Water Quality Test Kits Aquagenx	None		24-48 h
Coliform enzyme β -galactosidase and the <i>E. coli</i> enzyme β -glucuronidase based colorimetric assay	Colilert - IDEXX Laboratories	None		24 h or less

For instance, AqualVial™, a water testing kit sold by AquaBSafe (Ontario, Canada) for *E. coli* detection, is time-consuming and requires an incubation time of around 24 h before the results can be reported. Easy-to-use polymer-based optical biosensors have been reported previously, but they are either based on nonspecific electrostatic interactions between polymer chains and bacterial surface or peptides, which bind differentially to the components of bacterial surfaces. These sensing approaches are nonspecific with higher limits of detection.⁸⁸⁻⁹⁰ A summary of various commercially available colourimetric detection kits has been compiled and presented in Table 2.7.

Given the limitations described above, there is a need to develop a bacterial detection system that is rapid, easy-to-use, sensitive, and affordable. The colourimetric sensors for *E. coli* and other bacterial pathogens described in Table 2.7 give an advantage of being a functional assay and hence the LOD depends on the enzyme concentration and substrate to a great extent. These colourimetric assays are based on β -galactosidase enzyme and the fluorescent or coloured substrates of the enzyme. But since these enzymes are present inside the *E. coli*, the accessibility of the substrate to the enzyme is limited by the outer membrane of *E. coli*. Based on similar lines, various protease-based assays have been developed for different analytes, with a lower limit of detection as compared to affinity-based assays.

2.3. Protease-based assays

Proteases in nature have various functions ranging from regulating physiological process like defence mechanism, food digestion, blood clotting, cellular regulation etc.⁹¹ This makes proteases a potential biomarker to be used for developing assay and

sensors for various diseased states. Protease-based assays give an advantage over affinity-based immunoassays, in that, they are functional assays and are based on the protease function itself. Protease-based assays have two essential parts – protease's substrate and the sensing platform. The protease's substrate can be designed to make the assay more selective, specific, and also to define the assay time to some extent. Whereas, the choice of sensing platforms defines the limit of detection, response, and ease of use of the assays. The protease's substrate and substrate optimisation will be discussed in section 2.5. The section below focuses on the sensing platform for the assays.

Broadly speaking, protease assays can be divided into homogenous (*i.e.* substrate and samples present in an aqueous phase) and heterogeneous assays (*i.e.* protease substrate is immobilised on the surface, and the samples are present in solution) (Figure 2.6). The homogenous assays are generally grouped into colourimetric, spectrometry-based, and fluorescence resonance energy transfer (FRET) assays. Different types of nanomaterials have been used as reporter molecules like noble metal nanoparticles, quantum dots (QDs), and graphene oxide (GO). The heterogeneous assays are classified into electrochemical, surface-enhanced Raman scattering (SERS), surface plasmon resonance (SPR) and liquid crystal (LC) based assays. FRET-based assays have a short peptide substrate linked to a FRET donor and a FRET acceptor. When the donor and quencher are in proximity (1-10 nm), the fluorescence of donor is quenched by the acceptor. At large distances, no quenching occurs. These donor and acceptor pairs can be organic⁹¹ or nanomaterial-based. Previously gold and quantum dots (QDs) have been used as acceptor and donors respectively.

Carbon nanotubes (CNTs) based multiplex assay for detection of MMP-7, MMP-3 and other MMPs was reported by Huang *et al.*⁹² Specific substrate for different proteases were immobilised on the CNTs with different organic dyes.⁹³ Graphene oxide (GOs) has also been used as a FRET donor for the detection of chymotrypsin and MMP-2.⁹⁴ Along similar lines, gold nanoparticles have been used as FRET acceptors.⁹⁵⁻⁹⁷ Fluorescently labelled peptide on GNPs was used to detect MMP-7 by Park *et al.*⁹⁸ QDs have been extensively used as FRET donor because of high photostability, high molar absorption coefficient and wide excitation-emission peak separation.⁹⁹ Sapsford *et al.* used QDs for the detection of light-chain botulinum neurotoxin serotype-A using

labelled peptides.¹⁰⁰ Bioluminescence resonance energy transfer (BRET) was used by Dacres *et al.* to detect thrombin using a BRET substrate (green fluorescent protein).¹⁰¹ GNPs have been used to develop colourimetric assays based on their aggregation and dispersion. Chen *et al.* used peptide immobilised GNPs for detection of MMP-7. The peptide substrate prevented the aggregation of GNPs, but hydrolysis of the peptide substrate by MMP-7 resulted in the aggregation of GNPs resulting in a colour change.¹⁰²

Heterogeneous protease assays require peptide substrates to be immobilised on a solid surface. Since substrate corresponding to different proteases can be immobilised on the surface, this makes detection and distinguish multiple proteases on a single surface possible. Ji *et al.* developed an electrochemical sensor for the detection of thrombin using redox reporter molecule labelled substrate.¹⁰³ Surface plasmon resonance (SPR) sensors are a label-free technique for protease based assays. In this approach, the refractive index of the surface is measured, and the change in refractive index is correlated to the surface substrate concentration change.¹⁰⁴ In order to improve the signal, peptides are tagged with bulkier magnetic beads. Esseghaier *et al.* used a similar approach for HIV-1 protease and signal enhancement using magnetic beads.¹⁰⁵

Proteases have been extensively used for developing various assays, but they have not been explored for developing assays for bacterial pathogens. Various culture-dependent and advanced detection methods have been reported for bacterial pathogens, but they have their limitations. According to recent reports on food and water testing market there is huge potential growth for the market.^{106,107} Several global companies are currently involved in manufacture and supply of commercial kits for testing water and food for bacterial contamination. Even though there is such a huge market and extensive R&D for detection kits, these kits have various limitation (Table 2.1). The major bottlenecks in the detection of these pathogens are: a) sample preparation and pretreatment, b) development of easy to use and rapid microbiological test methods for detection. In order to take advantage of enzyme-based functional assays to attain lower LOD, this thesis focusses on proteases present on the outer membrane of bacterial pathogens. Use of these outer membrane proteases will also significantly reduce the issue of substrate accessibility to enzyme as in case of β -galactosidase based colourimetric assays.

Protease based assay	
Homogenous protease assays <ul style="list-style-type: none"> • FRET-based assays • Bioluminescence resonance energy transfer • Fluorescence polarisation • Gold nanoparticles based colorimetric assays 	Heterogeneous protease assays <ul style="list-style-type: none"> • Electrochemical sensors • Surface plasmon resonance sensors • Surface-enhanced Raman spectroscopy sensors • Enzyme-linked peptide protease assays • Liquid crystal based assays

Figure 2.6: Protease assays based on various different sensing platform are subdivided into homogenous and heterogeneous.⁹¹

2.4. Omptin family of proteases

Proteases also referred to as proteolytic enzymes, catalyse the breakdown of proteins by hydrolysing peptide bonds. So far, proteases are found in almost all organisms and are involved in various metabolisms. They have evolved to make optimal use of a wide range of conditions like pH, temperature, reductive environment etc.¹⁰⁸ Proteases can be classified based on the position of cleavage, their cleavage specificity and mechanism, the composition of their catalytic site, or on their homology.

Figure 2.7 gives a broad overview of the classification of proteases based on the position of protease cleavage into endopeptidases that cleave within the peptide sequence or exopeptidases that cleave the outermost residues. The exopeptidases can be further sub-classified into aminopeptidase that cleaves at the amino-terminal (N-terminal) and carboxypeptidases that cleave at the carboxyl-terminal (C-terminal).

Trypsin, one of the most well-known proteases, is a carboxypeptidase. Based on their catalytic specificity and mechanism, proteases can be further sub-classified into serine, cysteine, or threonine proteases (amino-terminal nucleophile hydrolases), or as aspartic, metallo, or glutamic proteases (with glutamic proteases being the only subtype not found in mammals so far).¹⁰⁹ MEROPS, an online database of proteolytic enzymes, their inhibitors, and substrates is a more widely accepted classification scheme for proteases. It is a classification scheme first published by Rawlings & Barrett in 1993 and based on their homology, it groups proteases into clans and families. Proteases with

similar sequences are in the same family and with related tertiary structure into the same clans. Omptin proteases are classified as aspartate proteases based on their cleavage specificity, and as Clan AF and Family A26 as per the MEROPS classification.¹¹⁰

Figure 2.7: A) Classification of proteases into various categories based on the position of cleavage and substrate preference. B) Illustration showing different types of proteases and their cleavage preferences (Reproduced with permission: Appendix-Permissions).¹¹¹

Within the cell, proteases are involved in regulating the fate, localisation, and activity of many proteins. They modulate protein-protein interactions, create new bioactive molecules, contribute to the processing of cellular information, and generate, transduce, and amplify molecular signals. Since proteases are involved in such a wide variety of regulations at the cellular level, they influence various functions such as DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodelling, heat shock and unfolded protein responses,

angiogenesis, neurogenesis, ovulation, fertilisation, wound repair, stem cell mobilisation etc.¹¹²

Bacterial proteases can be found in the cytoplasm, periplasm, and even in the lipid membranes. The membrane-associated proteases have various functions such as processing, quality control, and regulating proteins transported to plasma membrane or periplasm. The outer membrane of gram-negative bacteria contains mostly lipopolysaccharides and various outer membrane proteins (OMPs) which account for 50 % of the outer membrane mass.¹¹³ OMPs also include the proteases which play essential roles in bacterial pathogenesis by enhancing the adaptability of bacterial pathogens to various environments. These membrane proteases perform a critical function of cleaving the signal peptide from secretory pre-proteins. They also have an essential role as a virulence factor and help in evading the immune response, adhesions to the extracellular matrix and invasion into eukaryotic cells. In most cases, when a pathogenic bacterium invades the human gut, the cells lining the intestines begin to secrete antimicrobial peptides (AMPs). These AMPs, produced by the host, are critical components of the immune system to target and neutralise the bacterial pathogens. Most AMPs have hydrophilic and hydrophobic parts and are cationic (cationic AMPs - cAMPs) by nature. These cAMPs have different modes of action, but mostly they bind to the anionic bacterial surface. Upon binding to the surface, they integrate into the cytoplasmic membrane and form pores that eventually kill the bacterial pathogens. However, humans and the bacterial pathogens have evolved, and the race by bacterial pathogens to defend and invade the human body continues to evolve. Bacterial pathogens have developed various ways to overcome these cAMPs. These resistance mechanisms against cAMPs range from proteolytic degradation, sequestration by secreted proteins, cell surface/membrane alteration, and export by efflux pumps. For the bacteria, the outer membrane proteases are an effective way to negate the effects of cAMPs.

Omptins are a family of proteases located on the outer membrane of gram-negative bacteria and include proteases such as OmpT and OmpP from *E.coli*, PgtE from *Salmonella enterica*, Pla from *Yersinia pestis*, among others (Table 2.8).¹¹⁴ These proteases are characterised by their ten stranded beta barrel structure with their active sites facing the extracellular side. Omptins are known to cleave proteins and peptides

with narrow sequence specificities, and this specificity arises from the long extracellular loops connecting the strands of the β -barrel.¹¹⁵ Besides cleaving antimicrobial peptides (AMPs) and thus serving as a bacterial defence system, many of the omptin proteases are involved in other roles such as adhesion or invasion of bacteria, and hence contributing to their virulence.^{113,116}

Table 2.8: Properties of proteases of the Omptin family.

Protein	Organism	Identity to Ompt (%)	Protein Size (kDa)	Roles in pathogenesis
OmpT	<i>Escherichia coli</i> K12	100	33.4	Cleaves protamine, the antimicrobial peptide, secreted by the urinary tract epithelial cells; processes some autotransporter proteins
OmpP	<i>Escherichia coli</i> K12	73	33.1	Processes some autotransporter proteins
SopA/IcsP SopA2/So pA3	<i>Shigella flexneri</i> , enteroinvasive <i>Escherichia coli</i> , <i>Shigella dysenteriae</i>	60	33.4	Cleaves IcsA autotransporter, ensuring polar localization of IcsA; required for proper actin-based motility inside host eukaryotic cells
PgtE/ PgtE2	<i>Salmonella enterica</i>	50	32.8	Cleaves α -helical cationic antimicrobial peptides secreted by epithelial cells
Pla	<i>Yersinia pestis</i>	50	32.6	Activates plasmin; inactivates α 2-antiplasmin (plasmin inhibitor); cleaves Complement C3 component; mediates bacterial adhesion to ECM of eukaryotic cells; mediates bacterial invasion into eukaryotic cells.
PlaA	<i>Erwinia pyrifoliae</i>	47	32.3	N/A
Q8ZGQ6	<i>Yersinia pestis</i>	40	32.4	Contributes to infection establishment in <i>C. elegans</i>

Note: Adapted from Hritonenko, V. *et al.*, 2007.¹¹³

Figure 2.8: Sequence alignment of OmpT protease family. The percentage identity in amino acids sequence is shaded on a color scale, dark blue being highest and white the lowest. The four proposed catalytic residues are depicted with a *. The arrows represent the approximate position of the ten β -strands, and the extracellular loops are labelled L1–L5 (Reproduced with permission: Appendix-Permissions).¹¹⁴

The ompT proteases are sub-grouped into two groups –i) PgtE, Pla, and PlaA ii) OmpT, OmpP and SopA. Each group has a 60-84% sequence similarity within the members and have functional similarity among the members. PgtE and Pla have strong adhesion property and can cleave larger substrates. OmpT and SopA which form the second group have a similar function of processing the autotransporter proteins and are involved in virulence. The amino acids forming the catalytic, active, and LPS binding site are highly conserved which becomes evident from their sequence alignment analysis (Figure 2.8 and Figure 2.9). However, there are few mutations in the active site residues which defines the substrate preference of these proteases as well. Overall, the

catalytic site and proteolytic mechanism are highly conserved among the omptin proteases.

Table 2.9: Conserved amino acids in the omptin protease family at catalytic site, active site and LPS binding site.

	OmpT	OmpP	SopA	Pla/PlaA	PgtE
<i>Catalytic amino acids</i>					
Asp 83	+	+	+	+	+
Asp 85	+	+	+	+	+
Asp 210	+	+	+	+	+
His 212	+	+	+	+	
<i>Active site amino acids</i>					
Glu 27	+	+	+	+	+
Ser 40	+	+	+	+	+
Leu 42	+	+	+	+	+
Met 81	+	+	+	+	+
Asp 97	+	+	+	+	+
Ser 99	+	+	+	+	+
His 101	+	+	+	+	+
Ala 143	+	+	+	+	+
Tyr 150	+	+	+	+	+
Ile 170	+	+	+	+	+
Asp 208	+	+	+	+	+
Ser 223	+	+	Asp	Glu	Glu
Thr 263	+	+	+	+	+
Ala 280	+	+	+	+	+
Ile 282	+	+	+	+	+
<i>LPS binding amino acids</i>					
Tyr 134	+	+	+	+	+
Glu 136	+	+	+	+	+
Arg 138	+	+	+	+	+
Arg 175	+	His	Leu	+	+
Lys 226	+	Ile	+	Ser	Glu

2.4.1. PgtE - outer membrane protease of *S. enterica*

PgtE is another outer membrane protease of the omptin family that is present on *S. enterica* and is involved in virulence and resistance to antimicrobial peptides. PgtE is known to cleave plasminogen, a 92 kDa protein present in blood and acts as a precursor for plasmin protease which is involved in dissolution of fibrin blood clots. Plasminogen is activated by cleavage at Arg561-Val562. Like OmpT, PgtE also degrades short peptides and alpha-helical cationic AMP (i.e. LL37, C18G, Cap11, Cap18, Melittin

etc.).¹¹⁷ PgtE contributes to the survival of bacteria inside the host and is upregulated in *S. enterica*. Omptin family of proteases require LPS for their activity. But since long O-antigen chains of LPS deactivate PgtE, O-antigen length is reduced during the infectious stage and thereby leads to higher activity of PgtE.

2.4.2. Plasminogen activator (Pla) - outer membrane protease of *Y. pestis*

Pla is a membrane protease present on *Y. pestis* and is encoded by the pPCP1 plasmid. The plasmid is required for the circulation for *Y. pestis* in the host and spreading of pneumonic plague. It has been reported that the absence of the plasmid in some ancestral strains (i.e. Pestoides E and F) prevented these strains from causing infections.¹¹⁷ It was later shown that with the expression of Pla on *Y. pestis* the lethal dose (LD₅₀) of *Y. pestis* decreases by 10⁶, making Pla highly virulent.^{118–120} Hence, the expression of Pla has been associated with pneumonic plague and bubonic plague.^{118,121} Formation of fibrin clots is a common strategy adopted by the host cells to prevent the spread of infections. Pla activates plasmin by cleaving the plasminogen precursor which further dissolves fibrin clots, laminin, and extracellular matrix. Hence, facilitating the spread of bacterial infection. Pla induces a cascading activation of proteolysis in the host as it is not regulated by the host protease inhibitors.

2.4.3. SopA outer membrane protease of *S. flexneri*

SopA is an outer membrane protease present on *Shigella flexneri*. SopA cleaves autotransporter IcsA (VirG), between Arg₇₅₈ and Arg₇₅₉, releasing the passenger domain - IcsA α , from the bacterial surface.¹²² The accessible IcsA α is localised at one pole of the bacterial cell and is important for the formation of actin ‘comet’ tails on the bacterial surface.¹²³ The actin ‘comet’ tails are involved in propelling the bacterial pathogen within the host cells as well as enable them to spread to neighbouring cells.¹²³ Hence, SopA is an important virulence factor for *S. flexneri*, as it is involved in the localisation of IcsA.

2.4.4. OmpT- outer membrane protease of *E. coli*

OmpT is the most studied protease of the omptin family and is expressed in both nonpathogenic (*E. coli* K12) and human uropathogenic *E. coli* strains (UPEC). Although the OmpT gene is expressed in almost 50% of such *E. coli* isolated from urine

and wounds, they are seldom proteolytically active.¹¹³ On the other hand, 83-85% of *E. coli* express proteolytically active OmpT when isolated from samples from individuals suffering from urinary tract infection (UTI).¹²⁴ In the case of UPEC strains, protamine P1 is the natural substrate cleaved by OmpT, which is an AMP secreted by the epithelial cells lining the urinary tract. These peptides are secreted by the cells of the host organism as part of their defence mechanism to kill invading bacteria. The actual mechanism of the antimicrobial peptide is unknown, but the antibacterial peptides are expected to target phospholipids in the bacterial cytoplasmic membrane. Previous reports have shown that the peptides (defensin NP-1, magainin 1, cecropins etc.) are involved in disrupting bacterial protein synthesis, diminish cellular ATP content, inhibit amino acid transport, disrupt oxygen consumption, resulting in the leakage of intracellular components.¹²⁵ OmpT deactivates protamine P1 that helps *E. coli* survival inside the host and hence, contributes to the prevalence of bacterial infections. OmpT attached to epithelial cells results in the persistence of bacterial infection in the host.¹¹³ Since the OmpT acts as a defense mechanism for *E. coli* by degradation of cationic AMPs produced by human host¹²⁶, the expression of OmpT has been reported to increase in form of outer membrane vesicles.^{127,128} Previously it has been shown that Enterohaemorrhagic *E. coli* (EHEC) enhances the secretion of OmpT-loaded OMVs in coordination with the activation of virulence genes during infection and blocks bacterial cell attack by LL-37. EHEC cells degrade AMPs faster compared to enteropathogenic *E. coli* (EPEC) and is based on the higher expression of OmpT on the EHEC strain. Hence the expression of OmpT is dependent on their adaptation to their respective niches within the host, where the environmental cues and abundance of AMPs are different.¹²⁹ OmpT expression and activity has also been reported to increase in response to recombinant chloramphenicol acetyltransferase overexpression and heat shock in *E. coli*.¹³⁰ Hence, OmpT is classified as a virulence factor.

2.4.4.1. Structural characteristics of OmpT

At the time of writing this thesis, OmpT is the only protease from the Omptin family whose crystal structure is known (Figure 2.9).¹¹⁴ It is expressed as a 317 amino acid protein with the first 20 N terminus amino acids in its sequence acting as the signal peptide responsible for transporting the protein to the extracellular membrane. It has a transmembrane β -barrel structure with ten β -strands connected by four periplasmic

loops (T1-T4) and five longer extracellular loops (L1-L5). The β strands have an average length of 23 amino acids and a 37-45° tilt with respect to the barrel axis. The periplasmic loops do not have any known activity, but the extracellular loops form the grooves which form the active site of OmpT and other Omptin proteases.^{114,115} The amino acids involved in the catalytic activity of Omptins are conserved and form a negatively charged groove required for the binding of the cationic antimicrobial peptides. OmpT extends 40 Å above the membrane with aromatic amino acids presents at the boundary of the hydrophobic and hydrophilic area on the molecular surface are represented in yellow (Figure 2.9).

Figure 2.9: Structure representation of OmpT. (A) Ribbon representation of OmpT extracellular loops L1–L5. Aromatic residues (yellow), catalytic residues (red), and LPS binding site (purple) (B) OmpT and modelled LPS molecule at the putative binding site on OmpT. LPS is shown as thin grey lines. (Reproduced with permission: Appendix-Permissions).¹¹⁴

OmpT, like other members of the omptin family, prefers to cleave peptides at dibasic residues. Since OmpT has three dibasic sites present in its own sequence, it is difficult to express it *in vitro* as an intact protein because it undergoes autoproteolysis. Although it carries three di-basic sites (R37-K38, K217-R218 and K259-K260) in its sequence, only K217-R218 is prone to autoproteolysis.¹³¹ Located on a flexible extracellular L4 loop on top of the beta-barrel structure of OmpT, this exposed site makes it more

susceptible to autoproteolysis than the other two sites that are located on the body of the barrel.¹¹⁴

Two strategies have been adopted in the literature in order to overcome autoproteolysis during recombinant *in vitro* expression. First, the sequence of the protease was mutated in the vicinity of the autoproteolysis site, G216K and K217G.¹³¹ However, this mutant has a lower activity (~ 30% lower) as compared to the wild-type protease.¹³¹ Besides affecting the protease activity, this mutation alters OmpT's substrate preference.¹³² A second strategy to avoid autoproteolysis, which was adopted by Wu et al., 2012, and described in detail in Wu's PhD thesis, involves the addition of LPS to refolded OmpT immediately after cation exchange purification.^{133,134} In this case, the author suspected a protective role of LPS against OmpT autoproteolysis but did not characterize it any further. Strangely, autoproteolysis has never been reported for wild-type OmpT when it is expressed naturally on the outer membrane. It raises an interesting question as to why only the OmpT expressed *in vitro* as IBs and refolded *in vitro*, undergoes autoproteolysis? This thesis has looked into this aspect in the later sections.

2.4.4.2. OmpT activity and role of LPS

OmpT is one of the first enzymes that is known to require lipopolysaccharide (LPS) for its activity. Besides OmpT, Pla and PgtE have also been reported to require LPS for their activation.^{135,136} In the absence of LPS, OmpT shows no activity. It is possible that the activation of OmpT upon LPS binding is a safety mechanism, to ensure that the protease does not become active in the cytoplasm and periplasm causing degradation of other cytoplasmic protein with a dibasic site.¹³⁶ Once the mature OmpT is translocated and inserted into the outer membrane, LPS binds to the mature OmpT and makes it proteolytically active (Figure 2.9 A).¹³⁵

A single LPS molecule binds to OmpT for its activation, and this binding of LPS to OmpT does not cause major changes in OmpT's structure as confirmed by circular dichroism studies.¹³⁶ Amphipathic LPS molecules have a large hydrophobic lipid A part and a hydrophilic oligosaccharide region consisting of O Antigen-specific chain, outer core and inner core (Figure 2.10). LPS has a large negative charge because of the carboxylate/phosphate groups present on its sugar residues.¹³⁶ The phosphate of lipid A chain in LPS interacts with Arg138 and Arg175 of OmpT resulting in proper

alignment of the active site residues.¹³⁶ LPS must have a heptose bound phosphate and a fully acylated lipid A to help bring about the slight conformational change resulting in activation of mature OmpT.^{131,136} This subtle conformational change in OmpT occurs possibly in loop L4, leading to a change in the properties of the active site in such a way that it can hydrolyze substrates.¹³⁶

Figure 2.10: Schematic representation of the lipopolysaccharide structure showing: Lipid A, core oligosaccharide, and repeating O-antigen.¹³⁷

Although there is no available crystal structure of OmpT complexed with LPS, there is one available for FhuA complexed with LPS. FhuA from *E. coli* is an outer membrane protease with a transmembrane β -barrel structure similar to OmpT.^{138,139} Based on the binding site in FhuA, a putative LPS binding site was identified in OmpT. The binding

site consists of 4 basic amino acids that are conserved among prokaryotic and eukaryotic LPS-binding proteins.¹¹⁴ Of the four conserved basic amino acids, only three are found in OmpT (Arg138, Arg175 and Lys226) (Figure 2.9 B). The conserved fourth amino acid, Arg382, forming the binding site of FhuA is not present in the OmpT possibly because of the strong bending of OmpT at this position. Besides the four conserved amino acids, Glu304 and Phe302 are additional amino acids present in FhuA's LPS binding site. Similarly, OmpT has Glu136 and Tyr134 at the same positions. The five amino acids (Arg138, Arg175, Lys226, Glu304 and Phe302/Tyr134) binding to LPS are structurally conserved in OmpT and FhuA, and the amino acids are present at a similar position in the β -barrel with an RMSD of 1.1 Å for all atoms.¹¹⁴ Based on these similarities, Vandeputte-Rutten, L. *et al.* hypothesised that these amino acids are involved in LPS binding in case of OmpT as well.

It has been previously reported that LPS can perform a dual function: activation and inactivation of omptin proteins.^{135,140} Pla and PgtE are unable to cleave larger substrates because of steric hindrance from O-antigen in the outer core of LPS, whereas OmpT targets short cationic peptides which can easily escape the steric hindrance from the O-antigen.¹¹³ Gram-negative bacteria have approached this problem of steric hindrance in different ways. In the case of Pla, which is expressed on *Y. pestis*, O-antigen is not expressed.¹³⁵ Contrary to this, *S. enterica* expresses LPS with reduced length of O-antigen.¹⁴¹

2.4.4.3. OmpT - active site and proteolytic characteristics

Initially, OmpT was classified as a serine protease,¹⁴² since serine protease inhibitors partially inhibited OmpT activity and a Ser99Ala mutation results in 500 times lower activity. Later, OmpT was reclassified as aspartate protease based on its crystal structure. OmpT's unique mechanism has characteristics of serine and aspartate proteases (Figure 2.11).

The catalytic site Asp83-Asp85 and Asp210-His212 are similar to aspartate and serine-carboxyl protease, respectively. These 4 amino acids are highly conserved in the Omptin proteases. Since serine is absent in OmpT's catalytic site, a water molecule present at the catalytic site performs the role of serine.¹¹⁴ It has been proposed that the scissile bond between the dibasic amino acids residues present in the substrate,

undergoes a nucleophile attack by a water molecule, which is activated by the catalytic residue of OmpT. Hence, water molecule acts as an O γ (deprotonated -OH of serine) of catalytic serine in serine proteases while the Asp83-Asp85 contributes to a proton translocation or a stabilisation of an oxyanion intermediate.¹¹⁴ The proteolytic mechanism of Omptin family of proteases is conserved even if some homologs have mutations in their active site. The active site composed of amino acid residues in L1-L5 loops in the Omptin family is conserved, but by varying the amino acids in the active site, the specificity of the proteases can be changed. When Ser223 was changed to Arg223 in OmpT, the OmpTs preference changed from Arg-Arg to Ala-Arg.¹⁴³ The overall net negative charge of the catalytic site makes OmpT highly selective to basic amino acids as targets with a narrowly defined cleavage specificity. OmpT's natural substrate protamine P1 (which contains 24 arginines) and LL37 (which contains 2 dibasic sites) are rich in basic amino acids and hence, are easily cleaved by OmpT *in vivo*. Based on Schechter and Berger nomenclature, the cleavage site of a peptide substrate is represented as ...P2-P1-P1'-P2' ... and so on (Figure 2.12).

Figure 2.11: Illustration of the catalytic mechanisms of (a) serine-carboxyl proteases and (b) Omptins. For OmpT, nucleophilic water plays the role of the serine O γ in serine proteases. Catalytic residues (black), the substrate (red) and the putative electron translocation (blue arrows). (Reproduced with permission: Appendix-Permissions).¹⁴⁴

Figure 2.12: The Schechter and Berger nomenclature of the substrate specificity of the protease. Adapted from *Nomenclature of the substrate specificity of proteases*, Monash Bioinformatics Platform, (undated) at <https://prosper.erc.monash.edu.au/methodology.html>.¹⁴⁵

The P1-P1' represents the cleavage site where **P'** signifies residues carboxy-terminal to the scissile peptide bond and **P** as the amino-terminal residues. OmpT requires a basic amino acid at P1, but P1' is a bit flexible.¹⁴⁶ Arg and Lys are most preferred at P1-P1', but at P1' amino acids with small non-acidic side chains are acceptable. This flexibility at P1' makes it possible for OmpT to cleave plasminogen at the Arg-Val site.¹⁴⁷ OmpT has a different preference for amino acids at each position in the cleavage site (Table 2.10).^{113,146}

Table 2.10: OmpT's preference of amino acids (AA) at different positions in the cleavage site. The orange coloured rows are the sites of cleavage.

Position of substrate amino acid	Amino acids permitted in a descending order	Comments
P6	Lys, Arg>>Asn, Gln>Ala.	Positively charged amino acids are preferred; acidic amino acids are not allowed.
P4	Arg, Lys>>Gly.	Positively charged amino acids are preferred; acidic amino acids are not allowed.
P3	Arg>Trp>Val	
P2	Ala>Ile,Phe>Leu>Val>Pro>His>Gly>Arg.	Broad tolerance; acidic amino acids are not allowed.
P1	Arg or Lys.	Highly conserved.
P1'	Lys>Ile>His>Arg.	Acidic amino acids are not allowed.
P2'	Ile, Val>Ala.	Amino acids with small side chains are preferred.
P3'	Trp>Arg>Thr.	Amino acids with large side chains are preferred.

Note: Adapted from Hritonenko, V. et al., 2007.¹¹³

OmpT is selective at P1 and P1' but it diminishes as we move outwards from the cleavage site. OmpT at P2' prefers hydrophobic amino acids, while P2 prefers Ala and other non-acidic amino acids are tolerated at P2 as well. P3' position can accommodate amino acids with a large side chain. P4 and P6 again prefer basic amino acids. Since the catalytic site has an overall negative charge, acidic amino acids are not preferred in the peptide substrate. Substrate specificity varies within the members of the omptin family and can also be engineered by modifying the amino acids in the L1-L5 loops that form the active site.¹¹⁵ Several strategies have been used to express and purify the membrane proteases to study their structure, substrate preference.

Although OmpT and other Omptin proteases cleave at a dibasic site, efforts have been made to optimise the substrate sequence for them. Kramer *et al.* developed a FRET-based assay for kinetic analysis using the substrate Abz-Ala-Arg-Arg-Ala-Tyr(NO₂)-NH₂. Abz(o-aminobenzoyl) was used as the fluorophore, and Tyr(NO₂) (3-nitrotyrosine) was used as a quencher in the FRET assay. The intact peptide substrate whose fluorescence is quenched exhibited an increase in fluorescence when cleaved by OmpT between the two arginines. Dekker *et al.* used a similar approach to determine substrate specificity for OmpT.¹⁴⁶ Dekker *et al.*, 2001 used a library of 80 peptides of the form acetyl-Dap(dnp)-P2-P1-P1'-P2'-Lys (Abz)-Gly. Starting with an initial peptide ARRA, all four positions (P2, P1, P1' and P2') were substituted with 20 amino acids one at a time. The peptides were synthesized with a fluorophore (Abz), and quencher (Dap(DNP)) and the peptides were attached to a cellulose acetate membrane using a PEG linker. Peptide library for P2-P1-P1'-P2' positions were screened using OmpT followed by kinetic analysis. The findings suggest the presence of negatively charged amino acids at the P2-P2' position results in decreased activity. Since the catalytic site is negatively charged it suggests that long-range electrostatic interactions are important for the enzyme-substrate complex formation. By further modifying the amino acids in the vicinity of the cleave site the OmpT activity against the peptide substrate can be improved.

2.4.4.4. OmpT - expression, purification, and reconstitution in detergent micelles

The natural expression of OmpT protein is low, so purification of OmpT in large quantities would be difficult and would require very large quantities of bacterial culture.

The other approach for OmpTs expression is the recombinant expression of OmpT in *E. coli* in the membrane or the cytoplasm. For recombinant expression of OmpT in the membrane it is expressed with a signal peptide, further transported to the membrane and folded into mature protein. However, for recombinant expression in the cytoplasm, it is expressed without signal peptide in inclusion bodies (IBs). The expression in IBs has higher yield but OmpT is obtained in an unfolded, and aggregated state, whereas the recombinant expression in the membrane is properly folded but protein yield is reduced. Structural and functional studies of membrane proteins have clearly shown that they require a lipid-like hydrophobic environment for retaining their structure and activity. In the absence of a hydrophobic environment, the membrane proteins tend to aggregate and hence, lose their native structure and activity. Detergent micelles, liposomes, polymersomes, and other biomimetic systems have proven useful in replicating the environment provided by cellular membranes. Such systems have allowed for the detailed structural and functional analysis of many membrane proteins (pro- and eukaryotic).

For OmpT purification three different strategies for protein purification have been previously reported. The first approach involves the isolation of wild-type OmpT from the outer lipid membrane followed by ion-exchange and affinity chromatography-based purification.¹⁴⁸ The second strategy for OmpT purification is again the isolation of wild-type OmpT from the lipid membrane, but the extraction is done with Mg^{+} , EDTA, and octylglucoside.¹⁴⁹ However, both these OmpT purification strategies described above have a drawback since other membrane proteins will be copurified with OmpT. Also, the presence of endogenous lipids cannot be avoided. The third strategy involves expressing the membrane proteins, without the signal sequence, as inclusion bodies (IBs).^{150,151} The significant advantage of this approach is that the IBs are physically separated from the membrane lipids and proteins. Since the IBs can be produced in large quantities the yield of proteins that can be obtained from bacterial cells is very high. However, on the downside, the protein obtained is misfolded which needs to be unfolded before properly refolding the protein in the presence of detergent, to obtain an active protein. Various strategies have been used for membrane protein refolding. The membrane protein folding principles are significantly different from water-soluble protein refolding. Membrane proteins fold within a hydrophobic anisotropic environment, unlike polar, aqueous solvent. Membrane proteins expose their

hydrophobic residues to the membrane instead of burring them in the protein core. On the contrary, the hydrophilic residues are present on the outside of the membrane or at the membrane edges and even buried inside the protein core since membrane proteins exist in a complex environment with the interaction between the aqueous phase and the hydrophobic lipid environment. The lipid environment around the membrane proteins during refolding plays a significant role in the proper folding of membrane proteins. Since the native membrane environment is difficult to reproduce, membrane protein refolding is studied in much simpler detergent based *in vitro* systems.

In previous studies by Kramer *et al.*, 2001 different detergents like Tween-20, Triton X-100, N-Dodecyl-N,N-dimethyl-1-ammonio-3-propane sulphonate (DodMe₂NPrSO₃), octylpolyoxyethylene, and n-hexa-decylphosphorylcholine have been used for OmpT refolding.¹³¹ Even though OmpT refolded correctly in all the above-mentioned detergents at various concentration, it failed to exhibit any enzymatic activity. High concentrations of DodMe₂NPrSO₃ was used for refolding OmpT after the solubilization of OmpT IBs. Although the IBs were sufficiently pure, various ion exchange column-based purification steps were used by Kramer *et al.* after the refolding of OmpT was done. Kramer *et al.* 2000 used a two-step purification step, the first step of which involved OmpT purification using Fast Flow S-Sepharose column (cation exchange chromatography) followed by a second purification step using a DEAE column (anion exchange chromatography).¹³¹ Since LPS is essential to obtain active OmpT, the authors of the study incubated the purified OmpT overnight in a solution of LPS before a final purification step using anion exchange chromatography.

2.5. Peptide substrate optimisation

In order to develop an outer membrane protease-based assay, a substrate for which the protease has high catalytic activity is required. Better peptide substrates can be designed by optimising amino acids at cleavage site and in its vicinity. A “better” substrate can be loosely defined as any peptide that is capable of exhibiting higher affinity and catalytic efficiency than what is already reported in the literature. The rate of association of enzyme and peptide substrate is dictated by diffusion, which is governed by Brownian motion and generally has an association rate of 10^9 - 10^{10} M⁻¹s⁻¹. However, the stringent orientational constraints and activation energy barrier reduce the rate of enzyme-substrate complex formation to 10^5 - 10^6 M⁻¹s⁻¹.¹⁵² A faster association rate

constant is governed by favourable electrostatic intermolecular forces between enzyme and substrate. In principle, increasing the hydrophilic character of the substrate binding site by substituting charged amino acid may increase the preference for hydrophilic over hydrophobic substrates and vice versa. The electrostatic energy of interaction and kinetics of enzyme-substrate complex stabilise their charged transition states. Hence, surface charge and specific residues of the active site play an essential role in catalysis.¹⁵³ However, specificity is determined by complex and poorly understood electrostatic interactions, steric, and hydrophobic effects.¹⁵⁴

Enzymatic reactions depend on the interaction between the enzyme active site and the amino acid sequence surrounding the scissile bond in the substrate. Hence, electrostatic interactions between them have an essential role to play in the formation of the enzyme-substrate complex. The specificity information of amino acids at various positions can be used to design highly sensitive and specific synthetic substrate to be used for sensing and as potential inhibitors.

2.5.1. Design of peptide library

Although the synthesis of a peptide library can be expensive and require a high throughput screening technique to screen the peptide library, it provides a powerful tool for understanding the preference of enzymes for individual amino acids at specific positions within the peptide. It has also found tremendous use in the design of peptide-based drugs, and to study protein-protein interactions. As reported previously, a peptide library synthesised with different amino acids at P₂, P₁, P₁' and P₂, was screened for OmpT activity, where P₁ and P₁' are the cleavage site for the enzymes. Screening of the designed peptide library was done either by SPOT assays or FRET-based assays. Each of these four positions on the peptide was shown to have a different amino acid preference for OmpT. It has been reported that the short four-residue peptide, ARRA, is the most preferred substrate for OmpT exhibiting a catalytic efficiency as high as 10⁸ s⁻¹M⁻¹ with an upper limit of catalytic efficiency of enzymes being 10⁹ s⁻¹M⁻¹.¹³¹ The SPOT or FRET-based assays are not the ideal methods to evaluate peptide sequences for their catalytic efficiencies since these methods rely on labelling the peptides. Such labelling of the peptides can affect the binding and catalytic activity of the enzyme. Considering ARRA as the starting peptide sequence in the search for a better peptide, the individual positions P₂, P₁, P₁', and P₂ can be modified with 20 different amino acids.

There are two kinds of peptide libraries that can be generated by varying the amino acids at the different positions on the peptide: Positional scanning and combinatorial peptide libraries (Figure 2.13).

Figure 2.13: Peptide substrate optimisation and design of corresponding peptide libraries. In the peptide substrate, solid green circles represent position having fixed amino acids, solid red circles represent a position that needs to be optimised, and solid yellow circles represent a position that can be substituted with 20 different amino acids. (A) Positional scanning library – amino acids at a position are substituted one at a time, e.g. When three positions need to be optimised the library size is 60. (B) Combinatorial peptide library: amino acids at the positions are substituted simultaneously, e.g. When three positions need to be optimised, the library size is 8000.

A positional scanning library involves substituting amino acids at a given position, one at a time. Whereas, in the combinatorial peptide library, selected residues are randomly and simultaneously substituted with all 20 natural amino acids.¹³² There are two main approaches employed to generate peptide libraries: biological methods and synthetic methods. Biologically, peptide libraries can be constructed using filamentous phage display, plasmids, and polysomes.¹⁵⁵ Phage display is the most popular biological methods for generating peptide libraries. It involves expressing a library of peptides with an N-terminal fusion tag that helps the bacteriophage attach to a solid support and a C-terminal portion that is attached to the bacteriophage coat protein. Each peptide variant, from the peptide library, is presented on the surface of a bacteriophage and is bound to a solid surface via the N-terminal tag. During the screening, OmpT cleaves the peptide thereby releasing the bacteriophage from the solid surface. The free phages

are then amplified and undergo several rounds of screening before being sequenced to get the final peptide sequence. However, this method is laborious and does not provide an exhaustive evaluation of amino acids for all positions around the cleavage site. Solid phase peptide synthesis is a synthetic method for generating peptide libraries. Combinatorial peptide libraries have been used to identify substrate for human cysteine cathepsins¹⁵⁶, cysteine proteases, leader peptidase of *E. coli*¹⁵⁷ and other novel proteases.¹⁵⁷ The peptide libraries synthesised using solid phase synthesis approach can be screened using various techniques such as SAMDI-MS, SPOT assays, photolithography or particle-based assays.¹⁵⁸ All these techniques require immobilisation of peptide substrate on a solid substrates like gold, glass, nitrocellulose or PVDF membrane. These techniques mostly use radiolabeling, chemiluminescence, colourimetry, fluorescence, mass spectrometry or SPR based detection methods.¹⁵⁸

2.5.2. Screening of peptide array using SAMDI-MS

High throughput screening (HTS) techniques play a vital role in screening large molecular libraries against enzymes for developing active substrates and potential drug molecules. These HTS methods are based on fluorescence intensity (FLINT), fluorescence resonance energy transfer (FRET), time-resolved FRET (TR-FRET), and luminescence readouts. These approaches rely on fluorescent tags, radiolabels, and antibodies to measure enzyme activity. These approaches are prone to false positives which might be because of aggregation or interference from the molecules used for fluorescent tagging.^{159–161}

HTS techniques generally need immobilisation of peptide substrate on surfaces like glass, gold, nitrocellulose and PVDF membrane. For the detection of the peptides various labelling techniques (*i.e.* radiolabeling, fluorescence labelling, chemiluminescence, colourimetry) or non-labelling techniques (*i.e.* mass spectroscopy and surface plasmon resonance) can be used. The molecules used for peptide labelling are generally bulky aromatic fluorescent dyes, luciferase substrate, or a europium or terbium chelate. Although the molecules used for labelling can be detected rapidly with high sensitivity, they have various drawbacks such as labelling molecules interacting with the substrate itself can affect the overall activity and hence, increasing the false positives.^{161–164} Also, the radiolabels inherently pose a health risk, require special disposal methods which are expensive. The false positive rates can be as high as 80%

in a 1 million compound screening using fluorescence-based approaches.¹⁶⁵ In a recent study on caspase 6, a highly selective inhibitor which inhibited enzyme via uncompetitive mechanism was identified. But further studies suggested presence and character of fluorophore at P1' position of peptide substrate enhanced the enzyme-substrate interactions affecting the binding.¹⁶¹ It has also been reported that the presence of the fluorophore on the peptide substrate (i.e. Fluor-de-Lys) affects the activation of SIRT1, NAD⁺ dependent protein deacetylase, by resveratrol.^{162–164} The interaction of the labelling molecule, present on the substrate/inhibitor, with the enzymes increases the false positives. Hence, using label-free strategies will help reduce false positives and provide information about the molecular weight, purity, and degradation products. Thus, to reduce false positives various label-free detection methods based on biophysical properties like molecular weight (e.g., in microcantilever and MS), refractive index (e.g., in SPR, LSPR, and anomalous reflections) and molecular charge to monitor molecular presence or activity have been developed.¹⁶⁶ These techniques are quantitative at the same time label-free. Hence, substrates and products can be used in their native sequences without the need for labelling or the application of indirect detection methods.¹⁶⁴

Self-Assembled Monolayer Desorption Ionization (SAMDI) is a label-free HTS technology (Figure 2.14). It combines mass spectrometry (MS) with defined surface chemistries. The SAMDI label-free assay uses a self-assembled monolayer of alkane thiolates on gold which has a terminal maleimide group to immobilise substrates for enzymatic reactions including peptides, proteins, carbohydrates, metabolites and others. Cysteine-terminated peptide libraries can be selectively immobilised on the SAMDI array plate using maleimide-cysteine chemistry. Reactions containing enzymes and required cofactors are organised in standard micro-well plates and then robotically transferred to the monolayer array plate where enzymes can modify the immobilised peptide substrate. The array is rinsed to terminate the reactions and then analysed in a MALDI instrument, where irradiation of the spot with the laser results in cleavage of the gold-thiolate bond, and release of the intact alkanethiol, which then accelerates to the detector and has its mass determined. In this way, if the peptide substrate has been modified, the mass of the alkanethiol increases (or decreases) by a specific amount and that change is readily monitored by the MALDI instrument.

The activity of the enzymes is defined as:

$$\text{Activity} = \frac{AUC_{\text{product}}}{AUC_{\text{substrate}} + AUC_{\text{product}}} \times 100\%$$

Figure 2.14: Illustration of SAMDI-MS screening of peptide library for substrate optimization. (A) Immobilisation of peptide on SAMDI array and cleavage by the membrane protease. Enzyme activity is calculated from the peak intensities of each substrate and its product. (B) Enzyme activity for a positional scanning library starting from the sequence ARRA is represented as a heat map on a scale of 0-1, where 0 indicates least activity and 1, the highest.

The activity of each peptide can be represented as a heat map on a scale of 0-1 (Figure 2.14 B). SAMDI has been extensively used for assays of various enzymes like kinases, proteases, glycosyltransferases, methyltransferases,¹⁶⁷ lysine deacetylases,¹⁶⁸ and nucleic acid ligases.¹⁶⁹ Previously, SAMDI-Ms was used to screen 100,000 compounds to identify novel small molecules that might act as possible SIRT3 inhibitors which clearly demonstrates the HTS capabilities of the instrument.¹⁷⁰

2.6. Reporter molecule - Polythiophenes

Polythiophene (PTs) are conducting polymers formed by the polymerisation of thiophene rings (Figure 2.15 A, B). These thiophene rings are heterocyclic compounds

with the formula C_4H_4S (Figure 2.15 A), are interlinked through 2- and 5- positions of the rings, and can have different side chains attached at the 3- and 4- positions. PTs have delocalized electrons along the polymeric backbone, which give PTs their electrical and optical properties (Figure 2.15 C).

Figure 2.15: Chemical structure of A) Thiophene B) Polythiophene C) Resonance structures of polythiophene.

The conformations of the PT's backbone and how the chains are packed affects the delocalisation of electrons across the backbone (Figure 2.16). These confirmations can be planar or helical, which changes the effective conjugation length of the polymer backbone and also affects the delocalisation of electrons across the chain.¹⁷¹ However, the backbone conformation can be changed by varying the solvent, temperature, applied potential, pH, and side chain binding to other molecules. The optical response of PTs are tunable by modifying the side chains in polythiophene at 3- and 4- positions.¹⁷² Synthesizing PTs with sides chains that can specifically interact with certain type of molecules and bringing about a conformational change, makes it possible to use them for detection of these molecules. Recently, these polythiophenes have become a better choice for biological and analytical sensing applications because their electrical and optical properties are much more responsive as compared to small molecules.

Figure 2.16: Illustration of - A) π stacking of planar polythiophene chain.¹⁷³ Helical organisation of polythiophenes: B) Helical transoid (large pitch) C) Helical cisoid (small pitch). D) Helical packing of predominantly planar chains. (Reproduced with permission: Appendix-Permissions).¹⁷⁴

PTs have been employed in developing colourimetric and fluorometric assays for detecting adenosine triphosphate (ATP)¹⁷⁵, oppositely charged inorganic ions (CN^- , I^- , Cu^{+2} etc.)^{176–178}, microRNA¹⁷⁹, DNA¹⁸⁰ etc. Zhiyi *et al.* reported the use of anionic polythiophenes for the detection of protamines.¹⁸¹ The detection method is based on the electrostatic interaction between anionic polythiophene and the positively charged protamine molecules. Both colour change and fluorescence decrease upon the addition of protamine was reported as a signal readout for the sensing approach. Jinsong *et al.* used an antimicrobial peptide-polymer complex, negatively charged poly(para-phenylene ethynylene) (PPE), for discriminating 14 different bacteria in water and urine samples.⁸⁸ Pattern-based fluorescence recognition was used for generating cluster plots which were then used for distinguishing different strains. Marie-Pier Plante *et al.* reported the use of cationic polythiophene (cPT), Poly(3-alkoxy-4-methyl thiophene),

for the detection of *E. coli*. The cPT binds electrostatically to the negatively charged *E. coli* surface resulting in a decreased fluorescence.

The polythiophene-based sensing of various biomolecules is based on the electrostatic interaction between the PT and the biomolecules, which affects the conformation of the backbone changing the physical and chemical properties of PT. The side chains at the 3- and 4- position of the thiophene rings can be modified in accordance with the biomolecule being sensed. PTs show a color transition between deep violet ($\lambda_{\text{max}} = \sim 550$ nm) to bright yellow ($\lambda_{\text{max}} = \sim 400$ nm).¹⁸² This transition in colour is because of the planar-to-nonplanar (from highly conjugated to less conjugated) conformational transition of the backbone. The conjugated state of the backbone is related to the intermolecular and intramolecular (chain folding) aggregates. More importantly, the forces which define these interactions and backbone conformations of PTs are hydrogen bonding, nonpolar, and electrostatic interactions, leading to aggregation, gelation, or crystallisation.^{183,184} Chiral PTs show optical activity in the π - π^* transition region. But optically inactive non-chiral polythiophenes become chiral (show Induced Circular Dichroism – ICD) with the addition of chiral molecules. Formation of one-handed helical structure because of the acid-base complex formation between polythiophene and chiral molecules can result in the introduction of chirality in such optically inactive PTs.^{185–188}

It has been reported previously that combining optically inactive PT with helical biomolecules induces chirality in it PT, also changing the conformation of the PT backbone and optical properties.^{189,190} Whereas, Nilsson *et al.* showed induction of chirality by combining optically inactive PT and a synthetic peptide with a random-coil structure.¹⁷¹ This assembly was based only on electrostatic interactions and resulted in colourimetric and fluorometric changes. PTs have also been used for developing colourimetric assays for proteins. Leclerc *et al.* used cationic PTs and thrombin specific aptamer for the detection of thrombin.¹⁹¹ In the absence of thrombin, the aptamer forms complex with PT resulting in a red-shifted absorption maximum and reduced fluorescence. However, in the presence of thrombin, aptamer binds to it and results in the formation of a supramolecular structure. The PT binds around the aptamer-thrombin complex which prevents aggregation and planarization of the PT backbone resulting in orange colour and higher fluorescence. Since the conformational flexibility of PTs

gives a direct correlation between the geometric orientation of the PT backbone and the optical properties, their use for developing colourimetric assays where the biomolecules force the geometric orientation of the PT backbone to change is of great interest. The interaction of PTs and biomolecules resulting in a change in the backbone conformation brings about a change in the absorption and emission properties of the PT.

2.7. Scope of the thesis

Numerous nucleotide and immune assays-based approaches for *E. coli* detection have been developed, but they have their own limitations of ease of use, cost, assay time, and limit of detection. Developing an easy to use colourimetric assay for the detection of *E. coli* in water without compromising significantly on assay time and cost is the end goal of this thesis. The thesis focusses on exploring the idea of using membrane protease for the detection of *E. coli*. To achieve the desired goal, three important pillars need to be understood and explored: the protease (i.e. OmpT for *E. coli*), substrate optimisation for the protease, and reporter molecule for the sensing platform.

The scope of the thesis for the first pillar is to obtain the OmpT proteases in the pure form while dealing and overcoming the issues of protease self-cleavage (autoproteolysis). For pillar two, the thesis would focus on understanding OmpT's preference of amino acid at different positions on the peptide substrate in order to design a 14-16 amino acids long substrate for OmpT which is better than the present substrates. The third and final pillar focuses on the reporter molecules with colorimetric response, which in this particular case, is anionic polythiophene. The thesis will focus on understanding how the polythiophenes interact with the peptide substrates and bring about optical and conformational changes in both the molecules. Therefore, understanding the three pillars better, will help to design a more efficient sensing platform for detecting *E. coli* in water.

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Chapter 3

Experimental Methodology

The thesis has three crucial pillars: Protease (OmpT), Substrate (Peptide) and Reporter molecule (Polythiophene). For each of the pillars, various experiments and characterisation techniques have been used in the thesis. This chapter will cover all the sample preparation methods and experimental techniques used for the three pillars. Where applicable, the general theoretical principles for these techniques will be explained. The first section will talk about the experiments and characterisations related to the first pillar OmpT and obtaining it in sufficient quantities. This would cover techniques like protein expression, refolding, purification, FRET-based activity assays, and so forth. The second section will talk about the next pillar - peptide substrate, that covers peptide library design, synthesis, high throughput screening using SAMDI-MS, and kinetic analysis. The last part would include experimental details about the polythiophene which is the third pillar of the thesis and would focus on the assay development and optimisation strategies. Also, the experimental techniques used to study the characterisation of the interactions between the polythiophene and peptides will be discussed.

3.1. OmpT expression, purification and characterisation

OmpT protease is a member of Omptin protease family and is the most well-characterised protease of the family. Previously different approaches (i.e. expression in membrane and inclusion bodies) have been reported for the expression and purification of OmpT *in-vivo*.¹⁻³ However, the expression of OmpT in *E. coli* as inclusion bodies (IBs) has been considered because of a simple reason that a much higher protein yield is obtained and is easy to purify the protein. The only drawback of this approach is that the protein has to be unfolded and refolded. Different variants of OmpT were expressed as IBs and purified, which would be discussed in the following sections. The experiments in this section were designed keeping in mind the following goals:

- a. Recombinantly express and purify wild-type and mutant OmpT in *E. coli*.
- b. Explore the cause of autoproteolysis during *in vitro* refolding, a phenomenon that is absent during *in vivo* expression.
- c. Characterise the wild-type and mutant OmpT activity.

3.1.1. Materials

The plasmid construct, ompT_pET28a, for wild-type OmpT was provided by Prof Neil Kelleher (Northwestern University, IL, USA). The genes, mt_ompT_{DB}, mt_ompT_{LPS3}, mt_ompT_{LPS5} and sp_ompT were synthesised and subcloned in pET28 vectors and sequenced by Bio Basic Inc (Singapore) (Table 3.1). BL21(DE3) *E. coli* cells used for expressing wild-type and mutant OmpT as IBs, were purchased from Merck Millipore (Singapore). Lemo21 (DE3) competent *E. coli* cells for expressing OmpT in the membrane, were purchased from New England Biolabs Inc (Singapore). LB Broth and LB agar were purchased from BD Difco (MD, USA). Molecular biology grade kanamycin was purchased from Gibco by Life technologies (NY, USA). Isopropyl thio- β -D-galactoside (IPTG) was purchased from Gold Biotechnology (MO, USA). N-Dodecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate (DodMe₂NPrSO₃) and Lipopolysaccharides from *E. coli* 0111:B4 purified by phenol extraction (LPS) were purchased from Sigma (MO, United States). Urea was purchased from Affymetrix USB (Santa Clara, CA, USA). PD-10 desalting (prepacked Sephadex G-25 resin) columns were purchased from GE Healthcare Life Sciences (Singapore). Black flat bottom 384

well plates were purchased from Corning ® (NY, USA). All synthetic peptides were purchased as lyophilised powder and certified > 95% purity from GL Biochem (Shanghai, China) Ltd. Pierce™ BCA protein assay Kit was purchased from ThermoFisher Scientific (Singapore).

3.1.2. OmpT expression and purification

The *ompT* gene corresponds to a polypeptide of 317 amino acids whose first 20 amino acids act as a signal peptide responsible for transferring OmpT into the periplasm.⁴ The plasmid *wtompT_pET28a* had a construct where the mature OmpT sequence corresponding to 297 amino acids in addition to methionine and alanine at the N-terminal was cloned without the signal peptide sequence between NcoI and EcoRI into pET28a vector to produce *ompT_pET28a*. The N-terminal methionine, incorporated for initiation of translation, is eventually excised upon expression.⁵ The mutant *ompT* genes (Table 3.1) that were synthesised and subcloned into the XbaI and XhoI site into pET28a vector to produce *mtompT_{DB}_pET28a*, *mtompT_{LPS3}_pET28a* was purchased from Bio Basic Inc. (Singapore).

Table 3.1: List of OmpT variants expressed and purified

OmpT type	Mutation
<i>wtOmpT</i>	Wild-type OmpT
<i>wtOmpT_{SP}</i>	Wild-type OmpT with a signal peptide
<i>mtOmpT_{DB}</i>	OmpT with dibasic site mutated: G216K and K217G
<i>mtOmpT_{LPS3}</i>	OmpT with 3 LPS sites mutated: R138E, R175E, K226E
<i>mtOmpT_{LPS5}</i>	OmpT with 5 LPS sites mutated: Y134A, E136A, R138E, R175E, K226E

The wild-type and mutant OmpTs (298 amino acids) were expressed as IBs in *E. coli* Rosetta™ (DE3) cells using the method previously described by Kramer, R. A et al., 2000.³ Whereas, the wild-type OmpT with signal peptide was expressed in Lemo21(DE3) *E. coli*. Briefly, BL21(DE3) and Lemo21 (DE3) cells with different plasmids were grown separately overnight in 5 mL LB Broth with 50 µg/mL kanamycin at 37 °C in a shaker set to 220 RPM. For Lemo21(DE3), 25 µg/mL chloramphenicol was also added during culturing. This primary culture was used to inoculate 200 mL LB Broth secondary cultures that were later induced with IPTG (1 mM final concentration) when the OD₆₀₀ reached ~ 0.6-0.8. The cells were harvested after 4 h and washed with 50 mM Tris-HCl, pH 7.5 before suspending them in 5 mL lysis buffer (50 mM Tris-HCl, 40 mM EDTA, pH 8.0). These cells were kept on ice for 20 min

before adding a further 5 mL of pre-chilled lysis buffer, followed by a second incubation on ice for 20 min. After treatment with the lysis buffer, the cells were sonicated for 7 min (2 sec on, 4 sec off, and 50% amplitude) upon which, the solution turned viscous. This viscous solution was centrifuged at $4500 \times g$ for 30 min to separate the IBs as pellets which were then washed twice with 30 mL wash buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). During the final wash, the solutions were aliquoted into separate tubes and centrifuged to obtain IBs as pellets.

3.1.3. OmpT IBs refolding in the presence of detergent

OmpT IBs of wild-type and mutant were solubilised in a solubilising buffer (8 M urea, 50 mM glycine, pH 8.3) and kept on ice for 1 h. To this, pre-chilled refolding solution (20 mM Tris-HCl, 31 mM DodMe₂NPrSO₃ pH 7.5) was added such that the final concentration of protein and urea were between 0.8-1 mg/mL and 0.8 M, respectively. The samples were kept on ice for 3-4 h. For refolding IBs in the presence of LPS, 0.3 mM LPS was added to the refolding solution before adding the solution to solubilised IBs in urea. The refolding mixture with IBs was centrifuged at $10,000 \times g$ for 10 min to remove aggregates. The urea present in the protein sample was further removed using a PD-10 column. The columns were equilibrated with storage buffer (20 mM Tris, 10 mM DodMe₂NPrSO₃ pH 7.5) kept on ice to avoid protein degradation. Refolded protein was loaded on the PD-10 column and the eluted fractions were collected. The fractions were checked for protein concentration and samples with highest protein concentration were pooled together. Samples were aliquoted and stored at -20 °C until further use.

3.1.4. OmpT characterization

3.1.4.1. SDS-PAGE and molecular weight analysis

Protein samples were analysed for their purity and molecular weight using SDS-PAGE. SDS-PAGE is a technique used to separate charged molecules in a mixture based on their molecular weight (MW) under an external applied electric field. The protein sample to be analysed need to have a uniform charged distribution such that they can be separated just based on their MW. Hence SDS, a detergent with a strong protein-denaturing effect, is added such that it binds to the protein backbone giving the protein a constant molar ratio (Figure 3.1). The structure of the protein also plays an essential role in protein mobility in the gel. Tightly wound globular proteins move faster in

polyacrylamide gel as compared to same molecular weight long fibrous proteins.⁶ Hence, use of 2-mercaptoethanol and sample heating helps in unfolding the protein.

Figure 3.1: SDS-PAGE principle and parameters affecting protein movement: A) Effect of SDS and 2-mercaptoethanol on protein mobility. B) Separation of unfolded proteins with uniform charge distribution based on their MW.⁷

Sample preparation

Protein samples were mixed with SDS-Gel loading dye (as recommended by supplier) and heated on a heating block set to 95 °C for denaturing the samples for 5-40 min. Protein molecular weight markers were also run along with samples for protein MW estimation. The samples were run on a BIO-RAD protein electrophoresis unit at 90 V for 90-120 min. The 12 % acrylamide gels were rinsed with DI water three times for 15 min each with mild shaking. The gels were kept dipped in a commercially available CBB dye with mild shaking, for 30 min for staining the gel. The gels were rinsed with DI water several times to remove the background stains. The stained gels were scanned and kept for further analysis.

Gel analysis

Analysis of the gels, including percentage purity and fraction analysis, was done using GelAnalyzer2010 (<http://www.gelalyzer.com>).

3.1.4.2. BCA based protein concentration determination

Bicinchoninic acid (BCA) assay is a standard total protein quantification method. The protein concentration is calculated based on absorbance_{562nm} of protein standards. BCA protein assay is based on the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium, also known as the biuret reaction. The chelation of Cu^{+2} with protein results in the formation of a light blue complex. A peptide with 3 or more amino acids forms a coloured chelate complex with Cu^{+2} ions in an alkaline environment containing sodium potassium tartrate. In the next step, bicinchoninic acid is used for highly sensitive and selective colourimetric detection of the cuprous cation (Cu^{+1}) formed in the previous step. The chelation of two molecules of BCA with one Cu^{+1} results in the formation of an intense purple coloured product that shows strong linear absorbance (0-2 mg/mL) at 562 nm with varying protein concentration.

Sample preparation

Pierce™ BCA Protein Assay Kit was used for quantification of protein samples. The protocol recommended in the kit was followed. Briefly, the working reagent was prepared by mixing CBA reagent A and B in 50:1 ratio. The reactions were performed in duplicates. Protein standards were prepared by varying BSA protein standard concentration from 2000-25 $\mu\text{g/mL}$. 25 μL of each standard and unknown samples were pipetted in a 96 well plate. To this, 200 μL of working reagent was added and incubated at 37 °C for 30 min. After the incubation, absorbance at 562 nm was measured.

Concentration determination

A standard curve was plotted based on the absorbance of BSA standards vs protein concentration in $\mu\text{g/mL}$. The standard curve was used to determine the protein concentration of the unknown protein samples.

3.1.4.3. Liquid Chromatography-Mass Spectrometry (LC-MS) of OmpT

LC-MS is an analytical technique that is used for determining the molecular weight of molecules present in a sample. The sample molecules are first separated using liquid chromatography, and then the fractions are ionised via electron spray ionisation (ESI) generating charged ions of the molecules. The electromagnetic field present separates

these charged ions inside the analyser based on their charge to mass ratio. The separated molecular ions are detected quantitatively and presented as a mass spectrum. LC-MS is a highly sensitive analytical technique that can be used to detect unknown compounds or trace impurities in a sample based on their molecular weight. LC-MS has also been used to determine the purity of the samples.

Here, LC-MS was used to determine the molecular weight of intact and cleaved OmpT fragments. Accurate mass determination of OmpT sample was used to confirm if the two bands on SDS-PAGE corresponds to intact and cleaved fragments of OmpT or if it is just the effect of protein mobility in a polyacrylamide gel.

Sample preparation

LC-MS of the OmpT sample was performed by Dr. Kevin J Metcalf at Northwestern University. w_t OmpT samples were prepared for LC-MS analysis in two different ways. The frozen w_t OmpT sample was first thawed on ice. A fraction of the thawed w_t OmpT sample was diluted in water to a final concentration of $\sim 10 \mu\text{M}$ for injection to LC-MS system. The rest of the thawed w_t OmpT protein was used to prepare a sample for LC-MS analysis using methanol/chloroform/water precipitation method to remove detergent.⁸ In brief, 0.4 mL of methanol was added to 0.1 mL of the protein sample. The mixture was vortexed and centrifuged for 10 s at $9,000\times g$ to collect the sample. Then, 0.1 mL of chloroform was added and the samples were vortexed and centrifuged at $9,000\times g$ for 10 s. To this 0.3 mL of water was added, in order to phase separate the samples were vortexed and centrifuged at $9,000\times g$ for 1 min. Upper phase was discarded, and 0.3 mL of methanol was added to the lower phase. The sample was mixed and centrifuged at $9,000\times g$ for 2 min to precipitate the protein. The supernatant was discarded, and the pellet was dried for further experiments. The sample was resuspended in 5% acetonitrile and 0.1% formic acid (Thermo) in water to a concentration of $30 \mu\text{M}$ and diluted with water to a final concentration of $\sim 1 \mu\text{M}$. LC-MS analysis was performed on an Agilent 1200 series HPLC connected to an Agilent 6210A time-of-flight (TOF) mass spectrometer. A $10 \mu\text{L}$ injection of each sample was captured on a C18 trap column (Waters) and eluted using a gradient from 5% to 95% acetonitrile and 0.1% formic acid in water with a flow rate of 0.25 mL/min .

Data analysis

The mass spectrum of OmpT samples were deconvoluted and were analysed with Agilent MassHunter Qualitative Analysis B.04.00.

3.1.4.4. FRET-based functional assay for OmpT

Förster resonance energy transfer (FRET) is a phenomenon of transfer of energy between two light-sensitive chromophore molecules. One of the two molecules act as a donor, that is electronically excited. The other molecule acts as an acceptor, and the donor molecule transfers the energy to the receptor molecule through a nonradiative dipole-dipole coupling (Figure 3.2).

Figure 3.2: Schematic illustration of FRET-based protease assays. Gray beads represent the peptide substrate chain.

The efficiency of FRET is inversely proportional to the sixth power of the distance between the donor and the receptor molecule. FRET has been used for various kinds of protease assays. The FRET donor and acceptor are linked with a short peptide keeping them in close proximity (1-10 nm). This short distance results in an energy transfer between the FRET pair, however once the protease cleaves the peptides, distance between the FRET pair increase resulting in no FRET/higher fluorescence (Figure 3.2).

Sample preparation

For OmpT assays, a short synthetic peptide ARRA was used as previously reported by Kramer *et al.*³ ARRA peptide acted as a substrate for OmpT and has been reported as the best peptide substrate for it. 2-Aminobenzoyl (Abz) and 3-nitrotyrosine were used as the FRET donor and acceptor, respectively (Figure 3.3).

Figure 3.3: Schematic illustration of the FRET assay for OmpT using the tetrapeptide ARRA carrying 2-Aminobenzoyl (Abz) and 3-nitrotyrosine as the FRET donor (yellow) and acceptor, respectively (red).

All OmpT variants mentioned in Table 3.1 were tested for their protease activity using a synthetic peptide substrate, Abz-ARRA-Tyr(NO₂)-NH₂. A 0.5 mM stock solution of synthetic peptide substrate in Milli-Q water was diluted with 10x assay buffer (200 mM Bis-tris, 20 mM DodMe₂NPrSO₃, 50 mM EDTA, pH 6.7) to a final concentration of 50 μM for the assay. BCA assay was used to confirm the concentration of all 4 OmpT samples. A stock solution of 2 mg/mL of LPS from *E. coli* was prepared in water. All four samples of OmpT were diluted 80 times using the storage buffer and incubated with an equal volume of 20 μg/mL LPS for ~ 15 min. 10 μL of the OmpT-LPS mixture was added to 90 μL of 50 μM peptide substrate in assay buffer. The enzymatic reaction was performed at 37 °C in a 384 well back flat bottom plate. The increase in fluorescence intensity upon OmpT addition was collected over time using a TECAN infinite® M200 PRO (Männedorf, Switzerland) fluorescence spectrophotometer fitted with a plate reader. Fluorescence intensity signals were collected every 10 seconds for 60 min with the excitation wavelength set to 325 nm and emission at 420 nm.

Data analysis

All Data was plotted in Origin 9.0 (OriginLab Corporation, MA, USA). Specific activity was calculated for each OmpT sample based on the fluorescence data.

3.1.4.5. MALDI-TOF of peptide substrates

Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry is an ionisation technique in which laser energy is used to vaporise the matrix together with the sample. This technique is used for making ions from large molecules with minimal fragmentation. Time of Flight (TOF) mass spectrometry is then used to determine the ion's mass to charge ratio by using time of flight measurements. An external electric field accelerates the charged ions and the time required for the ions to reach the detector depends on the mass to charge ratio of the ions. The intensity of the ions vs m/z ratio of the ions is plotted to obtain the final results. MALDI-TOF gives qualitative data about the distribution of ion in the samples.

Sample preparation

Since peptide labelling can affect the activity of protease, unlabeled peptides were also used to confirm the activity of OmpT after refolding. Four hexameric peptides with dibasic sites (Ac-GARKVG-NH₂ and Ac-GARKIG-NH₂) and without dibasic sites (Ac-GLYGGGYL-NH₂ and Ac-GLYGGGYL-NH₂) were used for the reaction. A stock solution of 0.5 mM for each peptide was prepared and was stored at -20 °C. Each peptide was diluted using a 10x assay buffer to a final concentration of 10 μ M, to which OmpT was added at 1 μ g/mL. The reaction mixture was incubated at 37 °C for 1 hour with shaking at 500 RPM. HiPPR™ Detergent Removal Spin Column was used, as directed in product guide, to remove the detergent from the reaction mixture. The reaction samples for the four peptides and the matrix, α -Cyano-4-hydroxycinnamic acid were mixed (1:1), and 1 μ L of the mixture spotted on MALDI-TOF array plate. The matrix and reaction sample was allowed to dry before loading into the instrument. The samples were analysed using a Shimadzu Biotech Axima Performance Mass spectrometer (MSE, NTU) that contained a nitrogen UV laser. The intensity vs m/z spectrum was collected at a laser power of 100 mV in reflection hi-resolution mode, and other settings were adjusted for the best signal-to-noise ratio.

Data analysis

Mass spectrometry data was analysed using the software provided with Shimadzu Biotech Axima Performance. The graphs were exported as metafiles for future reference.

3.2. Designing and screening of peptides substrate library for OmpT using SAMDI-MS

3.2.1. Rational design of peptide substrate library

The specificity and selectivity of a protease-based sensing platform depend on how selective and specific the enzyme is towards the substrate. Also, the response time and limit of detection of the sensing platform depends to some extent on the enzyme and substrate. Hence, designing a peptide substrate for a protease becomes very important. Enzyme catalysis depends on two critical parameters: i) the rate at which the enzyme cleaves the substrate ii) the rate of association of enzyme and substrate. The rate at which an enzyme cleaves the substrate depends on the amino acids at the cleavage site. Whereas, the rate of association is governed by favourable electrostatic intermolecular forces between the residues lining the enzyme's active site and those on the substrate. Besides charge, the size of the amino acids (and the overall substrate) and the flexibility of the amino acid side chains also matter. The ability to form salt-bridges is yet another factor responsible for influencing catalysis. In principle, increasing the hydrophilic character of the binding site on substrate by substituting charged amino acids may increase the preference for hydrophilic over hydrophobic substrates and vice versa. Hence, to improve enzyme catalysis both these parameters need to be optimised.

The process of peptide substrate optimisation starts with the rational design of a peptide library composed of 76 individual peptides. The library design was based on a known tetrapeptide sequence (ARRA) where each position (Ac-G-**P2 P1 P1' P2'**-GC) was sequentially modified with one of nineteen different amino acids except for cysteine, which through its thiol group would be involved in the peptide immobilisation reaction (Table 3.2). The 76-peptide library was screened using SAMDI-MS.

Table 3.2: Position scanning library of 76 peptides starting with ARRA sequence.

Amino Acid			P2-P1-P1'-P2'			
			XRRA	AXRA	ARXA	ARRX
Alanine	Ala	A	ARRA	AARA	ARAA	ARRA
Arginine	Arg	R	RRRA	ARRA	ARRA	ARRR
Asparagine	Asn	N	NRRA	ANRA	ARNA	ARRN
Aspartic acid	Asp	D	DRRA	ADRA	ARDA	ARRD
Glutamic acid	Glu	E	ERRA	AERA	AREA	ARRE
Glutamine	Gln	Q	QRRA	AQRA	ARQA	ARRQ
Glycine	Gly	G	GRRA	AGRA	ARGA	ARRG
Histidine	His	H	HRRA	AHRA	ARHA	ARRH
Isoleucine	Ile	I	IRRA	AIRA	ARIA	ARRI
Leucine	Leu	L	LRRA	ALRA	ARLA	ARRL
Lysine	Lys	K	KRRA	AKRA	ARKA	ARRK
Methionine	Met	M	MRRA	AMRA	ARMA	ARRM
Phenylalanine	Phe	F	FRRA	AFRA	ARFA	ARRF
Proline	Pro	P	PRRA	APRA	ARPA	ARRP
Serine	Ser	S	SRRA	ASRA	ARSA	ARRS
Threonine	Thr	T	TRRA	ATRA	ARTA	ARRT
Tryptophan	Trp	W	WRRA	AWRA	ARWA	ARRW
Tyrosine	Tyr	Y	YRRA	AYRA	ARYA	ARRY
Valine	Val	V	VRRA	AVRA	ARVA	ARRV

The residues at the site P1-P1' were fixed based on the amino acid preference of OmpT at these positions. However, it is recognized that there is often an interdependence of amino acids at each position in the peptide sequence, and therefore arrays having two variable positions would be expected to reveal still more active substrates. A larger peptide library was designed to optimize amino acids in the immediate vicinity of the cleavage site. This peptide library was based on the peptide sequences described above, but the P2 and P2' positions were varied simultaneously, with amino acids at P1 and P1' (XX) positions obtained from position scanning library were kept as constant (Table 3.3). This array contained 361 peptides of the form Ac-GXRRZGC, where the X and Z residues are one of nineteen amino acids excluding cysteine. The peptides were synthesised and screened using SAMDI-MS. These optimised tetrapeptide substrates were substituted in LL37, a natural substrate for OmpT, and kinetic activity of OmpT for the substituted peptide was analysed using SAMDI-MS.

Table 3.3: Combinatorial peptide library of 361 peptides for P2'XXP2 substrate, where P2' and P2 represent the variable position of the substrate, with X as the optimised amino acids from positional scanning library.

	Amino Acid	P2-X-X-P2'																			
		Ala	Arg	Asn	Asp	Glu	Gln	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val	
		A	R	N	D	E	Q	G	H	I	L	K	M	F	P	S	T	W	Y	V	
P2'-X-X-P2'	Ala	A	AXXA	AXXR	AXXN	AXXD	AXXE	AXXQ	AXXG	AXXH	AXXI	AXXL	AXXK	AXXM	AXXF	AXXP	AXXS	AXXT	AXXW	AXXY	AXXV
	Arg	R	RXXA	RXXR	RXXN	RXXD	RXXE	RXXQ	RXXG	RXXH	RXXI	RXXL	RXXK	RXXM	RXXF	RXXP	RXXS	RXXT	RXXW	RXXY	RXXV
	Asn	N	NXXA	NXXR	NXXN	NXXD	NXXE	NXXQ	NXXG	NXXH	NXXI	NXXL	NXXK	NXXM	NXXF	NXXP	NXXS	NXXT	NXXW	NXXY	NXXV
	Asp	D	DXXA	DXXR	DXXN	DXXD	DXXE	DXXQ	DXXG	DXXH	DXXI	DXXL	DXXK	DXXM	DXXF	DXXP	DXXS	DXXT	DXXW	DXXY	DXXV
	Glu	E	EXXA	EXXR	EXXN	EXXD	EXXE	EXXQ	EXXG	EXXH	EXXI	EXXL	EXXK	EXXM	EXXF	EXXP	EXXS	EXXT	EXXW	EXXY	EXXV
	Gln	Q	QXXA	QXXR	QXXN	QXXD	QXXE	QXXQ	QXXG	QXXH	QXXI	QXXL	QXXK	QXXM	QXXF	QXXP	QXXS	QXXT	QXXW	QXXY	QXXV
	Gly	G	GXXA	GXXR	GXXN	GXXD	GXXE	GXXQ	GXXG	GXXH	GXXI	GXXL	GXXK	GXXM	GXXF	GXXP	GXXS	GXXT	GXXW	GXXY	GXXV
	His	H	HXXA	HXXR	HXXN	HXXD	HXXE	HXXQ	HXXG	HXXH	HXXI	HXXL	HXXK	HXXM	HXXF	HXXP	HXXS	HXXT	HXXW	HXXY	HXXV
	Ile	I	IXXA	IXXR	IXXN	IXXD	IXXE	IXXQ	IXXG	IXXH	IXXI	IXXL	IXXK	IXXM	IXXF	IXXP	IXXS	IXXT	IXXW	IXXY	IXXV
	Leu	L	LXXA	LXXR	LXXN	LXXD	LXXE	LXXQ	LXXG	LXXH	LXXI	LXXL	LXXK	LXXM	LXXF	LXXP	LXXS	LXXT	LXXW	LXXY	LXXV
	Lys	K	KXXA	KXXR	KXXN	KXXD	KXXE	KXXQ	KXXG	KXXH	KXXI	KXXL	KXXK	KXXM	KXXF	KXXP	KXXS	KXXT	KXXW	KXXY	KXXV
	Met	M	MXXA	MXXR	MXXN	MXXD	MXXE	MXXQ	MXXG	MXXH	MXXI	MXXL	MXXK	MXXM	MXXF	MXXP	MXXS	MXXT	MXXW	MXXY	MXXV
	Phe	F	FXXA	FXXR	FXXN	FXXD	FXXE	FXXQ	FXXG	FXXH	FXXI	FXXL	FXXK	FXXM	FXXF	FXXP	FXXS	FXXT	FXXW	FXXY	FXXV
	Pro	P	PXXA	PXXR	PXXN	PXXD	PXXE	PXXQ	PXXG	PXXH	PXXI	PXXL	PXXK	PXXM	PXXF	PXXP	PXXS	PXXT	PXXW	PXXY	PXXV
	Ser	S	SXXA	SXXR	SXXN	SXXD	SXXE	SXXQ	SXXG	SXXH	SXXI	SXXL	SXXK	SXXM	SXXF	SXXP	SXXS	SXXT	SXXW	SXXY	SXXV
	Thr	T	TXXA	TXXR	TXXN	TXXD	TXXE	TXXQ	TXXG	TXXH	TXXI	TXXL	TXXK	TXXM	TXXF	TXXP	TXXS	TXXT	TXXW	TXXY	TXXV
	Trp	W	WXXA	WXXR	WXXN	WXXD	WXXE	WXXQ	WXXG	WXXH	WXXI	WXXL	WXXK	WXXM	WXXF	WXXP	WXXS	WXXT	WXXW	WXXY	WXXV
	Tyr	Y	YXXA	YXXR	YXXN	YXXD	YXXE	YXXQ	YXXG	YXXH	YXXI	YXXL	YXXK	YXXM	YXXF	YXXP	YXXS	YXXT	YXXW	YXXY	YXXV
	Val	V	VXXA	VXXR	VXXN	VXXD	VXXE	VXXQ	VXXG	VXXH	VXXI	VXXL	VXXK	VXXM	VXXF	VXXP	VXXS	VXXT	VXXW	VXXY	VXXV

3.2.2. SAMDI-MS

SAMDI-MS is a mass spectroscopy-based label-free, high throughput screening technique. MS-based approaches are attractive substrate screening techniques since they do not require labelling and provide mass based specific information of the substrate. However, MS has limitations with tedious sample preparations protocols,

which increases the time and cost for high throughput screening. Prof. Milan Mrksich group at Northwestern University (Evanston, IL) has used self-assembled monolayers (SAMs) of alkanethiolates on gold with matrix-assisted laser desorption ionisation (MALDI) time of flight (TOF) MS, in short, SAMDI-MS for assays of enzyme activities. The SAMs also include tri(ethylene glycol) groups that prevent non-specific protein adsorption and are critical to realising clean and quantitative assays.^{9–12} SAMs alkanethiolates present maleimide groups¹³ or a 3- trifluoromethyl-3-phenyl-diazirine group¹⁴ which help in immobilising a wide variety of molecules in the SAM surface.

Figure 3.4: Illustration of SAMDI mass spectrometry assay. Red represents the peptides substrate for enzyme and blue is the product of cleaved peptides substrate.

The maleimide presenting SAMs assist in immobilising peptide substrate with cysteine in an array format. The substrate immobilisation on SAMs can be done before or after treatment with an enzyme and is referred to as on plate or pull-down approach for screening.¹³ The arrays are rinsed to remove salts and other non-immobilized components in the reactions followed by treatment with matrix and array analysis using a MALDI-TOF. Upon irradiation with LASER, the monolayers along with immobilised substrate are desorbed from the surface and give a spectrum reflecting the mass of the substrates and products present in the immobilised samples (Figure 3.4). In this way, if a molecule has been modified, the mass of the alkane thiolate increases/decreases by a

specific amount, and that change is readily monitored using the MALDI instrument. However, the substrate and product might have different ionisation efficiencies that would affect the activity and not give the actual yield of the reaction. For this the SAMs with tri(ethylene glycol) have an important role i.e. to act as an internal control. The ratios of peak intensities of the substrate and product peaks for each peptide relative to the tri (ethylene glycol)-terminated alkane thiolate (EG3) peak within each SAMDI spectra can be calculated to compare the ionisation efficiencies of substrate and product peptides. The values are within ~5%, it can be considered that the ionisation efficiencies are nearly identical for substrate and product. This is a critical step for using a technique typically considered as non-quantitative for determining relative concentrations. SAMDI-MS is a powerful approach to screen arrays and has been used to screen kinases, proteases, glycosyltransferases, methyltransferases,¹⁵ lysine deacetylases,⁹ nucleic acid ligases¹⁶ and 10,000 small molecules to identify inhibitors of the anthrax lethal factor.¹⁷

3.2.3. Materials

General laboratory chemicals and reagents were purchased from Sigma-Aldrich (USA). N, N-dimethylformamide (DMF) was purchased from BDH chemicals (USA). Filter plates, silicone plate covers, and filter sealing mats were obtained from ArcticWhite (USA). Rink amide linker lanterns were purchased from Mimotopes, and Fluorenylmethyloxycarbonyl chloride (Fmoc) rink amide MBHA resin was purchased from AnaSpec (USA). (Benzotriazol-1-yloxy)tripyrrolidinophosphonium (PyBOP) and 1-hydroxybenzotriazole monohydrate (HOBt) were purchased from AK Scientific (USA). Amino acids were purchased from ChemImpex (USA). Array plates were fabricated by electron-beam evaporation (Thermionics) of an adhesion layer of titanium (50nm) followed by a layer of gold (300nm) at a rate of 0.2-0.4 nm s⁻¹ with a pressure of 1.0x10⁻⁶ Torr. All the FAM labelled peptides (Figure 3.4) were purchased as a lyophilised powder and certified >95% purity from GL BIOCHEM (SHANGHAI) LTD, China. All the peptides were acetylated and amidated at the N and C terminal, respectively. Pierce™ Maleimide Activated Plates were purchased from ThermoFisher Scientific (Singapore).

3.2.4. Synthesis of peptide library

The peptide synthesis and screening using SAMDI-MS were performed by Dr. Sarah Wood in the laboratory of Prof. Mrksich at Northwestern University (Evanston, IL). Position scanning (Table 3.2) and combinatorial (Table 3.3) peptide libraries were synthesised using solid-phase organic synthesis by plating lanterns in plugged 96-well filter plates (Figure 3.5).

Figure 3.5: Illustration of the steps involved in peptide library synthesis. (A) Peptides are synthesised using lanterns, (B) Steps involved in peptide synthesis. (C) Steps involved in the cleavage process.

Peptide libraries were synthesised using solid-phase organic synthesis by plating lanterns in plugged 96-well filter plates. The N-terminal fluorenyl methyl oxy carbonyl (Fmoc) was deprotected with 20% piperidine in DMF for 20 min; the solvent was then filtered with a vacuum manifold. The lanterns were then washed five times with DMF. A solution containing 15 equivalents of amino acid, HOBt, and diisopropylcarbodiimide (DIC) was prepared, applied to each well, and incubated at room temperature for 2 h. The solutions were then filtered, washed 5 times with DMF, and then, the process was repeated for each amino acid. After the last step of coupling, the lanterns were transferred to a 96-well plate, and a cleavage cocktail was applied to the

lanterns containing 95% trifluoroacetic acid (TFA), 2.5% deionised ultra-filtered water (DIUF), and 2.5% triethyl silane (TES). The solutions were then evaporated by flowing nitrogen over the plate until the lanterns were dry. The residues were dissolved in 0.1% TFA and lyophilised. The remaining solids were suspended in 0.1% TFA and stored in a 384-well plate at -20 °C. Individual peptides were synthesised using solid-phase organic synthesis on Fmoc rink amide MBHA resin by first soaking the resin beads in plugged filter tubes for 20 min. The N-terminal Fmoc was deprotected with 20% piperidine in DMF for 20 min; the solvent was then filtered under vacuum. The resin was then washed five times with DMF. A solution containing four equivalents of amino acid, four molar equivalents of PyBOP, and eight molar equivalents of N-methyl morpholine was prepared, added to the tube, and incubated at RT for 20 min. The solutions were then filtered, washed five times with DMF, and then, the process was repeated for each amino acid. After the last step of coupling, the resin was transferred to a new tube and a cleavage cocktail was applied to the resin containing 95% TFA, 2.5% DIUF, and 2.5% TES, and the resin was incubated at room temperature for 2 hrs. The solution was then filtered to remove resin and peptides were precipitated in cold diethyl ether and evaporated by flowing nitrogen over the tube. The residues were dissolved in 0.1% TFA and lyophilised. The remaining solids were suspended in 0.1% TFA and stored at -20 °C.

3.2.5. OmpT activity assay of peptide library

SAMDI-MS array plates with 384 gold islands were modified with a monolayer solution of symmetric tri(ethylene glycol)-terminated and asymmetric maleimide and tri(ethylene glycol)-terminated disulfide, in which maleimide-terminated group is present at a relative fraction of 10%. $_{wt}OmpT$ or $_{mt}OmpT_{DB}$ was diluted in buffer (10 mM BisTris, pH 6.5) and supplemented with 1 mg/mL LPS to a final OmpT concentration of 2 µg/mL, then pre-incubated at 37 °C for 15 min. To initiate the reaction, peptide substrates were added at a final concentration of 25 µM. The reaction plate was incubated at 37 °C for 2 hr. Tris(2- carboxyethyl)phosphine (TCEP) reducing agent (100 µM) was added to each well and incubated at RT for 15 min. Small volumes of each reaction (2 µL) were transferred onto array plates in triplicate to allow for immobilisation of the peptide substrate and product. The monolayers were then rinsed

with DIUF and ethanol, dried under nitrogen, then treated with matrix (2,4,6-trihydroxy acetophenone, 10 mg/mL in acetone).

3.2.6. OmpT kinetic experiments for longer peptide using SAMDI-MS

w_t OmpT was diluted in buffer (10 mM BisTris, pH 6.5) and supplemented with 1 mg/mL LPS to a final OmpT concentration of 5.5 μ g/mL (Native LL37), 0.55 μ g/mL (FRRV-substituted LL37), or 0.11 μ g/mL (ARRA-substituted LL37), then pre-incubated at 37 °C for 15 min. To initiate the reaction, HPLC-purified peptide substrates at concentrations of 10 μ M – 150 μ M were added, and reaction mixtures were quenched at fixed timepoints with 0.1 mM ZnCl₂. TCEP reducing agent (100 μ M) was added to each well and incubated at RT for 15 min. Small volumes of each reaction (2 μ L) were transferred in quadruplicate onto an array plate to allow for immobilisation of the peptide substrate and product. The monolayers were then rinsed with DIUF and ethanol, dried under nitrogen, then treated with matrix (2,4,6- trihydroxyacetophenone, 10 mg/mL in acetone).

3.2.7. SAMDI-MS based screening of peptide arrays

The monolayers are analyzed by MALDI-TOF MS to obtain a mass spectrum for each spot. Mass analysis was performed using a 5800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). A 255 nm Nd:YAG laser was used as a desorption/ionisation source, and all spectra were acquired with a 10 kV accelerating voltage using positive reflector mode. The extraction delay was 450 ns; 3000 laser shots were applied, and the entire surface of the gold spot was sampled. The activity was calculated by measuring the area under each molecular ion peak (M^+) from each spectrum according to the equation:

$$Activity \% = \frac{AUC_{product}}{AUC_{substrate} + AUC_{product}} \times 100\%$$

3.2.8. Laboratory equipment and computer software.

A MultiDrop Combi Reagent Dispenser (ThermoScientific) was used to deliver the protease solution to each well on the microplate, and a Tecan EVO liquid handling system was used to transfer the peptide substrates to the microplate, and the reaction solution from the microplate to the SAMDI array plate. Data were analysed using

custom-written software that compares the location and magnitude of the peaks in the SAMDI spectra to a reference mass file that is unique to the set of peptides analysed to look for reaction profiles based on the characteristic mass shift that occurs following proteolysis. Further data analysis to identify trends was performed using Microsoft Excel and other commercial software. Kinetic data were fitted to a Michaelis-Menten model, of the form $V = ([S] - a) * V_{max} / ([S] - a + K_m)$, where a is a constant variable unique to each peptide substrate.

3.2.9. FAM labelled optimised peptide substrate assays

Fluorescein isothiocyanate, abbreviated as FAM, is an essential synthetic equivalent of fluorescein dye used for labelling peptides and nucleotides. FAM labelled peptides are used for fluorescence imaging, kinetic assays, or various sensing assays. Here, FAM peptides were used to study the catalytic activity of OmpT against various peptide substrates. Top 6 tetrapeptide hits obtained after screening 361 peptide library with OmpT were substituted into a 14 amino acids long LL37 peptides fragment (Table 3.4). the tetrapeptide sequence, ARRA was also substituted into LL37 fragment to compare OmpT activity against the optimised peptide substrate (Table 3.4). In order to study the catalytic activity of OmpT against substituted LL37 peptide substrate, these peptides were synthesised with a FAM molecule at N terminal and a cysteine at the carboxy-terminal (Table 3.4). Pierce™ Maleimide activated plates were used to capture the peptides on the plate surface using maleimide-thiol chemistry. Peptide substrates incubated with different concentrations of OmpT will show peptide cleavage to a different extent. Also, only the intact peptide will contribute to the fluorescence signal as for the cleaved peptide the FAM peptide fragment will be removed after the washing step. Hence, lower fluorescence corresponds to higher catalytic activity of OmpT against the peptide. Fluorescence intensities of the samples were measured using a TECAN infinite® M200 PRO (Männedorf, Switzerland) fluorescence spectrophotometer fitted with a plate reader.

Sample preparation

All 8 FAM labelled thiol peptides (Table 3.4) were dissolved in water at a stock concentration of 500 μ M such that the absorbance for all the stocks was the same. Pierce™ Maleimide Activated Plates were washed before use as recommended in the

kit. The peptides were further diluted to 1 μM in assay buffer (20mM Bis-Tris, 2 mM $\text{DodMe}_2\text{NPrSO}_3$ and 5 mM EDTA pH 6.7). OmpT was added to 100 μL of the peptide in different concentrations (3, 1.5, 0.75, 0.375, 0.187, 0.094 $\mu\text{g/mL}$) and allowed to react for 2 h at 37 $^\circ\text{C}$. After 2 h, the reaction mixture was transferred to a maleimide plate with two control wells for each peptide set as: only buffer and only peptide. The peptide in the reaction mixture was incubated in Pierce™ Maleimide Activated Plates for 3-4 h at room temperature with shaking at 500 RPM. The wells were further washed with wash buffer 3-4 times and 100 μL of phosphate buffer was added to wells. The wells were scanned (E_x 480 and E_m scan 510-700 nm) using a TECAN Infinite® 200 PRO.

Table 3.4: List of FAM peptides used.

Peptide code	FAM-LLGDFXRRYKEKIGC
LL37 _{FRRV}	FAM-LLGDFFRRVKEKIGC
LL37 _{FRRY}	FAM-LLGDFFRRYKEKIGC
LL37 _{ARRV}	FAM-LLGDFARRVKEKIGC
LL37 _{WRRV}	FAM-LLGDFWRRVKEKIGC
LL37 _{MRRRA}	FAM-LLGDFMRRAKEKIGC
LL37 _{MRRV}	FAM-LLGDFMRRVKEKIGC
LL37 _{ARRA}	FAM-LLGDFARRAKEKIGC
LL37 _{FRKS}	FAM-LLGDFFRKSKEKIGC

Data analysis

The fluorescence values at 530 nm for peptides substrate treated with OmpT (F_S) were normalised with the fluorescence of only buffer (F_B) and only peptide (F_P) samples (% Fluorescence = $(F_P - F_S)/(F_P - F_B) \times 100$). The data was plotted in Origin 9.0 as % fluorescence vs OmpT concentration used.

3.3. OmpT-Peptide-Polythiophene assay development for *E. coli* detection

In order to use the optimised peptides substrate described in the previous section for developing a OmpT protease based assay, a reporter molecule is required for generating a colourimetric response. Conjugated polymers (CPs) have been previously used for continuous and selective detection of biomolecules and have large potential for being used as biosensors and molecular electronics. CPs are very optically and electrically sensitive to even minor perturbations due to a combination of the electronically excited state (exciton) and ultrafast exciton mobility along the conjugated polymer backbone.^{18,19} The conjugated polymer backbone length can be manipulated by

changing the confirmations of the backbone, hence affecting the optical and electrical properties. Once such class of CP used for colourimetric assay development is polythiophene. The physical and chemical properties of the polythiophene can be modified by introducing the suitable side chains in the 3- or 4- position as described in section (Section 2.6). The forces between the polythiophene side chain and detection molecule that play an important role in bringing about the structural changes are hydrogen bonding and nonpolar and electrostatic interactions, leading to aggregation, gelation, or crystallisation. Since the optimised peptide substrate is a cationic peptide with a net positive charge (rich in arginine and lysine), anionic polythiophene with a carboxylic acid side chain was used. The anionic polythiophene, poly (thiophene-3-acetic acid) abbreviated PTAA, with acetic acid side chain will interact electrostatically with the optimised cationic peptide substrate, bring about the conformational change in the PT backbone. This will affect the optical and electrical properties of PTAA. So, in this section of the thesis, PTAA and peptide interactions are analysed and characterised in depth and further explored as a reporter molecule to develop a colourimetric assay for *E. coli* detection. Polythiophenes with carboxylic side group with varying side chain length were further screened as well.

3.3.1. Materials

PTAA, an anionic polythiophene, was provided by Assistant Prof. Umit Hakan Yildiz (Izmir Institute of Technology, Department of Chemistry, Urla, Turkey). It was synthesised by oxidative polymerisation of its monomer using the protocol reported previously.²⁰ PT with side chain length 3, 4, 5, and 6 were purchased from Rieke metals (Table 3.5).

Table 3.5: List of PTs purchased from Rieke metals

Polythiophene	Side chain length
(Poly[3-(Potassium-4-butanoate)thiophene-2,5-diyl])	3
(Poly[3-(Potassium-5-butanoate)thiophene-2,5-diyl])	4
(Poly[3-(Potassium-6-butanoate)thiophene-2,5-diyl])	5
(Poly[3-(Potassium-7-butanoate)thiophene-2,5-diyl])	6

Monobasic sodium phosphate, dibasic sodium phosphate, N-Dodecyl-N, N-dimethyl-1-ammonio- 3-propane sulphonate (DodMe₂NPrSO₃), Bis-tris were purchased from

Sigma. All the peptides (Table 3.6) were purchased as lyophilized powder and certified >95% purity from GL Biochem (Shanghai) Ltd, China.

Table 3.6: List of peptides used for assay development and characterisation

Peptide Name	Peptide Sequence	
LL37 _{FRRV}	Ac-LLGDFFRVKEKIG- NH ₂	FRRV substituted LL37 fragment
cLL37 _{FRRV}	CLLGDFFRVKEKIG- NH ₂	Cystine at N terminal of LL37 _{FRRV}
mLL37 _{FRRV}	MLLGDFFRVKEKIG- NH ₂	Methionine at N terminal of LL37 _{FRRV}
cLL37 _{ARRA}	CLLGDFARRAKEKIG- NH ₂	ARRA substituted LL37 fragment
AY11	Ac-AVSHTTHHAPY-NH ₂	Control peptide with no charge
cAY12	CAVSHTTHHAPY-NH ₂	Control peptide with no charge
frag1LL37 _{FRRV}	CLLGDFFR	Synthetic peptide fragments of LL37 _{FRRV}
frag2LL37 _{FRRV}	RVKRKIG	Synthetic peptide fragments of LL37 _{FRRV}

Peptides were acetylated and amidated at the N and C terminal, respectively. Transparent flat bottom 96 well plates were purchased from Corning ® (NY, USA). *E. coli* BL21(DE3) and Lemo21(DE3) were purchased from New England Biolabs Pte Ltd (Singapore). *E. coli* K12 ATCC® 700926™ was purchased from ATCC. Lemo21(DE3) cells were transformed with a pET-28a plasmid carrying OmpT gene under a T7 promoter whose expression was induced by the addition of IPTG. *E. coli* K12 ATCC® 700926™ and *E. coli* J96 ATCC® 700336™ were purchased from ATCC (USA) and supplied by Bio-REV Pte Ltd (Singapore). Ultrafree® - MC centrifuge device 0.2 µm PTFE membrane was purchased from Merck Millipore (Singapore).

3.3.2. Fluorescence and absorbance characterisation of PTAA-peptide complex

Stock solutions of PTAA were prepared at a concentration of 0.1 mg/mL in a reaction buffer containing 20 mM sodium phosphate buffer whose pH was adjusted to 8. cLL37_{FRRV} peptide stock solutions were prepared in the reaction buffer at a concentration of 500 µM. To study the effects of cLL37_{FRRV} on PTAA's absorbance and fluorescence intensity, different concentrations of cLL37_{FRRV} (150 - 6.25 µM) were added to PTAA (10 µg/mL). Mixing of PTAA and cLL37_{FRRV} was performed in the reaction buffer. All absorbance and fluorescence measurements were carried out on 100 µL samples in a flat bottom 96 well plate using a TECAN Infinite® 200 PRO (Switzerland). Absorbance measurements were collected from 330-650 nm.

Fluorescence scans were collected by exciting the samples at 420 nm and their emission spectrum recorded from 450-750 nm.

Data analysis

Fluorescence and absorbance data were plotted in Origin 9.0 . Fluorescence intensity values at emission maximum for all samples were taken, and % fluorescence increase was calculated with respect to the fluorescence of PTAA. % Fluorescence increase *vs* increasing concentration of all the peptides was plotted. The following formula was applied to convert fluorescence intensity (a.u.) to % fluorescence increase:

$$\% F.increase = \left(\frac{F_{PTAA+Peptide} - F_{PTAA}}{F_{PTAA}} \right) \times 100\%$$

3.3.3. Fluorescence and absorbance based characterization of PTAA complex with intact and cleaved peptide.

When OmpT digests the peptide cLL37_{FRRV}, it results in two peptide fragments of 7 and 8 amino acids. These two fragments (frag1LL37_{FRRV} and frag2LL37_{FRRV}) were purchased as separate peptides from GL BIOCHEM. Stock solutions of frag1LL37_{FRRV} and frag2LL37_{FRRV}, the two synthetic peptide fragments of cLL37_{FRRV} that are identical to the two cleaved fragments obtained when OmpT cleaves cLL37_{FRRV}, were prepared in the reaction buffer at a concentration of 1 mM (Table 3.6). To understand the effect of intact and cleaved fragments on the fluorescence intensity and absorbance of PTAA, intact and cleaved peptides were added in different ratios (10:0, 8:2, 6:4, 4:6, 2:8, 0:10) while keeping the total peptide concentration at 50 µM. The fluorescence intensity and absorbance spectra were measured, as mentioned before.

Data analysis

The % fluorescence Em₅₅₀ was normalised and calculated for both the peptides and different ratios based on the following formula:

$$\% F = \left(\frac{F_{Sample} - F_{PTAA}}{F_{PTAA+Peptide} - F_{PTAA}} \right) \times 100\%$$

The % fluorescence Em₅₅₀ was plotted for LL37_{FRRV} and LL37_{ARRA} *vs* ratio of intact: cleaved peptide fragments.

3.3.4. OmpT assay based on polythiophene and peptide complex formation

In order to evaluate if PTAA could be employed as a reporter molecule to detect changes in peptide length and thus develop it as part of an assay for *E. coli* detection, the peptides were first treated with OmpT and then added to PTAA. 50 μ M of the intact cLL37_{FRRV} was treated with varying concentrations (42, 8.4, 2.8, 1.68 and 0.84 nM) of recombinant OmpT that was reconstituted in detergent micelles. This OmpT was obtained by overexpressing the protein in *E. coli* BL21 as inclusion bodies using a protocol reported earlier.³ Briefly, the inclusion bodies were unfolded and solubilised using 8 M urea and subsequently refolded in the presence of DodMe₂NPrSO₃, a zwitterionic detergent. The refolded OmpT was purified and stored at 4 °C for further use. A 100 μ L reaction mixture was incubated at 37 °C with shaking at 500 RPM. After 2 h, 10 μ L of PTAA stock solution (0.1 mg/mL) was added to the 100 μ L reaction mixture. Fluorescence measurements and absorbance scans were collected as mentioned in 3.3.2.

Data analysis

The raw fluorescence (F.) intensity scans are reported as % fluorescence. The following formula was applied to convert fluorescence intensity Em₅₅₀ (a.u.) to % fluorescence increase :

$$\% F.increase = \left(\frac{F_{PTAA+Pept+OmpT} - F_{PTAA}}{F_{PTAA}} \right) \times 100\%$$

The % fluorescence increase vs OmpT concentration was plotted in Origin 9.0.

3.3.5. MALDI-TOF of OmpT treated optimised LL37 peptide fragments

MALDI-TOF of the LL37_{FRRV} peptide and after incubating peptide with OmpT was done to confirm the cleavage of the peptide.

Sample preparation

LL37_{FRRV} peptide was dissolved in 1x assay buffer at 10 μ M concentration to which OmpT was added at 1 μ g/mL. The reaction mixture was incubated at 37 °C for 1 h. The intact peptide and reaction mixture was mixed with the matrix, sinapinic acid, in 1:1 ratio and 1 μ L of these mixtures were spotted on a MALDI-TOF array plate. The matrix

and sample mix was allowed to dry before loading into the instrument. The samples were analysed using a Shimadzu Biotech Axima Performance that contained a nitrogen UV laser. m/z peaks were collected at a laser power of 100 mV in reflection hi-resolution mode and other settings were adjusted for the best signal-to-noise ratio.

Data analysis

The data was analyzed using the software provided with Shimadzu Biotech Axima Performance. The graphs were exported as metafiles for future reference.

3.3.6. Circular dichroism (CD) based characterization of PTAA-peptide complex

The following samples were prepared: (1) PTAA, (2) cLL37_{FRRV}, (3) PTAA mixed with cLL37_{FRRV}, (4) OmpT treated cLL37_{FRRV}, and (5) OmpT treated cLL37_{FRRV} mixed with PTAA. Final concentrations of PTAA, cLL37_{FRRV}, and OmpT were 10 µg/mL, 50 µM, and 1.4 µg/mL respectively. A high precision cell made of Quartz SUPRASIL from Hellma-Analytics with 10 mm pathlength was used to record the CD spectra. Samples were scanned from 500 - 190 nm using an AVIV 420 CD spectrophotometer (Lakewood, NJ, USA) with a step size of 0.5 nm. The obtained data was processed in Origin 9.0.

3.3.7. *E. coli* detection in water using PTAA and peptide

Four different strains of *E. coli* were employed for testing the efficacy of OmpT-Pep-PTAA assay for detecting *E. coli* in water (Table 3.7). *E. coli* BL21 is an OmpT deficient strain, which is mainly used for expression of recombinant proteins. The OmpT gene is removed from the BL21 strain, such that OmpT protein is not expressed on the membrane. This is done in order to prevent any degradation of the recombinant proteins being expressed in *E. coli* BL21. Whereas, the K12 strain is the most common *E. coli* strain and its presence in tap water is generally considered as confirmation of faecal contamination. *E. coli* J96 is associated with urinary tract infections. *E. coli* BL21 served as a negative control to rule out any interferences from intact *E. coli*. *E. coli* K12 and J96 acted as wild-type *E. coli* samples. In this particular instance, the *E. coli* Lemo21(DE3) were modified to overexpress OmpT on its surface and acted as a positive control.

Table 3.7: Strains of *E. coli* used to test the efficiency of OmpT-Pep-PT assay for *E. coli* detection in water and food samples.

<i>E. coli</i> strain	OmpT expression
<i>E. coli</i> BL21	Deficient in OmpT
<i>E. coli</i> K12 ATCC® 700926™	OmpT expressed on the outer membrane
<i>E. coli</i> J96 (ATCC® 700336™)	OmpT expressed on the outer membrane
<i>E. coli</i> Lemo21(DE3) transformed with pET-28a plasmid carrying OmpT gene under a T7 promoter	OmpT overexpressed on the outer membrane

3.3.7.1. *E. coli* detection using the pre-concentration method

The four different *E. coli* strains were grown overnight in LB media at 37 °C while shaking at 220 RPM. This primary culture was used for inoculating a secondary culture, which was further allowed to grow for 6 - 8 h. The Lemo21(DE3) cells with OmpT plasmid were induced with 1 mM IPTG at OD₆₀₀ = ~ 0.8. For OmpT overexpression in *E. coli* Lemo21(DE3), the *E. coli* Lemo21(DE3) cells were allowed to grow for 4 h after induction with IPTG. All four strains of *E. coli* were pelleted and then resuspended in the reaction buffer before serially diluting them to attain concentrations of 10⁸ - 10³ CFU/mL, considering OD₆₀₀=1 equivalent to 8 × 10⁸ CFU/mL. 200 µL, 2 mL and 20 mL of *E. coli* cells with 10⁸, 10⁷ and 10⁶ - 10³ CFU/mL, respectively, were filtered through a 0.2 µm PTFE centrifuge device. After filtering the sample, 100 µL of reaction buffer was added to the centrifuge device and was gently vortexed to resuspend the *E. coli* cells. 90 µL of the reaction mixture was retrieved from the centrifuge device to which 10 µL of 500 µM cLL37_{FRRV} stock solution was added and mixed. This 100 µL reaction mixture was incubated at 37 °C and shaking at 500 RPM for 1 h. After which, 10 µL of PTAA stock solution (0.1 mg/mL) was added to the reaction mixture and the sample's fluorescence intensity and absorbance recorded as mentioned before (3.3.2).

3.3.7.2. Preculture method

In order to establish the minimum pre-culturing time required for the PTAA-peptide method to detect 1 and 10 CFU of *E. coli* cells, three different *E. coli* strains were employed. *E. coli* J96, K12, and BL21 strains at an initial concentration of 1 and 10 CFU/mL were cultured, by adding LB media, for over a period of 3, 5, and 7 h respectively at 37 °C while shaking at 220 RPM. 200 µL of each of the cultured *E. coli*

cells were filtered through a 0.2 μm PTFE centrifuge device after 3, 5 and 7 h. To these filtered *E. coli* cells, 100 μL reaction buffer was added and vortexed gently to resuspend the *E. coli* cells. 90 μL of the reaction mixture was retrieved from the centrifuge device to which 10 μL of 500 μM LL37_{FRRV} stock was added and mixed. This 100 μL reaction mixture was then incubated at 37 °C and 500 RPM for 1 h. After which, 10 μL of PTAA stock solution (0.1 mg/mL) was added to the reaction mixture and the sample's fluorescence intensity and absorbance recorded as mentioned before (3.3.2).

3.3.8. Fluorescence and absorbance measurements of PT-peptide complex

Individual stock solutions of each of the five PTs (PTAA and Table 3.5) were prepared in a reaction buffer such that the absorbance at λ_{max} was ~ 0.14 . In order to study the effect of pH on PTs fluorescence with the addition of cLL37_{FRRV}, the PTs were mixed with 50 μM LL37_{FRRV} peptide in reaction buffer (20 mM sodium phosphate buffer) at three different pHs 6, 7, and 8. All absorbance and fluorescence measurements were carried out on 100 μL samples in a flat bottom 96 well plate using a TECAN Infinite® 200 PRO (Switzerland). Absorbance measurements were collected from 330-750 nm. Fluorescence scans were collected by exciting the samples at 420 nm and 495 nm, further recording their emission spectrum from 450-750 and 525-750 nm, respectively. The pH of the reaction buffer that showed the highest increase in fluorescence was selected for further experiments.

Stock solutions of the peptides (500 μM) were prepared in a reaction buffer (20 mM sodium phosphate buffer whose pH was adjusted to 8) and stored at -20 °C for further use. Different concentrations (150 - 6.25 μM) of the peptides (Table 3.6) were prepared from stock and mixed with PTs (Table 3.6) in order to study their effect on the absorbance and fluorescence of PTs. The PTs and peptides were mixed in the ratio of 10:90 in a 100 μL reaction. All absorbance and fluorescence measurements were done as described in the previous paragraph.

3.3.9. Circular Dichroism measurements of PT-peptide complex

The following samples were prepared: (1) PTAA, (2) cLL37_{ARRA}, (3) PTAA mixed with cLL37_{ARRA}, (4) OmpT treated cLL37_{ARRA}, and (5) OmpT treated cLL37_{ARRA} mixed with PTAA. The CD samples for PTAA, PT_{04CA}, PT_{05CA}, PT_{06CA} and PT_{07CA} with and without the three peptides cLL37_{FRRV} and cLL37_{ARRA} were prepared. PTs and

peptides were mixed in the ratio as mentioned in section 3.3.8. For instrumental details refer to the previous section (3.3.6).

3.3.10. *E. coli* detection in food samples

Green vegetables get contaminated very prominently at the farm, production plant and even at the store. The contamination can result from contact with contaminated water, animal faeces or even cross contamination among vegetables. Hence, a few different approaches were adopted to investigate these effects.

3.3.10.1. Approach -1: Soaking food samples in contaminated water

Briefly, store-bought bagged leafy vegetables- *lettuce, cabbage, and spinach* were cut into small squares of 22×22 mm (Figure 3.6) and soaked in *E. coli* cell suspension in water with 10^6 CFU/mL cells of *E. coli*. After soaking for 5 min, the samples were transferred into a small plastic tube containing growth media. After 15 min in the growth media with shaking at 220 RPM, the samples were removed, and the growth media incubated at 37 °C for 5 h to facilitate bacterial growth and multiplication. At the end of 5 h, the growth media's turbidity was measured as absorbance at 600 nm and this absorbance value was used to approximate the number of *E. coli* cells. Since turbidity can be caused by more than just bacterial growth, the samples were also assayed with the OmpT-Pep-PT assay described in previous sections. Glass coverslips coated with poly L-lysine were used as positive controls since the negatively charged *E. coli*'s surface is expected to adhere to the positively charged poly L-lysine. For negative control, vegetable samples were soaked in clean, uncontaminated, deionized (DI) water that was filtered through a 0.2 µm filter. An additional step was included after the first soaking step: Here, instead of discarding the samples after the first 5 min soak, they were transferred into a second tube containing growth media and allowed to soak for 15 min with shaking at 220 RPM, before finally discarding the samples and incubating the growth media at 37 °C for 5 h. This step was included to investigate if more than one wash is necessary to remove all or most of the adhering bacteria from the vegetable's surface.

Figure 3.6: Vegetable samples were cut into 22×22mm squares and tested for bacterial contamination.

3.3.10.2. Approach -2: Depositing contaminated water directly on the surface of food samples

The surface morphology of vegetables varies greatly, and *E. coli* can be expected to display a preference to adhere more strongly to one surface than to another. In order to determine if *E. coli* displayed such a variability in its adherence/release properties for the vegetables selected for this study, a known quantity of *E. coli* was deposited on each of the different vegetable's surface and the samples assayed. A similar experimental protocol, as described in approach-1, was followed but with slight modification. Instead of soaking the samples in *E. coli* cell suspension, 10 μL of *E. coli* at a concentration of 10^6 CFU/mL was deposited directly onto the vegetable's surface. These samples were allowed to sit at 4 °C overnight to allow the bacteria to adhere properly to the surface. The following day, the samples were transferred into plastic tubes containing growth media. The remaining steps were similar to those described in 3.3.10.1.

3.3.10.3. Approach -3: Increasing sampling area for analysis of food samples soaked in contaminated water

The amount of *E. coli* retrievable from the surface of a leafy vegetable is dependent on the concentration of *E. coli* on the surface as well as the surface area available for sampling. When more of the vegetable's surface area is sampled, the easier it is to detect *E. coli* contamination. To investigate this effect and to get an idea of the average surface area needed for sampling, 1, 2, 5, 8 and 10 small squares of lettuce measuring 22×22 mm were soaked in water deliberately contaminated with 10 CFU/mL *E. coli*. With the remainder of the steps being similar to those in approach-1, the growth medium at the end of 5-7 h was assayed using the OmpT-Pep-PT_{06CA} assay.

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Chapter 4

Recombinant OmpT refolded in vitro is resistant to autoproteolysis unless it is misfolded

This chapter focusses on refolding and autoproteolysis of Outer membrane protease (OmpT). OmpT is a 33.5 kDa aspartyl protease that cleaves at di-basic sites and is thought to function as a defence mechanism for E. coli against host generated cationic antimicrobial peptides. Since OmpT carries di-basic sites in its own sequence, it undergoes autoproteolysis in vitro resulting in an inactive protease. In the current study, SDS PAGE data of the ratio of intact and autoproteolysed OmpT correlated with the ratio of correctly refolded and misfolded protein, suggesting that only the misfolded protein undergoes autoproteolysis. Previously, it was reported that LPS might have a role in preventing autoproteolysis, but our data does not support this role for LPS since the proportion of autoproteolysis did not change significantly when OmpT was refolded in the absence or presence of LPS. Additional experiments aimed at evaluating the protective role of LPS revealed that the predicted LPS binding site on OmpT was not critical for OmpT activity. This latter result suggests that LPS might have more than one binding site on OmpT or that LPS activates OmpT via an as yet unknown mechanism.

4.1. Introduction

Outer membrane protease (OmpT) is a 33.5 kDa bacterial membrane protease belonging to the Omptin family of proteases and is found on the outer membrane of *E. coli*. It is thought to play an important role in the bacteria's defence system by cleaving cationic antimicrobial peptides produced by the host, making them ineffective.¹ OmpT has also been reported to be overexpressed in *E. coli* associated with urinary tract infections indicating a strong role in the pathogenicity of *E. coli* strains.^{2,3} OmpT preferentially cleaves host antimicrobial peptides at di-basic sites and requires lipopolysaccharides (LPS) for this proteolytic function.⁴⁻⁶ Since OmpT carries di-basic sites in its own sequence, it is prone to autoproteolysis when expressed *in vitro*. However, there is no report of OmpT autoproteolysis *in vivo*. A deeper understanding of OmpT's autoproteolytic activity along with the role of LPS can provide important clues to how this protease escapes autoproteolysis when sitting on the outer membrane of *E. coli*. Moreover, such an understanding can help target this protease for developing efficient antibiotics.

Recombinant OmpT can be expressed *in vitro* either in the *E. coli* outer membrane or overexpressed as cytoplasmic protein aggregates called inclusion bodies (IBs), which are easily isolated and subsequently unfolded, refolded, and purified. The latter method offers higher yields, but the protease exhibits autoproteolysis during refolding and purification. The presence of di-basic sites in its own sequence makes OmpT susceptible to autoproteolysis.⁷ Although it carries three di-basic sites (R37-K38, K217-R218 and K259-K260) in its sequence, only K217-R218 is prone to autoproteolysis.⁷ Located in a flexible extracellular L4 loop on top of the beta-barrel structure of OmpT, this exposed site makes it more susceptible to autoproteolysis than the other two sites that are located on the body of the barrel.⁸ To overcome autoproteolysis during recombinant *in vitro* expression, two strategies have been adopted in the literature. First, the sequence of the protease was mutated in the vicinity of the autoproteolysis site, G216K and K217G.⁷ However, this mutant had a lower activity (~ 30% lower) as compared to the wild-type protease.⁷ Also by mutating the amino acids G216K and K217G that are present in the vicinity of the autoproteolysis site, the substrate preference for OmpT is changed.⁹ A second strategy, which was adopted by Wu *et al.*, 2012, and described in detail in Wu's PhD thesis, involves the addition of LPS to

refolded OmpT immediately after cation exchange purification.^{10,11} In this case, the author suspected a protective role of LPS against OmpT autoproteolysis but did not characterise its role further.

This study concludes that a fully refolded OmpT does not undergo autoproteolysis, and it is only the unfolded/misfolded protein that undergoes proteolytic cleavage. Additionally, contrary to previous reports, the data confirms that LPS is not essential in protecting OmpT from autoproteolysis, nor is its binding to the predicted LPS-binding residues on OmpT critical for OmpT activity. These results indicate that it is unnecessary to work with a mutant form of OmpT which was designed to circumvent the problem of autoproteolysis. Instead, focusing on improving the yield of correctly refolded protease would be more beneficial since mutant OmpT has poor activity and an altered substrate specificity. Finally, the data also highlights the need to exercise caution when interpreting SDS-PAGE results of OmpT autoproteolysis since these are heat modifiable proteins and correct interpretation of autoproteolysis are obtained only upon prolonged heating of OmpT samples.

4.2. Experiments

4.2.1. OmpT expression and IBs isolation

OmpT and its various mutants were recombinantly expressed in *E. coli* as inclusion bodies (IBs). The detailed protocol is described in the previous sections 3.13. Briefly, the following OmpT variants were expressed and purified (Table 4.1)

Table 4.1: List of OmpT variants expressed and purified recombinantly in *E. coli*.

OmpT type	Mutation
_{wt} OmpT	Wild-type OmpT
_{wt} OmpT _{SP}	Wild-type OmpT with a signal peptide
_{mt} OmpT _{DB}	OmpT with dibasic site mutated: G216K and K217G
_{mt} OmpT _{LPS3}	OmpT with 3 of the 5 LPS binding residues mutated: R138E, R175E, K226E
_{mt} OmpT _{LPS5}	OmpT with all 5 LPS binding residues mutated: Y134A, E136A, R138E, R175E, K226E

4.2.2. OmpT IBs refolding and reconstitution in detergent micelles

OmpT IBs were refolded as described in section 3.1.3. Briefly, OmpT IBs were solubilised in 8 M urea and were refolded in the presence of a detergent while diluting the urea from 8 M to 0.8 M to facilitate refolding. Residual urea was removed using a PD10 desalting column. Samples were aliquoted and stored at -20 °C until further use. Protein concentration was determined using BCA assay as described previously in section 3.1.4.2.

4.2.3. OmpT protease activity assay

Based on a method previously described by Kramer *et al.*⁷, the four different OmpT samples mentioned in Table 4.1, were assayed for their protease activity. The fluorescence assays were performed using a synthetic peptide substrate, Abz-ARRA-Tyr (NO₂)-NH₂. The assay method is described in detail in section 3.1.4.4. Unlabelled peptides were also tested for OmpT activity and were analysed using MALDI-TOF. The sample preparation protocols are described in detail in sections 3.1.4.5.

4.2.4. Liquid Chromatography-Mass Spectrometry (LC-MS).

^wOmpT samples were prepared for LC-MS analysis as described in section 3.1.4.3. Briefly, methanol/chloroform/water precipitation was used for sample preparation. The samples were analysed on an Agilent 1200 series HPLC connected to an Agilent 6210A time-of-flight (TOF) mass spectrometer.

4.3. Results and discussion

4.3.1. Refolding efficiency of OmpT IBs and variable migration on SDS-PAGE gels

High yields and ease of separation from unwanted soluble proteins is an important advantage of expressing OmpT as IBs in *E. coli*.^{12,13} These IBs are solubilised using 8 M urea, completely unfolding the OmpT protein, and then refolded prior to their purification. OmpT and other gram-negative outer membrane proteins that adopt a beta-barrel structure are resistant to denaturation by SDS alone due to the extensive hydrogen bonding between the beta strands.¹⁴⁻¹⁶ As a result, during SDS-electrophoresis, OmpT samples treated with SDS retain a compact globular shape that allows them to migrate

further along the SDS-PAGE gel and appear as a lower molecular weight band (F Lane 2-4, Figure 4.1). However, misfolded OmpT and OmpT denatured with heat no longer retain a compact globular shape and migrate as a higher molecular weight band (UF Lane 1, 2-4, Figure 4.1). This variable migration of folded and unfolded OmpT is a useful method to determine the relative amounts of folded and unfolded protein in a sample and thus an indicator of refolding efficiency.¹⁷

Figure 4.1: Effect of heating on the mobility of $mtOmpT_{DB}$ on SDS-PAGE: *Lane 1* - $mtOmpT_{DB}$ IBs, *Lane M* - Protein molecular weight marker. $mtOmpT_{DB}$ after refolding was heated for 0 mins (*Lane 2*), 10 min (*Lane 3*), and 45 min (*Lane 4*). UF- Unfolded protein, F - Folded protein.

In the current study, SDS-PAGE data indicates a refolding efficiency close to 65% based on the relative intensities of the 33.5 kDa band, attributable to the unfolded/misfolded protein (Figure 4.2 A, Lane 0 min). However, this refolding efficiency varied between 50 -70% for different batches (data not shown) and was difficult to control using the protocol adopted from Krammer *et al.*⁷ Additionally, while Krammer *et al.* required only 5 min of boiling to completely unfold OmpT, the present study revealed a faint lower molecular weight band even after boiling the sample for 10 min (Figure 4.1, Lane 3).⁷ This faint band indicates that a small portion of the OmpT remained in the folded state despite the 10 min heating. This faint low molecular weight band disappeared only when the samples were heated for 45 min, indicating complete unfolding (Figure 4.1, Lane 4).

4.3.2. OmpT autoproteolysis

OmpT is known to undergo autoproteolysis due to the occurrence of dibasic sites in its own sequence. However, this autoproteolysis appears to be exclusive to *in vitro* systems since there is no report of OmpT autoproteolysis *in vivo*. To limit this autoproteolysis *in vitro*, it is important to first understand the reason why this is exclusive to *in vitro* systems. Since the intact OmpT and autoproteolysed OmpT have different molecular weights, it can be followed over a simple electrophoresis gel. However, the interpretation of the results is complicated by the fact that both these proteins migrate to the same molecular weight on an SDS-PAGE gel. In order to differentiate these proteins, the samples need to be completely unfolded, in which case, the autoproteolysed OmpT migrates as a lower molecular weight band and the intact unfolded OmpT migrates as a higher molecular weight band.

OmpT samples that were refolded from IBs and stored in 4 °C were collected at different time intervals and run on SDS-PAGE gel after no (Figure 4.2 A) and 45 min (Figure 4.2 B) of heating. Based on the ratio of the folded to unfolded OmpT bands, the refolding efficiency was close to 65% (Figure 4.2 A lane 0 min). However, it is interesting to note that the higher molecular weight band corresponding to the unfolded OmpT began to disappear with time and was reduced from ~ 35% to ~ 12% (Figure 4.2 A (Lanes - 0, 10 min, 24 h, 48 h and 72 h.)). This could indicate one of two things: either the unfolded OmpT is slowly refolding into the correct structure or the unfolded OmpT is undergoing autoproteolysis. To determine which of the two possibilities is true, the experiment was repeated, but this time with OmpT samples that were completely unfolded by heat denaturation. SDS-PAGE data shows that completely unfolded OmpT (Figure 4.2 B (Lane 0 min)) immediately after it was refolded into detergent micelles exhibited almost no autoproteolysis. However, with time, the lower molecular band, that corresponds to autoproteolysed OmpT, began to slowly appear as a band at 25 kDa (Figure 4.2 B (Lane 10 min, 24 h, 48 h and 72 h)) in a manner similar

Figure 4.2: Autoprolysis of OmpT over time: OmpT samples were collected at 0, 10 min, 24 h, 48 h, and 72 h after refolding. The samples were run on SDS-PAGE: (A) Without heating the samples (B) Samples were heated for 45 min before loading on SDS-PAGE. Red – represents the unfolded or misfolded protein. Green – represents folded or cleaved fragment of OmpT.

to the disappearance of the higher molecular weight band for unfolded OmpT described above. This autoprolysis band increased from 0 to ~ 33 % of the OmpT protein content (Figure 4.2 B). Taken together, this disappearance and appearance strongly suggests that it is the unfolded OmpT that undergoes autoprolysis.

Figure 4.3: LC-MS spectrum of $wtOmpT_{LPS}$ samples precipitated with methanol/chloroform/water precipitation protocol. : (A) The larger autoproteolysed fragment (24.4 kDa) and intact OmpT (33.5 kDa) after detergent removal via methanol:chloroform: water precipitation, and (B) the smaller proteolyzed fragment (9.1 kDa) from the soluble phase (i.e., without precipitation). (C-D) The methanol/chloroform/water precipitated sample showed good S/N for the two higher MW species, full length and peptide 1. The peptidic fingerprint in Fig A (peaks $> \sim 800$ m/z) is consistent with these two expected species. Deconvolution by maximum entropy shows these two species as the most prominent peaks. The mass of the peaks is presented in Table 1, with a comparison to the calculated mass.

MALDI-TOF and LC-MS analysis of these samples revealed peaks for the full-length protein at 33.5 kDa and two peaks at 9.1 kDa and 24.4 kDa for the autoproteolysed fragments confirming autoproteolysis as well as the site of autoproteolysis, which was between K217-R218 (Figure 4.3, and Table 4.2). The overall rate of autoproteolysis is rather slow and most ($\sim 67\%$) of the OmpT appeared to remain intact even after 72 h

(Figure 4.2 B (Lane 72 h)) suggesting that the correctly folded protein is resistant to autoprolysis. It is still not clear why this is the case.

Table 4.2: Molecular weight of intact and autoprolysed OmpT as determined by LC-MS.

OmpT Species	Calculated Mass (From aa sequence)	LC-MS	
		MCW ¹ precipitation	TCA ² precipitation
Full length (298 aa)	33548.79	33,548.18	n.d.
Peptide 1 (218 aa)	24462.81	24,461.77	n.d.
Peptide 2 (80 aa)	9,103.99	n.d.	9,104.62

¹ Methanol:Chloroform:Water, ² Trichloroacetic Acid

4.3.3. LPS does not prevent OmpT autoprolysis

OmpT is active only in the presence of LPS, which is thought to bind to the protease at a conserved LPS binding region. This LPS binding results in a slight structural change in the active site thus activating the protease.⁶ Since no LPS was added during the refolding stage, one would expect these proteases to be inactive and hence exhibit no autoprolysis until LPS is added externally. However, low levels of autoprolysis was observed and this is most likely due to contamination with residual LPS from the membrane during the isolation process. If this was the case, refolding OmpT in the presence of excess LPS should accelerate the autoprolysis process since a larger proportion of the OmpT population becomes active enough to cleave the unfolded protein. In order to verify if excess LPS during the refolding stage could accelerate autoprolysis, OmpT IBs were refolded in the presence of 0.3 mM LPS. SDS-PAGE data of samples refolded in the presence and absence of LPS, and collected at different time intervals (0 min, 10 min, 24 h, 48 h, and 72 h) did not show any significant difference, Figure 4.5. The protein bands at 33.5 kDa corresponding to unfolded/misfolded protein in non-heated (NH) lanes decreased over time for both, OmpT refolded without LPS (Figure 4.4 A Lanes NH) and with 0.3 mM LPS (Figure 4.4 B Lanes NH). While the 25 kDa protein band corresponding to cleaved band in the heated lanes increased over time for both, OmpT refolded without (Figure 4.4 A Lanes H) and with 0.3 mM LPS (Figure 4.4 B Lane H). SDS-PAGE data shows that autoprolysis profiles as well as refolding efficiencies were similar for OmpT samples refolded with and without any external addition of LPS. Since addition of LPS does not

accelerate autoproteolysis, it indicates that residual LPS from the bacterial membrane is not the reason for autoproteolysis of unfolded OmpT. In the work Wu *et al.* (described in detail in Wu's PhD thesis) they added LPS to refolded OmpT immediately after cation exchange purification.^{10,11} In this case, the author suspected a protective role of LPS against OmpT autoproteolysis but did not characterize it any further. However, the data presented herein indicates that adding LPS during the refolding stage neither protects OmpT from autoproteolysis nor does it affect the refolding process of OmpT in IBs. While these results do not explain the reason for autoproteolysis, it is clear that LPS does not play a significant role in protecting OmpT from autoproteolysis.

Figure 4.4: OmpT refolding in the presence and absence of LPS. (A) OmpT inclusion bodies refolded in absence of LPS. (B) OmpT inclusion bodies refolded in presence of 0.3 mM of LPS. Samples were collected at 0 min, 10 min, 24 h, 48 h and 72 h. Each sample was run after 45 min of heating (H) and non-heating (NH).

To clearly establish whether OmpT when bound to LPS is resistant to autoproteolysis, the predicted LPS binding site on OmpT (Arg138, Arg175, Lys226, Glu136 and Tyr134)⁸ was mutated with the expectation that LPS can no longer bind to OmpT. Such a mutant OmpT will be deficient in protease activity. If binding of LPS protected OmpT from autoproteolysis, the addition of wild-type OmpT to the mutant OmpT ($_{mt}$ OmpT), should result in the proteolytic cleavage of $_{mt}$ OmpT. Alternatively, if there is no proteolytic cleavage of $_{mt}$ OmpT, it will confirm that LPS binding does not protect OmpT from autoproteolysis. Surprisingly, the above experiment(s) could not be performed since $_{mt}$ OmpT that had three ($_{mt}$ OmpT_{LPS3} - Arg138, Arg175, Lys226) or all five ($_{mt}$ OmpT_{LPS5} - Arg138, Arg175, Lys226, Glu136 and Tyr134) amino acids of the predicted LPS-binding site mutated, exhibited excellent protease activity when LPS

was added. This indicates that either the predicted LPS binding site is not critical for OmpT activity or that there may be more than one LPS binding site on OmpT that is critical for its activity. ITC experiments to determine the number of LPS molecules binding to unfolded and refolded OmpT did not yield clear results due to technical difficulties in distinguishing between LPS binding to OmpT and LPS binding to the detergent micelles in which OmpT was refolded into (data not shown). However, previous studies with OprH, an outer membrane protein similar to OmpT, report that LPS binds non-specifically to OprH and it is possible that this is also the case with OmpT.^{18,19} However, due to technical difficulties, it was not possible to identify the LPS binding site that is most critical for OmpT activity, if such a site does exist.

4.3.4. Characterisation of refolded OmpT

To assay the activity of recombinantly produced wild-type or mutant OmpT, a previously reported synthetic peptide, Abz-ARRA-Tyr(NO₂)-NH₂ was employed. OmpT samples were incubated with LPS before adding to the peptide substrate. The ends of the peptide substrate were labelled with a fluorophore (2-Aminobenzoyl, Abz) and a quencher (3-nitrotyrosine - Tyr(NO₂)).⁷ When OmpT cleaves at the di-basic site, the fluorescence intensity of Abz at 425 nm will increase ~ 40 fold and this can be measured in a fluorescence spectrophotometer. The activity assay for the 4 variants of OmpT mentioned in Table 4.1 was performed (Figure 4.5).

Figure 4.5: Activity assay of all four variants of OmpT - wtOmpT, mtOmpT_{DB}, mtOmpT_{LPS3} and mtOmpT_{LPS5}.

The specific activity of $_{\text{mt}}\text{OmpT}_{\text{DB}}$ is lower than other variants of OmpT by a factor of ~ 10 whereas mutating the predicted LPS binding site did not affect the protease's specific activity. This clearly suggests that either the predicted LPS binding site is not critical for OmpT activity or that there may be more than one LPS binding to OmpT that is critical for its activity.

Table 4.3: Specific activity of the different OmpT variants

OmpT type	Mutation	Specific Activity ($\mu\text{M}/\text{min}/\text{mg}$)
$_{\text{wt}}\text{OmpT}$	Wild-type OmpT	1.3×10^5
$_{\text{mt}}\text{OmpT}_{\text{DB}}$	OmpT with dibasic site mutated: G216K and K217G	1.6×10^4
$_{\text{mt}}\text{OmpT}_{\text{LPS3}}$	OmpT with 3 LPS sites mutated: R138E, R175E, K226E	1.5×10^5
$_{\text{mt}}\text{OmpT}_{\text{LPS5}}$	OmpT with 5 LPS sites mutated: Y134A, E136A, R138E, R175E, K226E	1.6×10^5

Further, using MALDI-TOF analysis, it was confirmed that $_{\text{wt}}\text{OmpT}_{\text{LPS}}$'s specificity for di-basic sites was preserved (Figure 4.6 A-D).

Figure 4.6: (A-D) MALDI-TOF spectra of a short peptide with a di-basic site in the absence (A) and presence (C) of $_{\text{wt}}\text{OmpT}_{\text{LPS}}$. MALDI-TOF spectra of a short peptide lacking a di-basic site in the absence (B) and presence (D) of $_{\text{wt}}\text{OmpT}_{\text{LPS}}$.

When a short peptide (Seq: GARKVGV, MW 627; Figure 4.6 A) carrying a di-basic site in its sequence was treated with OmpT, the signal for the full-length peptide was no

longer visible in the MALDI spectra (Figure 4.6 C), indicating complete cleavage of the peptide. A control peptide (Sequence: GARIIG, MW 626; Figure 4.6B) lacking a di-basic site in its sequence was not cleaved by OmpT as seen from the peak arising from the full-length peptide (Figure 4.6 D).

4.4. Conclusions

Based on the above findings it is clear that only the unfolded/misfolded OmpT undergoes autoproteolysis *in vitro*. The correctly refolded OmpT remains resistant to autoproteolysis and this resistance is not due to LPS binding to the predicted LPS binding site on OmpT. Hence, rather than work with a mutant form of OmpT that is resistant to autoproteolysis but which shows poor activity and altered substrate preference, it is worthwhile to investigate methods to improve the refolding efficiency of recombinant wild-type OmpT. Furthermore, the predicted LPS binding site on OmpT is not critical for OmpT activity as previously proposed. It is possible that there might be multiple LPS binding sites present on OmpT or that LPS binding to OmpT is purely non-specific.

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Chapter 5

A bottom-up proteomic approach for OmpT's peptide substrate optimisation using SAMDI-MS

Peptide substrate is the second important pillar of the OmpT-Pep-PT assay for E. coli detection. Sensitivity, selectivity, limit of detection and assays time depends to a great extent on the substrate showing high catalytic activity for OmpT. Hence, identifying peptide substrates that are efficiently cleaved by proteases gives insights into substrate recognition and specificity, and improves assays sensitivity. Rationally designed peptide arrays by varying amino acids at P2, P1, P1' and P2' positions were designed, synthesized, and screened using SAMDI-MS, a high throughput screening technique. SAMDI-MS was used to identify a tetrapeptide substrate exhibiting high catalytic activity for OmpT. Analysis of protease activity for the preferred residues at the cleavage site (P1, P1'), nearest-neighbour positions (P2, P2'), and their positional interdependence revealed FRRV as the optimal peptide with the highest OmpT activity. Substituting FRRV into a 14 amino acid fragment of LL37, a natural substrate of OmpT, led to a greater than 400-fold improvement in OmpT catalytic efficiency, with a k_{cat}/K_m value of $6.1 \times 10^6 \text{ Lmol}^{-1} \text{ s}^{-1}$. Wild-type and mutant OmpT displayed significant differences in their substrate specificities, demonstrating that even modest mutations may not be suitable substitutes for the native enzyme.

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Peptide arrays ...

... are used to identify highly active substrates for the OmpT protease, as described by M. Mrksich, B. Liedberg, et al. in their Communication on page 16531 ff. By preparing the peptide arrays on self-assembled monolayers, mass spectrometry (the SAMDI method) was used to characterize the extent of cleavage of each peptide. A commonly used mutant form of the protease, although regarded as having identical activity, instead had an altered specificity for its substrates.

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5.1. Introduction

OmpT acts as a defence mechanism for *E. coli* by cleaving cationic anti-microbial peptides at dibasic sites. For developing an assay that is based on OmpT's catalytic activity, the peptide substrate plays a very critical role. The sensitivity, selectivity, limit of detection, and assay time depends to a great extent on how efficiently the peptide substrate is being cleaved by the protease. Hence, identifying peptide substrates that are efficiently cleaved by OmpT can help improving the sensitivity of peptide-based assays for *E. coli* detection in complex matrices as well as in the design of D-amino acid-based peptide inhibitors.

Peptide optimisation for target proteases can be achieved using various methods, including phage display,^{2,3} internally quenched fluorescent substrate libraries,^{4,5} positional scanning synthetic combinatorial libraries (PS - SCL),^{6,7} cellular libraries of peptide substrates (CLiPS),⁸ and in silico studies.⁹ However, detection of optimal protease targets using most of the above methods relies heavily on substrate labels or tags that can alter the substrate structure and influence reaction rates.¹⁰⁻¹² Self-assembled monolayers for matrix-assisted laser desorption ionisation mass spectrometry (SAMD-MS) is an efficient approach that avoids the many limitations of traditional label based assays and phage display methods.¹³ SAMD-MS uses self-assembled monolayers of long-chain alkane thiolates on gold coated surfaces to which peptides (or other enzyme substrates) are immobilized (Figure 5.1).¹³ SAMDI assay format is well-suited to quantitatively measure a broad range of enzyme activities and it is compatible with high throughput formats that use arrays in the common 384 and 1536 spot formats.¹⁴⁻²¹ SAMDI-MS was used to screen rationally designed peptide libraries for OmpT, without labelling the peptides.

This chapter focuses on the hypothesis that peptide substrate with high catalytic activity for a protease can be designed by understanding the amino acids preference of a protease at the cleavage site and in the vicinity. In order to understand the amino acid preference of OmpT, rationally designed position scanning and combinatorial peptide library were synthesised and screened using SAMDI-MS. This screening of a peptide library resulted in identifying a peptide substrate 400 times better than the natural peptide substrate for OmpT.

Figure 5.1: Illustration of the method used to identify peptide substrates for OmpT. Peptides from a library were individually pretreated with OmpT in 384 well plates. The solutions were then transferred to monolayer array plates, where the peptide substrate and cleaved product undergo immobilisation to the maleimide terminated monolayer. In SAMDI-MS, the monolayer is irradiated with a laser that dissociates the thiolate gold bond, resulting in the release of ionised alkane thiolate peptide species from the surface. The enzyme specificity and activity can be determined from characteristic mass shifts and relative peak intensities in the MS spectrum.

5.2. Experiments

5.2.1. Peptide synthesis

Both position scanning and combinatorial peptide libraries were synthesised using solid-phase organic synthesis by plating lanterns in plugged 96-well filter plates. The detailed protocol is described in 3.2.4.

5.2.2. OmpT activity assays with SAMDI-MS

To screen the synthesised peptide libraries, a pulldown approach was used. The 76 peptides of position scanning peptide library were incubated with $_{wt}$ OmpT and then immobilised on the SAMDI-MS array plates. Whereas the 361 peptides of the combinatorial peptide library were incubated with $_{wt}$ OmpT and $_{mt}$ OmpT_{DB} separately. The treated peptides were then immobilised on the SAMDI-MS array plates. The array plates were analysed using SAMDI-MS. For detailed experimental protocols refer to section 3.2.5.

5.2.3. OmpT kinetic experiments

OmpT's kinetic activity with FRRV and ARRA substituted LL37 peptide fragment was compared with the native peptide by using SAMDI-MS. The peptides were synthesised using solid-phase organic synthesis on Fmoc rink amide MBHA resin. The detailed protocol is described in section 3.2.6.

5.2.4. OmpT activity assays with FAM labelled substituted LL37 fragments

The sequence of the top 6 tetrapeptide hits obtained from screening the 361-peptide library with OmpT and ARRA were substituted into a 14 amino acids long LL37 peptides fragment (Table 3.4). These peptides were synthesised with FAM dye at N terminal and a cysteine at C terminal (FAM-LLGDFXRRYKEKIGC). Different concentrations of OmpT were added to 100 μ L of 1 μ M of FAM peptides. Pierce™ Maleimide activated plates were then used to capture the peptides on the plate surface using maleimide-thiol chemistry. The detailed protocol is described in section 3.2.9. The fluorescence of all the samples were measured with a plate reader and plotted as % fluorescence and OmpT concentration.

5.3. Results and discussion

5.3.1. Position scanning peptide library screening

In order to design peptides with high catalytic activity for OmpT, peptides libraries were designed and screened using SAMDI-MS. To start with, a position scanning peptide library of 76 peptides was synthesised based on a known tetrapeptide peptide substrate (ARRA) of OmpT with a dibasic site.²² In order to understand OmpT's

preference at P2, P1, P1' and P2' positions in a substrate, all the positions of ARRA were sequentially substituted with 19 amino acids except cysteine, which is used for endpoint immobilisation of the peptide substrate on the self-assembled monolayer of alkanethiols by maleimide chemistry. The peptide library was treated with both $_{wt}$ OmpT and $_{mt}$ OmpT_{DB} in order to understand if there is any change in the preference of amino acids at the cleavage site of the substrate.

After treatment of the peptides with $_{wt}$ OmpT and $_{mt}$ OmpT_{DB}, small volumes of each reaction (2 μ L) were transferred onto array plates having 384 gold islands that were modified with a monolayer presenting maleimide groups at a density of about 10% against a background of tri(ethylene glycol) terminated alkanethiolates.²³ The maleimide functionality allows for the selective immobilisation of cysteine-terminated peptides, and the glycol functionality is effective in preventing the non-specific adsorption of proteins.²⁴ The array plates were analysed by MALDI-TOF MS to obtain a mass spectrum for each spot, and the protease activity was calculated by measuring the relative area under each curve (AUC) for molecular ion peak (M^+) from each spectrum according to the equation:

$$Activity = \frac{AUC_{product}}{AUC_{substrate} + AUC_{product}} \times 100\%$$

Hence, the activity represents the percent yield in conversion of the substrate to the product, and though the activities provide a rank-ordering of the substrate turnovers, they do not provide a direct comparison of the substrates (which requires measurement of k_{cat} and K_m , as described in following sections).

The substrate and product might have different ionisation efficiencies that would affect the activity and not give the actual yield of the reaction. However, it was confirmed that this was not the case in the current study by using the peak for the tri(ethylene glycol)-terminated background alkane thiolate as an internal control; this analysis showed that the ionisation efficiencies for substrate and product differed by less than 2% (Table 5.1).

Table 5.1: Comparison of ionisation efficiencies of the top 5 peptide substrates identified in Ac-GXRRZGC peptide array.

Peptide Sequence	Substrate : EG ₃	Product : EG ₃
Ac-GFRRVGC	0.144	0.144
Ac-GFRRYGC	0.135	0.137
Ac-GARRVGC	0.095	0.096
Ac-GWRRVGC	0.067	0.068
Ac-GMRRAGC	0.041	0.040

***Note:** The ratios of peak intensities of the substrate and product peaks for each peptide relative to the tri (ethylene glycol)-terminated alkane thiolate (EG3) peak within each SAMDI spectra were calculated to compare the ionisation efficiencies of substrate and product peptides. The values are within 2%, demonstrating the ionisation efficiencies are nearly identical for substrate and product.

Figure 5.2: Wild-type OmpT activity screen on the 76-peptide library displayed as a heatmap. White areas correspond to no activity and dark areas correspond to high activity. The five most active peptide substrates are shown in the table together with the observed activity.

The results of the position scanning library were consistent with those reported by Dekker et al.²⁵ using *mt*OmpT_{DB}. The *wt*OmpT also has an absolute preference for charged basic residues at the P1 and P1' positions, though with a modest preference for Arg–Arg over a combination of Arg and Lys (Figure 5.2, rows 2 and 3). As expected, charged acidic residues are not favored at the cleavage site nor in its immediate vicinity. This can be explained by the acidic residues in the active site where the catalytic pairs consist of Asp83–Asp85 and His212–Asp210.²⁶ Beyond the cleavage site, the protease displays less stringent requirements for residues at the P2 position (Figure 2, row 4),

and has a preference for small hydrophobic residues like Ala and Val at the P2' position (Figure 2, row 1). Overall, these results are mostly in agreement with previous reports obtained using *mtOmpT*_{DB}, thus validating the use of SAMDI-MS in determining the substrate specificity for *wtOmpT*.^{27,28} Although the protease displayed a sufficiently high activity for ARRAs, we observed that three substrates - YRRA, PRRA, and ARRV- displayed higher activity, demonstrating the value of using arrays that include previously unknown substrates.

5.3.2. Combinatorial peptide library screening

The screening results of position scanning 76 peptide library was useful in determining the preferred residues at each of the four positions. However, it is recognized that there is often an interdependence of amino acids at each position in the peptide sequence, and therefore arrays having two variable positions would be expected to reveal still more active substrates. So, a larger peptide library was designed that was based on the sequences described above (Arg at P1 and P1'), but where the P2 and P2' positions were varied simultaneously, with arginine held constant at the P1 and P1' positions. This array contained 361 peptides of the form Ac-GXRRZG-C, where the X and Z residues are one of nineteen amino acids excluding cysteine.

The larger combinatorial peptide array of 361 peptides, was treated with *wtOmpT* and then analysed each spot with SAMDI-MS to determine the activity. The data was analysed and represented as a heat map (Figure 5.3). Some of the representative spectra are shown in Figure 5.4. Twelve tetrapeptides displayed higher activity for *wtOmpT* than did the ARRAs peptide, which had an activity of 91.0 %. Among these, FRRV exhibited the highest activity of any peptide in the array, at 98.9 %. Moreover, this larger peptide array also confirmed the preference for small hydrophobic residues Ala, Ile, and Val at the P2' position and for Ala and Phe at the P2 position. Interestingly, SAMDI-MS also revealed a strong positional interdependence for the P2' and P2 positions. For example, when the P2' position was occupied by a small hydrophobic residue and the P2 position was occupied by an aromatic residue or methionine, there is high activity, as is the case with the peptide substrate FRRV (Figure 5.3).

Figure 5.3: Wild-type OmpT activity screening of a combinatorial peptide library with substrate sequence Ac-GXRRZGC displayed as heatmap. The top five peptide substrates and corresponding OmpT activities are displayed in the table.

Figure 5.4: Representative SAMDI mass spectra of the best (A), somewhat good (B), and poor (C) peptide substrates before and after treatment with OmpT, where the substrate peptide sequence and mass are shown in red and the product peptide sequence and mass are shown in blue.

5.3.3. OmpT kinetic analysis of substituted and native LL37 peptide fragment.

To validate if FRRV was indeed a preferred substrate for the protease, this tetrapeptide sequence was substituted into LL37, a natural substrate for OmpT.^{29,30} LL37 is a cationic antimicrobial peptide that is secreted by the epithelial cells of lungs and intestines into the extracellular environment as part of innate immunity. To establish and maintain infection, OmpT present on pathogenic *E. coli* strains is known to cleave LL37 at the two dibasic sites present in its sequence, thus rendering them inactive (Figure 5.5 A).³⁰

Two variants of a fourteen amino acid N-terminal fragment of LL37 were synthesised in which the original FRKS sequence of LL37 was substituted with either FRRV or ARRA. For the unmodified/native LL37 fragment, $_{wt}$ OmpT exhibited a catalytic

efficiency of $1.4 \times 10^4 \text{ L mol}^{-1} \text{ s}^{-1}$ (Figure 5.5 C, Figure 5.6). However, when FRRV was substituted into this sequence, the catalytic efficiency significantly improved to $6.1 \times 10^6 \text{ L mol}^{-1} \text{ s}^{-1}$, which is approximately 400-fold higher than the original FRKS peptide (Figure 5.5 B, C and Figure 5.6). When the natural sequence was substituted with ARRA, wtOmpT exhibited a catalytic efficiency of $5.8 \times 10^5 \text{ L mol}^{-1} \text{ s}^{-1}$ that is approximately 40-fold higher than for the original peptide (Figure 5.5 C and Figure 5.6). These experiments establish that the protease is more active for FRRV than for ARRA or the natural sequence FRKS.

Figure 5.5: Kinetic comparison of peptide substrates that are inserted into the LL37 fragment. A) The LL37 sequence has 2 dibasic sites that can be cleaved by OmpT. A 14 amino acid peptide from the N-terminal of LL37 was substituted with the FRRV or ARRA sequences and compared to the native sequence having the FRKS motif. B) Kinetic data for cleavage of the FRRV substrate by wtOmpT). C) Best-fit values for K_m and k_{cat} were used to determine the k_{cat}/K_m turnover numbers for each substrate.

A sequence homology of LL37 in primates reveals that positions P2-P1-P1' (F-R-K) for the first dibasic site are highly conserved.²⁹ The P2' position is less conserved with

the occurrence of either Ala or Val, the same two non-polar residues that are highly favored at this position (Table 5.2). However, human LL37 appears to have evolved with Ser, a small polar residue, at the P2 position and for which the protease has a relatively poor cleavage preference.²⁹ It may be possible that this change is a consequence of positive selection pressure driven by exposure to various bacterial pathogens.

Figure 5.6: Kinetic data for the cleavage of the native LL37 fragment and the ARRA substituted fragment of LL37 by w_t OmpT.

Table 5.2: Sequence comparison of a portion of LL37, among different primates. A sequence comparison of the 14 amino acid fragment of LL37 that was used in this study, among different primates. P2, P1, and P1' residues for OmpT cleavage are highlighted for their conserved residues (yellow) and P2' is a less conserved residues (orange, green, and blue).

Uniprot Entry	Species	Sequence
J3KNB4	<i>Homo sapiens</i> (Human)	LLGDFFRKSKKEKIG
Q1KLX1	<i>Pan troglodytes</i> (Chimpanzee)	LLGDFFRKSKKEKIG
Q1KLX4	<i>Trachypithecus obscurus</i> (Dusky leaf-monkey)	RLGNFFRKAKKKIG
Q1KLY2	<i>Nomascus gabriellae</i> (Red-cheeked gibbon)	LPGNFFRKAREKIG
Q1KLY0	<i>Nomascus concolor</i> (Black crested gibbon)	LPGNFFRKAREKIG
Q1KLX8	<i>Hylobates moloch</i> (Silvery gibbon)	SLGNFFRKARKKIG
G1R3M3	<i>Nomascus leucogenys</i> (Northern white-cheeked gibbon)	LLGNFFRKAREKIG
Q1KLX5	<i>Trachypithecus cristatus</i> (Silvered leaf-monkey)	RLGNFFRKAKEKIG
Q1KLY3	<i>Gorilla gorilla gorilla</i> (Western lowland gorilla)	LLGDFFRKAKEKIG
Q1KLX2	<i>Pongo pygmaeus</i> (Bornean orangutan)	LLGDFFRKAREKIG
G8F302	<i>Macaca fascicularis</i> (Crab-eating macaque)	RLGNFFRKVKKEKIG
F7B5V0	<i>Macaca mulatta</i> (Rhesus macaque)	RLGNFFRKVKKEKIG
Q9GLV5	<i>Macaca mulatta</i> (Rhesus macaque)	RLGNFFRKVKKEKIG
Q1KLX7	<i>Macaca fascicularis</i> (Crab-eating macaque)	RLGNFFRKVKKEKIG
Q1KLX6	<i>Macaca tonkeana</i> (Tonkean macaque)	RLGNFFRKVKKEKIG
Q1KLX3	<i>Papio papio</i> (Guinea baboon)	RLGNFFRKVKKEKIG
A0A0D9RTQ0	<i>Chlorocebus sabaeus</i> (Green monkey)	RLGKFFRKVKKKIG
Q1KLY6	<i>Chlorocebus aethiops</i> (Green monkey)	RLGKFFRKVKKKIG

5.3.4. Screening of combinatorial peptide library with $mtOmpT_{DB}$

The larger combinatorial peptide array of 361 peptide was also treated with $mtOmpT_{DB}$, to directly compare the substrate specificities of $wtOmpT$ and $mtOmpT_{DB}$ (Figure 5.7A). Interestingly, the relative difference heatmap for the two proteases (computed as a difference in activity relative to the activity of $wtOmpT$ for the same substrate; Figure 5.7 B) reveal trends illustrating differences in their specificities.

Figure 5.7: Mutant OmpT displays differential substrate specificity compared to wild-type. A) Heatmap of global $mtOmpT_{DB}$ on the peptide array. B) Differences in specificity for $mtOmpT_{DB}$ were determined by dividing the difference in activity of the proteases on each peptide by the activity for the $wtOmpT$. Peptides that are coded green were more active for $mtOmpT_{DB}$ and peptides that are coded red showed greater activity for $wtOmpT$ C) Comparison of $mtOmpT_{DB}$ and $wtOmpT$ on LL37 peptide fragments.

Despite both OmpT variants exhibiting a high catalytic activity for FRRV, we observed substantial differences in the activities of the two proteases for many of the substrates. Most noticeably, $mtOmpT_{DB}$ displayed a higher activity for peptides substituted with

Ser at the P2' position (Figure 5.7 B, row-S). A similar comparison of wtOmpT and $_{mt}$ OmpT_{DB} activity on the LL37 peptide fragments again revealed a difference in their specificity. While both OmpT variants displayed similar activity for the FRRV and ARRA substituted LL37 peptide fragments (Figure 5.7 C), $_{mt}$ OmpT_{DB} displayed poor activity for the original natural sequence (FRKS). This was in contrast to the high activity of wtOmpT for the above peptide. Such a stark difference in properties of the wild-type and mutant forms of the protease illustrates the ways that even modest mutants (that is, interchanging two adjacent residues outside of the active site) can lead to distinct differences in substrate specificity.

5.3.5. FAM labelled optimised peptide substrate assays

The top 6 tetra-peptide hits of 361 peptide library screening with OmpT, for which OmpT showed highest activity were substituted in LL37 fragment with FAM labelling. This was done to ascertain that OmpT activity against the peptide had improved as compared to the native peptide. FAM labelled peptides were incubated with different concentrations of OmpT and then captured on a maleimide plate using cystine-maleimide chemistry. Higher the activity of OmpT against the peptide substrate, more peptide is cleaved resulting in lower fluorescence for better peptides (Figure 5.8).

Figure 5.8: Schematic illustration of the FAM-peptide and OmpT activity assay. FAM peptides mentioned in Table 3.4 were incubated with different OmpT concentrations and the peptides were captured on maleimide plates using cystine and thiol chemistry. The reduction in fluorescence was correlated to the added OmpT concentration.

Among the seven substituted LL37 peptides, six peptides (Figure 5.9 except WRRV) showed better activity as compared to the native peptide (Figure 5.9 FRKS magenta).

Figure 5.9: FAM-peptide based OmpT assay. The decrease in fluorescence corresponds to better activity of OmpT for the peptide substrate.

Using SAMDI-MS based approach for peptide substrate optimisation, LL37_{FRRV} showed the highest activity. However, when a fluorescence based assay was used to compare OmpT's activity against various top peptide substrates from SAMDI-MS, the results suggested LL37_{ARRV} being the best peptide substrate for OmpT. Further, OmpT showed a better activity against LL37_{ARRA} and LL37_{MRRA}, as compared to native and LL37_{FRRV} peptide. These findings clearly point towards the fact that fluorescent tags on the peptide substrate affect the binding of the peptide to the enzyme (K_m).³¹ The results make an important point to be considered while screening and optimising peptide substrates, that peptides can have different activity based on screening and assay formats used. Hence, the final substrate optimisation should be done in the assay format being used. Since LL37_{FRRV} showed 400 times better k_{cat}/K_m compared to native LL37 using a label-free SAMDI-MS approach and showed better activity compared to a native peptide in a FAM based assay, LL37_{FRRV} peptide was chosen as the optimized substrate of OmpT. Here the FAM peptides were used as a fluorimetric sensor for

OmpT. However, this approach was further developed into an assay for *E. coli* detection based on OmpT's activity to cleave the LL37_{FRRV}.

5.4. Conclusion

In conclusion, the use of SAMDI-MS and peptide arrays in this work revealed trends in OmpT activity and interplay between multiple positions in the peptide substrate sequence. Peptide arrays permit the unbiased evaluation of hundreds to thousands of enzyme substrates. In this example, it was demonstrated that peptide arrays and SAMDI-MS were able to discover new short peptide sequences that have catalytic turnover numbers that are approximately 400-fold greater than that for the native sequence and approximately 40-fold greater than that for the previously identified "best" ARRA substrate. Further, the use of peptide arrays revealed significant differences in substrate specificity for the wild-type and mutant proteases, showing that these forms of the OmpT protease cannot be used interchangeably, as they often have been in OmpT research. This approach will be important for enabling studies of a broader range of proteases, and potentially also for the development of an assay for detection of pathogenic bacteria, *E. coli*, see chapter 6.^{32,33}

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Chapter 6

6.1. Part A - OmpT-based *E. coli* detection with anionic polythiophene and unlabeled peptide substrate

E. coli and *Salmonella* are two of the most common bacterial pathogens involved in food and waterborne related deaths around the world. Conventional methods for detecting bacterial pathogens are time-consuming, labor intensive and laboratory-based. This chapter describes the development of a simple and rapid method to detect low levels of *E. coli* contamination in water by targeting the bacteria's surface protease activity. A polythiophene acetic acid (PTAA) was employed as a reporter molecule, and a short unlabeled peptide (cLL37_{FRRV}) was used as the substrate for OmpT, an outer membrane protease on *E. coli*. Mixing cLL37_{FRRV} with PTAA induces a helical conformation in both the peptide and the polymer resulting in an enhancement in the polymer's fluorescence. However, exposing cLL37_{FRRV} to *E. coli* or recombinant OmpT prior to mixing it with PTAA does not increase PTAA's fluorescence intensity. This ability of PTAA to distinguish between an intact cLL37_{FRRV} and its cleaved fragments was exploited to detect wild-type K12 and J96 *E. coli* strains in water at concentrations as low as 1 CFU/mL within 6 and 8 hours, respectively. These assay times are significantly shorter than most commercially available detection kits for *E. coli*.

6.1.1. Introduction

The present study describes the development of a simple, functional assay for detecting *E. coli* in contaminated water samples. It avoids labour-intensive steps that are often required in DNA/RNA isolation for nucleotide-based assays and circumvents stability and storage issues commonly faced with antibodies in immunoassays. It involves measuring changes in optical properties - fluorescence and absorbance, of the polythiophene (PT) reporter molecule that is mixed with an unlabelled peptide substrate previously exposed to *E. coli* expressing an outer membrane protease (Figure 6.1.1). It is possibly the first time an outer membrane protease has been targeted to develop a functional assay for detecting bacterial pathogens in water. Using this method, a limit of detection of 1 CFU/mL in 6 h with pre-culturing and 10^5 CFU/mL in 1 h without pre-culturing was achieved. This low limit of detection and short assay time is better than most commercially available detection kits for *E. coli*.

Figure 6.1.1: Schematic illustration of the OmpT-Pep-PT assay for *E. coli* sensing, with anionic polythiophene and unlabeled peptide substrate.

The method described herein relies on the enzymatic activity of OmpT, a membrane protease present on the surface of all wild-type *E. coli* strains.¹⁻³ OmpT is known to cleave short peptides preferentially at dibasic sites (-KK-, -KR-, -RK-, and -RR-).^{3,4} It acts as a defence mechanism for *E. coli* by cleaving cationic anti-microbial peptides

(cAMPs) that are secreted by the human body as a defence mechanism against bacterial pathogens.^{5,6}

Previously, a short peptide sequence was optimised at the preferred cleavage site for OmpT (refer to chapter 5).⁷ The optimised peptide sequence, FRRV, was substituted into a 15 residue peptide fragment derived from the N-terminal region of LL37.⁸⁻¹⁰ For the optimised substrate with the sequence CLLGDFFRRVKEKIG (cLL37_{FRRV}), *wt*OmpT exhibited a k_{cat}/K_m value that was 400 times higher than what was observed for the native LL37 fragment with the sequence CLLGDFFRKSKEKIG.⁷ Hence, cLL37_{FRRV} was chosen as the preferred peptide substrate in this study.

Poly-(thiophene acetic acid) (PTAA), an anionic PT (Figure 6.1.2 A) was chosen as the reporter molecule for the assay since the carboxyl functional group on the polymer can be expected to interact with the positively charged residues on cLL37_{FRRV}. PTs belong to a class of conjugated conducting polymers and have found applications in many chemical- and bio-sensing platforms in the recent years.^{11,12} The optical properties of PTs depend on the conformation of the polymer backbone.¹³⁻¹⁵ Hence, the optical properties can be tuned by modifying the side chains with different functional groups. These functional groups can interact with DNA, protein, small bio-analytes, metal ions, and surfactants resulting in changes in structural, electronic, and optical properties of the PT.

Figure 6.1.2: A) Chemical structure of polythiophene acetic acid (PTAA) used for assay development. B) cLL37_{FRRV} peptide sequence-red colour represents OmpT cleavage site (R8-R9).

When cLL37_{FRRV} is exposed to wild-type *E. coli*, the OmpT on its surface cleaves cLL37_{FRRV} between -R8-R9- resulting in two shorter peptide fragments (Figure 6.1.2 B).

Depending on the *E. coli* concentration and the time of incubation, a known amount of externally added cLL37_{FRRV} is partially cleaved or completely cleaved by OmpT. Upon mixing intact cLL37_{FRRV} with PTAA, the PTAA undergoes a structural change resulting in an increase in its fluorescence intensity. However, the shorter peptide fragments resulting from proteolytic cleavage are incapable of inducing a structural change in PTAA and hence do not cause any significant enhancement in PTAA's fluorescence intensity. This ability of PTAA to distinguish between an intact cLL37_{FRRV} and its cleaved fragments is the basis for detecting *E. coli* in contaminated water samples.

6.1.2. Experimental section

6.1.2.1. Fluorescence and absorbance characterisation of PTAA-peptide complex

To study the effects of cLL37_{FRRV} on PTAA's absorbance and fluorescence intensity, different concentrations of cLL37_{FRRV} (150 - 6.25 μ M) were added to PTAA (10 μ g/mL). Absorbance measurements were collected from 330-650 nm. Fluorescence scans were collected by exciting the samples at 420 nm and their emission spectrum recorded from 450-750 nm. For detailed experimental protocols refer to section 3.3.2.

Since the fluorescence intensity increase for PTAA saturated at 50 μ M of cLL37_{FRRV}, this peptide concentration was chosen for all further experiments. To understand the effect of intact and cleaved fragments on the fluorescence intensity and absorbance of PTAA, cLL37_{FRRV}, frag1LL37_{FRRV} and frag2LL37_{FRRV} were added in different ratios (10:0, 8:2, 6:4, 4:6, 2:8, 0:10) while keeping the total peptide concentration at 50 μ M. The fluorescence intensity and absorbance spectra were measured, as mentioned above. For detailed experimental protocols refer to section 3.3.3.

Besides mixing short synthetic peptide fragments of cLL37_{FRRV}, 50 μ M of the intact cLL37_{FRRV} was treated with varying concentrations of recombinant OmpT that was reconstituted in detergent micelles. To this reaction mixture PTAA was added and fluorescence measurements and absorbance scans were collected. For detailed experimental protocols refer to section 3.3.4.

6.1.2.2. MALDI-TOF based peptide mass characterisation

The intact cLL37_{FRRV} and cLL37_{FRRV} treated with OmpT samples were analysed using MALDI-TOF for their molecular weight. For detailed experimental protocols refer to section 3.3.5.

6.1.2.3. Circular dichroism (CD) based characterization of PTAA-peptide complex

The following samples were prepared: (1) PTAA, (2) cLL37_{FRRV}, (3) PTAA mixed with cLL37_{FRRV}, (4) OmpT treated cLL37_{FRRV}, and (5) OmpT treated cLL37_{FRRV} mixed with PTAA. For detailed experimental protocols refer to section 3.3.6.

6.1.2.4. *E. coli* detection using the pre-concentration method

Four different strains for *E. coli* were used to test the OmpT-Pep-PTAA assay for sensing *E. coli* in water samples. The four *E. coli* strains at various concentrations (10^8 , 10^7 , 10^6 - 10^3 CFU/mL) were pre-concentrated and incubated with cLL37_{FRRV}. The PTAA was added to the reaction mixture and the sample's fluorescence intensity and absorbance were recorded. For detailed experimental protocols refer to section 3.3.7.

6.1.2.5. *E. coli* detection using the pre-culture method

In order to establish the minimum pre-culturing time required for the OmpT-Pep-PTAA assay to detect 1 and 10 CFU of *E. coli* cells, three different *E. coli* strains were employed. *E. coli* J96, K12, and BL21 strains at an initial concentration of 1 and 10 CFU/mL were cultured, by adding LB media, for over a period for 3, 5, and 7 h respectively. The *E. coli* cells were incubated with cLL37_{FRRV} and PTAA was added to the reaction mixture post incubation. The sample's fluorescence intensity and absorbance were recorded. For detailed experimental protocols refer to section 3.3.7.

6.1.3. Results and Discussion

6.1.3.1. PTAA's fluorescence intensity increases upon addition of intact cLL37_{FRRV}, but not for the cleaved cLL37_{FRRV}

Figure 6.1.3: Absorbance spectra of PTAA and cLL37_{FRRV}. (A) Absorbance spectra of PTAA exhibiting a blue shift that corresponds to increasing cLL37_{FRRV} concentrations. Black arrow indicates the direction of the shift.

PTAA in an aqueous buffer has an absorption maximum at 450 nm, and upon adding cLL37_{FRRV} exhibits a blue shift that corresponds to a colour change from orange to yellow (Figure 6.1.3 and Figure 6.1.4). This blue shift increases linearly with increasing peptide concentration until it saturates at 50 μ M of the added peptide (Figure 6.1.3).

At sufficiently high PTAA concentration, addition of cLL37_{FRRV} peptide resulted in a visible colour change from orange to yellow, which was easily detected with the naked eye (Figure 6.1.4). However, when cleaved synthetic peptides were added to PTAA, no visible colour change was observed (Figure 6.1.4). When excited at 420 nm, PTAA exhibits a fluorescence emission maximum at 550 nm (Figure 6.1.5). The following formula was applied to convert fluorescence intensity (a.u.) to % F. increase:

$$\% \text{ F. increase} = \left(\frac{F_{PTAA+Peptide} - F_{PTAA}}{F_{PTAA}} \right) \times 100\%$$

The % fluorescence increase of PTAA on addition of LL37_{FRRV} increases linearly till 50 μ M and further shows an onset of saturation. The highest point in the linear dynamic range was selected, in order to have larger working window for change in fluorescence. Since the fluorescence intensity increase for PTAA saturated at 50 μ M of LL37_{FRRV}, this peptide concentration was chosen for all further experiments (Figure 6.1.5). At these concentrations, the overall increase in PTAA's fluorescence intensity is close to 200% (Figure 6.1.5 B). Separately, when cLL37_{FRRV} was treated with recombinantly expressed OmpT that was reconstituted into detergent micelles, it was cleaved at the dibasic site (-R₈-R₉- Figure 6.1.2 B) to yield two short fragments of eight and seven residues (Figure 6.1.6 and Figure 6.1.2 B). When these peptide fragments were mixed with PTAA, it did not result in a significant increase in fluorescence intensity, and the increase was no more than 20% at best (Figure 6.1.7 blue).

Figure 6.1.4: Color change for PTAA (0.4 mg/mL) from orange to yellow upon addition of cLL37_{FRRV} (100 μ M). No colour change is observed on the addition of synthesised peptides representing cleaved fragments of cLL37_{FRRV} (100 μ M).

Figure 6.1.5: Fluorescence spectra of PTAA and cLL37_{FRRV} (A) Fluorescence emission spectra of PTAA exhibiting an increase in fluorescence intensity that corresponds to increasing cLL37_{FRRV} concentrations. Black arrow indicates the direction of change. (B) Increase in fluorescence intensity of PTAA at 550 nm when mixed with increasing concentrations of intact cLL37_{FRRV}.

Figure 6.1.6: MALDI-TOF spectra for intact cLL37_{FRRV} and cleaved fragment frag1LL37_{FRRV}: A: Intact LL37_{FRRV} peptide (MW of 1780.19 Da) shows a m/z peaks at 1779.25 (monomer) and 3558.33 (dimer formed because of thiol bonds). B: Cleaved fragment frag1LL37_{FRRV} shows a peak at 1940.95, which corresponds to the dimer of this cleaved fragment.

Figure 6.1.7: Comparison of fluorescence spectra of PTAA (black), and when PTAA is mixed with either the intact cLL37_{FRRV} (red) or the cleaved fragments of cLL37_{FRRV} resulting from prior exposure to OmpT (blue).

As an additional control, two short synthetic peptides (frag1LL37_{FRRV} and frag2LL37_{FRRV}) that were identical to the two cleaved fragments obtained when OmpT cleaves cLL37_{FRRV} (Figure 6.1.2 B) were employed in this study. The two short synthetic fragments (Figure 6.1.2 B) were combined with intact cLL37_{FRRV} in different ratios and subsequently mixed with PTAA. The results indicate that the shorter peptides do not contribute much to the fluorescence intensity, but when mixed with increasing proportions of the longer intact peptide, showed an enhancement in the overall fluorescence intensity (Figure 6.1.8). The % fluorescence was normalized and calculated based on the following formula (the fluorescence intensity at emission maximum was used):

$$\% F = \left(\frac{F_{Sample} - F_{PTAA}}{F_{PTAA+Peptide} - F_{PTAA}} \right) \times 100\%$$

This clearly demonstrates that only the intact cLL37_{FRRV} contributes to the enhancement in fluorescence intensity while the shorter peptide fragments resulting from OmpT's enzymatic activity do not.

Figure 6.1.8: Fluorescence intensity of PTAA at 550 nm when mixed with different ratios of intact and cleaved LL37_{FRRV}.

The rate at which cLL37_{FRRV} is cleaved into shorter fragments is a function of OmpT concentration and time. Hence, with increasing OmpT concentrations, a larger proportion of cLL37_{FRRV} being cleaved into shorter fragments was expected. This process was monitored by measuring the end-point fluorescence intensity of PTAA that

was mixed with 50 μM of cLL37_{FRRV} exposed for 2 h to varying concentrations of OmpT (0.84 – 42 nM). There was an overall decrease in PTAA's fluorescence intensity that correlated with increasing OmpT concentration (Figure 6.1.9). At the highest OmpT concentration (42 nM), PTAA's % fluorescence increase was ~ 175% less than its % fluorescence increase with cLL37_{FRRV} in the absence of OmpT, suggesting that almost all of the cLL37_{FRRV} was cleaved into shorter fragments (Figure 6.1.9). To understand why PTAA's absorbance and fluorescence properties change in the presence of cLL37_{FRRV}, it is important to note that PTAA's initial absorbance maximum at 450 nm represents a planar configuration of the polymer backbone.^{13,14,16} The deprotonation of the carboxylic group on PTAA's side chain, when solubilised in the pH 8 reaction buffer, induces planarization and formation of a rod-like structure because of electrostatic repulsion between the side groups. The PTAA planar chains could also be grouped (stacked) together showing non-radiative de-excitation because of the contact between the polymer chains, which further contributes to a lowering of the fluorescence.¹³

Figure 6.1.9: Change in fluorescence intensity of PTAA when mixed with 50 μM cLL37_{FRRV} previously treated with varying concentrations of OmpT for 2 h.

Since cLL37_{FRRV} carries two arginines (R) and two lysines (K) in its sequence¹⁷, it is expected to interact electrostatically with the charged carboxylic acid groups of PTAA. These electrostatic interactions will force the polymer backbone to adopt a non-planar conformation, thereby decreasing the effective conjugation length of the polymer backbone.^{14,17} This conformational change in the PTAA backbone chain also alters the

optical properties - absorbance and emission spectra of PTAA, which was observed when cLL37_{FRRV} was mixed with PTAA (Figure 6.1.3 and Figure 6.1.5 A). The PTAA and cLL37_{FRRV} complex results in the separation of the PTAA polymer chains and induces a chirality in the PTAA backbone that was also confirmed by circular dichroism (CD) measurements.

6.1.3.2. CD measurements indicate structural changes in both PTAA and cLL37_{FRRV}

PTs are known to show split-type induced CD (ICD) in the π - π^* transition region (320-500 nm).^{18,19} The CD spectrum of PTAA in the pH 8 reaction buffer reveals that PTAA is optically inactive as it does not exhibit any characteristic ICD pattern in the π - π^* transition region (Figure 6.1.10 black). The absence of CD signals and an absorption maximum at 450 nm suggests that PTAA backbone adopts a planar achiral “rod-like” conformation. (Figure 6.1.10 and Figure 6.1.3 black). However, upon adding cLL37_{FRRV}, split-type ICDs in the π - π^* transition region was observed (Figure 6.1.10 blue).

Figure 6.1.10: CD Spectra of PTAA, cLL37_{FRRV}, PTAA-cLL37_{FRRV} complex, OmpT cleaved cLL37_{FRRV}, PTAA mixed with OmpT cleaved cLL37_{FRRV}, and PTAA mixed with synthesised cLL37_{FRRV} fragments.

Thus, the blue shift in absorption, increase in fluorescence of PTAA with the addition of LL37_{FRRV}, and ICD for PTAA-cLL37_{FRRV} all point towards a main-chain chirality such as a predominantly helical structure induced in PTAA backbone due to the interaction between PTAA and cLL37_{FRRV} (Figure 6.1.3, Figure 6.1.5 A and Figure

6.1.10 blue, respectively). The shape and sign of the ICD pattern for PTAA are characteristic of a left-handed helical form of PTs.²⁰ In the absence of PTAA, the CD spectra of cLL37_{FRRV} and the two shorter peptide fragments resulting from OmpT cleavage of cLL37_{FRRV} exhibit no defined secondary structure (Figure 6.1.10 red and magenta). However, in the presence of PTAA, cLL37_{FRRV} adopts a helical secondary structure as interpreted from the CD signal in the 190 – 240 nm range (Figure 6.1.10 blue and Figure 6.1.11 i).

Similar observations of unstructured peptides undergoing a conformational change upon interacting with PTs have been reported earlier.^{13,14} This conformational change is also reminiscent of the full-length LL37 which is known to adopt helical structures when exposed to lipid membranes.^{9,10} While both PTAA and the intact cLL37_{FRRV} adopt helical conformations when mixed together, there was no change in secondary structure observed for PTAA and an equimolar mixture of frag1LL37_{FRRV} and frag2LL37_{FRRV} which are the two synthesised peptides representing the two shorter fragments produced by OmpT when it cleaves cLL37_{FRRV} (Figure 6.1.10 purple and Figure 6.1.11 ii).

It is possible that frag1LL37_{FRRV} and frag2LL37_{FRRV} interact weakly with PTAA, but they are unable to bring about a conformational change in the PTAA backbone from a planar to a non-planar conformation resulting in poor or limited enhancement in fluorescence intensity (Figure 6.1.8). Also, when the shorter peptide fragments, resulting from OmpT cleavage of cLL37_{FRRV}, were added to PTAA, there was no change in PTAA's secondary structure. Thus, it is evident that mixing the intact cLL37_{FRRV} with PTAA induces helical conformations in both the peptide and polymer whereas mixing of the cleaved fragment of cLL37_{FRRV} with PTAA do not. A pictorial summary of the conformational changes in PTAA brought about by the addition of intact cLL37_{FRRV} and cleaved peptide fragments is shown in Figure 6.1.11.

Figure 6.1.11: Schematic illustration of the proposed mechanism of PTAA-peptide interaction in the presence and absence of OmpT. (i) Intact cLL37_{FRRV} and PTAA interact electrostatically and both molecules adopt a helical conformation. (ii) cLL37_{FRRV} cleaved by OmpT in detergent micelles may interact with PTAA without inducing any structural changes. Center panel illustrates the fluorescence intensity changes in PTAA corresponding to the scenarios in (i) and (ii).

6.1.3.3. *E. coli* wild-type strains detected with PTAA and cLL37_{FRRV} using the pre-concentrate method

From the results presented so far, it is clear that PTAA can be used to distinguish between intact and cleaved cLL37_{FRRV}. Since the proportion of intact and cleaved cLL37_{FRRV} in a sample is determined by OmpT concentration and time, PTAA can therefore be used indirectly to determine OmpT concentrations in a sample. However, it is more relevant to investigate the feasibility of using this approach to detect *E. coli* in water samples. For this, four different strains of *E. coli*: BL21, K12 (ATCC® 700926™), J96, and Lemo21(DE3) were employed. BL21 acted as a negative control as this strain lacks the genes necessary to synthesise OmpT and therefore does not have any OmpT present on its outer membrane. K12 and J96 are two wild-type strains of *E.*

coli expressing OmpT on the outer membrane, with the latter strain expressing OmpT at a slightly higher level.

Figure 6.1.12: A) Approaches to *E. coli* sensing using PTAA-cLL37_{FRRV}: Top - *E. coli* sensing involving pre-concentration method. Bottom - *E. coli* sensing involving pre-culture method.

A Lemo21(DE3) strain overexpressing recombinant OmpT on the outer membrane was used as a positive control. Milli-Q water samples were spiked with *E. coli* whose concentration ranged from 10^8 to 10^3 CFU/mL. The sample volumes required for testing differed for the various *E. coli* concentrations. For the highest *E. coli* concentration of 10^8 CFU/mL, a starting sample volume of 200 μ L was sufficient, but this sample volume had to be increased to 2 mL for 10^7 CFU/mL and 20 mL for concentrations between 10^6 - 10^3 CFU/mL. The water samples spiked with different *E. coli* concentrations were filtered through a 0.2 μ m PTFE filter to concentrate the *E. coli* cells and these *E. coli* concentrates were suspended in 90 μ L of the reaction buffer to which 10 μ L of 500 μ M cLL37_{FRRV} was added. After incubation for 1 h at 37 °C to allow the *E. coli* cells to interact with and cleave cLL37_{FRRV}, PTAA was added and the fluorescence intensity at 550 nm recorded. For the sake of convenience, this approach was called the “Pre-concentrate method” (Figure 6.1.12).

Figure 6.1.13: A) Detection of different strains of *E. coli* and their LOD. Fluorescence intensity of PTAA when mixed with cLL37_{FRRV} treated with different *E. coli* strains while using the pre-concentrate method. 200 μ L (light gray), 2 mL (gray) and 20 mL (dark gray) of sample volumes were used for detecting 10^8 , 10^7 , and 10^6 - 10^3 CFU/mL of *E. coli*, respectively. (B) Fluorescence intensity of PTAA when mixed with cLL37_{FRRV} treated with *E. coli* K12 or J96 wild-type strains using pre-culture method for detecting 1 and 10 CFU/mL of *E. coli*. Data in B & C were normalised for the highest PTAA fluorescence intensity at 550 nm and plotted as % fluorescence. Error bars in B & C indicate standard error of the mean for data points averaged from duplicates for n=3.

Results indicate a significant reduction in PTAA's fluorescence intensity for samples containing K12, J96, and Lemo21(DE3) strains, suggesting that these *E. coli* strains were able to cleave cLL37_{FRRV} (Figure 6.1.13 A blue, red, and magenta). Control samples containing the *E. coli* BL21 strain did not show any reduction in PTAA's fluorescence intensity (Figure 6.1.13 A black). Moreover, the reduction in PTAA's fluorescence is consistent with the different levels of OmpT expressed on the surface of different *E. coli* strains. Although it is tempting to exploit this differential OmpT expression to distinguish between specific strains of *E. coli*, it was concluded that the surface OmpT levels could not be regarded as a reliable marker since protein expression levels are influenced by the bacteria's environment and growth stage.²¹ For the two

wild-type strains K12 and J96, the limit of detection (LOD) defined here as the concentration causing a 20% change in fluorescence intensity, was $\sim 10^5$ and 10^6 CFU/mL, respectively (Figure 6.1.13 A blue and red). Thus, *E. coli* concentration could undoubtedly be detected at these concentration levels within 1 h. However, it would be much more useful to develop assays for the detection of *E. coli* at significantly lower concentrations.

6.1.3.4. LOD for *E. coli* detection was reduced to 1 CFU/mL by using the pre-culture method

In order to improve the LOD, the experiment was repeated with the two wild-type strains- K12 and J96, while including an additional pre-culturing step. For the sake of convenience, this approach is called the “Pre-culture method” (Figure 6.1.12 bottom part). In this step, water samples spiked with *E. coli* at 1 and 10 CFU/mL cells were first cultured in LB media for up to 7 h. 200 μ L of this pre-cultured sample was then analysed by following the steps described in the pre-concentrate method above. Results indicate that PTAA’s fluorescence intensity at 550 nm reduced by almost 35% and 44% for 1 and 10 CFU/mL of K12 strain, respectively, when pre-cultured for only 5 h (Figure 6.1.13 B green and purple). Similarly, a reduction in PTAA’s fluorescence intensity of 28% was observed for 10 CFU/mL of J96 strain when pre-cultured for 5 h (Figure 6.1.13 B magenta). It took 8 h of pre-culturing to detect 1 CFU/mL of the J96 strain, which showed a reduction of PTAA’s fluorescence intensity of almost 51% (Figure 6.1.13 B blue). In other words, by using the pre-culture method of detection, as low as 1 CFU/mL of K12 *E. coli* can be detected within 6 h. Moreover, for J96, the *E. coli* strain commonly seen in urine samples of patients suffering from UTI, 1 CFU/mL can be detected within 8 h. This is significant because most commercially available kits require almost twice or three times as much time to detect 1 CFU/mL of *E. coli*.

6.1.4. Conclusion

In conclusion, the OmpT-Pep-PTAA assay is a simple yet sensitive method to detect very low levels of *E. coli* in water. The assay employed PTAA as a reporter molecule which is able to distinguish between an intact and cleaved peptide that was optimised for OmpT. It is possibly this is the first time, an outer membrane protease has been targeted to develop a functional assay for *E. coli* detection and one in which the peptide

substrate is unlabelled. Using this method, it is possible to detect as low as 1 CFU/mL of the K12 and J96 wild-type *E. coli* strains in water within a total assay time of 6 (spectrometric readout) and 8 h (naked eye based), respectively. These assay times are significantly shorter than most commercially available detection kits for *E. coli*.

6.2. Part B – Improving assay sensitivity and scope by screening different anionic polythiophenes

Part A described the OmpT-Pep-PTAA assay and how it was used for detection of E. coli in water with visual detection at higher E. coli concentrations ($>10^7$ CFU/mL) and fluorescence-based detection at lower concentrations ($<10^6$ CFU/mL). This part focusses on optimising the assay conditions and screening new polythiophenes to improve the limit of detection for OmpT-Pep-PTAA assay and remove the need for fluorescence-based readout. Anionic PTs having carboxyl group at the 3 position with varying side chain length were screened with thiol containing peptides at three different pH. All PTs exhibited a higher overall fluorescence at pH 8 and among the PTs screened, PT_{06CA}, exhibited the highest increase in fluorescence (~130000 %) upon peptide binding. PT_{06CA} was chosen as the most optimal PT for OmpT-Pep-PT assay based on its optical response.

6.2.1. Effect of PT's side chain length on its fluorescence and secondary structure when mixed with short synthetic peptides containing N-terminal cysteine.

OmpT based *E. coli* sensing using anionic PTs and unlabeled cLL37_{FRRV} peptide is based on the PT's ability to distinguish between cleaved and intact peptide. Using this approach, as low as 1 CFU/mL of K12 and J96 wild-type *E. coli* strains were detected in water within a total assay time of 6 and 8 h, respectively. When using low concentrations of the PT ($\leq 10 \mu\text{g/mL}$), the assay results required a fluorescence readout. This can be overcome by identifying PTs that are capable of exhibiting higher fluorescence intensity changes or are capable of visual colour changes at low concentrations when bound to the peptide. The optical response of the PTs depends on structural changes in the PT backbone upon interacting with the peptide and such interactions can be influenced by the PT's side chain length, functional groups present on the side chain, pH of the solution, and molecules interacting with the PTs. Hence, by tailoring the side chain length and functional group on the PTs, it might be possible to improve the optical response in the visual range and at lower PT concentration. It was previously shown that such modifications to side chains of PTs led to enhanced optical properties.^{15,17,22} Since the structural changes in PT backbone and the peptide bring about an optical response, characterising the interactions between the peptide and polythiophene can help understand and thus help improving the assay sensitivity and optical response. Therefore, five different anionic polythiophenes having carboxyl group at position 3 and whose side chain length was varied, were screened to identify better PTs for optical sensing of *E. coli* (Figure 6.2.1). PTs with side chain length 3,4,5, and 6 were commercially purchased and screened. PT with side chain length 2 was not commercially available and hence could not be used for screening.

Further, six different synthetic peptides were designed and synthesised in order to characterise the effect of N-terminal thiol and presence/absence of peptide's positive charge, on the fluorescence of PTs when mixed with peptides. Based on preliminary data, the helicity and charge do affect the fluorescence of PTs (data not shown) but need further investigation.

Table 6.2.1: List of peptides used for polythiophene characterisation. Helicity of the peptides was determined using an online prediction algorithm Agadir (<http://agadir.crg.es/>).

Peptide Name	Peptide Sequence		% Helicity
LL37 _{FRRV}	Ac-LLGDFFRRVKEKIG-NH ₂	FRRV substituted LL37 fragment	3.39
cLL37 _{FRRV}	CLLGDFFRRVKEKIG- NH ₂	Cystine at N terminal of LL37 _{FRRV}	3.25
mLL37 _{FRRV}	MLLGDFFRRVKEKIG- NH ₂	Methionine at N terminal of LL37 _{FRRV}	3.13
cLL37 _{ARRA}	CLLGDFARRAKEKIG- NH ₂	Cystine at N terminal of ARRA substituted LL37 fragment	7.00
AY11	Ac-AVSHTTHHAPY-NH ₂	Control peptide with no charge	0.01
cAY12	cAVSHTTHHAPY-NH ₂	Control peptide with no charge	0.01

Figure 6.2.1: List of all the anionic PTs tested. The PTs vary in the length of their side chains with 2, 4, 5, 6 and 7 carbons. The PTs are named with the first two numbers in the subscript referring to the length of their side chain at position 3 and the last two letters in the subscript are abbreviations of the functional group (CA: carboxylic acid).

6.2.1.1. Experimental section

6.2.1.1.1. Peptide design for characterising the effect of cysteine

In part A, the peptide cLL37_{FRRV} was used to develop an OmpT based *E. coli* detection assay. The cLL37_{FRRV} peptide was optimised using SAMDI-MS for OmpT activity, with an N-terminal cysteine added in order to functionalize the peptide on the self-assembled monolayer of SAMDI-MS arrays. The peptides were synthesised with and without cysteine in order to see if dimerisation of peptide would affect its overall fluorescence. LL37_{FRRV} was also synthesised with an N-terminal methionine as a control peptide. A different set of control peptides, cAY12 and AY12 were designed and synthesised with a thiol group but without any positive charge.

6.2.1.1.2. Fluorescence and absorbance measurements of PT-peptide complex

In order to study the effect of pH on PTs fluorescence and absorbance with the addition of cLL37_{FRRV}, the PTs were mixed with cLL37_{FRRV} peptide at three different pH 6, 7, and 8 of the reaction buffer. The pH 8 of the reaction buffer showed the highest increase in fluorescence and was selected for further experiments. Further different concentrations (150 - 6.25 μ M) of the peptides (Table 6.2.1) were mixed with PTs (Figure 6.2.1) in order to study their effect on the absorbance and fluorescence of PTs. Absorbance and fluorescence measurements were carried out and for detailed experimental protocols refer to section 3.3.8.

6.2.1.2. Results

6.2.1.2.1. Fluorescence and absorbance-based characterization of PTs and peptide interactions

6.2.1.2.1.1. Highest change in PT's absorbance and fluorescence when mixed with cLL37_{FRRV} at pH 8

Anionic PTs mixed with cationic cLL37_{FRRV} showed an optical response with colour and fluorescence change. Being polyelectrolytes, the PTs exhibit optical properties that are heavily influenced by their environment, and pH is capable of significantly altering its optical properties. When the absorbance and fluorescence of PTs were compared in three different pH (6, 7, and 8), the colour changed from purplish to yellow or orange

(Figure 6.2.2 B) for PT_{04CA}, PT_{05CA}, PT_{06CA}, and PT_{07CA}. Whereas, the colour for PTAA changed from light orange to yellow (Figure 6.2.2 B). The colour change for all the PTs with the addition of peptide cLL37_{FRRV} at pH 8 is much more prominent when observed under UV₃₆₅ (Figure 6.2.2 C). All five PTs exhibited a blue shift in absorbance maximum with the addition of cLL37_{FRRV} (Figure 6.2.3).

Figure 6.2.2: Fluorescence intensity changes of PTs when mixed with 50 μ M cLL37_{FRRV} at pH 6, 7 and 8: A) Maximum fluorescence increase for all PTs when excited at 495 and 420 nm. B) The observed visual colour change, and C) Color change under UV_{365nm}.

The absorbance maximum of PTs at a longer wavelength ($\sim$ 440 nm for PTAA and $>$ 520 nm for PT_{04CA} - PT_{07CA}) represents a certain degree of planarization of polymer backbone (Figure 6.2.3 solid lines).¹⁴ The electrostatic repulsive forces between the carboxylate side groups of the PTs or the hydrogen bonding within the PT chains might result in PTs attaining a stretched planar confirmation forming a π -stack, hence reduce the electrostatic repulsion between the carboxylate side groups.^{14,18,23} When positively

charged cLL37_{FRRV} peptide is mixed with negatively charged PTs, they interact via electrostatic interaction, causing a blue shift in absorbance maximum for all the PTs (Table 6.2.2 and Figure 6.2.3 dashed lines).^{13,14}

The blue shift observed in the absorption maximum of PTs with the addition of cLL37_{FRRV} suggests a decrease in effective conjugation length of the PTs backbone and formation of a nonplanar conformation of the polymer backbone (Table 6.2.2 and Figure 6.2.3).^{13,16,17} The blue shift seen upon addition of cationic peptides to anionic PTs suggests that electrostatic interaction between carboxylate groups of PT and positively charged arginine (R) and lysine (K) forces the PTs backbone to adopt a nonplanar conformation.¹⁴ This change from a planar to nonplanar conformational in the PTAA backbone chain also alters the emission spectra of PTs by increasing the fluorescence (Figure 6.2.4 solid lines increased to dotted line). The blue shift in absorbance of PTs is much larger (appx. > 50 nm) at pH 8 as compared to pH 6 (Table 6.2.2). This points towards the fact that the larger fractions of carboxylic groups on the PTs are deprotonated at pH 8 as compared to pH 6 thus being more prone to interact with the peptides as reflected in the larger blue shift. Also, the addition of cLL37_{FRRV} to the PTs increased the intensity of the fluorescence emission because of the polymer backbone adopting a more nonplanar confirmation (Figure 6.2.2 A, Figure 6.2.4). PTs and cLL37_{FRRV} form a complex structure as a result of the electrostatic interactions and hydrogen bonding between the negatively charged carboxyl groups of the PTs side chain and the positively charged groups of arginine and lysine of cLL37_{FRRV}. These electrostatic interactions result in the separation of the PTs polymer chains and induce chirality in the PT backbone, which was also confirmed by CD experiments. The PTs planar chains are not separated from each other showing non-radiative deexcitation because of the contact between polymer chains, contributing to lower PT fluorescence.¹³ However, the interactions between the positively charged side chains of cationic cLL37_{FRRV} and negatively charged carboxylate groups of PTs, disrupt the electrostatic repulsive forces between the PTs hence, resulting in increased fluorescence.

Table 6.2.2: Absorption maxima for the individual PTs and when mixed with cLL37_{FRRV} at three different pH 6,7 and 8 in 20 mM Phosphate buffer.

	pH 6		pH 7		pH 8	
	PT	PT + Peptide	PT	PT + Peptide	PT	PT + Peptide
PTAA	440	400 (-40)	440	400 (-40)	440	400 (-40)
PT _{04CA}	520	500 (-20)	520	420 (-100)	520	400 (-120)
PT _{05CA}	520	500 (-20)	530	460 (-70)	470	450 (+20)
PT _{06CA}	550	550 (0)	550	500 (-50)	550	480 (-80)
PT _{07CA}	550	510 (-40)	550	500 (-50)	550	490 (-60)

PTAA and PT_{04CA} exhibited a higher increase in fluorescence when excited at 420 nm as compared to 495 nm (Figure 6.2.2 A). Whereas, the PTs with longer side chain: PT_{04CA}, PT_{05CA}, PT_{06CA} and PT_{07CA} exhibited a 1.5, 2.5, and 3 times higher increase in fluorescence, respectively, when excited at 495 nm compared to 420 nm (Figure 6.2.2 A). The increase in fluorescence with the addition of peptides also depends on the excitation wavelength used (Figure 6.2.2 A). Hence, the fluorescence increase in PTs might not correlate entirely with the visual response under UV₃₆₅ as the PTs and PT-peptide complexes were excited with 365 nm light. The increase in fluorescence of PTs with the addition of cLL37_{FRRV} is maximum at pH 8 which is in agreement that the carboxylic acids side chains of PT are deprotonated at pH 8 and thus interacting efficiently with the positively charged side groups of arginine and lysine. Since the reaction buffer at pH 8 shows the highest change in fluorescence and absorbance peak shift, phosphate buffer at pH 8 was chosen for further studies.

Figure 6.2.3: Absorbance spectra of PTs (solid line) and PTs mixed with 50 μM cLL37FRRV (dotted line) at pH 6, 7 and 8. A) PTAA B) PT₀₄CA C) PT₀₅CA D) PT₀₆CA E) PT₀₇CA.

Figure 6.2.4: The fluorescence spectrum of PTs (solid line) and PTs mixed with 50 μ M cLL37FRRV (dotted line) at pH 6, 7 and 8. A) PTAA B) PT₀₄CA C) PT₀₅CA D) PT₀₆CA E) PT₀₇CA.

6.2.1.2.1.2. *Introduction of a cysteine residue to the N-terminus of LL37_{FRRV} significantly increased PT's fluorescence when mixed together*

In Part A, cLL37_{FRRV} was used to develop an assay for *E. coli* detection. cLL37_{FRRV} was the sequence optimised from SAMDI and the role of cysteine was to help immobilise the peptide on to the gold surface. Interestingly, when the peptide was synthesised without the N-terminal cysteine, the maximum increase in fluorescence attained upon mixing with PTAA was much lower ~100 % as compared to 180 % for the thiolated peptide (A). The following formula was applied here after to convert fluorescence intensity (a.u.) to % Fluorescence increase (the fluorescence intensity at emission maximum was used):

$$\% \text{ Fluorescence increase} = \left(\frac{F_{PTAA+Peptide} - F_{PTAA}}{F_{PTAA}} \right) \times 100\%$$

Since the peptide is capable of dimerising via the thiol groups on the cysteine, it can result in a longer peptide that could contribute to the increased fluorescence observed in the PTs. Attempts to isolate this parameter (increased peptide length) as the main source of the fluorescence change were met with technical difficulties. Reducing agents such as DTT and TCEP were added to the samples to prevent dimerisation, but these agents on their own interfered with the PT's fluorescence, thus making the results difficult to interpret. Hence, an alternative approach was attempted where the cysteine was substituted with a methionine such that no dimerisation was possible. A similar trend of a relatively higher increase in fluorescence of PT's for cLL37_{FRRV} and mLL37_{FRRV} as compared to LL37_{FRRV} (Figure 6.2.5 and Table 6.2.3) was observed.

This effect of higher saturation values for % fluorescence increase with cLL37_{FRRV} is also evident in the optical response of PTs with N-terminal cysteine and methionine as compared to LL37_{FRRV} (Table 6.2.4). Although the peptides containing methionine did not result in a consistent increase in fluorescence across all PT's as compared to cLL37_{FRRV}, suggesting that peptide dimerisation might have an important role to play in improving the PT's overall fluorescence increase (Table 6.2.3). However, the phenomenon needs to be examined further to understand the reason for thiolated peptides showing higher fluorescence. Also, the decreasing absorbance at 600 nm for PT for the addition of these three peptides, (Table 6.2.4) suggests that it is dispersion of PT chains in the solution that causes the increased fluorescence.

Figure 6.2.5 : Percentage fluorescence increase (solid lines) and absorbance₆₀₀ (dotted lines) of PTs with addition of cLL37_{FRRV} (Red), mLL37_{FRRV} (blue) and LL37_{FRRV} (Black) : A) PTAA B) PT_{04CA} C) PT_{05CA} D) PT_{06CA} and E) PT_{07CA}.

Table 6.2.3: Maximum % fluorescence increase PTs with the addition of LL37_{FRRV}, cLL37_{FRRV} and mLL37_{FRRV}. Values in red represent % increase in cLL37_{FRRV} and mLL37_{FRRV} as compared to LL37_{FRRV}.

	LL37 _{FRRV}	cLL37 _{FRRV}	mLL37 _{FRRV}
PTAA	121	163 (35 %)	105 (-13 %)
PT04CA	1800	3200 (78 %)	1900 (6%)
PT05CA	2800	3200 (14 %)	2700 (4 %)
PT06CA	65000	130000 (100%)	76000 (17%)
PT07CA	2300	3900 (70%)	3600 (57%)

Table 6.2.4: Maximum % change in absorbance at 600 nm of PTs with the addition of LL37_{FRRV}, cLL37_{FRRV}, and mLL37_{FRRV}.

	LL37 _{FRRV}	cLL37 _{FRRV}	mLL37 _{FRRV}
PTAA	-12	-12	-10
PT04CA	-45	-46	-44
PT05CA	-58	-58	-58
PT06CA	-47	-60	-50
PT07CA	-54	-65	-55

6.2.1.2.1.3. Positive charge is crucial for an increase in PTs fluorescence

To determine the importance of positive charge on the peptide in increasing the fluorescence of PTs, two peptides (AY11 and cAY12) were designed such that there were no positively charged residues in the peptide sequence. The addition of AY11 and cAY12 to the PTs results in a negligible increase in PT's fluorescence as compared to cLL37_{FRRV} (Figure 6.2.6, Figure 6.2.7, and Table 6.2.5).

Figure 6.2.6: Optical response of the 5 PTs under UV₃₆₅ when mixed with AY11, cAY12 and cLL37_{ARRA}.

Table 6.2.5: Maximum % fluorescence increases in PTs with the addition of AY11, cAY12 and cLL37_{ARRA}.

	AY11	cAY12	cLL37 _{ARRA}
PTAA	28	58	168
PT04CA	125	280	1650
PT05CA	100	250	3000
PT06CA	173	3100	120000
PT07CA	17	350	2600

There is no detectable optical response observed under UV365 lamp and also negligible change in the absorbance at 600 nm of PT samples upon the addition of peptide AY11 and cAY12 (Table 6.2.6). These results suggest the PT's optical response and the secondary structure change in the PTs backbone with the addition of peptides, is dependent on the electrostatic interactions between the charged side groups of PT and peptide. Similar to the results observed with cLL37_{FRRV}, the cAY12 consistently showed higher fluorescence of PTs as compared to AY11 (Table 6.2.5). The peptide cLL37_{ARRA} showed a lower increase in fluorescence compared with cLL37_{FRRV} (Table 6.2.3 and Table 6.2.5). Also, the optical response for PTs with cLL37_{ARRA} under UV365 was much lower as compared to PTs when mixed with cLL37_{FRRV} (Figure 6.2.6 and Figure 6.2.6). As shown in Chapter 5, cLL37_{FRRV} is not only a better catalytic substrate for OmpT compared to cLL37_{ARRA}, but also gives a better optical response when added to PTs. Hence, cLL37_{FRRV} is the better peptide substrate to be employed in the OmpT-Pep-PT assay.

Table 6.2.6: Maximum % change in absorbance at 600 nm of PTs with the addition of AY11, cAY12 and cLL37_{ARRA}.

	AY11	cAY12	cLL37 _{ARRA}
PTAA	-9	-7	-12
PT04CA	2	0	-44
PT05CA	-2	-18	-53
PT06CA	3	10	-52
PT07CA	0	1	-46

Figure 6.2.7: Percentage fluorescence increase (solid lines) and absorbance (dotted lines) of PTs with the addition of cAY12 (Red), cLL37_{ARRA} (blue) and AY11 (Black): A) PTAA B) PT_{04CA} C) PT_{05CA} D) PT_{06CA} and E) PT_{07CA}.

6.2.1.2.2. Secondary structure characterisation of PT-peptide complex using CD spectroscopy

6.2.1.2.2.1. CD measurements indicate PTAA adopting a left-handed helical secondary structure with all LL37_{FRRV} variants.

As explained in previous sections, the interaction of cLL37_{FRRV} with PTAA brings about a structural change in both the peptide and PT secondary structure. PTAA backbone tends to adopt a nonplanar chiral conformation, which is evident with split-type ICDs in the π - π^* transition region for PTAA (Figure 6.2.8). Similar structural changes were observed when LL37_{FRRV} and mLL37_{FRRV} are added to PTAA, which suggest that the addition of cysteine or methionine does not affect the secondary structure of the peptide (Figure 6.2.8). All three peptides: LL37_{FRRV}, mLL37_{FRRV}, and cLL37_{FRRV} adopt a helical secondary structure from an undefined secondary structure with the addition of PTAA (Figure 6.2.8).

Figure 6.2.8 : CD measurements of PTAA, LL37_{FRRV}, mLL37_{FRRV}, cLL37_{FRRV} and the PTAA-peptide complex.

6.2.1.2.2.2. No secondary structural changes in PTAA and cLLC37_{ARRA}

Addition of cLL37_{ARRA} to PTAA resulted in an increase in fluorescence with a blue shift in the absorbance spectrum, suggesting a structural change in PTAA from a planar confirmation to a nonplanar conformation. However, no split-type ICDs in the π - π^* transition region was observed with cLL37_{ARRA} (Figure 6.2.9). The reasons for the same are yet not understood but might be because of the racemic mixture of left and right-handed ICD in PTAA.

Figure 6.2.9: CD measurements of PTAA, cLL37_{ARRA}, OmpT cleaved cLL37_{ARRA}, PTAA-cLL37_{ARRA} complex and PTAA mixed with OmpT cleaved cLL37_{ARRA}.

6.2.1.2.2.3. cLL37_{FRRV} preferentially induces left-hand helix in PTs and itself adopts a helical secondary structure.

Based on previous results, the electrostatic interactions between PTAA and cLL37_{FRRV} bring about structural changes in the PTAA backbone (observed in the form of ICD Figure 6.2.8 and absorbance blue shift Figure 6.2.11) and cLL37_{FRRV}. The cLL37_{FRRV} peptide was also mixed with different PTs (PT_{04CA} – PT_{07CA}) to characterise the changes in the secondary structure (Figure 6.2.10). The CD spectra of all five PTs suggest PTs are optically inactive as they do not exhibit any characteristic ICD pattern in the π - π^* transition region (Figure 6.2.10 B dotted lines). Taken together, the absence of CD signals (Figure 6.2.10 B dotted line) and an absorption maximum at longer wavelengths (Table 6.2.2) suggests that PT's backbone adopts a planar achiral conformation in the

Figure 6.2.10: CD measurements of cLL37_{FRRV} and its complex with different PTs (i.e. PTAA, PT_{04CA} – PT_{07CA}). A) Combined spectra of cLL37_{FRRV} with PTs B) cLL37_{FRRV} spectrum with individual PTs.

pH 8 reaction buffer. For both PTAA and PT_{04CA} a split-type ICDs in the π - π^* transition region was observed, but for PTs with a longer side chain (i.e. PT_{05-07CA}) no ICD was observed.

Figure 6.2.11: Absorbance of PTs (black) and PT-cLL37_{FRRV} complex (Red). The absorbance peaks show a blue shift for all.

However, cLL37_{FRRV} showed a transition from undefined secondary structure to adopting a helical secondary structure as interpreted from the CD signal in the 190 – 240 nm range. The blue shift in absorption, increase in fluorescence of PTs with the addition of cLL37_{FRRV}, and ICD for PTAA/PT_{04CA}-cLL37_{FRRV} all indicate that it is a result of main-chain chirality such as a predominantly helical structure induced in PTs backbone due to the interaction between PTs and cLL37_{FRRV}. The shape and sign of the ICD pattern for PTs are characteristic of a left-handed helical form of PTs.^{18,20} Also, cLL37_{FRRV} results in left-handed helix formation of the PTs, which suggests positive or negative ICD would depend on the peptide sequence and not the PT side chains.

6.2.1.2.2.4. *cLL37_{ARRA} adopts a helical secondary structure when mixed with PTs*

Figure 6.2.12: CD measurements of cLL37_{ARRA} and its complex with different PTs (i.e. PTAA, PT_{04-7CA}). A) Combined spectra of cLL37_{ARRA} with PTs B) cLL37_{ARRA} spectrum with individual PTs.

Based on helicity prediction by Agadir, cLL37_{ARRA} has lower helicity as compared to cPep_{HHCD} (Table 6.2.1), this also evident from the CD data (Figure 6.2.12 dotted purple line). Although the addition of cLL37_{ARRA} results in an increase in fluorescence and

blue shift in absorbance, no ICD signal was observed for PTAA, PT_{04CA} and PT_{06CA}. The CD signal for cLL37_{ARRA} after mixing with PTAA and PT_{04CA} does not represent a proper helix signal. However, the cLL37_{ARRA} peptide with PT_{05-07CA} shows a CD signal corresponding to helix formation (Figure 6.2.12). Also, cLL37_{ARRA} showed a split-type ICDs in the π - π^* transition region (420- 500nm) for PT_{05/07CA} (Figure 6.2.12 solid red and pink).

Figure 6.2.13: Absorbance of PTs (black) and PT-cLL37_{ARRA} complex (Red). The absorbance peaks show a blue shift for all.

The blue shift in absorption (Figure 6.2.13) and increase in fluorescence of PTs with the addition of cLL37_{ARRA} (Figure 6.2.7 solid blue lines) indicate that it is a result of PT backbone adopting a non-planar conformation. This results in the peptide adopting a helical secondary structure with all PTs. Although main-chain chirality, such as a

predominantly helical structure induced in PT's backbone, for PTs was not observed but would be investigated further for the absence of the signal.

6.2.1.3. Discussion

The absorbance and fluorescence data from the screening of PTs suggested that the PTs in solution have a planar achiral conformation (Figure 6.2.14).^{24,25}

Figure 6.2.14: Illustration representing the assembly of PTs and PT-Peptide complex resulting in reduced and enhanced fluorescence.

The side chain length influences the PT backbone planarization further affecting the optical and electronic properties of the PTs in solution.^{15,25–27} This stacked structure results in reduced fluorescence and higher absorbance at 600 nm. However, with the addition of positively charged peptides to the stacked anionic PTs, they undergo a structural change from planar achiral conformation to a non-planar chiral confirmation. This results in dissociation of the stacked assembly of PTs reducing the non-radiative de-excitation of PTs chains, resulting in an enhanced fluorescence signal with a blue shift in emission wavelength.¹³ This secondary structure change in PT is accompanied by peptides adopting a secondary structure. However, the data suggests that mixing excess of the cLL37_{FRRV} peptide results in decreased fluorescence with an increase in absorbance at 600 nm. This is possible because of the PT-peptide complex forming a

stacked assembly resulting in increased size (higher absorbance at 600 nm) and reduced fluorescence (Figure 6.2.14). These optical changes are also accompanied by secondary structure changes, which are evident from the CD presented before. The direction of ICD left or right-hand helix depends on the chiral molecule the PT is interacting with.^{20,28–30} As Katsuhiro *et al.*, 2001 has reported that induced helix sense (right or left handed) depends on the acid-base complexations between the side chains of the conducting polymer and the added chiral molecules (Figure 6.2.15).³⁰

Figure 6.2.15: Proposed structures in acid-base complexations of poly-2 with carboxylic acids (A) and α -hydroxy acids (B) and the relationships between the induced helix sense (right-handed or left-handed) and the absolute configurations of carboxylic acids and α - hydroxy acids. Reproduced with permission (Appendix - Permissions).³⁰

Based on the results obtained in the previous section, it appears that cLL37_{FRRV} induces left-hand helicity in PTs, but as the side chain length of PTs increases, the ICD signal is not observed. Similarly, for cLL37_{ARRA}, the ICD signal is not observed even with shorter side chains of PTs (PTAA and PT_{04CA}). However, there is sufficient data to suggest that cLL37_{ARRA} peptide interacting with PTs resulting in fluorescence change and peptides attaining a secondary helix structure. The data suggests that with the increase in side chain length, the ICD signal is not observed (Figure 6.2.10), but the change in peptide's structure from undefined secondary structure to a helical secondary structure is observed (Figure 6.2.10 and Figure 6.2.12). cLL37_{ARRA} not showing ICD signals can be a result of a racemic mixture of left and right-handed helix because of the symmetry of ARRA in the peptide (Figure 6.2.16 A, B). The similar racemic mixture of left and right helix might be formed with PTs having longer side chains (PT_{04CA-07CA}) (Figure 6.2.16 C, D). These findings need further characterisation and

experimental data. Based on these observations and screening of the PTs, PT_{06CA} showed the highest increase in fluorescence with a visual response under UV₃₆₅. Hence, PT_{06CA} was used further for testing *E. coli* in food samples, as described in the part C of chapter 6.

Figure 6.2.16: Illustrations representing positively charged amine side groups of peptide interacting with negatively charged PTs. A) cLL37_{ARRA}-PT complex. B) cLL37_{FRRV}-PT complex C-D) cLL37_{FRRV}-PT – represents PTs with different side chain length interacting with cLL37_{FRRV} (Blue – PTs and Gray – Peptides with positively side groups. 2 Diamond red arrows represent arginine in ARRA of cLL37_{ARRA}. Diamond red and solid circle red arrows represent arginine in FRRV of cLL37_{FRRV}. Blue arrows represent other positively charged amino acids).

6.2.1.4. Conclusion

Based on the results obtained from screening five different anionic polythiophenes of varying side chain length (ranging from 2-7 carbon atoms except for three carbons, at position 3) with six different peptide variants whose cysteine and the overall charge was varied, the following conclusions can be made:

- i. Presence of positive charge on the peptides is required for the increase in fluorescence of the PTs as observed for cLL37_{FRRV}, cLL37_{ARRA} etc. and no increase in fluorescence is observed for AY11 and cAY12 which do not have a positive charge.
- ii. Presence of a cysteine at the N-terminal of the peptide results in a higher increase in fluorescence; however, the reasons for this needs further studies.
- iii. When both anionic PT and the cationic peptides are mixed, they undergo conformational changes in their secondary structure. The PTs go from a non-chiral planar configuration to an achiral/chiral nonplanar configuration. The peptides go from an undefined secondary structure to a helical secondary structure depending on their propensity to form a helix.

6.3. Part C – Testing food samples for *E. coli* contamination

Part B established that by using PT with different side chains the optical response can be tuned. PT_{06CA} was selected for further assay development based on the improved optical response. This chapter further focuses on developing strategies to detect E. coli present on the surface of food samples. The food samples like green leafy vegetables are highly prone to E. coli contamination. So the detection of E. coli contamination of these samples was performed by capturing the E. coli on the vegetable's surface into a liquid medium and later assay it using the OmpT-Pep-PT_{06CA} assay. Using a different polythiophene PT_{06CA} a detection limit of 10 CFU/mL in 7 h, with pre-culturing was achieved using cLL37_{FRRV}.

6.3.1. *E. coli* testing in food samples (leafy vegetables)

The OmpT-Pep-PTAA assay described in Chapter 6 - Part A, was primarily used for detecting pathogenic bacteria in contaminated water samples. However, once the assay was established in water samples, it was further tested to see if it can also be used to detect pathogenic bacteria present on the surface of food samples (Figure 6.3.1) that are prone to *E. coli* contamination.

Figure 6.3.1: Food samples tested for the presence of *E. coli* on the surface, based on OmpT-Pep-PT assay.

6.3.1.1. Ability to detect *E. coli* in contaminated fresh leafy vegetables (lettuce, cabbage, and spinach)

Considering the sources of *E. coli* contamination on farm products (Table 6.3.1), the majority of *E. coli* contamination is restricted to the vegetables' outer surface.³¹ Hence, it is possible to detect bacterial contamination on vegetables by first releasing the bacteria from the vegetable's surface and capturing it into a liquid medium. This liquid medium can then be assayed for detecting or quantifying bacterial contamination. Since obtaining contaminated fresh vegetables is extremely difficult, store-bought green leafy vegetables were exposed to water deliberately contaminated with *E. coli*. After the brief exposure, the now contaminated vegetables were transferred into a growth medium to capture any *E. coli* released from the vegetable's surface. At this point, if any *E. coli* is released from the vegetable's surface, it would be too few to assay. Hence, the growth media was incubated at 37 °C for 5 h to facilitate *E. coli* growth and multiplication. After 5 h, the growth medium was assayed using the OmpT-Pep-PTAA method to

detect *E. coli*. Since it can be suspected that the method in which the bacterial contamination is introduced on to the vegetable's surface, the surface itself, and the amount of sample area analysed might affect the LOD. Hence, a few different approaches were adopted to investigate these effects. These are described briefly in the following experimental section.

Table 6.3.1 : Source of *E. coli* contamination in farm produce.

Farm	<ul style="list-style-type: none"> • Via direct contact with animal faeces • Contaminated irrigation water, bird faeces, and manure
Production Plant	<ul style="list-style-type: none"> • Contaminated water used to wash produce • Via equipment used to cut up leafy veggies
Store	<ul style="list-style-type: none"> • Cross-contamination from raw foods • Not bagged

6.3.1.2. Experimental section

6.3.1.2.1. Effect of cleaved fragments on PT_{06CA} fluorescence

Based on the screening of 5 different PTs with various peptides, it was evident that PT_{06CA} showed the highest increase in fluorescence with the addition of cLL37_{FRRV}. In order to see the effect of cleaved peptide fragments when added to PT_{06CA} the experiment explained in section 3.3.3 was repeated with PT_{06CA}.

6.3.1.2.2. Approach -1: Soaking food samples in contaminated water

Briefly, store-bought bagged leafy vegetables- *lettuce, cabbage, and spinach* were cut into small squares and soaked in water deliberately contaminated with 10⁶ CFU/mL cells of *E. coli*. The samples were kept in growth media with shaking for 15 mins. The samples were removed and growth media incubated at 37 °C for 5 h to facilitate bacterial growth and multiplication. For detailed experimental protocols refer to section 3.3.10.1

6.3.1.2.3. Approach -2: Depositing contaminated water directly on the surface of food samples

The surface morphology of vegetables varies greatly, and *E. coli* can be expected to display a preference to adhere more strongly to one surface than to another. In order to

determine if *E. coli* displayed such a variability in its adherence/release properties for the vegetables selected for this study, a known quantity of *E. coli* was deposited on each of the different vegetable's surface and the samples assayed. For detailed experimental protocols refer to section 3.3.10.2

6.3.1.2.4. Approach - 3: Increasing sampling area for analysis of food samples soaked in contaminated water

The amount of *E. coli* retrievable from the surface of a leafy vegetable is dependent on the concentration of *E. coli* on the surface as well as the surface area available for sampling. When more of the vegetable's surface area is sampled, the easier it is to detect *E. coli* contamination. To investigate this effect and to get an idea of the average surface area needed for sampling different numbers of small squares of samples were used. For detailed experimental protocols refer to section 3.3.10.3

6.3.1.2.5. OmpT based assay for *E. coli* detection using PT_{06CA}

PT_{06CA} was further used as a reporter molecule for the pre-concentration approach as explained in the section 3.3.7.1. Three different strains of *E. coli* BL21, J96 and K12 have been used for the pre-concentration approach.

6.3.1.3. Results and discussion

6.3.1.3.1. Effect of cleaved fragments on PT_{06CA} fluorescence

The two short synthetic fragments were combined with intact cLL37_{FRRV} in different ratios and subsequently mixed with PT_{06CA}. The results indicate that the shorter peptides do not contribute to the fluorescence intensity when added to PT_{06CA} (Figure 6.3.2) as compared to minor contribution when added to PTAA (Figure 6.1.8).

Figure 6.3.2: Fluorescence intensity of PT_{06CA} at 600 nm when mixed with different ratios of intact and cleaved cLL37_{FRRV}.

Also, when mixed with increasing proportions of the longer intact peptide, showed an enhancement in the overall fluorescence intensity (Figure 6.3.2). This demonstrates that only the intact cLL37_{FRRV} contributes to the enhancement in fluorescence intensity while the shorter peptide fragments resulting from OmpT's enzymatic activity do not.

6.3.1.3.2. Approach - 1: Soaking food samples in contaminated water

The results of the approach 1 are presented in Figure 6.3.3 B and is a plot of the absorbance values of the growth media at 600 nm for the different samples and controls. As expected, control samples of lettuce and cabbage exhibit very low absorbance values indicating no bacterial growth. However, the spinach control shows a high absorbance value indicating *E. coli* contamination. It appears that the spinach used in this experiment was already contaminated before its packaging was opened. Since the experiment was not carried out under aseptic conditions, it is difficult to confirm the actual source of contamination. After the first soak of 5 min, all vegetable samples and the positive control show high absorbance values indicating bacterial growth.

Figure 6.3.3: A) Absorbance measurements at 600 nm indicate bacterial growth, indicating that *E. coli* from vegetable's surface was captured into growth media. *E. coli* introduced onto the vegetable surface by soaking samples in contaminated water. B) Soaking samples in contaminated water.

When compared to the second wash where very little bacterial growth is observed, it is clear that most of the *E. coli* is released into the growth media during the first 15 min. From the above results, it is not possible to comment on the capture efficiency of adhered *E. coli* into the liquid media since the initial amount of *E. coli* adhering to the vegetable's surface during the 5 min soaking step cannot be controlled. Moreover, it is expected that *E. coli* adheres variably to the different vegetable surfaces. From these results, it is clear that *E. coli* from the vegetable's surface was successfully captured into the liquid medium.

6.3.1.3.3. Approach - 2: Depositing contaminated water directly on the surface of food samples

Figure 6.3.4: A) Absorbance measurements at 600 nm indicate bacterial growth, indicating that *E. coli* from vegetable's surface was captured into growth media. B) *E. coli* introduced onto the vegetable surface by depositing contaminated water directly on vegetable's surface.

Results presented in Figure 6.3.4 A once again indicate that control samples that were not exposed to the contaminated *E. coli* water did not show any bacterial growth except for the spinach samples. However, absorbance values of samples exposed to contaminated water, indicate that a significant amount of *E. coli* came-off the vegetable's surface. However, the amount of *E. coli* cells coming-off the surface is not the same. This is an indication that the different surfaces adsorb or release *E. coli* at different levels. Despite the difference in levels of *E. coli* released from the vegetable's surface, enough *E. coli* was captured to enable a proper assay of the sample.

6.3.1.3.4. Approach - 3: Increasing sampling area for analysis of food samples soaked in contaminated water

Results of % fluorescence reduction plotted in Figure 6.3.5 A, indicate that hardly any *E. coli* was detectable when only 1 or 2 lettuce squares were used. However, when 5 or more of the squares were used, there is a significant reduction in the fluorescence intensity indicating the presence of *E. coli*.

Figure 6.3.5: *E. coli* detection improved when a greater number of the vegetable sample was analyzed. A) The OmpT-Pep-PT_{06CA} assay used to detect *E. coli* captured from varying no of vegetable samples analyzed; B) OmpT-Pep-PT_{06CA} assay samples under UV₃₆₅.

Control samples where ten squares of lettuce were soaked in DI water instead of the *E. coli* contaminated water, indicated no *E. coli* as seen from the absence of any significant reduction in % fluorescence. More *E. coli* could be captured and detected from the vegetable's surface when the sampling area was increased. Also using the PT_{06CA} in the OmpT-Pep-PT assays for detection of *E. coli* makes it possible to observe the results under the UV₃₆₅ instead of using a fluorescence reading (Figure 6.3.5 B).

6.3.1.3.5. *OmpT* based pre-concentrate assay for *E. coli* detection using PT_{06CA}

From the results presented so far, it is clear that PT_{06CA} can be used to distinguish between intact and cleaved LL37_{FRRV}. Hence three different strains of *E. coli*: BL21, K12 (ATCC® 700926™) and J96 were used for sensing. The samples were prepared as explained in the previous section (6.1.2.4). To these samples, PT_{06CA} was added and the fluorescence intensity at 600 nm recorded.

Figure 6.3.6: A) Detection of different strains of *E. coli* and their LOD. Fluorescence intensity of PT_{06CA} when mixed with LL37_{FRRV} treated with different *E. coli* strains while using the pre-concentrate method. 200 μ L (light gray), 2 mL (gray) and 20 mL (dark gray) of sample volumes were used for detecting 10^8 , 10^7 , 10^6 - 10^3 CFU/mL of *E. coli*, respectively. B) The samples observed under UV₃₆₅.

Results indicate a significant reduction in PT_{06CA}'s fluorescence intensity for samples containing K12 and J96 strains, suggesting that these *E. coli* strains were able to cleave LL37_{FRRV} (Figure 6.3.6 A blue and red). Control samples containing the *E. coli* BL21 strain did not show any reduction in PT_{06CA}'s fluorescence intensity (Figure 6.3.6 A black). Moreover, the reduction in PT_{06CA}'s fluorescence is consistent with the different levels of OmpT expressed on the surface of different *E. coli* strains. For the two wild-type strains, K12 and J96, the limit of detection (LOD) which is defined here as a 20% change in fluorescence intensity, was 10⁵ and 10⁶ CFU/mL, respectively (Figure 6.3.6A blue and red). These are no doubt, high *E. coli* concentrations, and it would be much more useful to detect low or very low *E. coli* concentrations. However, by using a different polythiophene, PT_{06CA}, makes it possible to achieve these limits of detection using a UV lamp. This not only makes the OmpT-Pep- PT_{06CA} assay easy to use and fast but also the cost of the instrument is further reduced.

6.3.1.4. Conclusions

Studies with store-bought lettuce, cabbage, and spinach confirm that bacterial contamination can be detected on these vegetables when they are exposed to contaminated water with *E. coli* levels as low as 10 CFU/mL in 7 h. This was achieved by simply capturing the *E. coli* on the vegetable's surface into a liquid medium that was later assayed using the OmpT-Pep-PT_{06CA} assay. With further studies, the LOD could be reduced to 1 CFU/mL. It was also demonstrated that *E. coli* from the surface of vegetables could be easily captured into a liquid medium without introducing any complex processing steps and at the same time without significantly increasing the total assay time. The new screened PT, PT_{06CA}, anionic polythiophene with a slightly longer carbon tail that is more sensitive than PTAA was used for OmpT-Pep-PT_{06CA} assay. By using this new material, it was possible to slightly improve the LOD for the pre-concentration method. Most significantly, with this new material, the need for a spectrophotometer to read the assay results is completely eliminated. The results of the assay can be easily detected with the naked eye when the samples are viewed under visible or UV light (Figure 6.3.5 and Figure 6.3.6). This is a significant improvement over the previous material.

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Chapter 7

Summary, Benchmarking and Future work

This thesis has investigated the feasibility of using OmpT protease for developing an assay for E. coli detection in water and food. The three pillars of this OmpT-Pep-PT assay: i) OmpT protease ii) Optimised peptide substrate iii) Polythiophene have been studied and characterised in previous chapters. This chapter briefly summarises the findings and work performed in this thesis. This chapter further benchmarks the OmpT-Pep-PT assay with commercially available kits and lab-based detection methods for E. coli detection in water and food samples. This chapter also highlights the future research work proposed on the OmpT-Pep-PT assay and multiplexing.

7.1. Summary

The focus of this thesis was to understand the substrate preferences of an omptin membrane protease, in order to develop a protease-based assay for detecting bacterial pathogens such as *E. coli* and *Salmonella*. These pathogenic bacteria are significant contributors to foodborne infections throughout the world. This thesis approached the assay development in a way that it built on three critical inter-related pillars i) Protease - OmpT ii) Peptide substrate - cLL37_{FRRV} and iii) A reporter polymer - Polythiophene. The hypothesis and findings related to these pillars have been described in detail in Chapter 4, 5, and 6, respectively. These three pillars were combined to develop the OmpT-Pep-PT based assay for *E. coli* detection which is fast, easy to use, and cheap when compared to most commercially available kits. A similar approach can be used for developing assays for *Salmonella* which has PgtE on the outer membrane and which belongs to the same family of proteases as OmpT.

Chapter 4, which focusses on the first pillar – OmpT protease, describes the expression, purification, autoproteolysis, and LPS binding aspects of OmpT. Findings of this chapter suggest that only the misfolded/unfolded OmpT undergoes autoproteolysis. Hence, the reduction or elimination of OmpT autoproteolysis depends on better-refolding strategies. A refolding efficiency of 60-70 % was achieved for OmpT in this thesis. The refolding efficiency can be improved using different detergents or lipid environments, which represents its natural environment for folding.¹ The **stated problem** of understanding autoproteolysis and limiting it **was partially achieved**. Based on the findings it is now understood that OmpT autoproteolysis is only restricted to unfolded population of OmpT. However, this autoproteolysis could not be eliminated because 100 % protein refolding efficiency can't be achieved. Another critical factor for OmpT is LPS, which is critical for its activity. An LPS binding site composed of five amino acids was previously predicted for OmpT. Although LPS is critical for OmpT's activity, the findings of this chapter suggest the predicted LPS binding site is not critical for OmpT's. However, it cannot be excluded that there are multiple LPS binding sites that are involved in OmpT's activity. This needs to be further characterised using NMR or biophysical techniques that can help elucidate the structure-activity relationship of OmpT and LPS.

Chapter 5 focusses on the substrate optimization for OmpT by screening rationally designed peptide libraries using a high throughput screening technique, SAMDI-MS. The screening of the position scanning library consisting of 76 peptides confirmed an absolute preference for charged basic residues at the P1 and P1' position. Further, by screening a combinatorial peptide library composed of 361 different peptides, 12 tetrapeptides were identified for which OmpT exhibited better activity compared to a previously designed ARRA peptide.² When the best tetrapeptide sequence was substituted into a more extended peptide sequence, it lead to a 400-fold improvement in OmpT catalytic efficiency, with a k_{cat}/K_m value of $6.1 \times 10^6 \text{ mol}^{-1} \text{ s}^{-1}$, as compared to the original peptide substrate. This chapter further makes an important conclusion that wild-type and mutant OmpTs displayed significant differences in their substrate specificities, demonstrating that even modest mutants may not be suitable substitutes for the native enzyme. The optimized peptide cLL37_{FRRV} was further used for developing an assay for *E. coli* detection in water. While position P2, P1, P1' and P2' were optimized for OmpT, modifying amino acids on either side of the cleavage site (P3, P3' and beyond) could potentially yield even better peptide substrates. SAMDI-MS can be further used for optimizing such peptides and can also be used to identify peptide sequences that inhibit OmpT activity. The **stated problem** of optimization of peptides substrate for OmpT was **achieved** by identifying a tetra peptide FRRV which when inserted into a natural substrate resulted in a 400-fold improvement in OmpT catalytic efficiency.

Chapter 6 describes the development of OmpT based assay for *E. coli* detection using an optimised peptide substrate (cLL37_{FRRV}) and polythiophene (PTAA) as reporter. Part A of the chapter deals with the development of the assay towards *E. coli* detection in water samples. The OmpT-Pep-PTAA assay was able to detect wild-type K12 and J96 *E. coli* strains in water at concentrations as low as 1 CFU/mL within 6 and 8 h, respectively. Part B of the chapter focusses on improving the OmpT-Pep-PTAA assay. Hence, several PTs with varying side chain length were screened with different peptide sequences that varied in their overall charge, helicity, and composition. Initial findings concerning these parameters are reported in this section, but still, detailed studies are required to understand the mechanism of peptide and PT interactions fully. Presence of a cysteine at the N-terminal of the peptide showed a consistent increase in fluorescence as compared to a non-thiolated peptide. Based on the findings, a PT with a longer side

chain (PT_{06CA}) was used for OmpT-Pep-PT_{06CA} assay. Part C of this chapter focused on the strategies that helped retrieve and detect *E. coli* from the surface of green leafy vegetables, which are most prone to contamination. The use of PT_{06CA} as a reporter molecule in detecting *E. coli* retrieved from the surface of leafy vegetables improved the overall assay as it was now possible to observe the results under UV₃₆₅ with the naked eye, instead of relying on a fluorescence readout. While these results are still preliminary, further testing with more food samples is required to conclude on the most optimal strategy to retrieve *E. coli* from food samples in order to make *E. coli* detection methods more robust. The OmpT-Pep-PT assay is easy to use, fast and cheap, which can be further improved with a potential to develop into a household *E. coli* detection kits. Optimization of the parameters such as PTs, peptides for multiplexing, and prototype development are the milestones to be achieved next. The **stated target** of developing an assay for detection of 1 CFU/mL of *E. coli* in water within 10 h was **achieved** using OmpT-Pep-PT assay.

7.2. Benchmarking and future work

7.2.1. Prototype development for OmpT-Pep-PT assay for *E. coli* detection

The OmpT-Pep-PT assay has been tested for detection of *E. coli* in water and some food samples like green leafy vegetables. *E. coli* contamination is mostly related to these green leafy vegetables, and since the assay is easy to use, cheap and fast, it has the potential to be used in the field. In order to understand the commercial potential of the OmpT-Pep-PT assay for *E. coli* detection, the results of the assay were compared with the results reported for the commercially available *E. coli* detection kits as well as the lab-based detection methods. The comparison was based on four different parameters: price, assay time, the limit of detection and ease of use. Price was majorly based on the instrumentation, chemical and human resource requirement and categorized into cheap, moderate and expensive. Ease of use for an assay was defined by the size of the sphere used for representation, see Figure 7.1 and Figure 7.2. Smaller spheres represented the need of the trained user to perform the assay and larger assays indicate as easy to use, and no trained user was required.

While considering PCR based detection approaches available in the market (Figure 7.1 black spheres), their LOD is good but at the same time the cost, ease of use, and time

required for assaying are on the higher side. The lateral flow assays or colourimetric sensors are easy to use, but the assay time is comparatively larger (Figure 7.1 green and cyan solid sphere). The OmpT-Pep-PT preconcentration assay has a higher limit of detection compared to other approaches (Figure 7.1 dark red sphere). However, the LOD was reduced to 1 and 10 CFU/mL by introducing a pre-culture step with a total assay time of 6-8 h.

Figure 7.1: Comparison of commercially available kits for *E. coli* detection in water and food samples.

When compared to the OmpT-Pep-PT assay, most of the lab-based detection methods (PCR, immuno, electrochemical etc.) had a slightly lower overall assay time, but these assays are expensive and at the same time, require a trained user to handle the instrument and interpret the results. The OmpT-Pep-PT assay with the preculture step is easy to use, cheap, sensitive with a 1-10 CFU/mL LOD, and with competitive assay times of 6-8 h. The OmpT-Pep-PT assay has the potential for commercialization and development of a prototype is the next step. For this, the assay will be further tested with various fruits and vegetables to optimize the strategies to retrieve *E. coli* from the surface (swabbing samples, vortexing, etc.). The two main strategies to test are: 1) Use of ziplock bag to collect the water used to rinse the food samples followed by filtration of the sample to retrieve *E. coli*; 2) Use a swab to retrieve *E. coli* from the surface of samples and preculture in media before testing using OmpT-Pep-PT assay.

Figure 7.2: Comparison of the lab-based detection methods for *E. coli* detection in water and food samples.

The outer membrane protease, OmpT based detection of a bacterial pathogen has been established for *E. coli* detection in water and specific food samples. The OmpT based *E. coli* sensing method is a sensitive and rapid approach, with a detection limit of 1 CFU/mL in 6-8 hrs. However, the specificity of the assay is also an important aspect. Use of bacterial pathogen-specific proteases for detecting them gives an advantage of being more specific by designing better/specific peptides substrates. Designing specific peptides substrate for different omptin proteases like PgtE and Pla is being worked on as the part of future work. Developing specific peptide substrate for proteases present on various bacterial pathogens will help in developing sensitive and specific detection assays. Since *E. coli* and *Salmonella* are one of the major causes of food borne infections, they are the major focus of initial research. The assay can be easily modified with use of different peptides substrates specific to different bacterial pathogen proteases. Bacterial pathogens such as *Salmonella* is also a major contributor to food and waterborne infections. They have outer membrane protease PgtE, which belongs to Omptin family of protease. Hence, the next step would be to expand the knowledge gained from OmpT, to develop a similar assay for the detection of *Salmonella* and extend the assay to a multiplex system for simultaneous detection of *E. coli* and *Salmonella*.

The solution assays for *E. coli* detection will be further integrated with smartphone and app based approaches for field testing and better signal readout. This thesis envisions to develop a small tube or Ziplock bag based chambers for pre-culturing step before *E. coli* testing in case of very low concentrations. The samples will be rinsed with water inside the tube or Ziplock bag to detach the *E. coli* present on the surface. The samples will be kept for preculturing in order to increase the concentration of *E. coli* before testing. The peptide substrate digestion will be performed in these chambers followed by PT addition. The signal readout under a portable or handheld UV lamp/torch will be performed using smartphone camera. Also, the use of a small attachment on the phone camera with fixed excitation and emission wavelength can be employed for signal readout using the app. The phone based app will analyze the color and fluorescence change between the sample and control to give the results, which could be further uploaded alerting the required agencies for control measures in case of positive contamination found. Similar approach can be used for *Salmonella* testing in fielding by designing substrates specific to PgtE. This would be developed as an array format assay which would be analyzed using mobile phone app and use pattern analysis tools to confirm the presence of *E. coli*, *Salmonella* or both.

7.2.2. PgtE based assay development for *Salmonella* detection

PgtE is another outer membrane protease of the omptin family present on *Salmonella* and is involved in virulence and resistance to antimicrobial peptides. PgtE also degrades short peptides and α -helical cationic AMP (i.e. LL37, C18G, Cap11, Cap18, Melittin).³ For developing an assay for *Salmonella* based on PgtE, like OmpT-Pep-PT assay, an optimized peptide substrate is required. Hence PgtE protease would be recombinantly expressed in IBs, refolded and purified as was performed in the case of OmpT. In order to improve the refolding of the PgtE, different detergents and lipid environment will be tested. Preliminary results suggest successful refolding in detergent micelles using the protocol employed for OmpT refolding (data not shown). With successful expression and purification of PgtE next comes the designing of peptide substrate library. Since PgtE belongs to Omptin family of proteases, the peptide substrate library design would begin with ARRA. The screening of a position scanning library would give a better understanding of PgtE preference for amino acids at different positions (P2-P2'). Based on which a combinatorial peptide library will be designed to optimize the amino acids

in the vicinity of PgtE cleavage site in the substrate. The natural substrate for PgtE includes the antimicrobial peptides and complement factors B (B) and H (H).⁴ In order to improve the K_m , PgtE interaction with the substrate, the interaction between the enzyme active site and the amino acid sequence surrounding the scissile bond in the substrate need to be improved. Hence, the optimised short peptides substrate from combinatorial peptide library will be substituted in PgtE's natural substrate to improve PgtE's catalytic efficiency. By designing substrate specific to OmpT and PgtE respectively, it is possible to develop a multiplex system capable of detecting both *Salmonella* and *E. coli*. Substrate specific to each protease will be designed in order to achieve multiplexing. The multiplexing approach will be tested in solutions first and further translated into lateral flow assays for easy to use household kits.

As shown in chapter 6 part B, PTs interact with the various peptides differently resulting in a different fluorescence response for each peptide. This fluorescence response depends on the peptide sequence, which defines the properties such as helicity, charge, and enzyme's catalytic activity. An array of peptides against which OmpT and PgtE have different catalytic efficiency will be developed. Since both the enzyme will have different catalytic activity against the peptides in the array, they will be cleaved at different rates. The peptide arrays treated with OmpT and PgtE when mixed with PTs will show an optical response which is envisioned to act as a fingerprint for each enzyme. This fingerprint approach based on the enzyme's catalytic activity against a peptide and PT optical response when mixed with these peptides would be explored for translation to *E. coli* and *Salmonella* multiplexing.

7.2.3. Screening of different conducting polymers for visual sensing and improved optical response

The OmpT-Pep-PT assay is based on the interaction of peptide and PTs via electrostatic interactions. The anionic functional groups present on PT side chains in the present study are carboxylic groups but varying the groups with sulphate or phosphate, can change the binding of peptides and PTs. As shown in chapter 6 part B, the side chain length does affect the fluorescence increase, hence different functional group involved in the interactions between PT-peptide can help in designing better PTs with an improved optical response. The Liedberg group has previously reported the use of tailored polythiophene for detection of nucleic acids by varying the PT's side groups

and by combining homo and copolymers.⁵ More of these variants of PTs will be explored for developing the multiplex assay for *E. coli* and *Salmonella* in an array format.

7.2.4. Characterisation of PTs with different peptides to understand the effect of N-terminal cysteine, charge, and helicity of peptide

As reported in chapter 6 part B, N-terminal cysteine has resulted in an enhanced fluorescence increase. The reason for the enhanced fluorescence response is not known but might be due to dimerisation of peptide or due to the thiol group from cysteine interacting with the PT. This will be investigated further to understand the reason for the enhanced fluorescence increase with the addition of thiol. Peptides will be dimerised and reduced to different levels to see if there is any relation between the increase in fluorescence and the amount of dimerised peptide.

Chapter 6, part B also indicated that the presence of positive charge on the peptide is an absolute necessity for the interaction between PT and peptide. However, preliminary results suggest (data not shown) excess charge on peptide results in aggregation and the concomitant decrease in fluorescence. Thus, charge optimisation might help designing peptides with a higher optical response. The effect of positive charge will be studied by screening peptides with increasing net positive charge with the different PTs to identify the amount of net positive charge required for maximal fluorescence increase.

The ICD data shown in chapter 6 part A and B, suggests the peptides adopt a secondary helix structure on mixing with PTs, which in turn suggests the propensity of a peptide to form helix can significantly affect the fluorescence. Peptides with increasing helicity will be designed with a fixed charge and screened against different PTs to characterise their effect. Understanding how peptide charge and helicity affect the optical response would help design a peptide substrate with better optical response with PTs.

Since N-terminal cysteine, helicity, and charge of a peptide can affect the interaction of the peptides with PTs, nano Isothermal Titration Calorimetry (nano-ITC) will be used to understand the binding between PTs and peptides along with fluorescence and absorbance spectroscopy. This would help characterise how the interactions between

peptides and PTs are correlated to N-terminal thiols, helicity, and charge, which in turn influence the absorbance and fluorescence changes in PTs.

7.2.5. Inhibitor studies for *E. coli*

Inhibitors for Omptin proteases can play an essential role in designing antimicrobial peptides against these bacterial pathogens. The screening of 361 peptide library with SAMDI-MS for OmpT identified several peptides that despite having a dibasic site did not show any activity. These peptides showing no activity are possible inhibitors for OmpT protease present on the *E. coli* and will be tested if they exhibit competitive or non-competitive inhibition. A complementary approach will also be explored by using the peptides showing good activity with OmpT (i.e. FRRV) but instead of L form of arginine, D forms of the amino acids will be used in the peptide. So, enzyme substrates can be converted into an inhibitor by making a dibasic site (Arg-Arg) to D form of arginine. These inhibitors can be developed into therapeutic targets along with cationic antimicrobial peptides.

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8. APPENDIX

8.1. List of *E. coli* detection kits

Table 8.1: - Commercial *E. coli* detection kits available in market

S. no	Method	Name	Target organism	Food matrix/ water	Pre- treatment/separation/ concentration	Limit of detection	Time	Company	Storage
1	PCR	MicroSEQ™ <i>E. coli</i> O157: H7 Detection Kit	<i>E. coli</i> O157: H7	Any Swab Type, Food, Water Samples	Enrichment and DNA extraction	1-3 CFU/25 g sample	8-18 h depending on enrichment media and volume	Thermofisher	Storage at 2-8 °C
2	LFI	Singlepath® & Duopath® Lateral Flow Tests for Immunological Detection	<i>E. coli</i> O157/EHEC/STEC/ VTEC, <i>Salmonella</i> , <i>Campylobacter</i>					Merck	
3	PCR	BAX® System PCR Assay for <i>E. coli</i> O157: H7 MP	<i>E. coli</i> O157: H7	Raw ground beef, beef trim, produce	Culture enrichment for 8-24 h	10 ⁴ CFU/mL, after enrichment	8-24 h culture and 3.5 h PCR	Hygiena	Storage at 2-8 °C

4	RT PCR	BAX® System Real-Time PCR Assay for <i>E. coli</i> O157: H7	<i>E. coli</i> O157: H7	Raw ground beef, beef trim, produce	8-10 h, depending on food type, sample volume and enrichment media.	10 ⁴ CFU/mL, after enrichment	8-10 h culturing and 1 h PCR	Hygiena	Storage at 2-8 °C
5	Immuno Assay	FOODCHEK™ – <i>E. COLI</i> O157 TEST KIT	<i>E. coli</i> O157	Ground beef and beef trims, Carcass sponge	7 h of enrichment, filtration, temperature - controlled incubation	1CFU/375 g beef, 0.05 CFU/100 cm ²	~ 10-12 h total	FOODCHEK™	FOODCH EK™ reader required
6	PCR	iQ-Check® <i>E. coli</i> O157:H7 PCR Detection Kit	<i>E. coli</i> O157:H7		Media enrichment	1 – 10 CFU/ 25 g sample	12 h after enrichment	Bio-Rad	
7	DIP sticks	O157 Coli-Strip	<i>E. coli</i> O157:H7	Stool specimens	Broth enrichment	10 ⁴ CFU/mL	15 min after enrichment	Corisbio	
8	Plating - Chromogenic media	RAPID'E.coli O157:H7 Medium	<i>E. coli</i> and <i>E. coli</i> O157:H7				48 h	Bio- rad	
9	ELISA	Solus <i>E. coli</i> O157 ELISA	<i>E. coli</i> O157: H7	Raw beef meat products (seasoned or not) raw milk and dairy products, vegetables and environmental samples	Enrichment	10 ⁴ - 10 ⁵ CFU/mL	18-22 h (2 h for ELISA)	Solus scientific	

10	LFI	Reveal® for <i>E. coli</i> O157:H7	<i>E. coli</i> O157:H7		8 - 20 h culture enrichment	1 CFU/25 g; 1 CFU/375 g	15 minutes (after 8/20-h enrichment)	Neogen	2-30 °C
11	ELISA	VIDAS® UP ECPT	<i>E. coli</i> O157: H7	Raw beef and beef trim, irrigation water, raw vegetables	7 to 24 h culture enrichment for raw meat; 15-24 h other matrices		50 min for assay + enrichment time 7-24 h	Biomérieux-industry	
12	LFI	VIP® Gold - EHEC	<i>E. coli</i> O157: H7	Foods and environmental samples	Enrichment 8 h minimum			Biocontrol – MiliiporeSigma	Room Temperature
13	PCR	3M™ Molecular Detection Assay <i>E. coli</i> O157	<i>E. coli</i> O157:H7	Food samples	Enrichment	1-5 CFU	~ 1-2 h for PCR	3M	2-8 °C
14	PCR	Foodproof® <i>E. coli</i> O157 Detection Kit	<i>E. coli</i> O157: H7	Raw meat, soybean sprouts or raw milk	Enrichment 18-24 h	10 ³ - 10 ⁴ CFU/mL in enrichment cultures	PCR ~1.5 h	Biotecon Diagnostic	Store kits 15 - 25 °C
15	Real-time PCR	GENE-UP®	<i>E. coli</i> O157:H7, <i>Salmonella</i>		Enrichment 8-24 h			Biomérieux-industry	
16	PCR	<i>E. coli</i> O157 Food Safety Kit	<i>E. coli</i> O157: H7		Enrichment 10 h, Extract DNA	1 CFU/mL	~ 2 h	Instant Labs	2-8 °C
17	PCR	ANSR® for <i>E. coli</i> O157: H7	<i>E. coli</i> O157: H7	Raw beef, spinach and sprout irrigation water		1CFU/analytical unit 10 ⁴	12-24 h	Neogen	

						CFU/mL post enrichment			
18	PCR	GeneDisc® Technologies - <i>E. coli</i> O157(:H7)	<i>E. coli</i> O157(:H7)		Enrichment 8 h and DNA extraction	1 CFU/25 g of food sample 1 CFU/375 g raw beef	~2 h	Pall Corporation	
19	PCR	Assurance GDS® for <i>E. coli</i> O157: H7	<i>E. coli</i> O157(: H7)	Validated for ground beef, beef trim, leafy greens, juices, and sprout irrigation water	Enrichment and IMS		8 h for 25 g samples 10 h for composite 375 g samples	BioControl	2-8 °C
20	RT PCR	Mericon VTEC stx1/2 Kit	Shiga toxin - <i>E. coli</i>	Food, animal feed, and pharmaceutical products.	Enrichment culture and DNA isolations (~24 h)		18-27 h complete process (PCR 2 h)	Qiagen	
21	LFI and anti- <i>E. coli</i> O157 antibodies and colloidal gold conjugate on surface	Lateral Flow System for <i>E. coli</i> O157	<i>E. coli</i> O157(: H7)	Ground beef and boneless beef	8 h for 25 g samples 10-15 h for 375-g samples	1-4 CFU/25g sample	10-15 min after enrichment	Hygiena	15-30°C

22	LFI	RapidChek® <i>E. coli</i> O157	<i>E. coli</i> O157	Foodstuffs (ground beef and boneless trim beef) and environmental applications			8-18 h including enrichment	Romerlabs	
23	Enzyme Immunoassay	TRANSIA® AG	<i>E. coli</i> O157: H7	Meat, dairy, poultry, fruit, nuts, and more	Enrichment culture		Beef in 10 h and 20 h for matrix	Biocontrolsys	2 - 8°C
24	LIQUID CRYSTAL BIOSENSORS	<i>E. coli</i> O157 Antibody Kit	<i>E. coli</i> O157	Beef and spinach	Enrichment culture 10-15 h, Immunomagnetic separation			Crystal Diagnostics	
25	Latex agglutination	Pro-Lab Diagnostics™ Prolex™ - Blue <i>E. coli</i> O157 Latex Test Reagent Kits	<i>E. coli</i> O157			One colony is sufficient		Fisher scientific	2 - 8°C
26	Latex agglutination	DRYSPOT <i>E. coli</i> O157 TEST KIT	<i>E. coli</i> O157		Plating of the samples is required			Thermo Scientific	
27	Colourimetric assay	AquaVial <i>E. coli</i> and Coliform Water Test Kit	<i>E. coli</i> O157:H7	Water	None	1 CFU/ mL	24 h at 37 °C	Aquabsafe	
28		WaterSafe Bacteria Test	Coliform bacteria	Water	None		48 h	Watersafe	

29	Colourimetric assay	MicroSnap™ <i>E. coli</i> Test Kit	<i>E. coli</i>	Water	None	< 10 CFU in 8 h	8 h	Hygiena	
30	Colorimetric assay	Total Coliform/ <i>E. coli</i> Indicator Bacteria Test Kit	<i>E. coli</i> and Coliform bacteria	Water	None	1 CFU/100 mL and non- <i>E. coli</i> coliform 10 CFU/100 mL	24-48 h	LaMotte Company	
31	Colourimetric assay	Drinking Water Test Kit (<i>E. coli</i> / Coliform Test)	<i>E. coli</i> and Coliform bacteria	Water	None	1 CFU/100 mL	18-24 h	Rakiro	
32	Colourimetric assay	Compartment Bag Test (CBT) Quantitative <i>E. coli</i> Water Quality Test Kits	<i>E. coli</i>	Water	None		24-48 h	Aquagenx	
32	Coliform enzyme β -galactosidase and the <i>E. coli</i> enzyme β -glucuronidase based colorimetric assay	Colilert	Total coliforms and <i>E. coli</i>	Water	None		24 h or less	IDEXX Laboratories	

Table 8.2 : - Lab based *E. coli* detection methods

Method	Target organism	Food matrix / water	Pre-treatment/separation/concentration	Limit of detection	Detectable range in food	Time	Ref
Nucleotide based assays							
PCR	<i>Salmonella enteritidis</i> and <i>Listeria monocytogenes</i>	Milk	Magnetic nanoparticles	8-13 CFU/mL	-	-	¹
PCR	<i>E. coli</i> O157:H7	Ground beef	Centrifugation and titanous hydroxide for bacterial immobilization	10 ³ CFU/g of ground beef			²
PCR	<i>E. coli</i> O157:H7	Ground beef	1) Filtration and centrifugation and lysed using enzymatic digestion and successive freeze/thaw cycles. 2) DNA extraction by phenol/chloroform extraction	10 ³ CFU/g of ground beef		12 h	³
Multiplex PCR	<i>S. aureus</i> , <i>E. sakazakii</i> , <i>E. coli</i> O157:H7, <i>V. parahaemolyticus</i> , <i>Salmonella spp</i>	Milk, chicken	8 h enrichment/preculture	1 CFU/mL milk or chicken meat homogenate		18 h	⁴
Multiplex PCR	<i>Salmonella spp.</i> , <i>L. monocytogenes</i>	Whole milk, Cheese, Ice cream, Beef	Immunomagnetic beads	10-10 ⁴ CFU/mL			⁵

Quantitative PCR	<i>S. enterica</i>	Chicken rinse	Two step filtrations	7.5×10 ² CFU/100 mL		3 h	⁶
Quantitative PCR	<i>E. coli</i> O157:H7	Apple juice Raw milk Beef	None	10 ⁴ CFU/mL 10 ³ CFU/mL 10 ⁵ CFU/g	10 ⁴ -10 ⁹ CFU/mL 10 ³ -10 ⁹ CFU/mL 10 ⁵ -10 ⁹ CFU/g		⁷
Quantitative PCR	<i>E. coli</i> O157:H7 <i>L. monocytogene</i>	Ground beef Hot Dog	None	10 ³ -10 ⁸ CFU/mL 10 ⁴ -10 ⁸ CFU/mL	1 CFU/g raw ground beef (4 h enrichment) 1 CFU/g in beef hotdogs (30 h enrichment)		⁸
Quantitative PCR	<i>S. aureus</i>	Raw milk	None	10 CFU /reaction	-	6 h	⁹
Multiplex quantitative PCR	<i>E. coli</i> O157:H7 <i>Salmonella</i> spp. <i>S. aureus</i>	Spinach Lettuce		10 ³ CFU/g	-		¹⁰
Multiplex quantitative PCR	<i>E. coli</i> O157:H7 <i>S. enterica</i> <i>Typhimurium</i> <i>L. monocytogenes</i>	Lettuce Tomato Ground beef	Immunomagnetic separation	10 ² in pure culture 10 ³ spiked food		5 h	¹¹
Quantitative reverse transcription RT- PCR	<i>L. monocytogenes</i> <i>S. enterica</i>	Whole milk (WM) Ice cream (IC)	Two step centrifugation and Zirconium hydroxide for bacterial immobilization	10 ² CFU/mL (WM) 10 CFU/mL (IC)	-		¹²

Loop mediated isothermal amplification	<i>STEC E. coli</i>	Lettuce (iceberg and romaine), Sprouts (alfalfa, clover, mung bean), Spinach (baby, savoy, semi-savoy)	6-8 h enrichment 1 CFU/25 g of sample	10 ⁵ -10 ⁶ CFU/25 g	-	-	¹³
Loop mediated isothermal amplification	<i>Salmonella spp.</i>	Cantaloupe, Spinach, Tomato	Propidium monoazide for LAMP	3.4 - 34 CFU/g pure culture 6×10 ³ -10 ⁴ CFU/g spiked food samples	-	3 h	¹⁴
Loop mediated isothermal amplification	<i>ETEC E. coli</i>	Raw milk	None	547 CFU/mL	-	1 h	¹⁵
Nucleic acid sequence-based amplification	<i>E. coli</i>	Drinking water	Heat shock to cells (5-40 min)	40 CFU/mL		4 h	¹⁶
Nucleic acid sequence-based amplification	<i>S. aureus</i>	Milk	None	1-10 CFU/mL		3-4 h	¹⁷
Microfluidic lab on a chip	<i>S. enterica</i> , <i>E. coli O157:H7</i> , <i>L. monocytogenes</i>	Banana Milk Hotdog	DNA extraction	3.72×10 ⁴ DNA copies 3.58×10 ⁴ DNA copies 1.79×10 ⁴ DNA copies		14 min for PCR	¹⁸

Immuno based assays

Polyacrylonitrile fiber-based sandwich enzyme-linked immunosorbent assay (ELISA)	<i>S. typhimurium</i>	Milk and juice	None	$10^6 - 10^7$ CFU/mL		2 h	¹⁹
Single-walled carbon nanotubes enhanced direct and sandwich ELISA	<i>S. typhimurium</i>	Fat-free and 9% fat milk	None	Fat free milk: 10^3 (direct) and 10^4 (sandwich) CFU/mL; 9% milk: 10^4 CFU/mL		Direct – overnight , Sandwich – 3 h	²⁰
Gold nanoparticle (AuNP) network ELISA	<i>E. coli</i> O157:H7 and <i>S. Typhimurium</i>	Reduced-fat milk, ground beef, and pineapple juice	Immunomagnetic separation (IMS)	<i>E. coli</i> O157:H7: 3 CFU/mL in milk and beef; <i>S. Typhimurium</i> : 15 CFU/mL in juice		~ 2 h	²¹
AuNP-enhanced ELISA	<i>E. coli</i> O157:H7	Vegetable, milk and beef	Immunomagnetic separation (IMS)	6.8×10^2 CFU/mL in vegetable and milk, and 6.8×10^3 CFU/mL in ground beef		3 h	²²
Sandwich chemiluminescent immunoassay	<i>E. coli</i> O157:H7 and <i>S. Typhimurium</i>	Milk		10^5 – 10^6 CFU/mL for the <i>E. coli</i> O157:H7 and 10^6 – 10^7 CFU/mL for <i>Salmonella</i>		4 h	²³

Sandwich fluorescent immunoassay	<i>E. coli O157:H7</i> , <i>S. Typhimurium</i> and <i>L. monocytogenes</i>	Spinach, chicken, and milk		< 10 CFU/mL		2 h	²⁴
Enzyme-linked antibody aptamer sandwich assay	<i>S. Typhimurium</i>	Milk	Aptamer-modified magnetic particles	10 ³ CFU/mL	10 ³ to 10 ⁸ CFU/mL	3 h	²⁵
Multiplex LFI	<i>E. coli O157:H7</i> and <i>E. coli BL21</i>	Milk, orange juice and lettuce	Immunomagnetic separation and cell lysis	<i>E. coli</i> BL21: 10 ² CFU/mL in milk and juice and 10 ⁶ CFU/mL in lettuce; <i>E. coli</i> O157:H7: 10 CFU/mL in milk and juice and 10 ⁵ CFU/mL in lettuce		30 min	²⁶
Biosensor based assays							
SPR immunosensor	<i>E. coli</i> O157:H7	Milk, apple juice, and ground beef	None	10 ² -10 ³ CFU/mL		30 min after sample injection	²⁷
Sandwich SPR immunosensor (enzymatic signal enhancement)	<i>E. coli</i>	Spinach leaves	Centrifugation	10 ³ -10 ⁴ CFU/mL		2.5 h	²⁸
SPR immunosensor	<i>S. Enteritidis</i>	Chicken extract	Homogenized and filtered	10 ⁶ CFU/mL		~20 min	²⁹

Sandwich multi-channel SPR immunosensor	<i>E. coli O157:H7</i> , <i>S. Typhimurium</i> , <i>L. monocytogenes</i> and <i>C. jejuni</i>	Apple juice (pH 3.7 and pH 7.4)	Heat-kill and ultrasonication of cells, and pH adjustment of apple juice pH 7.4	3.4×10^3 - 1.2×10^5 CFU/mL		-	30
SPR biosensor	<i>E. coli O157:H7</i>	Cucumber and ground beef	Homogenised and filtered	3.0×10^4 CFU/mL in cucumber and 3.0×10^5 CFU/mL in beef sample		-	31
Colorimetric biosensor	<i>S. aureus</i> and <i>E. coli</i>	Apple juice	None	10^8 CFU/mL			32
Light scattering immunosensor	<i>E. coli O157:H7</i>	Iceberg lettuce	Homogenised and filtered	10 CFU		<6 min	33
FRET-based optical fiber biosensor	<i>S. Typhimurium</i>	Pork		10^5 CFU/mL		5 min	34
Fluorescence nucleic acid biosensor	<i>S. enteritidis</i>	Milk	Centrifugation and lysis of cells	1.5×10^2 CFU/mL		5.5 h	35
Amperometric immunosensing strip	<i>E. coli O157:H7</i>	Milk	Centrifugation, Lipid and protein removal	5×10^3 CFU/mL		1 h	36
Voltammetric sandwich immunosensor	<i>E. coli O157:H7</i>	Apple juice	Immunomagnetic separations and pH adjustment	5×10^3 CFU/mL		80 min	37
Voltammetric sandwich immunosensor	<i>E. coli O157:H7</i> , <i>Campylobacter</i> and <i>Salmonella</i>	Milk	None	400, and 800 CFU/mL	-	-	38

Voltammetric sandwich immunosensor	<i>Salmonella</i>	Milk	Immunomagnetic separation	10 ³ CFU/mL	10 ³ to 10 ⁶ CFU/mL	1 h	³⁹
Potentiometric aptamer biosensor	<i>E. coli</i> CECT 675 (surrogate for <i>E. coli</i> O157:H7)	Milk and apple juice	Filtration and washing	6 CFU/mL in milk and 26 CFU/mL in apple juice	10 ⁴ CFU/mL		⁴⁰
Impedance immunosensor	<i>E. coli</i> O157:H7	Grounded beef	Immunomagnetic separations	7.4 × 10 ⁴ Culture media and 8.0 × 10 ⁵ CFU/mL in Grounded beef		35 min	⁴¹
Capacitive immunosensor	<i>E. coli</i> O157:H7	Pasteurized milk, ground beef, and spinach	Filtration	10 ³ , 10 ⁴ and 10 ³	10 ² - 10 ⁵ CFU/mL	1 h	⁴²
Impedimetric immunosensor	<i>S. Typhimurium</i>	Fat-free milk	None	5×10 ³ CFU/mL	10 ³ - 10 ⁷ CFU/mL		⁴³
Impedimetric immunosensor	<i>E. coli</i> O157:H7	Whole milk	none	83.7 CFU/mL	10- 10 ⁵ CFU/mL		⁴⁴
Impedimetric immunosensor	<i>E. coli</i> O157:H7	Ground beef and cucumber	Homogenised and filtered	1.5×10 ² CFU/mL	1.5×10 ² -10 ⁷ CFU/mL		⁴⁵
QCM DNA biosensor	<i>E. coli</i> O157:H7	Apple juice, milk and grounded beef	Centrifugation, DNA extraction and PCR amplification	5.3×10 ³ CFU/mL	10 ² - 10 ⁶ CFU/mL		⁴⁶
MIP based QCM and SPR	<i>E. coli</i>	Apple juice		10 ⁸ CFU/mL			⁴⁷
Cantilever immunosensor	<i>E. coli</i> O157:H7	Grounded beef	None	10 CFU/mL		10 min	⁴⁸

Magnetoelastic (ME) phage-based biosensor	<i>S. Typhimurium</i>	Tomato, cantaloupe and spinach leaf	None	5×10^5 CFU/mL		~30 min	49–51
Hydrogel based colorimetric assay	<i>E. coli</i>	Water (100 mL sample)	None	4×10^6 - 10^5 CFU/mL in 5 min and 4×10^4 - 400 CFU/mL in 1 h			52
Polymer and peptide	<i>Discriminate 14 bacteria</i>	Water	None	$\sim 10^6$ CFU/mL			53

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