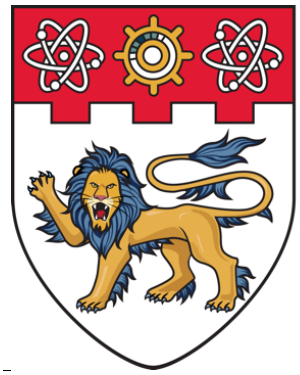

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

DEVELOPMENT OF BIOSENSORS FOR DETECTION OF BACTERIAL TOXINS AND CONTAMINANTS IN COMPLEX FOOD MATRICES

NEVENA KLISARA

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

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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.

13.08.2019.
Date

Klisara Nevena
NEVENA KLISARA

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.

13.08.2019

Date

Bo Gunnar Liedberg

Authorship Attribution Statement

This thesis contains material from 2 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 4 is published as Klisara, Nevena, et al. Functional fluorescence assay of botulinum neurotoxin A in complex matrices using magnetic beads. *Sensors and Actuators B: Chemical* 281 (2019): 912-919. DOI: 10.1016/j.snb.2018.10.100

The contributions of the co-authors are as follows:

- Prof Bo Gunnar Liedberg and Dr Palaniappan provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts along with Dr Palaniappan. The manuscript was revised by Dr Willem Haasnoot, Mr Jeroen Peters and Prof Michel W.F. Nielen.
- I co-designed the study with Dr Palaniappan and Dr Willem Haasnoot and performed all the laboratory work at the Centre for Biomimetic and Biosensor Science (CBSS), NTU and Institute of Food and Safety (RIKILT), Wageningen University. I also analyzed the data.
- Results were discussed with Dr Palaniappan, Dr Haasnoot and Dr Peters.

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The contributions of the co-authors are as follows:

- Prof Liedberg and Dr Palaniappan initiated the project and edited the manuscript.
- I prepared the manuscript drafts along with Dr Palaniappan.
- I co-designed the study with Dr Palaniappan. The experiments were performed by me and FYP student Yo Mei You in the Centre for Biomimetic and Biosensor Science (CBSS).
- Results were discussed with Prof Bo Liedberg and Dr Palaniappan.

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The contributions of the co-authors are as follows:

- Prof Liedberg and Dr Palaniappan initiated the project and edited the manuscript.
- I prepared the manuscript drafts along with Dr Palaniappan.
- I co-designed the study with Dr Palaniappan. The experiments were performed by me in the Centre for Biomimetic and Biosensor Science (CBSS).
- Results were discussed with Prof Bo Liedberg and Dr Palaniappan.

13.08.2019.
Date

Klisara Nevena
NEVENA KLISARA

Abstract

This study attempts to merge novel sampling strategies and biomolecular sensing tools for the development of next generation of rapid and sensitive bioassays for detection of bacterial toxins and contaminants in complex food matrices. State of the art sampling, extraction and biosensor technologies are combined to develop prototype assays for onsite detection of toxins/proteases in contaminated food products. Considering the vast number of foodborne diseases and caused deaths, the assay for the fast and reliable analysis could assist in the early detection and subsequent prevention of foodborne illnesses. The conventional methodologies are tedious and cumbersome with requirements for trained personnel and expensive instruments. Thus, there is an urgent need to develop on-site assay to meet requirements such as low cost, fast response, selective and sensitive with no need for sophisticated instrumentations.

In this study, emphasis will be on botulinum neurotoxin (BoNT) as a model target for the development of assays, one of the most toxic substances known to man (lethal dose for humans 3-10 ng/kg). Several outbreaks of infant botulism in Australia, Italy, UK and Ireland due to contaminated dairy products and other food-botulism outbreaks have raised demands for efficient diagnostic tools to detect the toxin or the toxin-producing clostridia spores. The focus of this thesis was to develop assays where efforts emphasized on the use of synthetic peptide as recognition molecule which can achieve sensing performance sufficient enough to be utilized in the food safety monitoring and overcome limitations of the gold standard assay – the mouse bioassay. On the other hand, utilizing the peptide as a substrate provides easy and more precise detection, higher degree of robustness and stability compared to conventional approaches using antibodies. The sensing strategy is based on observing the change in fluorescent/colorimetric response due to the reduction in the number of reporter molecules (in solution or on the membrane) upon the cleavage of peptide by targeted protease. As for assaying in foods, the sample preparation plays a crucial role in successful target detection, especially at low concentration levels, therefore the magnetic beads were used as facilitators in the sample clean-up process due to their easy manipulation by the external magnetic field. Utilizing the peptide and magnetic beads,

two assays were developed. In the first assay – the solution-based assay – the reduction in the fluorescence intensity upon cleavage was observed depicting the LOD of 0.5 - 1 nM (25-50 ng/mL) in ~6h of total assay time. In order to simplify the assay by excluding the instrument for the signal read-out and unstable fluorescence dye, the solution-based assay was translated to dipstick assay with naked eye detection. In this assay magnetic beads served as mediators in the clean-up process as well as reporter molecules simplifying the overall assay performance. The dipstick assay yielded LOD of 1 nM (50 ng/mL). Further improvements in assay sensitivity, a reduction of overall assay time with more simple testing approach was accomplished with the development of sorbent incorporated membrane-based and bulb/tip clean-up integrated within dipstick assay. In this alternative approach, the dipstick assay offered clean-up and detection as one step, and better sensitivity (0.1 nM; 5 ng/mL) thanks to the stable fluorescence nanobeads. A potential application of sorbents integrated dipstick test across a wide range of foods was proven by testing liquid, solid and canned food in this study.

Assays responses for BoNT/A protease met requirements for analytically relevant levels (below that of oral toxicity; 1 µg/kg) in food matrices. Developed assays offer the avenue for excluding conventional long and tedious sample pre-treatments with utilization of magnetic beads and/or sorbents. The proposed assays deliver new approaches for on-site food safety monitoring capable of overcoming threats related to accidental contamination in the food supply chain due to improper handling of food products as well as from deliberate threats as terrorist attacks.

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Figure 5.5 Visual detection of the test line without amplification (1), with MAL-MBs

Abbreviations

AB	Antibody
ALLISA	Assay with a Large Immuno-sorbent Surface Area
BCA	Bicinchoninic Acid Protein Assay
BoNT/A	Botulinum Neurotoxin A
BoNT/A LC	Botulinum Neurotoxin A Light Chain
BoNT/E LC	Botulinum Neurotoxin E Light Chain
BSA	Bovin Serum Albumin
CA	Cellulose Acetate
CDC	Centers for Disease Control and Prevention
CIE	International Commission of Illumination
DI	Distilled Water
DIG-ELSA	Digoxigenin-Enzyme Linked Immunosorbent Assay
EC	Capillary Electrophoresis
EDC	1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide
ELICA	Enzyme-Linked Coagulation Assay
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionization
FDA	Food and Drug Administration
FNP	Fluorescence Nanoparticles
FRET	Fluorescence Resonance Energy Transfer
FTIR	Fourier Transform IR
GC-MS	Gas Chromatography Mass Spectrometry
GCB	Graphitized Carbon Black
GF	Glass Fiber
GNP	Gold Nanoparticle
HC	Heavy Chain
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LFA	Lateral Flow Assay

LLE	Liquid Liquid Extraction
LOD	Limit of Detection
LPS	Lipopolysaccharides
LSPR	Localized Surface Plasmon Resonance
MAL-MB	Maleimide Magnetic Bead
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
MES	2-(N-morpholino) Ethanesulfonic Acid
MF	Microfiltration
MNP	Magnetic Nanoparticle
NC	Nitrocellulose
NF	Nanofiltration
NHS	N-hydrox- ysulfosuccinimide
NIR	Near Infrared Spectroscopy
NP	Nanoparticle
OTA	Ochratoxin
PAGE	Polyacrylamide Gel Electrophoresis
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate-Buffered Saline
PCB	Polychlorinated Biphenyls
PCDD	Polychlorinated Dibenzo-p-Dioxins
PCDF	Dibenzofurans
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PES	Polyether Sulfone
POPs	Persistent Organic Pollutants
PSA	N-[3-(trimethoxysilyl) propyl] ethylenediamine
PVDF	Polyvinylidene Difluoride
QD	Quantum Dot
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RT	Room Temperature
SA-MBs	Streptavidin Magnetic Beads

SAMDI-MS	Self-Assembled Monolayers for Matrix-assisted Laser Desorption/Ionization Mass Spectrometry
SAPE	Streptavidin-R-phycoerythrin
SEB	Staphylococcal Enterotoxin B
SEM	Scanning Electron Microscopy
SNAP-25	Synaptosomal-Associated Protein 25
SNARE	Soluble N-Ethylmaleimide-Sensitive-Factor Attachment Protein Receptor
SPE	Solid Phase Extraction
SPR	Surface Plasmon Resonance
ST-1	Strategy 1
ST-2	Strategy 2
TLC	Thin-Layer Chromatography
TTX	Tetrodotoxin
UF	Ultrafiltration
UV/VIS	Ultraviolent- visible Spectroscopy
VAMP	Vesicle- Associated Membrane Protein
VFA	Vertical Flow Assay
ZP	Zeta Potential
μ-PAD	Microfluidic Paper-based Device

Chapter 1

Introduction

Over the past few decades, necessity for biosensors has expanded for food quality monitoring, since various foodborne illnesses have been caused by numerous harmful contaminants and toxic compounds. Therefore, emphasis was laid on development of rapid detection techniques for prevention and elimination of threats. The aim of this thesis is develop novel biomolecular sensing tools for rapid, sensitive and selective detection of toxins and contaminants in complex food matrices. This chapter provides a short overview of the thesis including hypotheses/problem statements, objectives and scope of this study and a brief description of each chapter of this thesis.

1.1 Background and Significance

Food safety has been a major concern for several decades as any contamination adversely affects the health of consumers. Improperly processed, stored and handled food could cause fatal diseases upon intake, either from microbial or chemical contamination. These diseases are of significant concern because of their high mortality rate, if not treated immediately and properly. Foodborne disease are diverse in their types and severity, where symptoms related to foodborne diseases differ in intensity from mild (vomiting, nausea, diarrhoea) to severe (neuronal disease, paralysis, cancer), which can lead to the death. Billions of individuals of all ages are at risk associated with unsafe food consumption, especially children under 5 years, pregnant women, and elderly. Over the past decades, food borne outbreaks have been addressed with reported numbers of casualties. Most of these outbreaks are related to foods contaminated with pathogens, bacterial toxins, mycotoxins, viruses etc. For instance, *E.coli* outbreak caused by contaminated bean and seed sprout (50 casualties[1]), listeriosis outbreak in cantaloupes (30 casualties)[2], salmonellosis outbreak in peanuts (9 casualties)[3], botulism outbreak in home-canned potatoes (1 casualty)[4], nacho cheese (1 casualty)[5], canned hot dog chili sauce[6], etc., have been reported. Therefore, continuous monitoring of foods is required at all stages of processing, from the raw food to the ready-to-eat food, in order to diminish contamination caused by microbial and chemical agents[7]. Moreover, concerns related to food safety and quality have been increased recently due to the globalization of food production as well as climate change, which will further continue to modify food safety risk regulations and requirements for contaminants detection.

As for the food safety assessment at laboratory level, various methodologies have been utilized including conventional microbial culturing followed by sophisticated analysis using techniques such as HPLC, TLC, LC-MS, GC-MS, etc., with varying sensitivity and accuracy of detection. The main limitation of conventional methods is the time consuming analysis, lasting from several hours to few days, usually requiring pre-treatment processes. Additionally, these methodologies are laborious, expensive, space consuming, and require trained personnel. Since the conventional methods are inadequate for rapid screening in

food analysis, development of fast, facile, miniaturized and cost-effective biosensors is required. One of the major concerns in the development of biosensors for food quality analysis is the limit of detection (LOD). The sensitivity of biosensors, and thereby the LOD, is influenced by the properties of materials they are made of, such as the transducers (electrodes, nanoparticles, dyes) and recognition molecules (antibodies, peptides, aptamer, etc.)[8]. Besides, sensitivity and LOD of biosensors are limited by interferences from complex food matrices. Since rapid and on-site food sample assays are desirable, state-of-the-art extraction processes involving tedious protocols may not be applicable. Therefore developing alternative simple extraction and clean-up protocols are of significant importance for on-site assaying.

Food contaminants such as pathogens, fungus, bacterial toxins, mycotoxins, etc., have been detected by various assays such as enzyme-linked immunosorbent assays (ELISA) with good sensitivity[9,10]; PCR-based assay[11,12] microarrays[13], electrochemiluminescence-based assay[14], FRET-based assay (e.g. for proteases activity detection)[15] have been reported to be rapid. Even though these platforms are frequently utilized, most of these approaches still require sophisticated instrumentation and trained personnel to conduct the analysis. Paper-based sensing is a cheaper option with minimal sample requirement and possibility to be used by non-professionals in resource-limited settings. This approach has been welcomed in food analysis due to their good performances, reliability and good correlation to laboratory experiments. Different types of paper-based assays include dipstick assay, lateral flow assay (LFA)[16,17], vertical flow assay (VFA)[18] and microfluidic paper-based device (μ-PAD)[19] have been reported. Depending on target analytes and the food matrices, the type of materials used in fabrication of paper based device and the reporter molecules for transduction are optimized for improving the limit of detection (LOD) . Another crucial component that influences sensitivity and selectivity is the bio-recognition element. Currently, LFAs are operating with affinity-based mechanism, relying on specific antibody-antigen interaction. However, the thermal instability of the antibodies for on-site assaying is the main drawback limiting practical applications. Therefore, the assay may yield false positive results, especially in the case of proteases detection (e.g. bacterial toxins with proteolytic activity), where the activity of toxins is more accurately addressed based

on their catalytic function compared to detection based on their epitope distribution and affinity. In an attempt to overcome these issues, peptides have been evaluated as recognition element as a replacement for antibodies[20]. Their robustness, stability, easy to manipulate and modify according to specific target could improve the overall assay's performance. Additionally, chemical synthesis of peptides is rapid and cost effective without batch-to-batch variations as compared to antibody production.

The overall performance of the biosensor relies to a large extent on the procedures used to remove the interferences and to extract the target molecule from the food matrices. Preparation and pre-treatment steps are very important for assay reliability, sensitivity and selectivity. Most of the adopted extraction techniques are laboratory-based and time-consuming with the utilization of organic or inorganic solvents that adversely affect the yield of the target analyte. Commonly utilized extraction techniques are liquid-liquid extraction, solid phase extraction, solid supported liquid extraction, solid phase micro-extraction and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique. Besides conventional approaches, membrane-based techniques (microfiltration, nanofiltration, ultrafiltration, chromatography membrane based on affinity, etc.) have been reported for separation of target analytes in food analysis[21]. Membrane-based extraction avoids high solvent usage, but they are sensitive to solid particles and in some cases, their efficiency in removing interferents depends on the type of food. Thus, alternative approaches for extraction are required for efficient removal interferents. For instance, utilization of sorbent materials for extraction and clean-up and their incorporation into polymer membranes would simplify the process of extraction and pre-concentration, upon retaining interferences such as carbohydrates, vitamins, strong pigments, lipids, preservatives, etc. On the other hand, magnetic bead based extraction is an alternative approach to avoid centrifugation, filtration, and other pre-treatment steps. Their chemical and mechanical stability, possibility for diverse functionalization, biocompatibility, low toxicity, low background interference, inertness and easy manipulation with external magnetic field attract immense attention for their application in food analysis[22–24].

This thesis will focus on developing colorimetric paper based assays for rapid, facile and on-site analysis of complex food samples by utilizing the advantages of peptides and facile clean-up process including magnetic beads and membranes. The simplicity of this colorimetric paper based assay together with the use of robust molecular components (peptides as receptors/substrates) offers an attractive route for development of low-cost assays with a longer shelf-life and an easy read-out for on-site applications. An early detection of toxic contaminants avoiding standard measurements will help to prevent the occurrence of foodborne illnesses and outbreaks. Apart from food screening, the proposed methodology is also applicable for environmental monitoring and national security applications.

1.2 Hypothesis/Problem Statement

1. The presence of active botulinum neurotoxin (BoNT) in food samples can be detected at concentrations well below the lethal dose (1 μg/kg) by substituting the current gold standard (mouse bioassay) with a synthetic peptide as recognition molecule/substrate.
2. Synthetic peptides can be used in combination with magnetic beads for facile extraction of substrates from complex matrices for subsequent detection, without using tedious standard procedures and sophisticated instrumentation.
3. Polymer membranes incorporated with sorbent materials could be integrated into a paper-based strip for efficient removal of interferents in complex food matrices for detection of active BoNT (functional-based assay) at competitive concentration levels of ELISA assay (affinity-based assay).

1.3 Objectives and Scope

The main objective of this thesis is to develop a portable device/dip-stick by merging extraction, clean-up and pre-concentration steps with catalytic sensing strategies for target detection and quantification. The developed device/dip-stick is expected to facilitate food quality monitoring. The proposed assay involves peptide for the target recognition/digestion and magnetic beads for rapid and facile clean-up of interferents in

complex food matrices. The proposed system could be easily transferred to the LFA device further simplifying the detection process by excluding the use of instruments, enabling naked eye detection. The developed system could be a good asset for proteases screening in food samples for an early detection and subsequent prevention of related food-borne diseases.

The following specific objectives will be explored in this thesis:

1. To investigate the catalytic sensing strategy with the implementation of peptides as recognition/substrate molecules and magnetic beads for the food sample clean-up for analysis in complex food matrices.
2. To fabricate a paper-based device for monitoring toxins (e.g. BoNT) on-site.
3. To investigate the influence of polymer membranes incorporated with different sorbent materials for removal of interfering matrix components.

1.4 Dissertation Overview

This thesis is comprised of 8 chapters. Each of them are briefly explained in the following paragraphs.

Chapter 1 provides a rationale for the research and outlines the goals and scope. The significance of this thesis is briefly introduced, followed by proposed hypotheses/problem statements, objectives of this research along with the scope. Finally, a short summary on the overview of the thesis is presented.

Chapter 2 reviews the literature on the food safety problems, pathogens, toxins and other agents associated with foodborne diseases. This chapter also reviews the conventional separation and detection methods as well as current state-of-the-art technologies including optical, current on-site assays and membrane-based detection utilizing different nanomaterials (e.g., magnetic beads) for the food safety monitoring. Then, the potential of membranes and magnetic beads for sample pre-treatment and separation is discussed.

Subsequently, a highly toxic protease – botulinum neurotoxin A (BoNT/A) – as a model system - has been used to evaluate the sensing platforms and the rationale for its needy detection is provided.

Chapter 3 describes experimental procedures of sensing strategies both in solution and on the membrane-based platform. This chapter also provides the principles of characterization techniques used and methods of data analysis. Finally, fabrication of dipstick test and membrane-based clean-up integration with paper-based assay are described.

Chapter 4 elaborates detection of active BoNT/A LC in complex food matrices using the magnetic bead-based fluorescence assay. The results demonstrate that the presence of active BoNT/A can be detected by employing corresponding peptide as a recognition molecule at the concentration level below that of oral toxicity.

Chapter 5 describes the development of colorimetric paper based-assay for detection of active BoNT/A LC for the on-site food safety monitoring. In this chapter, it is demonstrated that translation from the solution-based sensing, presented in chapter 4, to the paper-based sensing is feasible, further facilitating the detection by excluding sophisticated instruments.

Chapter 6 introduces the integration of membrane-based clean-up with paper-based sensing. The results revealed that graphitized carbon black (GCB) incorporated in the membrane retains interferences from carrot juice (e.g. pigments) successfully. Herein, clean-up process and detection are combined as a single step, unlike the methodology presented in the chapter 5, where the clean-up process was performed in the solution state by employing the magnetic beads and external magnetic field.

Chapter 7 elaborates on application of the membrane-based clean-up dipstick assay for testing of complex food matrices such as meat and canned food.

Chapter 8 presents the summary and the future outlook of this dissertation.

1.4 Findings and Outcomes/Originality

This research led to several outcomes:

1. Functional fluorescence-based assay employing magnet beads and peptide as a recognition element is successfully developed and applied in both buffer and food matrix (carrot juice) for the detection of protease (e.g. BoNT/A LC), with an LOD below that of oral toxicity (1 μg/kg) and with good selectivity.
2. The dipstick assay has been devised for the detection of proteases with the colorimetric response observed by the naked eye, which is more facile compared to the conventional detection methods and applicable for the on-site assaying.
3. The membrane-based clean-up has been integrated within dipstick assay, merging the clean-up and detection in one step process further providing easier sensing of protease in complex food matrices at low LOD and high specificity.

References

1. Outbreaks of E. coli O104:H4 infection. Available at: http://www.euro.who.int/en/countries/germany/outbreaks-of-e.-coli-o104h4-infection. (Accessed: 17th March 2019)
2. Listeria deaths rise to 29 in cantaloupe outbreak. Available at: https://edition.cnn.com/2011/11/02/health/cantaloupe-deaths/index.htmL. (Accessed: 17th March 2019)
3. Multistate Outbreak of Salmonella Typhimurium Infections Linked to Peanut Butter. doi:10.1056/NEJMoa1011208
4. Home-canned potatoes to blame for botulism outbreak - CNN. Available at: https://edition.cnn.com/2015/04/28/health/potatoes-to-blame-for-botulism/index.htmL. (Accessed: 17th March 2019)
5. Settlement Reached in California Botulism Outbreak | Food Poison Journal. Available at: https://www.foodpoisonjournal.com/foodborne-illness-outbreaks/settlement-reached-in-california-botulism-outbreak/. (Accessed: 17th

March 2019)

6. Juliao, P. C. *et al.* National outbreak of type a foodborne botulism associated with a widely distributed commercially canned hot dog chili sauce. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **56,** 376–82 (2013).
7. Chiou, Jiachi, Arthur Ho Hon Leung, Hang Wai Lee, and W. W. Rapid testing methods for food contaminants and toxicants. *Journal of integrative agriculture* **14,** 2243–2264 (2015).
8. Jafarizadeh-Malmiri, H., Sayyar, Z., Anarjan, N. & Berenjian, A. in *Nanobiotechnology in Food: Concepts, Applications and Perspectives* 81–94 (Springer International Publishing, 2019). doi:10.1007/978-3-030-05846-3_6
9. Moon, J. *et al.* A Gold Nanoparticle and Aflatoxin B1-BSA Conjugates Based Lateral Flow Assay Method for the Analysis of Aflatoxin B1. *Materials* **5,** 634–643 (2012).
10. Wang, L. *et al.* A bare-eye-based lateral flow immunoassay based on the use of gold nanoparticles for simultaneous detection of three pesticides. *Microchimica Acta* **181,** 1565–1572 (2014).
11. Kawasaki, S. *et al.* Multiplex Real-Time Polymerase Chain Reaction Assay for Simultaneous Detection and Quantification of *Salmonella* Species, *Listeria monocytogenes* , and *Escherichia coli* O157:H7 in Ground Pork Samples. *Foodborne Pathogens and Disease* **7,** 549–554 (2010).
12. Salazar, J. K., Wang, Y., Yu, S., Wang, H. & Zhang, W. Polymerase chain reaction-based serotyping of pathogenic bacteria in food. *Journal of Microbiological Methods* **110,** 18–26 (2015).
13. Os, A. T., Uce, R. & Cher, R. Methods for Detecting Botulinum Toxin with Applicability to Screening Foods. *Concise Reviews / Hypotheses in Food Science* **70,** 121–130 (2005).
14. Rivera, V. R., Gamez, F. J., Keener, W. K., White, J. A. & Poli, M. A. Rapid detection of Clostridium botulinum toxins A, B, E, and F in clinical samples, selected food matrices, and buffer using paramagnetic bead-based electrochemiluminescence detection. *Analytical Biochemistry* **353,** 248–256 (2006).

15. Wang, Y., Liu, X., Zhang, J., Aili, D. & Liedberg, B. Time-resolved botulinum neurotoxin A activity monitored using peptide-functionalized Au nanoparticle energy transfer sensors. *Chem. Sci.* **5,** 2651–2656 (2014).
16. Huang, Z. *et al.* A novel method based on fluorescent magnetic nanobeads for rapid detection of Escherichia coli O157:H7. *Food Chemistry* **276,** 333–341 (2019).
17. Wang, D.-B. *et al.* Detection of Bacillus anthracis spores by super-paramagnetic lateral-flow immunoassays based on "Road Closure". *Biosensors and Bioelectronics* **67,** 608–614 (2015).
18. Chen, P. *et al.* Paper-based Vertical Flow Immunoassay (VFI) for detection of bio-threat pathogens. *Talanta* **191,** 81–88 (2019).
19. Lu, Y., Shi, W., Qin, J. & Lin, B. Fabrication and Characterization of Paper-Based Microfluidics Prepared in Nitrocellulose Membrane By Wax Printing. *Analytical Chemistry* **82,** 329–335 (2010).
20. Shi, J. *et al.* A graphene oxide based fluorescence resonance energy transfer (FRET) biosensor for ultrasensitive detection of botulinum neurotoxin A (BoNT/A) enzymatic activity. *Biosensors and Bioelectronics* **65,** 238–244 (2015).
21. Jönsson, J. Å. & Mathiasson, L. Membrane-based techniques for sample enrichment. *Journal of Chromatography A* **902,** 205–225 (2000).
22. Chen, Y. T., Kolhatkar, A. G., Zenasni, O., Xu, S. & Lee, T. R. *Biosensing using magnetic particle detection techniques*. *Sensors* **17,** (2017).
23. Chen, Y. T. *et al.* Specific Detection of Proteins Using Exceptionally Responsive Magnetic Particles. *Analytical Chemistry* **90,** 6749–6756 (2018).
24. Marín, T., Montoya, P., Arnache, O. & Calderón, J. Influence of Surface Treatment on Magnetic Properties of Fe3O4 Nanoparticles Synthesized by Electrochemical Method. *Journal of Physical Chemistry B* **120,** 6634–6645 (2016).

Chapter 2

Literature Review

This chapter reviews and summarizes the background about food safety, toxins and contaminants presented in foods; related problems and challenges; current techniques and on-site assays for the food monitoring applications. Firstly, a brief introduction of food safety issues and most frequent pathogens and contaminants are given. Furthermore, various developed methods and strategies for the early detection of toxins and contaminants are also recapped. The chapter also explains the significance of the matrix clean-up by conventional and non-conventional methods (e.g. magnetic beads) in order to provide more accurate detection and better reliability of the biosensing system. Finally, the research model – Botulinum neurotoxin– used in this research study is explained in details. PhD in context of literature with questions to answer based on literature are clarified as well.

2.1.1 Food Safety

Food safety is one of the most important segment related to the human health, given that over 70% of the total internal contamination of humans is caused by ingestion of contaminated foods, giving the sense that food safety is an imperative in preserving the health. As the toxic substances in food can cause a whole range of health disorders such as biochemical, physiological, reproductive and pathological changes, and in extreme cases can cause death of the individual, the food safety has become a matter of global importance. Given the wide-spread presence of harmful contaminants and toxins, the risks associated with them in all their forms can only be limited, but not completely eliminated.

The complexity in the food safety and analysis is increasing with a food market globalization across the world[1]. Additionally, some food share the same production lines and storage spaces, containing multiple ingredients from different parts of the world as well as a consumption of raw food and ready-to-eat food further challenge fast monitoring of toxins and contaminants[2]. Food contaminations can occur via numerous ways. Typically, via environmental sources including fertilization and pesticide application; food processing (e.g. polycyclic aromatic hydrocarbons - PAH), improper handling and storage (e.g. bacterial growth, bacterial toxins), additives and adulterants; and biological sources include bacteria, fungi, viruses, naturally presented toxins[3]. The other complexity in implementing the food safety and analysis comes from contaminants diversity (toxins, chemicals, allergens, etc.), and their rapid increase. In addition to this, a constant growth of human population (estimated to be 9 billion by 2050) poses demands for a massive food production which will require even more strict regulations to be applied during every step from harvesting, processing, storage, transportation, till consumption[4]. The food safety monitoring has never been more challenging. Even though the restrictive regulations have been established by Codex Alimentarius Commission for European Community[5] and Food and Drug Administration for United States[6], demands for faster, cheaper, and environmental friendly tools and procedures for the traceability of food are increasing. Besides, food industries must be more aware and honest in implementing the established rules and good practices as well as in delivering the safe food products.

However, uncontrolled contaminations with microorganisms, fungi, biotoxic substances, non-biological agents and other hazardous compounds lead to food waste, food toxicity and foodborne diseases. Considering the estimation numbers of 600 million affected individuals and 420 thousands deaths annually, the monitoring of the global food safety is the key for an early prevention of food poisoning and related foodborne diseases[7].

2.1.1.1 Toxins and Contaminants

Sources of food toxicity, from the field to the plate, are diverse including foodborne pathogens, food allergens and toxins (mycotoxins, bacterial toxins), antibiotics in food, chemical (disinfectants, plastics, detergents) and environmental (persistent organic pollutants (POPs)) contaminants, and polycyclic aromatic hydrocarbons (PAHs) which are considered a big source of sever illnesses and death.

Global prevention, control and elimination of foodborne pathogens resulted in a dramatical change of foodborne infectious spectrum, and emergence of new ones which are more antimicrobial resistant due to the genetic variations. The most common emerging foodborne pathogens are *Salmonella spp., Vibrio spp., Campylobacter spp., Escherichia coli* O157, *Staphylococcus aureus,* and *Listeria monocytogenes* that are involved in outbreaks[8–10] and severe illnesses affecting millions of people annually[11]. Many of our foods are good substrates and source of nutrients for pathogens growth and their further transmission, but usually they appear in under processed diary and meat products, eggs, fresh fruits and vegetables, and contaminated water. They are causing typical symptoms such as fever, headache, vomiting, diarrhea, and abdominal pain. By growing under favorable conditions, some bacteria produce their virulence products, biotoxins, which can be categorized as endotoxins (released after lysis of bacteria) and exotoxins (secreted by bacteria into surroundings). These extremely poisonous substances cause the intoxication of humans by digestion of contaminated food. Several biotoxins are very well known for their low lethal doses such as shiga toxin (*Shigella dysentariae*), cholera toxin (*V. cholerae*), botulinum toxins (*C. botulinum*), enterotoxin (*S. aureus*), and tetrodotoxin (*Vibrio alginolyticus*) causing typical symptoms, based on the nature and quantity of toxin,

such as vomiting, diarrhea, fever, weakness, kidney, and respiratory failure, etc. that in the most severe cases can lead to death[12]. Unlike bacteria, that can be killed by pasteurization, increasing acidity and salt content, or reducing the water activity, most of bacterial toxins and spores are resistant to environmental changes. Even though the processed foods are less prone to be a source of infection and intoxication than fresh foods, their safety and stability depends on the good hygienic practices and manufacturing process[13].

Toxic fungi are other pathogens frequently involved in foodborne diseases. Generally, they are classified as filed fungi such as *Fusarium graminearum* and *Fusarium moniliforme* affecting plants before harvesting, and storage fungi such as *Penicillium verrucosum* and *Asspergillus flavus* occurring after harvesting[14]. Their secondary metabolites called mycotoxins are extremely poisonous chemical compounds which are resistant to heat and cannot be completely destroyed under normal cooking process, unlike bacterial toxins (proteins) which unfold and loose activity in cooking process. Mycotoxins are associated with chronic and cumulative effects on the human health, and acute with rapid symptoms of serious illnesses, whereas bacterial toxins as multifunctional proteins are self-programmed to reach the targeted organ where they act. The appearance of most alarming mycotoxins such as zeralenon, aflatoxin, orchatoxin, nivalenon, fumonisin and patulin in animal feeds, human foods and soil is related to high temperature, moisture content, and prolong time of transport and storage with poor hygienic conditions[15].

Environmental and chemical pollutants are other risk associated food contaminants which entering the food chain via soil, air and contaminated industrial water and they are changing continuously due to the industrial development, climate change, and different agriculture practices[16,17]. As the base of our food chain, plants (vegetables and fruits) are prone to absorb toxic substances and subsequently upon consumption to trigger different response of human health such as hormonal disbalance, allergies, immune suppression, reproductive defects, cancer, metabolic degradation, kidney damage, brain injuries and disabilities, etc[3]. On the list of emerging contaminants due to their accumulative effects in the body and their relation to various health disorders are POPs[18] (polychlorinated biphenyls (PCB), polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF)), heavy metals

(cadmium, lead, arsenic), pesticides[16], and carcinogenic and genotoxic PAHs which occurs in foods during processing or if the food being exposed to air, solids or water contaminated with PAHs[19]. As a measure of control, FDA determined the minimum levels of chemicals allowed to be present in foods[18]. However, as persistent pollutants over longer period of time have higher concentrations with longer decay time and in that way magnifying bioaccumulation, the effective surveillance and more strict regulations are required[20]. Additionally, better response systems need to be established to prevent chemicals from entering the food chain especially in underdeveloped countries where legislation and regulations might be weak, and appearance of new non-regulated chemicals that escape detection are of additional concern.

Since the food adulteration has never been easier considering that many toxins and contaminants are lethal at a low concentration level causing quickly developed foodborne diseases within a few hours, their sensitive and rapid detection on-site is urgent. Due to the huge diversity of toxins and contaminants, and the complexity of food matrices, it is impossible to detect them using one analytical technique, which forces scientists to establish methodologies and techniques for facile and on-site detection. In this sense, biosensor technologies stand for a cutting-edge in the food safety and security field. Moreover, they can provide the simultaneous detection of multiple analytes within the same matrix as they utilize biological recognition elements. The improvement in the sensor technologies has been based on the demand over the past few years, with the current focus not only on the analyte detection but also to deliver tools that are applicable onsite and friendly to use.

2.1.2 Current Techniques for Food Safety Monitoring

From the past century till today, techniques and methodologies for food analysis have changed impressively from wet chemistry towards modern instrumentation and miniaturized devices, and have been improving continuously upon requests for more robust, efficient, cost-effective, selective, sensitive and friendly-to-use tools.

2.1.2.1 Laboratory-Based Techniques

Over the 20th century food analysis has been based on application of analytical techniques such as chromatography, spectrometry, and spectroscopic methods (Fig. 2.1; Table 2.1). Chromatography has been extensively utilized for the separation of molecules based on differences in structure, composition and charge by moving a sample along a stationary phase. The most commonly utilized are gas chromatography (GC) for analysis of volatile and semi-volatile and derivatized compounds[21]; high performance liquid chromatography (HPLC) with normal and reversed-phase column for the separation of compounds based on their polarity in complex matrices in a short period of time; and thin layer chromatography (TLC) for analysis of many samples simultaneously and economically. Besides, multidimensional chromatography provides better separation and reduces sample preparation steps, therefore its application increasing in food analysis domain.

Mass spectrometry (MS) coupled with chromatography techniques, tandem-MS or MS/MS (e.g. LC-MS, HPLC-MS, HPLC-ESI-MS, GC-MS), were largely applied for identification and quantification of various food contaminants such as antibiotics, pesticides, POPs, allergenic proteins, bacterial and fungal metabolites[22–25]. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are commonly used for detection of intact peptides, proteins, carbohydrates, etc. enabling rapid screening through samples[26]. Additionally, the sensitivity of chromatographic techniques, which is reported to be from micromole to picomole, was enhanced with spectroscopy and spectrometry techniques down to picomole level[27]. Besides high throughput spectrometry methods, spectroscopy methods are also used to obtain fast quantitative data in complex matrices owing to software developments substituting and overcoming long and tedious wet chemistry. Near infrared spectroscopy (NIR) and Fourier transform IR (FTIR) instruments have been applied for detection of melamine in infant formula, liquid and powder milk[28–30], and soya bean meals[31]. Other separation tools frequently used for detection of adulterants in foods are capillary electrophoresis (EC), mono-dimensional and two-dimensional polyacrylamide gel electrophoresis (PAGE).

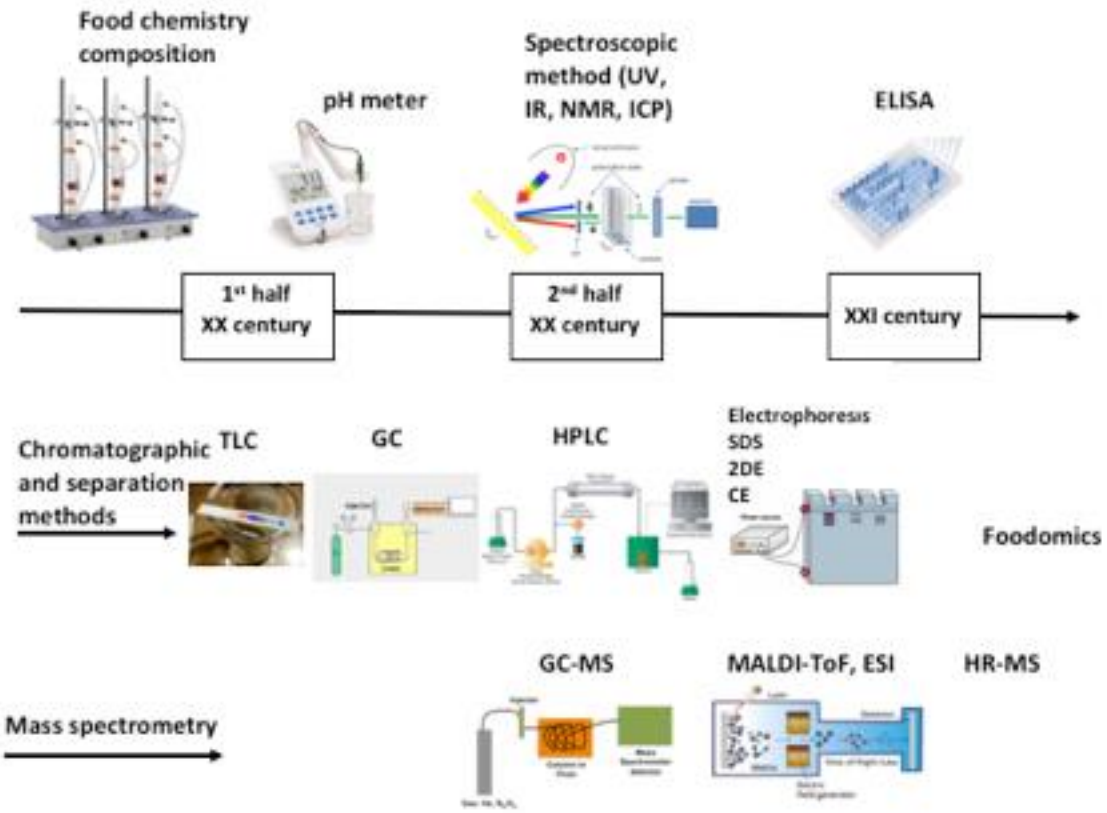


Figure 2.1. The evolution of laboratory-based technique for food analysis and food safety monitoring.

As an alternative to the traditional microbiological tests (conventional culturing approaches), polymerase chain reaction (PCR)[32] and real-time polymerase chain reaction (qPCR)[33] have been developed. The rapid and specific detection is due to the ability of this method to generate tens of billions of copies of targeted DNA fragments involving 4 steps: denaturation by heat, annealing prime to target sequence, extension, and the end of the first cycle. Detection of foodborne pathogens in eggs, meat and dairy products, and ready-to-eat products with different limit of detection (depends on the complexity of food matrices) has been reported using PCR-based methods[34]. Apart from pathogens, bacterial toxins have been detected as well. For instance, botulinum neurotoxin type B was detected at attomolar level in honey using the sialyllactose-DNA conjugate which serve as a detection probe (bind to the toxin) and as a template for qPCR, where the DNA marker was amplified[35].

Even though those techniques are precise and accurate with good separation ability, still they are time and space consuming, laborious and required trained personnel making them inconvenient in fast screening in the sample analysis. Additionally, the new challenges in analysis laboratories posed by green chemistry concept including the use of environmental friendly solvents, solvent-less techniques, easier and cleaner separation methodologies, miniaturization led to development of the new spectrum of assays (see below) with expectations to deliver fast and reliable results without compromising accuracy, sensitivity and selectivity in respect to the existing analytical techniques[36].

Table 2.1. Examples of laboratory based techniques and methods used in food analysis.

Analyte	Sample (LOD)	Technique
Mycotoxins	brazilian peanut kernel (4.2-600 μg/kg, aflatoxin)[37]	HPLC-MS
Pesticides	fruits and vegetables (0.001-1 mg/kg, LOQ)[38]	
Adulteration	goat milk and cow milk[39]	
Veterinary drug	bovine whole milk (9.9–18.4 μg/kg, coccidiostats)[40]	
Mycotoxins	wine (0.02ug/L, aflatoxin[41]; 0.01-0.21 ng/ml[42], 0.1-3μg/L[43], ochratoxin), milk and milk product (0.1ug/kg, aflatoxin)[44], rice sample (0.11 ng/g, citrinin; 0.08 ng/ml, ochratoxin)[45], cereals (1 ng/g, zeralenon)[46]	HPLC-UV/VIS
PAHs	pork meat (0.01-11.63 ng/g)[47]	
Allergens	peanuts protein (2 ppm)[48], milk (1.25 ppm)[49]	LC-MS
Bacterial toxins	apple juice (80-100 ng/ml, staphylococcal enterotoxin B – SEB)[50]	
Pesticides	fruits and vegetables (50 ng/ml)[51]	GC-MS
POPs	fatty food (>5g/ml, 6.3 pg/ml, 200 ng/ml)[52]	
Adulteration	milk[53]	EC-MS
Mycotoxins	apples and pears (120-130 μg/kg, patulin)[54], corn and barley (50 μg/kg, citrinin)[55]	TLC

Pathogens	ground meat, beef, pork, fish, shrimp, cheese, canola leaf and cabbage (*Listeria monocytogenes, Staphylococcus aureus, Salmonella enterica and Escherichia coli* O157:H7)[56]; *seafood, meat and ready-to-eat products (Salmonella, Shigella* and *L. monocytogenes)[57];* fish, meat, water, vegetables, ready-to-eat, bivalves, boiled mussels (*Salmonell, Escherichia coli* O157, *L. monocytogenes)[58]*	PCR
Toxins	cholera toxin and botulinum neurotoxin A[59] canned mushroom soup, full fat cream, cottage cheese, mozzarella cheese, full fat hard cheese, and sliced ham (staphylococcal enterotoxins (SEs))[60]	

Enhancements in food analysis came through applications of nanotechnology and nanomaterials intended to provide cost-effective assays with shorter analysis time and multi-analyte detection, and help in overcoming challenges encountered in detection of food contaminants and toxins[61].

2.1.2.2 Optical Assays

An intense research and commercial interest have been focused on the development of optical assays. The principle of these assays is based on the change in optical properties upon biospecific interaction (e.g. in refractive index, absorption and fluorescence properties). This type of interaction can be monitored in real time, and is a generally applicable to reactions such as antibody-antigen, DNA-protein, enzyme-substrate, and many more. The presence of analyte can be measured directly (UV absorption, SPR, surface-enhanced Raman scattering -SERS, fiber optics, etc.)[62–64] or indirectly (via optical probes)[65,66]. The signal can be easily amplified in order to enhance an assay sensitivity by applying different nanomaterials such as magnetic beads, gold and silver nanoparticles, alloy nanoparticles, dyed beads, etc[67]. They are capable of detecting wide range of and multiple analytes non-distractively at very low concentration level. Regardless of valuable

sensitivity and specificity, sophisticated instruments for the signal read out are still required. These assays are discussed in the following section.

2.1.2.2.1 Affinity-Based Detection

Enzyme-linked immunosorbent assay (ELISA; Fig. 2.2A) is the most commonly used affinity-based assay in food analysis. This assay quantitatively determines the analyte concentration by employing antibodies (monoclonal or polyclonal), where the signal from the reporter molecule (e.g. fluoroprobe) is spectroscopically obtained within 5-6 h. ELISA has been widely applied for analysing of various analytes in diverse foods and feeds such as mycotoxins (0.1 – 5 µg/kg)[67], toxins (BoNT, 2 pg/ml – 2 ng/ml[68,69]; SEB, 3.9-18.5 ng/ml[70]; TTX, 4.44 ng/ml[71]), pathogens (*E. coli* O157:H7, 10^5–10^8 cfu/mL[72]; *Salmonella typhi*, 10^4–10^5 cfu/ml[73]), allergens[74], etc. The main drawbacks of this assay are cross-reactivity with others interfering molecules, instability of antibodies and their batch-to-batch variations. In order to improve assay time and stability, other entities such as aptamers, nucleic acids have been attempted. The other commonly utilized assay for monitoring specific bio-interactions in real time, generally antigen-antibody reactions, is based on surface plasmon resonance (SPR) phenomena, where the increase in refractive index at the surface of the sensors is detected after bio-interactions happen (Fig. 2.2B). The high detection sensitivity at picomolar and femtomolar level has been reported after enhancing the signal by introducing nanomaterials (carbon nanomaterials, gold, silver and magnetic nanoparticles)[75]. Since 2000, Biacore Q has been employed in food analysis for direct detection of pathogens, toxins, veterinary drugs and nutritional additives with better reported sensitivity compared to that of indirect ELISA assay, where multiple steps prior obtaining the response are needed[76].

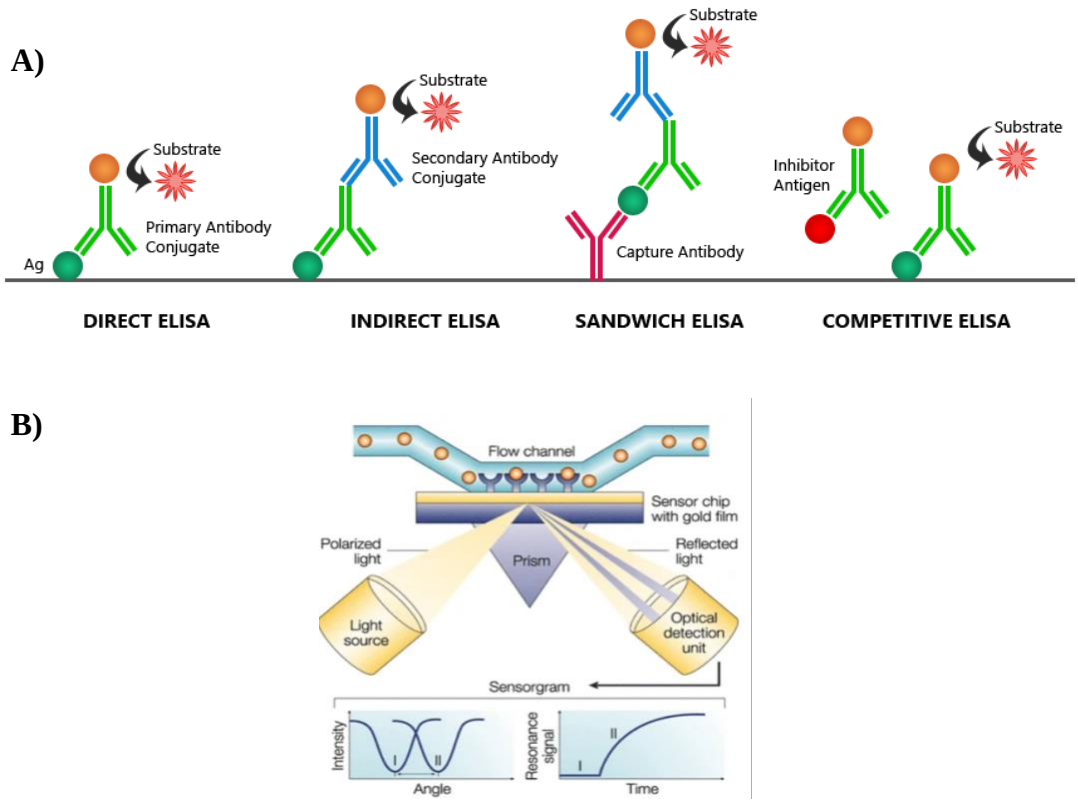


Figure 2.2. Different types of ELISA assay with their working principles[77](A), and SPR biosensor principal (B)[78].

Besides antibodies as main recognition entities in affinity-based assays, antimicrobial peptides have been employed for the detection of pathogen bacteria such as *E.coli*, *Listeria monocytogenes*, *Salmonella* Typhimurium, etc. Highly stable antimicrobial peptides bind to bacteria surfaces by electrostatic and hydrophobic reaction, where the detection method can be electrochemical, colorimetric, fluorescent, SPR (change in the refractive index), and mass change[79]. Apart from this, peptides have been used in recognition of viruses, for instance, the peptide-based molecular beacon in FRET-based format reacts with a hemagglutinin (a specific region on the virus) causing the strong fluorescence[80].

2.1.2.2.2 Functional-Based Detection

Functional-based assays are utilizing the peptide substrates as a recognition molecule to be hydrolysed or cleaved in a specific manner (at the cleavage point) by enzymes (e.g. proteases such as botulinum toxins, tetanus and anthrax) with catalytic functions. The main advantage of these assays is that they detect the active population of the toxins, unlike the affinity-based assays that merely detect the presence and/or concentration of the toxin (regardless of their activity). For instance, in a functional-based assay, proteases can be detected by cleaving a substrate and characterizing the substrate's cleaved parts by mass spectrometry (MALDI-ToF or MS/MS)[81], or indirectly by employing antibodies against the cleaved substrate parts followed by detection with sophisticated instruments such as Luminex technology[82]. On the other hand, the presence of enzymes can be identified via changes in optical properties of transducers measured as a change in absorbance, fluorescence, luminescence, refractive index, etc. Among numerous assay platforms developed for the enzymes activity detection, commonly applied are usually fluorometric in nature. For instance, substrate peptides are labelled with a fluorophore and quencher (FRET-pair), where their cleavage by enzymes resulting in a fluorescence intensity increase owning to the increase in the distance between donor and acceptor[83]. In addition to this, nanomolar sensitivity has been reported by gold nanoparticles-based assays, where the enzyme activity upon cleavage of corresponding peptide elicits LSPR shift[84]. On the other hand, the fluorophore-labelled peptide can be immobilized on the surface (e.g. gold), where upon the digestion, the intensity of accumulated cleaved parts of peptide containing the fluorophore is measured to reveal the enzyme concentration[85].

In our research group, synthetic peptides have been successfully utilized to detect other proteases such as MMP-7, besides BoNT/A. In both cases, peptides are specifically designed to be precisely digested at the cleavage point by tested proteases. So far, synthetic peptides have been used in the FET-based device[86] and optical-based sensing (colorimetric[87,88] and fluorescent[89]).

Despite the advantage of being able to directly measure enzymes activity, the functional-based assay face certain drawbacks, for instance, independent performance of these assays might yield different results due to the temperature instability of enzymes and their activity is dependent on defined assay conditions (pH, nature and strength of ions, purity and stability of other assay components, different substrates). Since the enzymes show the highest activity at their optimum conditions, the assay's protocols are based on the features of individual enzymes, which further posing difficulties in comparison studies[90]. The main differences between peptides and antibodies are shown in Table 2.2.

Table 2.2. Comparison between peptide and antibody.

Peptide	**Antibody**
Produced *in vitro* (chemical synthesis)	Produced *in vivo* (cell culture)
Cheaper and faster production, less laborious	Tedious and very expensive production
Longer shelf life, more stable	Shorter shelf life, less stable
For detection of enzymatically active targets	For detection of targets regardless of their activity. Recognizes surface epitopes on the target molecule.
Easy to modified with other molecules, optimization of design sequence is possible	Demanding and expensive site-specific and quantitative modification.
More uniform production owing to chemical synthesis, chromatographically pure	Batch to batch variations
Specific and selective detection (careful design of peptide is required to avoid the cleavage by other proteases that may be present in the sample)	Prone to cross-reactivity, unspecific binding

2.1.3 On-Site Assays for Food Safety Monitoring

Astonishing work has been invested so far to deliver precise analytical techniques intended to analyse different types of food and related contaminations. Even though in this powerful era of instrumentations, hundreds of foodborne infections occur worldwide with a constant increase in the number of contaminants and toxins. Therefore, to ensure the global food safety more suitable and faster detection approaches are required. Especially in developing counties where the lack of essential instrumentations, consumables and trained personnel are major problems, the on-site portable devices/test for analysis are crucial considering that they are fast, cheap, easy to use and require small sample volume. Additionally, food industries are in a need for rapid screening methods in order to avoid traditional and instrumentation methodologies as conformational tools unless the positive results are obtained[81]. Some of them are explained in the following subsections.

2.1.3.1 Nanoparticle-Based Assay

Nanoparticles have been extensively used in development of sensing platform owing to their high surface area to volume ratio, easy functionalization via numerous bioconjugation strategies, magnetic and electronic properties, which contributes to improved sensitivity of targeted analytes. The most utilized nanoparticles (NPs) in biosensing are gold, silver, carbon, magnetic nanoparticles, quantum dots, etc. Their diversity in size and shape, and good stability enables their integration with other analytical properties. Some examples of NP-based assays applied for the food monitoring is shown in Table 3. For instance, gold nanoparticles (GNP) have been extensively used as transducers in colorimetric biosensing due to their tuneable optical properties. In these assays, the colour change is caused by a bio-affinity intereaction[92] (e.g. antigen-antibody, protein-DNA, peptide-sugar) or substrate cleavage[84,93] (BoNT/A protease-peptide), where the colour change is related to inter particle distance (aggregation-purple/dispersion-red) observable by naked eye. On the other hand, the adsorption of analyte/molecules on the particle surface can be monitored as a localized surface plasmon resonance (LSPR) peak shift by UV/VIS spectrometer[94] or as the change in Brownian motion which declines with increasing the particles volume[95].

Additionally, GNPs have been employed in FRET-based assay owing to their quenching properties over the wide range of wavelength. For an example, the substrate for the detection of botulinum neurotoxin A light chain (BoLcA) serves as a linker between GNPs and fluorescent dye providing the fluorescence quenching by GNPs. In the presence of BoLcA the substrate is cleaved allowing the dye to distance form GNPs. In this way the recovery of fluorescence signal is observed and proportional to the BoLcA concentrations. The assay principal is shown in Fig. 2.3.

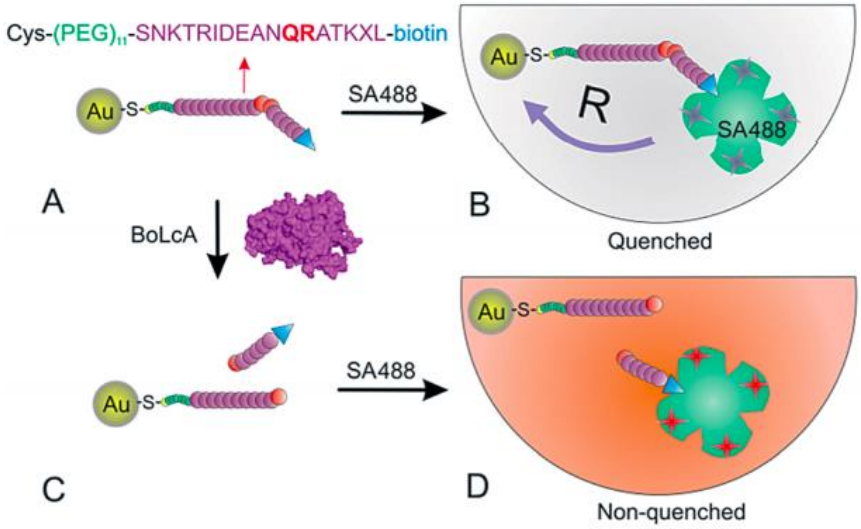


Figure 2.3. FRET-based assay with GNPs as a quencher for the detection of BoLcA[89].

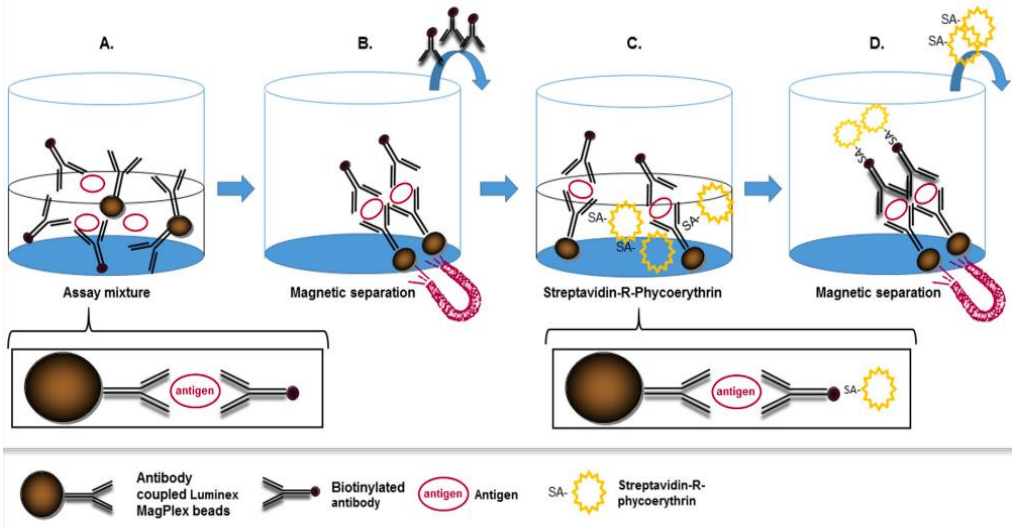
In spite reported sensitivity at the nanomolar and picomolar level, the colloidal stability of particles suspension is still a challenge. Uncontrollable aggregation may be triggered by sample interferences, ionic strength and acidity leading to the false results[96]. Besides an extensive use of GNPs, fluorescence nanoparticles (FNPs), known as quantum dots (QD), have been utilized in order to increase sensitivity and multiplexing capabilities due to their broad absorption and narrow emission spectra, less photobleaching (compared to fluorescence dyes) and high quantum yields. Usually, FNPs are employed in immunoassays conducted in a 96-well microtiter plate (SEB[97], BoNT/A[98]) with demonstrated multiplexing detection (cholera toxin, ricin, shiga-like toxin 1, and SEB)[99], and FRET-based format detection[100]. Additionally, FNPs are utilized in lateral

flow/dipstick assays for the sensitive detection of BoNT/A at nanomolar level[101]. Wang et al. were the first one to use multicolour FRET NPs for multiplex monitoring of *E.coli* species with high sensitivity and specificity[102].

Recently, magnetic nanoparticles (MNPs) appear as a useful component in bio-sensing since they have shown excellent stability in homogenous as well as in heterogenous assays. The high sensitivity is achieved due to the density of binding sites per nanoparticles and excellent diversity of functionalized groups (e.g. carboxyl, amine, maleimide, epoxy, streptavidin, biotin, A/G protein) giving the avenue for numerous approaches for assays setup[103]. In food analysis, MNPs have gained an immense interest used in separation owing to their easy manipulation by external magnetic field and inertness towards matrix interferences, thus shortening time analysis by avoiding tedious sample pre-treatments. For instance, in a bio-assay (Fig. 2.4), MNPs can be functionalized with antibody, peptides, DNA, proteins, etc. according to the analyte of interests, where the detection happens upon introducing the secondary antibody labelled with fluorescence dyes or gold nanoparticles. Kim at al. developed a surface-enhanced Raman scattering (SERS)-based immunoassay technique for the sensitive detection of BoNT (5.7 ng/mL) by employing MP-functionalized antibody to capture the toxin and Nile Blue A-GNP-antibody complex for the recognition[104]. Furthermore, magnetic beads have been utilized in multiplexing analysis for more efficient monitoring of food contaminants showing a potential for the on-site assaying[105]. Additionally, MNPs are used as labels for detection as well as amplifiers of signal response, which, in respect to their properties and very short read-out time, has a huge potential to replace optical and fluorescence detection methodologies and have displayed a potential to be integrated within miniaturized detecting system e.g. lab-on-the-chip systems[103]. Orlov et al. developed multiplex sensing platform for the detection of BoNT types simultaneously using magnetic nanolabels in the lateral flow immunoassay format, where the signal of the test line is read out by magnetic particles quantification reader[106]. Additionally, magnetic beads as labels were used for the detection of *Bacillus anthracis* spores, where the complex MPs-spores are retained near sample pad generating a retention line ("Road Closure"), which is absent if the spores are not presented in the sample[107]. Moreover, magnetic nanoparticle-based assay do not suffer from bleaching and

spectral overlapping like fluorescence-based sensing. An review of MNPs used in microfluidics for the detection of bacterial toxins, mycotoxins, allergens, pathogens and heavy metal in food has been published by Weng at al[108].

A)



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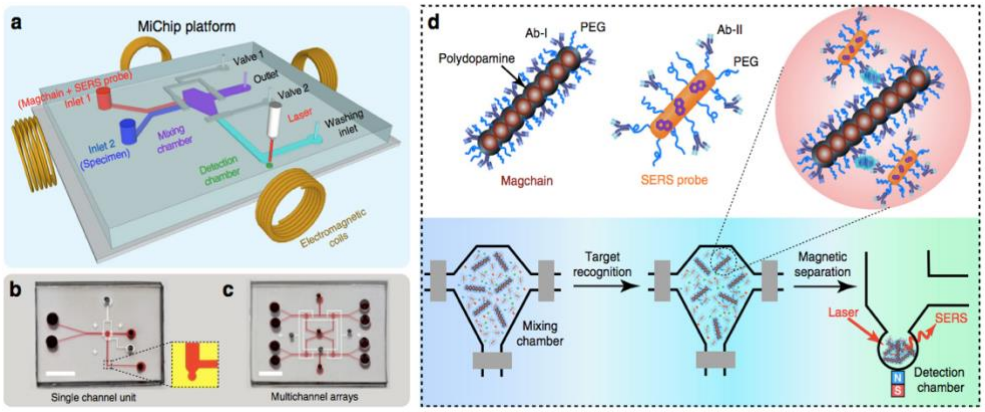


Figure 2.4. Schematic illustration of MNP-based assay with fluorescence detection (A)[109] and incorporation of MNP as a magnetic nanochain within a microfluidics biochip for multiplex bacteria detection (B)[110].

Table 2.3. Some examples of NP-based assays used in food analysis.

NPs	Detection method	Analyte/LOD	Assay time
GNP	Absorbance	Aflatoxin (25 pg/ml)[111] BoNT/A (5-0.1 nM)[84] *L. monocytogenes* (2.82 CFU/ml)[112] LPS (0.3 nM)[113] Melamine (1mg/ml)[114]	15 min 4h ND* 5 min 10 min
	Fluorescence	Pathogen bacteria (ND*)[115] *S. aureus* (1 nM)[116]	30 min 4h
	Chemiluminescence	SEB (0.01 ng/ml)[117]	2h
MNP	Fluorescence	BoNT/A (0.5 nM)[118], OTA (0.005-7.2 nM)[119]	6h 3h
	Magnetic field	Allergenic proteins (5nM)[120] P. pachyrhizi fungus (90 ng/ml)[121] Allergens (1.5-7ng/ml)[122]	2.5h 1.5h 2.5h
	Absorbance	*Salmonella* (100 CFU/ml)[123] Pesticides (1 nM- 5µM)[124]	1 h 15 min
FNP	Fluorescence	Cholera-shiga toxin-ricin (30 ng/ml), SEB (2ng/ml), BoNT/A (31pM) *E.coli and S. typhimutium* (CFU/ml)[125]	3.5 h 2h

ND*- not defined

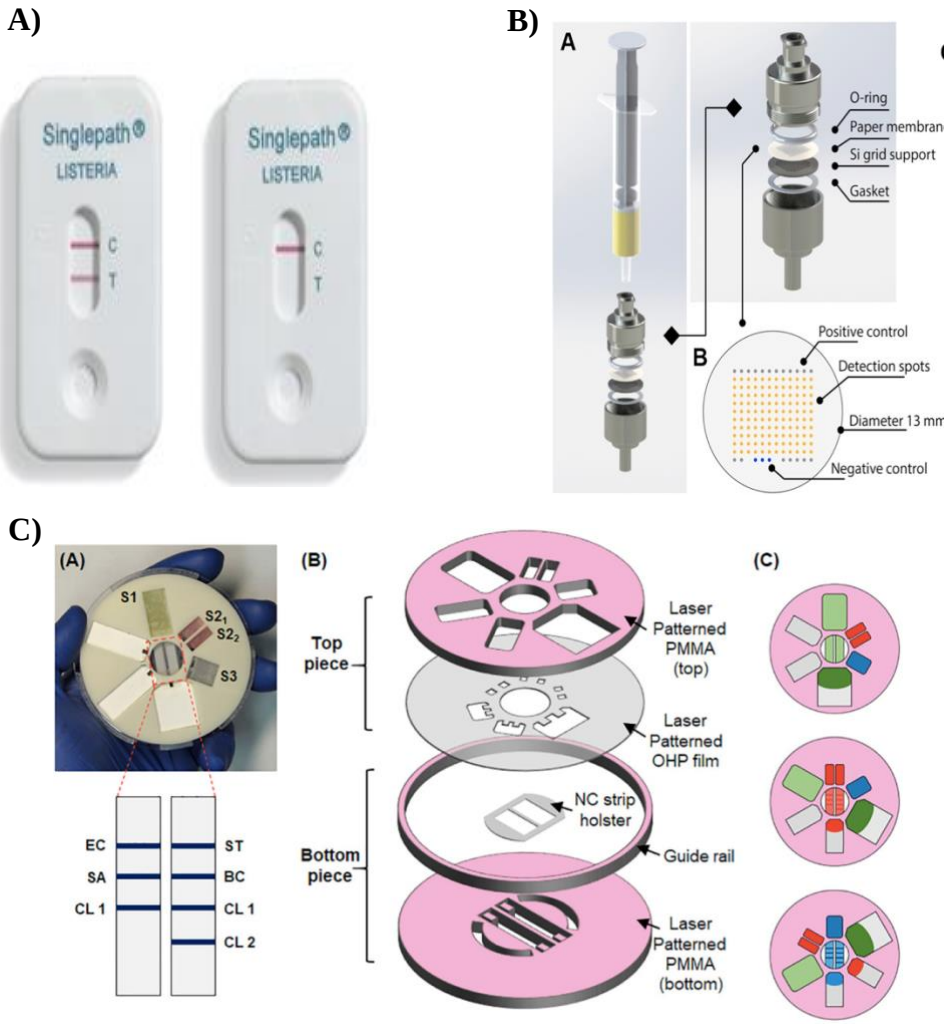
2.1.3.2 Paper-Based Assays

Membranes are widely used materials in analytical and clinical applications as a filter and as a support structure. Diversities of membranes (nitrocellulose, cellulose, glass fiber, nylon, polyvinylidene difluoride (PVDF), polyether sulfone (PES), and cellulose acetate (CA)) and their advantages such as low cost, biocompatibility, biodegradability, easy to manufacture, functionalize, abundance, and chemically inertness are unique properties that launched membranes as one of the highly promising materials for the on-site assays development. The major morphological characteristics such as pore size, porosity and uniformity affect the membranes binding/capturing capacity of molecules (e.g. protein, nucleic acids), which can be bound onto membranes via hydrophobic, electrostatic, and

van der Waals interactions. The immobilization of biological or chemical reagents can be performed by physical adsorption, chemical coupling, dip casting, contact and non-contact (ink-jet) printing. Given these characteristics, membranes with a large surface area (small pore size and higher pore density) display slower wicking rate (more time for the interaction), therefore accommodating a high protein immobilization further providing the better sensitivity. Additionally, membranes can be easily coated to be hydrophilic enabling the flow without external forces and patterned by many technique such as wax printing, laser treatment, screen printing, which makes them a perfect platform for portable and low-cost devices.

Among mentioned membranes, the nitrocellulose is most frequently utilized membrane in the paper-based assaying due to the outstanding properties such as high surface area and high uniformity, lateral flow consistency, range of pore size, excellent binding properties (80-100 μg/cm$_2$), commercial availability, cost-effectiveness, environmental friendly, simple, disposable, and compatibility with many detection methods. The first utilization of nitrocellulose membranes for RNA-DNA complexes adsorption was demonstrated by Nygaard and Hall in 1963[126]. Afterwards, nitrocellulose membranes were intensively utilized to study the interaction between nucleic acids and other biomolecules, which resulted in the development of Southern Blot, Northern Blot and Western Blot technique. In recent years, nitrocellulose is well applied in lateral (LFA) and vertically flow (VFA) assay, dipstick assay and paper microfluidic devices (μPAD) (Fig. 2.5). A simple LFA is made of four parts: sample pad, conjugation pad, detection pad, and absorption pad with reagents deposited onto the membrane. Once the sample is dropped on the sample pad, capillary forces move sample through the conjugation pad containing bio-receptors where conjugation occurs. Conjugates are drawn towards the imprinted test line and control line. The conjugates are always captured on the control line indicating that assay works, and they are captured on the test line only in the presence of an analyte which is observed as a colour change by a naked eye. A dipstick assay works by dipping in the liquid sample followed by observation of the colour change. To facile the screening through samples contaminated with various analytes, multiplex LFAs for simultaneous detection have been reported[127]. Since the depletion of sample in multiplex detection results in the low signal

intensity of a test line, additional loading of enhancers[128,129] (multiple step assay; LFA-ELISA) have demonstrated sensitive analyte detections which can be further simplified for practical on-site applications using functional packaging systems that provides a sequential release of reagents to the paper strip[130]. In VFA, a sample is applied vertically on the surface of microarray membranes placed in the syringe filter holder, where the flow rate is control by a syringe pump[131]. At the beginning of their development, these assays were qualitative providing yes/no detection with poor LODs. However, quantitative analysis and better LODs came with the integration of analytical detection methods (colorimetry, fluorescence, electrochemistry, chemiluminescence, etc.) with imaging devices, strip readers, smartphones, and other technologies. For instance, in the colorimetric analysis, the quantification of signal (color variation) can be detected with the RGB (red, green and blue) analysis.



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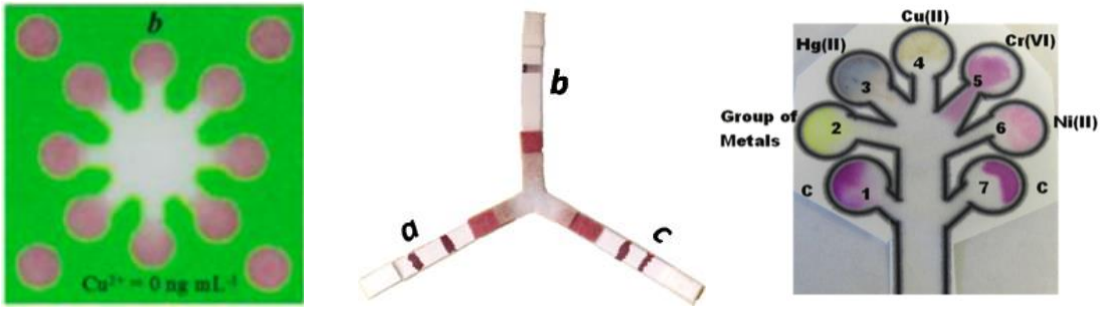

Figure 2.5. Membrane-based assay presented in LFA format (A)[132], VFA format (B)[131], handheld multiplex LFA (C)[130], and µPAD (D)[133–135].

The classical formats of paper-based assays have been improved by technological development resulted in µPAD, firstly introduced by Whitesides' group in 2007. µPAD is designed by forming hydrophobic channels, points, areas on the hydrophilic membrane surface by many techniques such as photolithography, wax printing, wax dipping, plotting, inkjet printing, and plasma etching. The pattering of the paper into hydrophilic channels with integrated hydrophobic barriers has been done by using materials such as polydimethylsiloxane (PDMS), photoresist, wax and polystyrene. Further, the conventional format of µPADs has changed towards different geometries (2D, 3D, flower-like and tree-like shape), since the shape of the paper can enhance the sensitivity, allow multiplexing and multifunctionality of the device. Colorimetric µPADs have been successfully employed for the detection of pesticides[136], neurotoxins (paraoxon and aflatoxin B1)[137], heavy metals[138], ricin[139], etc. Nowadays, µPADs are commercialized owning to their simplicity, ease-of-use, cost-effectiveness, rapidness, improved sensitivity and the ability to quantify the response signal.

The main problem of these assays is that a sample needs to be in the liquid state or sufficiently viscous to ensure a proper flow throughout the paper. If not, the pores can be

clogged by interfering compounds and a higher concentration of the analyte can adversely affect the detection leading to over-saturation and false results[140].

2.1.4 Sample Pre-treatment and Separation

Sample preparation techniques play an important role when it comes to the qualitative and quantitative analysis of toxins and contaminants. Food samples typically undergo pre-treatment procedures based on physical, physio-chemical and chemical approaches[141]. Due to the matrices complexity, usually more than one method is required to achieve a cleaner sample and a better analytical outcome. Besides this, choosing the appropriate ratio between the amount of sample and the operating volume remains challenging due to the fact that just a small amount of samples is exposed to the real detection assay. Since now, there are numerous extraction techniques applied in food analysis aiming to obtain the maximum recovery of analyte as well as simultaneous extraction many of them which regarding their physicochemical properties (solubility, stability, size, polarity, etc.) causing difficulties to generalized extraction procedures.[142] Thus, this remains a challenging task for the development of better sample preparation techniques for proper handling of food samples of complex nature. Commonly adopted techniques in food sample analysis are liquid-liquid extraction (mycotoxins[143]), solid phase extraction (pesticides[144]), solid supported liquid extraction, solid phase micro-extraction (PAHs, acetic drugs, pesticides[145]) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique (pesticides[146])[147]. For example, conventional method as liquid-liquid extraction (Fig. 2.6A) applies two immiscible solvents where an analyte migrates into extraction solvent upon the equilibrium is reached and the analyte of interest can be concentrated by evaporating the solvents. The extraction of analytes can be done by solid phase extraction (SPE; Fig. 2.6B) where the analyte and interfering compounds are separated between a mobile and stationary phase. Many sorbents including ethyl (C2), octyl (C8), octadecyl (C18), cyclohexyl (CH), phenyl (PH) cyanopropyl (CN), diol (2OH), aminopropyl (NH2) are consumed in a removal of co-extracted compounds[148]. Beside this, for the specific sample preparation and pre-concentration SPE-based imprinted polymer has attracted considerable interest in food and environmental analysis, e.g. for cyanotoxins[149]. However, regarding

the long extraction time, expensive high purity solvents, long evaporation time, limited selectivity, expensive sorbent, the conventional methods have been replaced by non-conventional methods.

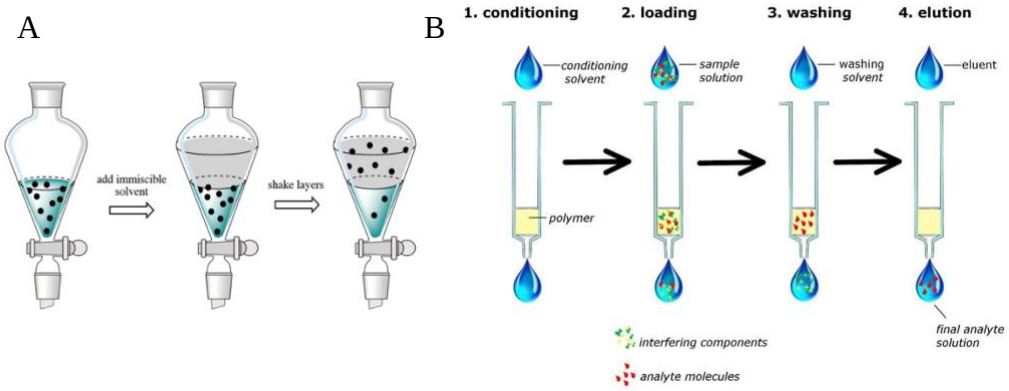

Figure 2.6. Commonly used methods, LLE (A) and SPE (B), for the food sample extraction and clean-up[150,151].

Change in food testing has come along with immunoaffinity columns and recently they have been used widely due to their high selectivity towards a given analyte (e.g. pathogens, seafood toxins, allergens)[152], whereas matrix components are removed in a washing step[153]. Unlike in urine and other simple body fluids, the complexity of food samples may be problematic for immunoassays, especially in direct assays where the better purification is required with no cross-reactions. Additionally, QuEChERS technique has gained immense popularity during the recent years mainly due to its simplicity and cost-effectiveness, especially for pesticide samples. This technique utilizes organic or inorganic solvents for dissolution of sample matrices followed by cleaning using sorbents that retain interferences[154,155]. Many other extraction methods, in respect to the type of sample and the toxic compound, are well used in food and environmental analysis such as microwave-assisted extraction, pulsed electric field assisted extraction, supercritical fluid ultrasound assisted extraction, to mention a few. However, urgent need for a simple sample preparation method, with a solvent-free approach allowing simultaneously extraction and pre-concentration (enrichment) of the analyte, has led scientists to the membrane-based technique. Membranes serve as a selective barrier based on the mass transfer of compounds

through it. Many different membranes have been successfully engineered with different morphology (porous and nonporous), shape (planar, tubes, sheets, etc.), surface areas, physical and chemical properties[156]. Membrane-based techniques such as microfiltration (MF), electrically enhanced microfiltration, ultrafiltration (UF), charged ultrafiltration, nanofiltration membranes (NF), chromatography membranes based on affinity, etc. have been reported for separation and purification of target analytes (e.g. bacteria[157]) in complex food matrices[158]. Unfortunately, it possesses low efficiency and is time-consuming, and is sensitive to solid particles (membranes pores are clogged due to interfering compounds)[156]. Along this line, for an easy analyte separation from other interferences in complex food matrices, magnetic beads have been widely explored. They are advantageous over centrifugation, filtration, solid phase extraction and membrane separation techniques due to efficient, clean, fast, and specific separation managed just by an external magnetic field. Magnetic beads coated with specific recognition biomolecules e.g. antibodies have been used to capture and pre-concentrate analytes (bacterial toxins[159], mycotoxins[160], hepatitis A virus[161], etc.) at low concentration presented in food matrices, which further enables more sensitive detection with increased reliability and signal-to-noise ratio due to the interferences removal in washing steps (Fig. 2.4A). Apart from immunoaffinity columns, membranes, and magnetic beads used for preconcentration of analyte at low concentration level, molecular imprinted solid phase extraction has been utilized for isolation of pathogen bacteria and microbial toxins[162]. Recently, a rapid and easy-to-use pre-concentration/isolation of an analyte at femtomolar level is possible with developed immunoaffinity micropipette tips such as MSIA™ D.A.R.T.'S™[163,164].

2.1.5 Research model - Botulinum Neurotoxin A

Exotoxins known as botulinum neurotoxins (BoNTs) are produced by an anaerobic, spore-forming, rod-shaped and gram-positive bacteria *Clostridium botulinum*[165,166]. Naturally, bacteria occur in solids, water, widely distributed in anaerobic environments, the intestinal tract of animals from where it transfers itself to humans system[167]. Transferring ways into the human system are via inhalation, injection, mucous membranes, through eyes, orally or wound. Accordingly, there are different modes of botulism such as food-borne botulism,

wound botulism, infant botulism, adult intestinal colonization botulism and iatrogenic botulism[166,168]. Foodborne botulism occurs after consuming contaminated food (improperly processed food such as canned food). Once spores reach the intestinal tract they can germinate into bacteria followed by releasing of the toxin and its transmission into the bloodstream. Infant botulism also occurs trough intestinal mucous layer in infants under 12 months and together with food-borne botulism are considered prevalent forms in humans[169]. Infection of wounds by bacteria and secretion of BoNT into bloodstream leads to wound botulism, whereas iatrogenic botulism is a consequence of using too high doses of BoNT in cosmetics application[170]. BoNT is considered one of the most lethal compounds known to the world so far. The lethal dose of BoNT depends on the way of exposure, for instance, 1-2 ng/kg intravenously but it is more than 10 times less toxic when inhaled[166,168]. Consequently, its extreme toxicity, effectiveness, and availability marked this toxin as the highest risk bioterror weapon according to US Centers for Disease Control and Prevention (CDC)[171]. Besides, the toxin has been the major concern for a long time in the food and medical science.

It is 150 kDa toxic protein, named holotoxin, composed of two polypeptides, 50 kDa light chain (LC) and 100 kDa heavy chain (HC), linked by a single disulfide bond and a peptide belt[84] (Fig. 2.7). The light chain is considered as a catalytic domain while the heavy chain possesses binding and translocation properties[89,172]. BoNT LC is zink-metalloproteases which play an important role in the activity of the toxin. Other Zn-dependent proteases, including BoNT, contain conserved HExxH structure (His(222)-Glu(223)-Xaa- Xaa-His(226)),[168] where two His together with Glu 261 help in coordinating the active Zn atom and Glu 223 manages the water molecule[173]. There are seven different serotypes of BoNTs, BoNT/A – BoNT/G, plus a recently reported type H[174] produced by different strains of *C. botulinum*. Serotypes A, B, E and F are considered as agents for causing the neuroparalytic disease botulism in humans while serotypes C and D causing botulism in animals. Each of them has the affinity towards different proteins in SNARE complex, for instance, synaptosomal associated protein (SNAP-25) is a substrate for BoNT/A/E/C cleaved at different position Q-R, R- I, R-A, recspectively[175]. The vesicle- associated membrane

protein (synaptobrevin; VAMP) is cleaved by BoNT B/D/F and Syntaxin is cleaved by BoNT/C176.

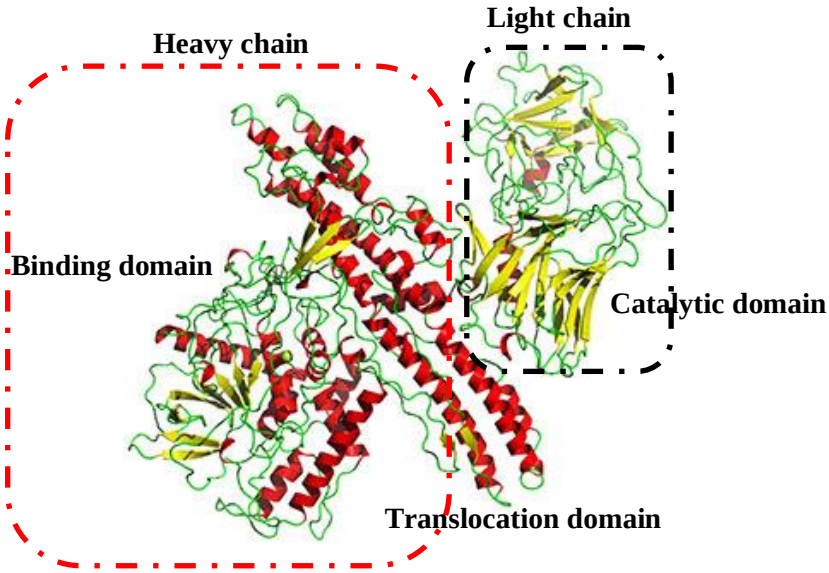

Figure 2.7. Molecular structure of Botulinum Neurotoxin A. Heavy chain (HC) containing binding and translocation domain. Light chain (LC) containing the catalytic domain177.

Once BoNTs is ingested into human organism, it goes through digestion process reaching the intestinal track, subsequently. After its absorption through intestinal track enters the bloodstream and moves towards neuromuscular junction where it acts and damages/cleaves vesicular/membrane peptides. BoNT intoxication can be divided into four phases including all three functional domains178 (Fig. 2.8):

1. Binding of the toxin to the targeted neuronal cell, pre-synaptic nerve ending.
2. Endocytosis into a vesicle.
3. Translocation of the LC through lipid membrane into the cytosol.
4. Cleavage of the SNARE proteins.

It has been investigated that the toxin linkage to neuronal cell happens via two binding localities in the HC, binding domain, which includes ganglioside and protein receptors. Protein receptor reacts after ganglioside binding motif (conserved sequence in

BoNT/A/B/E/F/and G) has encored the toxin on the cell membrane and both contributes to binding affinity and specificity providing endosomal internalization. Once internalized, BoNT exhibits in acidic compartments where it cannot be blocked with antidote anymore. At this stage, it is believed that pH affects conformational changes of translocation part resulting in higher hydrophobicity followed by inducing a pore in the lipid membrane, therefore enabling the entrance for the LC into cytosol. Catalytic domain, in the cytosol, targets and cleaves SNARE (soluble N- ethylmaleimide- sensitive-factor attachment protein receptor) proteins in a highly precise way and blocks the release of neurotransmitter acetylcholine further causing the flaccid paralysis.

As a consequence of the cleaved SNARE proteins, botulism occurs within 18 to 36 hours starting with the facial muscular paralysis followed by double vision, drooping eyelids, difficulty in speaking, weakness, vertigo and continues downwards into extremities[169]. The final stage of botulism is a paralysis of respiratory muscles and death.

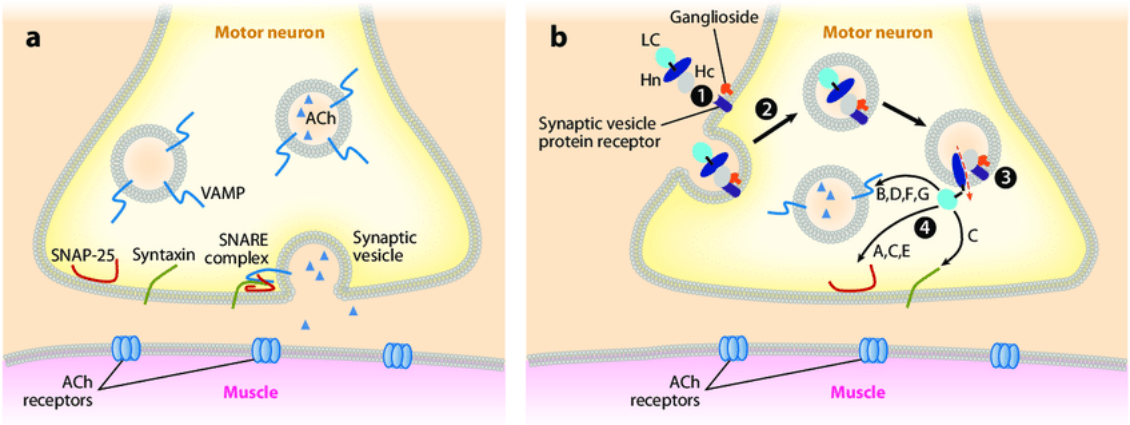

Figure 2.8. Schematic illustration of BoNT/A toxicity mechanism. Neurotransmitter, acetylcholine is packed in vesicles. Normally, vesicle is docked via group of proteins called the SNARE proteins and subsequently the acetylcholine is released into the synaptic cleft (left image). In the case of botulism, the toxin bound to the vesicle enters the cytosol where it cleaves the SNARE proteins disabling the fusion and blocking the release of acetylcholine (right part of image)[179].

The mouse bioassay is the standard method for the detection of BoNT, where symptoms

are observed after 2-4 days upon contaminated sample is injected into mice. Alternative approaches *in vitro* such as immunoassays are well utilized for the BoNT detection[180]. Even though, immunoassays have demonstrated low LODs, they are slow, require extensive labor work, and rarely provide information on the BoNT enzymatic activity.

The detection of BoNT/A has been demonstrated in various food matrices using different detection methods, as shown in Table 4.1. Performances of developed assays depend on the food complexity and in general, these assays possess the potential for testing complex food matrices upon utilization of appropriate sample clean-up protocols (*e.g.* using sorbent materials, magnetic beads). In this thesis, the chosen food samples, either carrot juice or milk could represent a complex food matrix as they contain high levels of interferents. For instance, fluorometric and colorimetric assaying of BoNT/A in carrot juice is challenging as it contain pigments that interfere with the optical responses. Hence, sample clean-up protocols have been investigated in this thesis for assaying BoNT/A in carrot and milk samples. It is not possible to generalize the clean-up process due to the diversity of food matrices. However, specific clean-up protocols can be developed for food matrices that are categorized based on their content.

2.2 Question to be answered: Can synthetic peptides, as recognition element/substrate provide more accurate and robust detection of proteases compared to antibodies detection methodologies?

Antibodies, as a recognition element, have been widely used in biosensors due to the binding affinity and fast recognition of a target. Nevertheless, antibody-based biosensors showed some limitations:

- Antibody recognizes a target based on the structure of the target, which can lead to cross-reactivity among different serotypes and their subtypes which are similar in structure, further causing imprecision detection.
- Antibodies are unable to distinguish between active and inactive protease which is crucial for addressing the food toxicity and further could lead towards the false-

positive results.

- Their tedious production procedure, thermal stability issues, short shelf life, and lack of reproducibility have been reported in antibody-based biosensors.

Therefore, this research study explores solution and paper-based biosensors using synthetic peptide as recognition element in order to overcome the limitations mentioned above.

2.3 Question to be answered: Are functional-based assays *in vitro* capable of substituting the conventional methods and mouse bioassay for the food safety applications?

The current methods for food analysis such as chromatography and spectroscopic methods as well as mouse bioassay are sensitive but time/space consuming and require trained personnel for analysis. Additionally, most of these approaches require laboratory facilities and a range of reagents for extraction of analytes of interest prior analysis, limiting on-site analysis. Nevertheless, one promising approach for on-site fast analysis are bioassays owing to their low cost, easy of fabrication with minimal or no use of instrumentation for analysis. Depending on the targeted analyte, approaches for sensing can be different. In this research toxic proteases presented in foods are detected via functional-based assays, which are capable of delivering more precise detection (compared to the affinity-based assay and most of conventional methods) by mimicking the action mode of targeted proteases. Apart from this, time required for overall detection process is much less compared to time needed to perform some of analytical methods or mouse bioassay. Thus, functional-based assays for proteases detection in food matrices as faster, more precise and reliable tools have a potential to substitute the conventional methods in rapid screening through many samples for on-site assaying.

2.4 PhD in context of literature

Unsafe and unregulated food is the main contributor to foodborne illnesses across the world. In this era of globalization, food industries face incredible growth opportunities, but brought bigger risk exposures that cannot be ignored. In addition to this, climate change,

developed truism, market liberalization, and a trend towards affordable and fast-foods increase the complexity of food safety monitoring. Consumers are more than ever concerned with unhealthy food related issues since tracking the complex supply chain have never been more challenging. Unsuitability of conventional methods to provide on-site fast screening check-ups opens the opportunity for sensitive and portable biosensors to take a role in assessing the food safety. As sources of food contamination are diverse and intensities of symptoms-related foodborne diseases as well, and harms to human health are different, the detection emergency is placed on the most potent agents, for instance, proteolytic enzymes (BoNTs, tetanus, anthrax) and pathogens (*E.coli*, *Salmonella*, *Listeria monocytogenes*, etc.). In order to address some of the emergence requirements, this research proposes the functional-bioassays that are promising sensing tools for the specific detection of toxic proteases *e.g.* BoNT/A. Quantitative/qualitative detection have been achieved in solution and paper-based assays employing peptide, magnetic beads, and sorbents for matrix clean-up. The proposed assays yielded LODs below that of oral toxicity with a good specificity. Additionally, the portability, ease-of-use and long-term robustness make them suitable for the on-site applications.

Reference

(1) Uroš, A.; Martina, G. Š.; Dajana, G. S.; Tamara, M.; DjuroJ, O. Foodomics and Food Safety : Where We Are. *Food Technol. Biotechnol.* **2017**, *55* (3), 290–307.

(2) Raz, S. R.; Haasnoot, W. Multiplex Bioanalytical Methods for Food and Environmental Monitoring. *Trends in Analytical Chemistry* **2011**, *30* (9), 1526–1537.

(3) Rather, I. A.; Koh, W. Y.; Paek, W. K.; Lim, J. The Sources of Chemical Contaminants in Food and Their Health Implications. *Frontier in Pharmacology* **2017**, *8*, p.830.

(4) Majeed, A. Food Toxicity : Contamination Sources , Health Implications And Prevention. *Journal of Food Science and Toxicology* **2017**, *1*, 1–2.

(5) Food and Agriculture Organization of the United Nations, W. H. O. *The Science of Food Standards : The Road from Codex Alimentarius Commission 39 to 40*; Food & Agriculture Org., 2018.

(6) Donal, Laurie C., Ray A. Matulka, G. A. B. Naturally Occurring Food Toxins. *Toxins* **2010**, *2*, 2289–2332.

(7) Food safety https://www.who.int/news-room/fact-sheets/detail/food-safety (accessed Feb 14, 2019).

(8) Yeni, F.; Yavaş, S.; Alpas, H.; Soyer, Y.; Yava¸s, S. Y.; Alpas, H.; Ye¸, Y.; Soyer, Y. Most Common Foodborne Pathogens and Mycotoxins on Fresh Produce: A Review of Recent Outbreaks Most Common Foodborne Pathogens and Mycotoxins on Fresh Produce: A Review of Recent Outbreaks. *Critical Reviews in Food Science and Nutrition* **2015**, *56* (9), 1532–1544.

(9) Käferstein, F. K.; Motarjemi, Y.; Bettcher, D. W. Foodborne Disease Control: A Transnational Challenge. *Emerging infectious diseases* **1997**, *3* (4), 503–510.

(10) Callejón, R. M.; Rodríguez-Naranjo, M. I.; Ubeda, C.; Hornedo-Ortega, R.; Garcia-Parrilla, M. C.; Troncoso, A. M. Reported Foodborne Outbreaks Due to Fresh Produce in the United States and European Union: Trends and Causes. *Foodborne Pathogens and Disease* **2015**, *12* (1), 32–38.

(11) Addis, M.; Sisay, D. Diseases A Review on Major Food Borne Bacterial Illnesses. *Journal of Tropical Diseases* **2015**, *3* (4).

(12) Sikorski, Z. *Toxins in Food*; 2005.

(13) Nout, M. J. R. Fermented Foods and Food Safety. *Food Research International* **1994**, *21*, 291–298.

(14) Tola, M.; Kebede, B. Occurrence, Importance and Control of Mycotoxins : A Review. *Cogent Food & Agriculture* **2016**, *21* (1), 1–12.

(15) Murphy, P. A.; Hendrich, S.; Landgren, C.; Bryant, C. M. Food Mycotoxins: An Update. *Journal of Food Science* **2006**, *71* (5), R51–R65.

(16) Kantiani, L.; Llorca, M.; Sanchís, J. Emerging Food Contaminants : A Review. *Analytical and Bioanalytical Chemistry* **2010**, *398*, 2413–2427.

(17) Kim, Y. S.; Park, K. H.; Chun, H. S.; Choi, C.; Bahk, G. J. Correlations between Climatic Conditions and Foodborne Disease. *Food Research International* **2015**, *68*, 24–30.

(18) Mortimer, D. N.; Agency, F. S. *Persistent Organic Pollutants in Foods : Science , Policy and Regulation*; Woodhead Publishing Limited, 2013.

(19) Janoszka, B. HPLC-Fluorescence Analysis of Polycyclic Aromatic Hydrocarbons (PAHs) in Pork Meat and Its Gravy Fried without Additives and in the Presence of Onion and Garlic. *Food Chemistry* **2011**, *126* (3), 1344–1353.

(20) Jones, K. C.; Voogt, P. De. Persistent Organic Pollutants (POPs): State of the Science. *Environmental Pollution* **1999**, *100*, 209–221.

(21) Lehotay, S. J.; Hajšlová, J. Application of Gas Chromatography in Food Analysis. *TrAC Trends in Analytical Chemistry* **2002**, *21* (9–10), 686–697.

(22) Martinović, T.; Andjelković, U.; Gajdošik, M. Š.; Rešetar, D.; Josić, D. Foodborne Pathogens and Their Toxins. *Journal of Proteomics* **2016**, *147*, 226–235.

(23) Turner, N. W.; Subrahmanyam, S.; Piletsky, S. A. Analytical Methods for Determination of Mycotoxins: A Review. *Analytica Chimica Acta* **2009**, *632* (2), 168–180.

(24) Stefano, V. Di; Avellone, G.; A, D. B.-… of C.; 2012, U. Applications of Liquid Chromatography–Mass Spectrometry for Food Analysis. *Journal of Chromatography A* **2012**, *1259*, 74–85.

(25) Careri, M.; Mangia, A.; Musci, M. Applications of Liquid Chromatography-Mass Spectrometry Interfacing Systems in Food Analysis: Pesticide, Drug and Toxic Substance Residues. *Journal of Chromatography A* **1996**, *727* (2), 153–184.

(26) Monica Gallo; Pasquale Ferranti. The Evolution of Analytical Chemistry Methods in Foodomics. *Journal of Chromatography A* **2016**, *1428*, 3–15.

(27) Robert J. McGorrin. One Hundred Years of Progress in Food Analysis. *ournal of Agricultural and Food Chemistry* **2009**, *57* (18), 8076–8088.

(28) Zhang, L.-G.; Zhang, X.; Ni, L.-J.; Xue, Z.-B.; Gu, X.; Huang, S.-X. Rapid Identification of Adulterated Cow Milk by Non-Linear Pattern Recognition Methods Based on near Infrared Spectroscopy. *Food Chemistry* **2014**, *145*, 342–348.

(29) Balabin, R. M.; Smirnov, S. V. Melamine Detection by Mid- and near-Infrared (MIR/NIR) Spectroscopy: A Quick and Sensitive Method for Dairy Products Analysis Including Liquid Milk, Infant Formula, and Milk Powder. *Talanta* **2011**, *85* (1), 562–568.

(30) Mauer, L. J.; Chernyshova, A. A.; Hiatt, A.; Deering, A.; Davis, R. Melamine Detection in Infant Formula Powder Using Near- and Mid-Infrared Spectroscopy.

Journal of Agricultural and Food Chemistry **2009**, *57* (10), 3974–3980.

(31) Haughey, S. A.; Graham, S. F.; Cancouët, E.; Elliott, C. T. The Application of Near-Infrared Reflectance Spectroscopy (NIRS) to Detect Melamine Adulteration of Soya Bean Meal. *Food Chemistry* **2013**, *136* (3–4), 1557–1561.

(32) De Medici, D.; Kuchta, T.; Knutsson, R.; Angelov, A.; Auricchio, B.; Barbanera, M.; Diaz-Amigo, C.; Fiore, A.; Kudirkiene, E.; Hohl, A.; et al. Rapid Methods for Quality Assurance of Foods: The Next Decade with Polymerase Chain Reaction (PCR)-Based Food Monitoring. *Food Analytical Methods* **2015**, *8* (2), 255–271.

(33) Rodríguez-Lázaro, D.; Cook, N.; Hernández, M. Real-Time PCR in Food Science: PCR Diagnostics. *Curr Issues Mol Biol* **2013**, *15* (2), 39–44.

(34) Maurer, J. *PCR Methods in Foods*; Springer Science & Business Media, 2006.

(35) Kwon, S. J.; Jeong, E. J.; Yoo, Y. C.; Cai, C.; Yang, G.-H.; Lee, J. C.; Dordick, J. S.; Linhardt, R. J.; Lee, K. B. High Sensitivity Detection of Active Botulinum Neurotoxin by Glyco-Quantitative Polymerase Chain-Reaction. *Analytical Chemistry* **2014**, *86* (5), 2279–2284.

(36) Cifuentes, A. Food Analysis : Present , Future , and Foodomics. *ISRN Analytical Chemistry* **2012**.

(37) Gonçalez, E., Nogueira, J.H., Fonseca, H., Felicio, J.D., Pino, F.A. and Corrêa, B. Mycobiota and Mycotoxins in Brazilian Peanut Kernels from Sowing to Harvest. *International Journal of Food Microbiology* **2008**, *123* (3), 184–190.

(38) Soler, C.; Y Pico. Recent Trends in Liquid Chromatography-Tandem Mass Spectrometry to Determine Pesticides and Their Metabolites in Food. *TrAC Trends in Analytical Chemistry* **2007**, *26* (2), 103–115.

(39) Chen, R.-K.; Chang, L.-W.; Chung, Y.-Y.; Lee, M.-H.; Ling, Y.-C. Quantification of Cow Milk Adulteration in Goat Milk Using High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometry. *Rapid Communications in Mass Spectrometry* **2004**, *18* (10), 1167–1171.

(40) Nebot, C.; Iglesias, A.; Regal, P.; Miranda, J.; Control, C. F.-F.; 2012, U. A Sensitive and Validated HPLC–MS/MS Method for Simultaneous Determination of Seven Coccidiostats in Bovine Whole Milk. *Food control* **2012**, *27* (1), 29–36.

(41) Jaimez, J.; Fente, C. .; Vazquez, B. .; Franco, C. .; Cepeda, A.; Mahuzier, G.;

Prognon, P. Application of the Assay of Aflatoxins by Liquid Chromatography with Fluorescence Detection in Food Analysis. *Journal of Chromatography A* **2000**, *882* (1–2), 1–10.

(42) Brera, C., J. M. Soriano, F. Debegnach, and M. M. Exposure Assessment to Ochratoxin A from the Consumption of Italian and Hungarian Wines. *Microchemical Journal* **2005**, *79* (1–2), 109–113.

(43) Jornet, D., O. Busto, and J. G. Solid-Phase Extraction Applied to the Determination of Ochratoxin A in Wines by Reversed-Phase High-Performance Liquid Chromatography. *Journal of Chromatography A* **2000**, *882* (1–2), 29–35.

(44) Kok, W. T. Derivatization Reactions for the Determination of Aflatoxins by Liquid Chromatography with Fluorescence Detection. *Journal of Chromatography B: Biomedical Sciences and Applications* **1994**, *659* (1–2), 127–137.

(45) Nguyen MT, Tozlovanu M, Tran TL, P.-L. A. Occurrence of Aflatoxin B1, Citrinin and Ochratoxin A in Rice in Five Provinces of the Central Region of Vietnam. *Food Chemistry* **2007**, *105* (1), 42–47.

(46) Tanaka T, Hasegawa A, Matsuki Y, Lee US, U. Y. Rapid and Sensitive Determination of Zearalenone in Cereals by High-Performance Liquid Chromatography with Fluorescence Detection. *Journal of Chromatography A* **1985**, *328*, 271–278.

(47) Janoszka, B. HPLC-Fluorescence Analysis of Polycyclic Aromatic Hydrocarbons (PAHs) in Pork Meat and Its Gravy Fried without Additives and in the Presence of Onion and Garlic. *Food Chemistry* **2011**, *126* (3), 1344–1353.

(48) Kevin J. Shefcheck, *; John H. Callahan, A.; Musser, S. M. Confirmation of Peanut Protein Using Peptide Markers in Dark Chocolate Using Liquid Chcromatography−Tandem Mass Spectrometry (LC-MS/MS). *J. Agric. Food Chem* **2006**, *54* (21), 7953–7959.

(49) Dorcas Weber, *, †; Philippe Raymond, ‡; Samuel Ben-Rejeb, † and; Lau†, B. Development of a Liquid Chromatography−Tandem Mass Spectrometry Method Using Capillary Liquid Chromatography and Nanoelectrospray Ionization−Quadrupole Time-of-Flight Hybrid Mass Spectrometer for the Detection of Milk Allergens. *J. Agric. Food Chem* **2006**, *54* (5), 1604–1610.

(50) Callahan, J. H.; Shefcheck, K. J.; Williams, T. L.; Musser, S. M. Detection, Confirmation, and Quantification of Staphylococcal Enterotoxin B in Food Matrixes Using Liquid Chromatography−Mass Spectrometry. *Analytical Chemistry* **2006**, *78* (6), 1789–1800.

(51) Kateřina Maštovská, †; Steven J. Lehotay, *, A.; Anastassiades‡, M. Combination of Analyte Protectants To Overcome Matrix Effects in Routine GC Analysis of Pesticide Residues in Food Matrixes. *Analytical Chemistry* **2005**, *77* (24), 8129–8127.

(52) Pirard, C.; Focant, J.-F.; De Pauw, E. An Improved Clean-up Strategy for Simultaneous Analysis of Polychlorinated Dibenzo- p -Dioxins (PCDD), Polychlorinated Dibenzofurans (PCDF), and Polychlorinated Biphenyls (PCB) in Fatty Food Samples. *Analytical and Bioanalytical Chemistry* **2002**, *372* (2), 373–381.

(53) Műller, L.; Barták, P.; Bednář, P.; Fryšová, I.; Ševčík, J.; Lemr, K. Capillary Electrophoresis-Mass Spectrometry – a Fast and Reliable Tool for the Monitoring of Milk Adulteration. *ELECTROPHORESIS* **2008**, *29* (10), 2088–2093.

(54) Martins, M. L.; Gimeno, A.; Martins, H. M.; Bernardo, F. Co-Occurrence of Patulin and Citrinin in Portuguese Apples with Rotten Spots. *Food Additives and Contaminants* **2002**, *19* (6), 568–574.

(55) Gimeno, A. Determination of Citrinin in Corn and Barley on Thin Layer Chromatographic Plates Impregnated with Glycolic Acid. *Journal-Association of Official Analytical Chemists* **1984**, *67* (1), 194–196.

(56) Zhang, D.; Zhang, H.; Yang, L.; Guo, J.; … X. L.-J. of F.; 2009, U. SIMULTANEOUS DETECTION OF LISTERIA MONOCYTOGENES, STAPHYLOCOCCUS AUREUS, SALMONELLA ENTERICA AND ESCHERICHIA COLI O157:H7. *Journal of food safety* **2009**, *29* (3), 348–363.

(57) Garrido, A.; Chapela, M.; Román, B.; … M. F.-… F. R. and; 2012, undefined. Development of a Multiplex Real-Time PCR Method for Simultaneous Detection of Salmonella Enterica, Shigella Flexneri and Listeria Monocytogenes in Processed. *Springer*.

(58) Garrido, A.; Chapela, M.-J.; Román, B.; Fajardo, P.; Vieites, J. M.; Cabado, A. G.

In-House Validation of a Multiplex Real-Time PCR Method for Simultaneous Detection of Salmonella Spp., Escherichia Coli O157 and Listeria Monocytogenes. *International Journal of Food Microbiology* **2013**, *164*, 92–98.

(59) Mason, J.; Xu, L.; Sheng, Z.; He, J.; Protocols, T. O.-N.; 2006, U. Liposome Polymerase Chain Reaction Assay for the Sub-Attomolar Detection of Cholera Toxin and Botulinum Neurotoxin Type A. *Nature Protocols* **2006**, *1* (4).

(60) McLAUCHLIN, J.; NARAYANAN, G. L.; MITHANI, V.; O'NEILL, G. The Detection of Enterotoxins and Toxic Shock Syndrome Toxin Genes in Staphylococcus Aureus by Polymerase Chain Reaction. *Journal of Food Protection* **2000**, *63* (4), 479–488.

(61) Mayra Granda Valdés &; Arístides Camilo Valdés González &; Josefa Angela García Calzón & Marta Elena Díaz-García. Analytical Nanotechnology for Food Analysis. *Microchim Acta* **2009**, *166*, 1–19.

(62) Citartan, M.; Gopinath, S. C. B.; Tominaga, J.; Tang, T.-H. Label-Free Methods of Reporting Biomolecular Interactions by Optical Biosensors. *The Analyst* **2013**, *138* (13), 3576.

(63) Brogan, K.; Biology, D. W.; 2005, undefined. Optical Fiber-Based Sensors: Application to Chemical Biology. *Current Opinion in Chemical Biology* **2005**, *9* (5), 494–500.

(64) Driscoll, A. J.; Harpster, M. H.; Johnson, P. A. The Development of Surface-Enhanced Raman Scattering as a Detection Modality for Portable in Vitro Diagnostics: Progress and Challenges. *Physical Chemistry Chemical Physics* **2013**, *15* (47), 20415.

(65) Simonova, M. A.; Valyakina, T. I.; Petrova, E. E.; Komaleva, R. L.; Shoshina, N. S.; Samokhvalova, L. V.; Lakhtina, O. E.; Osipov, I. V.; Philipenko, G. N.; Singov, E. K.; et al. Development of XMAP Assay for Detection of Six Protein Toxins. *Analytical Chemistry* **2012**, *84* (15), 6326–6330.

(66) van Oordt, T.; Stevens, G. B.; Vashist, S. K.; Zengerle, R.; von Stetten, F. Rapid and Highly Sensitive Luciferase Reporter Assay for the Automated Detection of Botulinum Toxin in the Centrifugal Microfluidic LabDisk Platform. *RSC Advances* **2013**, *3* (44), 22046.

(67) Ye, H.; Xia, X. Enhancing the Sensitivity of Colorimetric Lateral Flow Assay (CLFA) through Signal Amplification Techniques. *Journal of Materials Chemistry B* **2018**, *6* (44), 7102–7111.

(68) Stanker, L.; Scotcher, M.; Cheng, L.; Ching, K.; McGarvey, J.; Hodge, D.; Hnasko, R.; Stanker, L. H.; Scotcher, M. C.; Cheng, L.; et al. A Monoclonal Antibody Based Capture ELISA for Botulinum Neurotoxin Serotype B: Toxin Detection in Food. *Toxins* **2013**, *5* (11), 2212–2226.

(69) Stanker, L. H.; Merrill, P.; Scotcher, M. C.; Cheng, L. W. Development and Partial Characterization of High-Affinity Monoclonal Antibodies for Botulinum Toxin Type A and Their Use in Analysis of Milk by Sandwich ELISA. *Journal of Immunological Methods* **2008**, *336* (1), 1–8.

(70) Sapsford, K. E.; Francis, J.; Sun, S.; Kostov, Y.; Rasooly, A. Miniaturized 96-Well ELISA Chips for Staphylococcal Enterotoxin B Detection Using Portable Colorimetric Detector. *Analytical and Bioanalytical Chemistry* **2009**, *394* (2), 499–505.

(71) Ling, S.; Chen, Q.-A.; Zhang, Y.; Wang, R.; Jin, N.; Pang, J.; Wang, S. Development of ELISA and Colloidal Gold Immunoassay for Tetrodotoxin Detetcion Based on Monoclonal Antibody. *Biosensors and Bioelectronics* **2015**, *71*, 256–260.

(72) Feng, M.; Yong, Q.; Wang, W.; Kuang, H.; Wang, L.; Xu, C. Development of a Monoclonal Antibody-Based ELISA to Detect Escherichia Coli O157:H7. *Food and Agricultural Immunology* **2013**, *24* (4), 481–487.

(73) Kumar, S.; Balakrishna, K.; Batra, H. V. Enrichment-ELISA for Detection of Salmonella Typhi From Food and Water Samples. *Biomedical and Environmental Sciences* **2008**, *21* (2), 137–143.

(74) Gomaa, A.; Boye, J. Simultaneous Detection of Multi-Allergens in an Incurred Food Matrix Using ELISA, Multiplex Flow Cytometry and Liquid Chromatography Mass Spectrometry (LC–MS). *Food Chemistry* **2015**, *175*, 585–592.

(75) Mohammadzadeh-Asl, Saeideh, Ahmad Keshtkar, Jafar Ezzati Nazhad Dolatabadi, and M. de la G. Nanomaterials and Phase Sensitive Based Signal Enhancment in Surface Plasmon Resonance. *Biosensors and Bioelectronics* **2018**, *110*, 118–131.

(76) Karlsson, R. SPR for Molecular Interaction Analysis: A Review of Emerging

Application Areas. *Journal of Molecular Recognition* **2004**, *17* (3), 151–161.

(77) ELISA Fundamental Principle, How ELISA Works - Immunoassays https://www.bosterbio.com/protocol-and-troubleshooting/elisa-principle (accessed Feb 18, 2019).

(78) Cooper, M. A. Optical Biosensors in Drug Discovery. *Nature Reviews Drug Discovery*. July 2002, pp 515–528.

(79) Qiao, Z.; Fu, Y.; Lei, C.; Li, Y. Advances in Antimicrobial Peptides-Based Biosensing Methods for Detection of Foodborne Pathogens: A Review. *Food Control* **2020**, 107116.

(80) Lim, E. K.; Guk, K.; Kim, H.; Chung, B. H.; Jung, J. Simple, Rapid Detection of Influenza A (H1N1) Viruses Using a Highly Sensitive Peptide-Based Molecular Beacon. *Chemical Communications* **2016**, *52* (1), 175–178.

(81) Kalb, S.; Boyer, A.; Barr, J. Mass Spectrometric Detection of Bacterial Protein Toxins and Their Enzymatic Activity. *Toxins* **2015**, *7* (9), 3497–3511.

(82) von Berg, L.; Stern, D.; Pauly, D.; Mahrhold, S.; Weisemann, J.; Jentsch, L.; Hansbauer, E. M.; Müller, C.; Avondet, M. A.; Rummel, A.; et al. Functional Detection of Botulinum Neurotoxin Serotypes A to F by Monoclonal Neoepitope-Specific Antibodies and Suspension Array Technology. *Scientific Reports* **2019**, *9* (1).

(83) Sun, H.; Panicker, R. C.; Yao, S. Q. Activity Based Fingerprinting of Proteases Using FRET Peptides. *Peptide Science: Original Research on Biomolecules* **2007**, *88* (2), 141–149.

(84) Liu, X.; Wang, Y.; Chen, P.; Wang, Y.; Zhang, J.; Aili, D.; Liedberg, B. Biofunctionalized Gold Nanoparticles for Colorimetric Sensing of Botulinum Neurotoxin A Light Chain. *Analytical Chemistry* **2014**, *86* (5), 2345–2352.

(85) Frisk, M. L.; Tepp, W. H.; Johnson, E. A.; Beebe, D. J. Self-Assembled Peptide Monolayers as a Toxin Sensing Mechanism within Arrayed Microchannels. *Analytical Chemistry* **2009**, *81* (7), 2760–2767.

(86) Chen, H.; Chen, P.; Huang, J.; Selegård, R.; Platt, M.; Palaniappan, A.; Aili, D.; Tok, A. I. Y.; Liedberg, B. Detection of Matrilysin Activity Using Polypeptide Functionalized Reduced Graphene Oxide Field-Effect Transistor Sensor. *Analytical*

Chemistry **2016**, *88* (6), 2994–2998.

(87) Chen, P.; Selegård, R.; Aili, D.; Liedberg, B. Peptide Functionalized Gold Nanoparticles for Colorimetric Detection of Matrilysin (MMP-7) Activity. *Nanoscale* **2013**, *5* (19), 8973–8976.

(88) Chen, P.; Liu, X.; Goyal, G.; Tran, N. T.; Shing Ho, J. C.; Wang, Y.; Aili, D.; Liedberg, B. Nanoplasmonic Sensing from the Human Vision Perspective. *Analytical Chemistry* **2018**, *90* (7), 4916–4924.

(89) Wang, Y.; Liu, X.; Zhang, J.; Aili, D.; Liedberg, B. Time-Resolved Botulinum Neurotoxin A Activity Monitored Using Peptide-Functionalized Au Nanoparticle Energy Transfer Sensors. *Chemical Science* **2014**, *5* (7), 2651–2656.

(90) Bisswanger, H. Enzyme Assays. *Perpectives in Science* **2014**, *1* (1–6), 41–55.

(91) Mandal, P.; Biswas, A.; Choi, K.; Food, U. P.-A. J. O.; 2011, U. Methods for Rapid Detection of Foodborne Pathogens: An Overview. *American Journal Of Food Technology* **2011**, *6* (2), 87–102.

(92) Kanjanawarut, R.; Su, X. Colorimetric Detection of DNA Using Unmodified Metallic Nanoparticles and Peptide Nucleic Acid Probes. *Analytical Chemistry* **2009**, *81* (15), 6122–6129.

(93) Liu, X.; Wang, Y.; Chen, P.; McCadden, A.; Palaniappan, A.; Zhang, J.; Liedberg, B. Peptide Functionalized Gold Nanoparticles with Optimized Particle Size and Concentration for Colorimetric Assay Development: Detection of Cardiac Troponin I. *ACS Sensors* **2016**, *1* (12), 1416–1422.

(94) Park, J.-H.; Byun, J.-Y.; Mun, H.; Shim, W.-B.; Shin, Y.-B.; Li, T.; Kim, M.-G. A Regeneratable, Label-Free, Localized Surface Plasmon Resonance (LSPR) Aptasensor for the Detection of Ochratoxin A. *Biosensors and Bioelectronic* **2014**, *59*, 321–327.

(95) Cheng, H.-P.; Chuang, H.-S. Rapid and Sensitive Nano-Immunosensors for Botulinum. *ACS Sensors* **2019**, *4* (7), 1754–1760.

(96) Anna Laromaine, †; Liling Koh, †; Muthu Murugesan, †; Rein V. Ulijn, *, A.; Molly M. Stevens*, †. Protease-Triggered Dispersion of Nanoparticle Assemblies. *Journal of the American Chemical Society* **2007**, *129* (14), 4156–4157.

(97) Goldman, E. R.; Anderson, G. P.; Tran, P. T.; Mattoussi, H.; Charles, P. T.; Mauro,

J. M. Conjugation of Luminescent Quantum Dots with Antibodies Using an Engineered Adaptor Protein To Provide New Reagents for Fluoroimmunoassays. *Analytical Chemistry* **2002**, *74* (4), 841–847.

(98) Warner, M.; Grate, J.; Tyler, A.; … R. O. Quantum Dot Immunoassays in Renewable Surface Column and 96-Well Plate Formats for the Fluorescence Detection of Botulinum Neurotoxin Using High-Affinity. *Biosensors and Bioelectronics* **2009**, *25* (1), 179–184.

(99) Goldman, E. R.; Medintz, I. L.; Mattoussi, H. Luminescent Quantum Dots in Immunoassays. *Analytical and Bioanalytical Chemistry* **2006**, *384* (3), 560–563.

(100) Shi, L.; De Paoli, V.; Rosenzweig, N.; Rosenzweig, Z. Synthesis and Application of Quantum Dots FRET-Based Protease Sensors. *J. AM. CHEM. SOC* **2006**, *128*, 10378–10379.

(101) Klisara, N.; Palaniappan, A.; Liedberg, B. Sorbent-Incorporated Dipstick for Direct Assaying of Proteases. *Analytical and Bioanalytical Chemistry* **2020**.

(102) Wang, L.; Zhao, W.; O'Donoghue, M. B.; Tan, W. Fluorescent Nanoparticles for Multiplexed Bacteria Monitoring. *Bioconjugate Chemistry* **2007**, *18* (2), 297–301.

(103) Gijs, M. A. M. Magnetic Bead Handling On-Chip: New Opportunities for Analytical Applications. *Microfluidics and Nanofluidics* **2004**, *1* (1), 22–40.

(104) Kim, K.; Choi, N.; Jeon, J. H.; Rhie, G.; Choo, J. SERS-Based Immunoassays for the Detection of Botulinum Toxins A and B Using Magnetic Beads. *Sensors* **2019**, *19* (19), 4081.

(105) Peters, J.; Cardall, A.; Haasnoot, W.; Nielen, M. W. F. Aj. **2014**.

(106) Orlov, A. V.; Znoyko, S. L.; Cherkasov, V. R.; Nikitin, M. P.; Nikitin, P. I. Multiplex Biosensing Based on Highly Sensitive Magnetic Nanolabel Quantification: Rapid Detection of Botulinum Neurotoxins A, B, and E in Liquids. *Analytical Chemistry* **2016**, *88* (21), 10419–10426.

(107) Wang, D.-B.; Tian, B.; Zhang, Z.-P.; Wang, X.-Y.; Fleming, J.; Bi, L.-J.; Yang, R.-F.; Zhang, X.-E. Detection of Bacillus Anthracis Spores by Super-Paramagnetic Lateral-Flow Immunoassays Based on "Road Closure." *Biosensors and Bioelectronics* **2015**, *67*, 608–614.

(108) Weng, X.; Neethirajan, S. Ensuring Food Safety: Quality Monitoring Using

Microfluidics. *Trends in Food Science & Technology* **2017**, *65*, 10–22.

(109) Mechaly, A.; Vitner, E.; Levy, H.; Weiss, S.; Bar-David, E.; Gur, D.; Koren, M.; Cohen, H.; Cohen, O.; Mamroud, E.; et al. Simultaneous Immunodetection of Anthrax, Plague, and Tularemia from Blood Cultures by Use of Multiplexed Suspension Arrays. *Journal of Clinical Microbiology* **2018**, *56* (4), e01479-17.

(110) Xiong, Q.; Lim, C. Y.; Ren, J.; Zhou, J.; Pu, K.; Chan-Park, M. B.; Mao, H.; Lam, Y. C.; Duan, H. Magnetic Nanochain Integrated Microfluidic Biochips. *Nature Communications* **2018**, *9*, 1743.

(111) Luan, Y.; Chen, J.; Xie, G.; Li, C.; Ping, H.; Ma, Z.; Lu, A. Visual and Microplate Detection of Aflatoxin B2 Based on NaCl-Induced Aggregation of Aptamer-Modified Gold Nanoparticles. *Microchimica Acta* **2015**, *182* (5–6), 995–1001.

(112) Wachiralurpan, S.; Sriyapai, T.; Areekit, S.; Sriyapai, P.; Augkarawaritsawong, S.; Santiwatanakul, S.; Chansiri, K. Rapid Colorimetric Assay for Detection of Listeria Monocytogenes in Food Samples Using LAMP Formation of DNA Concatemers and Gold Nanoparticle-DNA Probe Complex. *Frontiers in chemistry* **2018**, *6*, 90.

(113) Sun, J.; Ge, J.; Liu, W.; Wang, X.; Fan, Z.; Zhao, W.; Zhang, H.; Wang, P.; Lee, S.-T. A Facile Assay for Direct Colorimetric Visualization of Lipopolysaccharides at Low Nanomolar Level. *Nano Research* **2012**, *5* (7), 486–493.

(114) Zhou, Q.; Liu, N.; Qie, Z.; Wang, Y.; Ning, B.; Gao, Z. Development of Gold Nanoparticle-Based Rapid Detection Kit for Melamine in Milk Products. *Journal of Agricultural and Food Chemistry* **2011**, *59* (22), 12006–12011.

(115) Phillips, R. L.; Miranda, O. R.; You, C.-C.; Rotello, V. M.; Bunz, U. H. F. Rapid and Efficient Identification of Bacteria Using Gold-Nanoparticle–Poly(Para-Phenyleneethynylene) Constructs. *Angewandte Chemie International Edition* **2008**, *47* (14), 2590–2594.

(116) Shi, J.; Chan, C.; Pang, Y.; Ye, W.; Tian, F.; Lyu, J.; Zhang, Y.; Yang, M. A Fluorescence Resonance Energy Transfer (FRET) Biosensor Based on Graphene Quantum Dots (GQDs) and Gold Nanoparticles (AuNPs) for the Detection of MecA Gene Sequence of Staphylococcus Aureus. *Biosensors and Bioelectronics* **2015**, *67*, 595–600.

(117) Yang, M.; Kostov, Y.; Bruck, H. A.; Rasooly, A. Gold Nanoparticle-Based Enhanced

Chemiluminescence Immunosensor for Detection of Staphylococcal Enterotoxin B (SEB) in Food. *International Journal of Food Microbiology* **2009**, *133* (3), 265–271.

(118) Klisara, N.; Peters, J.; Haasnoot, W.; Nielen, M. W. F.; Palaniappan, A.; Liedberg, B. Functional Fluorescence Assay of Botulinum Neurotoxin A in Complex Matrices Using Magnetic Beads. *Sensors and Actuators B: Chemical* **2019**, *281*, 912–919.

(119) Hayat, A.; Mishra, R. K.; Catanante, G.; Marty, J. L. Development of an Aptasensor Based on a Fluorescent Particles-Modified Aptamer for Ochratoxin A Detection. *Analytical and Bioanalytical Chemistry* **2015**, *407* (25), 7815–7822.

(120) Cadková, M. C.; Metelka, R.; Holubová, L.; Horák, D.; Dvořáková, V.; Bílková, Z.; Korecká, L. Magnetic Beads-Based Electrochemical Immunosensor for Monitoring Allergenic Food Proteins. *ANALYTICAL BIOCHEMISTRY* **2015**, *484*, 4–8.

(121) Mendes, R. K.; Laschi, S.; Stach-Machado, D. R.; Kubota, L. T.; Marrazza, G. A Disposable Voltammetric Immunosensor Based on Magnetic Beads for Early Diagnosis of Soybean Rust. *Sensors & Actuators: B. Chemical* **2012**, *166–167*, 135–140.

(122) Ng, E.; Nadeau, K. C.; Wang, S. X. Giant Magnetoresistive Sensor Array for Sensitive and Specific Multiplexed Food Allergen Detection. **2016**.

(123) Joo, J.; Yim, C.; Kwon, D.; Lee, J.; Shin, H. H.; Cha, H. J.; Jeon, S. A Facile and Sensitive Detection of Pathogenic Bacteria Using Magnetic Nanoparticles and Optical Nanocrystal Probes. *The Analyst* **2012**, *137* (16), 3609.

(124) Liang, M.; Fan, K.; Pan, Y.; Jiang, H.; Wang, F.; Yang, D.; Lu, D.; Feng, J.; Zhao, J.; Yang, L.; et al. Fe3O4 Magnetic Nanoparticle Peroxidase Mimetic-Based Colorimetric Assay for the Rapid Detection of Organophosphorus Pesticide and Nerve Agent. *Analytical Chemistry* **2013**, *85* (1), 308–312.

(125) Wen, C.-Y.; Hu, J.; Zhang, Z.-L.; Tian, Z.-Q.; Ou, G.-P.; Liao, Y.-L.; Li, Y.; Xie, M.; Sun, Z.-Y.; Pang, D.-W. One-Step Sensitive Detection of Salmonella Typhimurium by Coupling Magnetic Capture and Fluorescence Identification with Functional Nanospheres. *Analytical Chemistry* **2013**, *85* (2), 1223–1230.

(126) Nygaard, A. P.; Hall, B. D. A Method for the Detection of RNA-DNA Complexes. *Biochemical and Biophysical Research Communications* **1963**, *12* (2), 98–104.

(127) Song, S.; Liu, N.; Zhao, Z.; Njumbe Ediage, E.; Wu, S.; Sun, C.; De Saeger, S.; Wu,

A. Multiplex Lateral Flow Immunoassay for Mycotoxin Determination. *Analytical Chemistry* **2014**, *86* (10), 4995–5001.

(128) Yang, W.; Li, X.; Liu, G.; Zhang, B.; Zhang, Y.; Kong, T.; Tang, J.; Li, D.; Wang, Z. A Colloidal Gold Probe-Based Silver Enhancement Immunochromatographic Assay for the Rapid Detection of Abrin-A. *Biosensors and Bioelectronics* **2011**, *26* (8), 3710–3713.

(129) Fu, E.; Liang, T.; Houghtaling, J.; Ramachandran, S.; Ramsey, S. A.; Lutz, B.; Yager, P. Enhanced Sensitivity of Lateral Flow Tests Using a Two-Dimensional Paper Network Format. *Analytical Chemistry* **2011**, *83* (20), 7941–7946.

(130) Shin, J. H.; Hong, J.; Go, H.; Park, J.; Kong, M.; Ryu, S.; Kim, K.-P.; Roh, E.; Park, J.-K. Multiplexed Detection of Foodborne Pathogens from Contaminated Lettuces Using a Handheld Multistep Lateral Flow Assay Device. *Journal of Agricultural and Food Chemistry* **2018**, *66* (1), 290–297.

(131) Chen, P.; Gates-Hollingsworth, M.; Pandit, S.; Park, A.; Montgomery, D.; AuCoin, D.; Gu, J.; Zenhausern, F. Paper-Based Vertical Flow Immunoassay (VFI) for Detection of Bio-Threat Pathogens. *Talanta* **2019**, *191*, 81–88.

(132) Singlepath® Listeria Lateral Flow Tests from Merck KGaA, Darmstadt, Germany | SelectScience https://www.selectscience.net/products/singlepath-listeria-lateral-flow-tests/?prodID=195034 (accessed Feb 23, 2019).

(133) Chaiyo, S.; Siangproh, W.; Apilux, A.; Chailapakul, O. Highly Selective and Sensitive Paper-Based Colorimetric Sensor Using Thiosulfate Catalytic Etching of Silver Nanoplates for Trace Determination of Copper Ions. *Analytica Chimica Acta* **2015**, *866*, 75–83.

(134) Li, C.; Vandenberg, K.; Prabhulkar, S.; Zhu, X.; Schneper, L.; Methee, K.; Rosser, C. J.; Almeide, E. Paper Based Point-of-Care Testing Disc for Multiplex Whole Cell Bacteria Analysis. *Biosensors and Bioelectronics* **2011**, *26* (11), 4342–4348.

(135) Hossain, S. M. Z.; Brennan, J. D. β-Galactosidase-Based Colorimetric Paper Sensor for Determination of Heavy Metals. *Analytical Chemistry* **2011**, *83* (22), 8772–8778.

(136) Hossain, S. M. Z.; Luckham, R. E.; McFadden, M. J.; Brennan, J. D. Reagentless Bidirectional Lateral Flow Bioactive Paper Sensors for Detection of Pesticides in Beverage and Food Samples. *Analytical Chemistry* **2009**, *81* (21), 9055–9064.

(137) Hossain, S. M. Z.; Luckham, R. E.; Smith, A. M.; Lebert, J. M.; Davies, L. M.; Pelton, R. H.; Filipe, C. D. M.; Brennan, J. D. Development of a Bioactive Paper Sensor for Detection of Neurotoxins Using Piezoelectric Inkjet Printing of Sol−Gel-Derived Bioinks. *Analytical Chemistry* **2009**, *81* (13), 5474–5483.

(138) Hossain, S. M. Z.; Brennan, J. D. β-Galactosidase-Based Colorimetric Paper Sensor for Determination of Heavy Metals. *Analytical Chemistry* **2011**, *83* (22), 8772–8778.

(139) Cunningham, J. C.; Scida, K.; Kogan, M. R.; Wang, B.; Ellington, A. D.; Crooks, R. M. Paper Diagnostic Device for Quantitative Electrochemical Detection of Ricin at Picomolar Levels. *Lab on a Chip* **2015**, *15* (18), 3707–3715.

(140) Quesada-González, D.; Merkoçi, A. Nanoparticle-Based Lateral Flow Biosensors. *Biosensors and Bioelectronics* **2015**, *73*, 47–63.

(141) Latif, S.; Pfannstiel, J.; Makkar, H.; Chemistry, K. B. Amino Acid Composition, Antinutrients and Allergens in the Peanut Protein Fraction Obtained by an Aqueous Enzymatic Process. *Food chemistry* **2013**, *136* (1), 213–217.

(142) Moreno-Bondi, M. C.; Marazuela, M. D.; Herranz, S.; Rodriguez, E. An Overview of Sample Preparation Procedures for LC-MS Multiclass Antibiotic Determination in Environmental and Food Samples. *Analytical and Bioanalytical Chemistry* **2009**, *395* (4), 921–946.

(143) Andrade, P. D.; da Silva, J. L. G.; Caldas, E. D. Simultaneous Analysis of Aflatoxins B1, B2, G1, G2, M1 and Ochratoxin A in Breast Milk by High-Performance Liquid Chromatography/Fluorescence after Liquid-Liquid Extraction with Low Temperature Purification (LLE-LTP). *Journal of Chromatography A* **2013**, *1304*, 61–68.

(144) Picó, Y.; Fernández, M.; Ruiz, M. J.; Font, G. Current Trends in Solid-Phase-Based Extraction Techniques for the Determination of Pesticides in Food and Environment. *Journal of Biochemical and Biophysical Methods*. March 10, 2007, pp 117–131.

(145) Naing, N. N.; Tan, S. C.; Lee, H. K. Micro-Solid-Phase Extraction. In *Solid-Phase Extraction*; Elsevier, 2020; pp 443–471.

(146) Li, G.; Yu, W.; Xiao, Z.; Long, M.; Tong, L.; Qiu, Y. A Modified QuEChERS/GC–MS for Simultaneous Determination of 16 Pesticide Adjuvant Residues in Fruits and Vegetables. *SN Applied Sciences* **2020**, *2* (1), 35.

(147) Bueno, D.; Istamboulie, G.; Muñoz, R.; Marty, J. L. Determination of Mycotoxins in Food: A Review of Bioanalytical to Analytical Methods. *Applied Spectroscopy Reviews* **2015**, *50* (9), 728–774.

(148) Rahmani, A.; Jinap, S.; Soleimany, F. Qualitative and Quantitative Analysis of Mycotoxins. *Comprehensive Reviews in Food Science and Food Safety* **2009**, *8* (3), 202–251.

(149) van Apeldoorn, M. E.; van Egmond, H. P.; Speijers, G. J. A.; Bakker, G. J. I. Toxins of Cyanobacteria. *Molecular Nutrition & Food Research* **2007**, *51* (1), 7–60.

(150) Sandoval Riofrio, M. A.; Alexandra, M. Extraction of Phorbol Esters (PEs) from Pinion Cake Using Computationally-Designed Polymers as Adsorbents for Solid Phase Extraction. **2017**.

(151) 4.1: Overview of Extraction - Chemistry LibreTexts https://chem.libretexts.org/Bookshelves/Organic_Chemistry/Book%3A_Organic_Chemistry_Lab_Techniques_(Nichols)/4%3A_Extraction/4.1%3A_Overview_of_Extraction (accessed Apr 27, 2019).

(152) Dzantiev, B. B.; Byzova, N. A.; Urusov, A. E.; Zherdev, A. V. Immunochromatographic Methods in Food Analysis. *TrAC - Trends in Analytical Chemistry*. Elsevier B.V. 2014, pp 81–93.

(153) Şenyuva, H. Z.; Gilbert, J. Immunoaffinity Column Clean-up Techniques in Food Analysis: A Review. *Journal of Chromatography B* **2010**, *878* (2), 115–132.

(154) Tsai, W.; Huang, T.; Huang, J.; A, Y. H.-… of C.; 2009, undefined. Dispersive Solid-Phase Microextraction Method for Sample Extraction in the Analysis of Four Tetracyclines in Water and Milk Samples by High-Performance Liquid. *Journal of Chromatography A* **2009**, *1216* (12), 2263–2269.

(155) Mattarozzi, M.; Milioli, M.; Bianchi, F.; Cavazza, A.; Control, S. P. Optimization of a Rapid QuEChERS Sample Treatment Method for HILIC-MS2 Analysis of Paralytic Shellfish Poisoning (PSP) Toxins in Mussels. *Food Control* **2016**, *60*, 138–145.

(156) Jakubowska, N.; Polkowska, Ż.; Namieśnik, J.; Przyjazny, A. Analytical Applications of Membrane Extraction for Biomedical and Environmental Liquid Sample Preparation. *Critical Reviews in Analytical Chemistry* **2005**, *35* (3), 217–235.

(157) Benoit, P. W.; Donahue, D. W. *Methods for Rapid Separation and Concentration of Bacteria in Food That Bypass Time-Consuming Cultural Enrichment*; 2003; Vol. 66.

(158) Jönsson, J. Å.; Mathiasson, L. Membrane-Based Techniques for Sample Enrichment. *Journal of Chromatography A* **2000**, *902* (1), 205–225.

(159) Klisara, N.; Yu, Y. M.; Palaniappan, A.; Liedberg, B. Towards On-Site Visual Detection of Proteases in Food Matrices. *Analytica Chimica Acta* **2019**, *1078*, 182–188.

(160) Xuan, Z.; Ye, J.; Zhang, B.; Li, L.; Wu, Y.; Wang, S. An Automated and High-Throughput Immunoaffinity Magnetic Bead-Based Sample Clean-Up Platform for the Determination of Aflatoxins in Grains and Oils Using UPLC-FLD. *Toxins* **2019**, *11* (10), 583.

(161) Wu, R.; Xing, X.; Corredig, M.; Meng, B.; Griffiths, M. W. Concentration of Hepatitis A Virus in Milk Using Protamine-Coated Iron Oxide (Fe3O4) Magnetic Nanoparticles. *Food Microbiology* **2019**, *84*, 103236.

(162) Saini, S. S.; Kaur, A. Molecularly Imprinted Polymers for the Detection of Food Toxins: A Minireview. *Advances in Nanoparticles* **2013**, No. 2, 60–65.

(163) Pilolli, R.; Chaudhari, R.; Palmisano, F.; Monaci, L. Development of a Mass Spectrometry Immunoassay for Unambiguous Detection of Egg Allergen Traces in Wines. *Analytical and Bioanalytical Chemistry* **2017**, *409* (6), 1581–1589.

(164) Smits, N. G. E.; Blokland, M. H.; Wubs, K. L.; Nessen, M. A.; Van Ginkel, L. A.; Nielen, M. W. F. Monolith Immuno-Affinity Enrichment Liquid Chromatography Tandem Mass Spectrometry for Quantitative Protein Analysis of Recombinant Bovine Somatotropin in Serum. *Analytical and Bioanalytical Chemistry* **2015**, *407* (20), 6041–6050.

(165) Worbs, Sylvia, Uwe Fiebig, Reinhard Zeleny, Heinz Schimmel, Andreas Rummel, Werner Luginbühl, and B. D. Qualitative and Quantitative Detection of Botulinum Neurotoxins from Complex Matrices: Results of the First International Proficiency Test. *Toxins* **2015**, *7* (12), 4935–4966.

(166) Rossetto, O.; Pirazzini, M.; Montecucco, C. Botulinum Neurotoxins: Genetic, Structural and Mechanistic Insights. *Nature Reviews Microbiology* **2014**, *12* (8), 535–549.

(167) Os, A. T.; Uce, R.; Cher, R. Methods for Detecting Botulinum Toxin with Applicability to Screening Foods. *Concise Reviews / Hypotheses in Food Science* **2005**, *70* (8), 121–130.

(168) Singh, B. R. Botulinum Neurotoxin Structure, Engineering, and Novel Cellular Trafficking and Targeting. *Neurotoxicity Research* **2006**, *9* (2–3), 73–92.

(169) Le Maréchal, C.; Woudstra, C.; Fach, P. Botulism. In *Clostridial Diseases of Animals*; John Wiley & Sons, Inc: Hoboken, NJ, 2016; pp 303–330.

(170) Kammerer, R. A.; Benoit, R. M. Botulinum Neurotoxins: New Questions Arising from Structural Biology. *Trends in Biochemical Sciences* **2014**, *39* (11), 517–526.

(171) Čapek, P.; Dickerson, T. Sensing the Deadliest Toxin: Technologies for Botulinum Neurotoxin Detection. *Toxins* **2010**, *2* (1), 24–53.

(172) Lam, K.-H.; Yao, G.; Jin, R. Diverse Binding Modes, Same Goal: The Receptor Recognition Mechanism of Botulinum Neurotoxin. *Progress in Biophysics and Molecular Biology* **2015**, *117* (2–3), 225–231.

(173) Thomas Binz, *,‡; Steffen Bade, ‡; Andreas Rummel, ‡; Astrid Kollewe, ‡ and; Alves§, J. Arg362 and Tyr365 of the Botulinum Neurotoxin Type A Light Chain Are Involved in Transition State Stabilization†. **2002**.

(174) Maslanka, S. E.; Lúquez, C.; Dykes, J. K.; Tepp, W. H.; Pier, C. L.; Pellett, S.; Raphael, B. H.; Kalb, S. R.; Barr, J. R.; Rao, A.; et al. A Novel Botulinum Neurotoxin, Previously Reported as Serotype H, Has a Hybrid-like Structure with Regions of Similarity to the Structures of Serotypes A and F and Is Neutralized with Serotype A Antitoxin. *Journal of Infectious Diseases* **2016**, *213* (3), 379–385.

(175) Peng, L.; Liu, H.; Ruan, H.; Tepp, W. H.; Stoothoff, W. H.; Brown, R. H.; Johnson, E. A.; Yao, W.-D.; Zhang, S.-C.; Dong, M. Cytotoxicity of Botulinum Neurotoxins Reveals a Direct Role of Syntaxin 1 and SNAP-25 in Neuron Survival. *Nature Communications* **2013**, *4* (1), 1472.

(176) Binz, T.; Sikorra, S.; Mahrhold, S.; Binz, T.; Sikorra, S.; Mahrhold, S. Clostridial Neurotoxins: Mechanism of SNARE Cleavage and Outlook on Potential Substrate Specificity Reengineering. *Toxins* **2010**, *2* (4), 665–682.

(177) Uses of Botulinum Toxin and Botulinum Toxin Testing - Pacific BioLabs https://pacificbiolabs.com/uses-of-botulinim-toxin-and-botulinum-toxin-t/ (accessed

Mar 4, 2019).

(178) Tighe, A. P.; Schiavo, G. Botulinum Neurotoxins: Mechanism of Action. *Toxicon* **2013**, *67*, 87–93.

(179) Masuyer, G.; Chaddock, J. A.; Foster, K. A.; Acharya, K. R. Engineered Botulinum Neurotoxins as New Therapeutics. *Annual Review of Pharmacology and Toxicology* **2014**, *54* (1), 27–51.

(180) Hobbs, R. J.; Thomas, C. A.; Halliwell, J.; Gwenin, C. D. Rapid Detection of Botulinum Neurotoxins—A Review. *Toxins* **2019**, *11* (7), 418.

Chapter 3

Experimental Methodology

This chapter describes materials, developed protocols and characterization techniques used in this study. Firstly, the choice of materials used in this study is explained followed by protocols, sample preparations and the fabrication of dipstick (colorimetric and fluorimetric) used in the assay development. Then, characterization techniques such as UV/VIS (for fluorescence measurements), MALDI-TOF (for revealing the peptide cleavage and characterization of clean-up protocol), ZP (for determination of particles charge), BCA assay (for determination of the amount of antibodies), and HPLC (for characterization of the membrane clean-up process) are described. Finally, a brief overview of methodologies is given.

*****Materials and protocols were taken from: Klisara, Nevena, et al. "Functional fluorescence assay of botulinum neurotoxin A in complex matrices using magnetic beads." *Sensors and Actuators B: Chemical* 281 (2019): 912-919; Klisara, Nevena, et al. "Towards on-site visual detection of proteases in food matrices." *Analytica Chimica Acta* (2019); Klisara, Nevena, et al. "Sorbent Incorporated Dipstick for Direct Assaying of Proteases." *Analytical and Bioanalytical Chemistry* (2020).

3.1 Rationale for selection of Methods and Materials

3.1.1 Rationale for Material Selection

3.1.1.1 Rationale for Selection of Magnetic Beads

In recent years, a huge attention was attracted by magnetic particles (MP) ranging from nanometers to micrometers due to their features such as biocompatibility, low toxicity, facile preparation processes and functionalizability, low background interference, superparamagnetic behaviour, and roles such as carrier, reporter, and amplifier[1]. Magnetic particles have been used for various purposes such as imaging agents, drug delivery carrier, transducers, signal amplifiers, in separation fields, etc[2]. The utilization of MPs in biosensing applications is favourable due to the particle size, their surface and colloidal stability in different media and they are not affected by reagent chemistry, a high surface to volume ratio that accommodates a high density of functional moieties, simple biofunctionalization, and commercial availability[3]. The chemistry of surface functionalization such as the presence of carboxyl group, amine group, maleimide group, streptavidin, A protein is one of the key parameter to diverse bioconjugation (small molecules, drugs, nucleotides, proteins)[4]. One of the major advantage of MPs is that they can be magnetically probed and manipulated using external magnetic field, which is very well utilized in clean-up processes substituting the laborious and time consuming separation techniques[5]. Additionally, it is possible to obtain and control desired morphology and size according to the particular application thanks to different methods of synthesis. In the first part of this thesis, monosized superparamagnetic beads (micron-sized) were used for the clean-up due to their colloidal stability, which is accomplished by using a highly cross-linked polystyrene with magnetic material (iron oxide) precipitated within the pores and evenly distributed throughout the particles. Apart from this, a hydrophilic layer of glycidyl ether is used to coat the particles providing the MNPs with a smooth surface, which enable more uniform degree of functionalization, which is one of the critical parameter for achieving assay repeatability. It has been reported that larger beads containing more magnetic material enable better separation[6]. However, for the development of LFA/dipstick

assay, micron-sized MNPs were not successfully employed because of a slow migration and larger physisorption of beads on the LFA membrane due to their bulky size, which affected the response and sensitivity. These limitations were overcome by employing nanometer-sized MNPs (200 nm), which migrate faster and enable the sensitive detection due to the increased bead density across the LFA test line. However, rapid migration of MNPs on LFA membrane may lower the sensitivity of an assay due to the reduced reaction time between antigen and antibody, which is immobilized in the conjugation pad. Therefore, the MNPs size should be chosen based on the assay requirements[7].

It is well known that MPs tend to aggregate which results in increased particles size as a consequence of large surface energy, dipole-dipole interactions and van der Waals attraction forces that restricts their application. Nevertheless, the aggregation and colloidal instability are overcome by electrostatic (ionic liquids; repulsive forces), steric (polymers and surfactants; increases the distance between particles, while magnetic properties decreases) and electrosteric (polyelectrolytes, ionic surfactants) stabilization approaches[8]. MPs have been widely applied in biosensor science for detections of proteins, enzymes, bacteria, toxins, viruses, etc. enabling even complex multiplex analysis, which placed them as an important asset in these developments[9].

3.1.1.2 Rationale for Selection of Peptides

Peptides, short-chain amino acid oligomers, have been widely utilized in diagnostic and biosensing applications as recognition elements[10]. Many peptides have been found in natural sources: nervous system, cardiovascular system, intestine, etc., and can be easily and cost effectively synthesized in large scale with the solid phase synthesis. Additionally, it is possible to vary the number and positions of 20 amino acids in the sequence in many ways, obtaining various chemical and biological properties. Moreover, modifications of the peptide ends via N-amine and C-carboxyl terminal is an easy process, which results in a large number of unique peptide substrates[11]. Peptide's modifications including attachments of small organic molecules (ligands) can lead to enhanced binding affinity between peptides and targets. Besides, the peptide's design can be projected according to

the required biomimetic function. Apart from this, the synthesis of these synthetic molecules is highly reproducible placing them in the spot of intense studies. Compared to antibodies, peptides are smaller with low molecular weight and also more chemically stable which gives them advantage to be used in harsh physicochemical conditions (pH and temperature). The extraordinary stability comes from the short-chain peptide structure, therefore peptide-based sensors show better shelf-life compared to the antibodies-based sensors[12]. As a biorecognition molecule employed in bioassays, they are specific and selective towards the target, which enables more precise detection. Regarding the mentioned, peptides are an attractive option to be used as recognition entities in biosensing applications. So far there are many peptide-based sensors developed for detection of various targets ranging from living cells, active proteases, pathogens, toxins, viruses, biomarkers to small inorganic ions.

3.2 Biosensing using the magnetic bead-based assay in solution

3.2.1 Superparamagnetic bead-antibody conjugation

The stock solution of superparamagnetic beads was vortexed for 5 min in order to re-suspend the superparamagnetic beads and an aliquot of 100 µl was pipetted into a vial. The beads were placed in the magnetic rack (DynaMag™-2, Invitrogen; 2.8 µm) for 2 min and the supernatant was discarded. The beads were washed twice with 200 µl of 25 mM MES (pH = 5). The beads were then re-suspended in 50 µl of EDC and 50 µl of NHS solution, both at a concentration of 50 mg/mL and dissolved in 25 mM MES. The beads were incubated at room temperature (RT) in the dark for 20 min with intermittent vortexing for 5 s. After incubation, the beads were washed twice with 200 µl of 25 mM MES buffer. For covalent antibody coupling, 500 µl of the antibody solution at a concentration of 60 µg/mL dissolved in 25 mM MES buffer was utilized. The reaction was conducted at RT in the dark over 2 h with constant shaking and rotating. After incubation, the beads were washed with 500 µl of the storage/blocking buffer (PBS + 0.1% BSA + 0.05% Azide, pH 7.4). The beads were then captured using a magnet; the supernatant was discarded and the beads were blocked with 500 µl of the storage/blocking buffer for 30 min at RT in the dark with

constant shaking and rotating. After blocking, the antibody-coupled beads were re-suspended in 100 µl of the storage/blocking buffer and stored at 4 ºC in the dark.

3.2.2 Detection of BoNT/A LC using Strategy 1 (ST -1)

The peptide-containing PEG_{11} unit (100 nM; 290.14 ng/mL) was bound to anti-PEG functionalized beads (approximately 10^4 beads/assay) in a 96-well plate at RT (Corning) under constant shaking for 30 min (400 rpm, Thermo Shaker Incubator, Hangzhou Miu Instruments) in HEPES buffer (50 mM HEPES + 0.1% BSA, pH 7.4) followed by separation of the peptide-bead complexes from the unbound peptides using a magnet (LifeSep™-96F). After the washing steps (twice), the peptide-bead complexes were incubated (digested) with different concentrations of the BoNT/A LC (0.3, 0.5, 1.0, 2.0, 2.5, 5.0 and 10.0 nM) for 5 h at 37 ^oC in HEPES buffer (50 mM HEPES + 0.1% BSA). The beads were then washed with PBS buffer and incubated with the SAPE dye (50 µl/well at a concentration of 1 µg/mL) in PBS buffer for 30 min at RT in the dark for conjugation to the biotinylated end of the peptide substrate. Subsequently, the beads were separated from the excess of dye and washed in PBS buffer. The final volume for the fluorescence scan was 100 µl/well (Tecan Infinite M200 Pro Plate Reader, Tecan, Research Triangle Park, NC). The schematic illustration of the ST-1 steps is depicted in Fig. 4.1, the upper part of the scheme (Chapter 4).

3.2.3 Detection of BoNT/A LC using Strategy 2 (ST -2)

In the second strategy (ST-2), the peptide (100 nM) was mixed with different concentrations of BoNT/A LC (same conditions as for ST-1) and incubated for 5 h at 37 ^oC. After digestion, an aliquot of 10 µl of anti-PEG-functionalized beads (approx.10^4 beads/well) were added and incubated with the suspension containing the cleaved and uncleaved peptides for 30 min. Washing was performed in PBS buffer (twice) followed by incubation with SAPE dye (50 µl/well; 1 µg/mL) for 30 min at room temperature in the dark. Subsequently, the SAPE-modified beads were thoroughly washed in PBS buffer. The fluorescence intensity (100 µl/well) was measured by plate reader. The schematic

illustration of the ST-2 steps is depicted in Fig. 4.1, the lower part of the scheme (Chapter 4).

3.2.4 Preparation of spiked food samples

Carrot juice was purchased at a local supermarket. The pH was adjusted to 7.4. Three different approaches were adopted for the BoNT/A LC assay in carrot juice. First, 10 µl of the carrot juice was spiked with the BoNT/A LC in order to obtain a final concentration of 1 µM. The spiked carrot juice was then serially diluted with HEPES buffer to obtain the required test concentrations of BoNT/A LC. Second, the carrot juice was diluted 5 times prior to spiking with the BoNT/A LC followed by serial dilutions with HEPES buffer to obtain the test BoNT/A LC concentrations. Third, carrot juice was filtered and subsequently spiked with the BoNT/A LC and serially diluted with HEPES buffer. An aliquot of 10 µl of skim milk, prepared by dissolving the skim milk powder (1 g) in DI water (10 mL), was diluted 5 times in HEPES buffer prior to spiking with the BoNT/A LC followed by subsequent dilutions as mentioned above. The spiked samples were tested using the proposed protocol.

Carrot juice was chosen as a food matrix due to the high content of pigments and colorants, that interferes with colorimetric assay responses both in homogeneous solution and paper based assays. Apart from this, foodborne botulism associated with consumption of contaminated beverages has already been reported[13].

3.2.5 Specificity test

Botulinum neurotoxin E light chain (BoNT/E LC) was used to determine the specificity of proposed protocol. A peptide aliquot of 100 nM (100 µL) was spiked with different concentrations of BoNT/E LC (final concentrations were 0 nM, 0.3 nM, 0.5 nM, 1 nM, 2.5 nM and 10 nM) and incubated for 5h at 37$_{o}$C. After digestion, an aliquot of 10 µl of anti-PEG-functionalized beads (approx.10$_{4}$ beads/well) were added and incubated with the suspension containing the cleaved and uncleaved peptides for 30 min. Washing step was performed twice with PBS buffer. Subsequently, SAPE dye was added (50 µL/well; 1

µg/mL) and incubated for 30 min in the dark. After washing the excess of dye (twice), the wells were scanned by plate reader.

3.3 Biosensing using dipstick assay

The substrate (10 nM; 100 µL) was spiked with different concentrations of BoNT/A LC (final concentrations were 10 nM, 5 nM, 2.5 nM, 1 nM) in HEPES buffer (50 mM HEPES + 0.1% BSA) and incubated for 5 h at 37 ^{o}C. For assaying in carrot juice, the pH was adjusted to 7.4 prior spiking with BoNT/A LC. Aliquots of 10 µl of the carrot juice were then spiked with BoNT/A LC in order to obtain the final concentrations of 1, 2.5, 5 and 10 nM. The spiked carrot juice was diluted 9x with the substrate (10 nM; 100 µl) for digestion of BoNT/A LC. Upon digestion for 5h, an aliquot of 2 µL (20 µg mL^{-1}) of SA-MBs was added, vortexed and incubated for 30 min at room temperature with constant shaking (400 rpm, Thermo Shaker Incubator, Hangzhou Miu Instruments). SA-MBs are introduced in order to capture the biotinylated fragment of the substrate and to isolate SA-MB-substrate complex from the cleaved PEGylated part of the substrate, BoNT/A LC and the sample matrix using a magnetic separator (DynaMag^{TM}-2, Invitrogen) for 1.5 min. Subsequently, the supernatant was discarded followed by washing step with HEPES buffer (100 µL, twice). Finally, the complexes were re-suspended in the HEPES buffer and tested with dipstick, where the concentration of BoNT/A LC is evaluated visually.

3.3.1 Fabrication of dipstick assay

2 mm-wide nitrocellulose strips were cut by a paper cutter. The LFA dipstick consists of two components: a cellulose absorption pad that is attached to the nitrocellulose strip with an overlap of 2 mm to facilitate the flow of the solutions during the assay, and a nitrocellulose strip (NC) for a deposition of antibodies. The anti-PEG antibodies (2 µl; 0.25 µg mL^{-1}) are deposited manually on the NC placed 5 mm from the absorption pad as a test line, followed by air drying at room temperature for 1 h. Subsequently, the strips were blocked with blocking buffer (PBS + 1% BSA; 60 µl) at 37 ^{o}C for 1 h followed by washing step with PBS buffer and drying at 37 ^{o}C for 1 h.

3.3.2 RGB analysis

The digital images of LFA dipsticks were captured using a Samsung Note 8 mobile phone camera (12.2MP; 4032 x 3024)/high resolution camera Sonyα7R (30 MP). Subsequently, images in JPEG format file were imported into ImageJ software for further processing. Five area (20x20 pixels) of the test line was utilized to create the digital array. Finally, RGB values were extracted from ImageJ software to quantify the colorimetric response.

3.3.3 Signal amplification for dipstick assay

Maleimide coated magnetic beads (MAL-MBs) and a linker/peptide were used to improve the contrast of the test lines generated by magnetic beads. The linker/peptide is modified with biotin and cysteine on either termini in order to conjugate with SA-MBs and MAL-MBs. The optimized concentration of MAL-MBs (3 µL, 30 µg/mL) were coupled to the optimized concentration of linker (100 µL; 100 nM) for 1 h at room temperature under constant shaking (700 rpm). The excess of the linker/peptide was separated from the complex using the magnetic separator for 1.5 min followed by washing with 50 mM HEPES + 0.1% BSA buffer (100 µL, twice). MAL-MBs coupled to the linker/peptide were added to the SA-MB-substrate complex (after digestion and washing) and incubated for 30 min at room temperature under constant shaking (400 rpm). In this process, the free streptavidin molecules on the reporter magnetic bead surface accommodates MAL-MB-linker complexes via streptavidin-biotin interactions. Subsequently, signal amplification was evaluated by visual inspection and further verified by luminance analysis.

3.3.4 Specificity test

The substrate (10 nM; 100 µL) was spiked with different concentrations of BoNT/E LC (10 nM, 5 nM, 2.5 nM, 0 nM before, and 10 nM, 5 nM, 2.5 nM, 1 nM, 0.5 nM, 0 nM after amplification) in HEPES buffer (50 mM HEPES + 0.1% BSA) and incubated for 5 h at 37 $_{o}$C. Upon digestion, aliquots of SA-MBs/MAL-MBs (before/after amplification) were added, vortexed and incubated for 30 min at room temperature with constant shaking (400

rpm). Subsequently, the excess of peptides were washed away (100 µL; HEPES buffer) by applying the magnet separation for 1.5 min. Finally, the complexes were re-suspended in the HEPES buffer and tested with dipstick, where the concentration of BoNT/E LC is evaluated visually.

3.4. Sorbents and polymer membranes for food matrix clean-up

3.4.1 Solution-based clean-up using sorbents materials

One mL of spiked carrot juice (1 µM of BoNT/A LC; the pH has been adjusted to 7.4) were mixed with different sorbents such as PSA (50 mg; N-[3-(trimethoxysilyl) propyl] ethylenediamine), GCB (0.5 mg; Graphitized Carbon Black), $MgSO_4$ (150 mg; magnesium sulphate) and Q-sep (20 mg; commercially available powder; a mixture of sorbents such as PSA/GCB/$MgSO_4$ and C-18), and their combinations in order to evaluate the removal of pigments and other interferents (organic acids, fatty acids & sugar molecules, anthocyanins). The tubes were then centrifuged at 10000 rpm for 3 min, and supernatants are examined visually to observed the colorant removal. Afterwards, the colourless solution were characterized by MALDI-ToF (section 3.4.2.2).

3.4.2 Membrane-based clean-up in combination with sorbents

The acidity of carrot juice has been adjusted from pH 4.5 to 7.4 in order to conserve the structure of toxin. Three mL of carrot juice sample was spiked with the final concentration of 7 µM of BoLcA. The spiked sample was incubated with mixing for 15 min at room temperature. For the clean-up of interferences and extraction of toxin combinations of PVDF membrane with varying porosity (0.45, 0.22, 0.1 µm) were utilized. Additionally, GCB (0.5 mg) and Q-Sep (20 mg) sorbents in combination with PVDF membranes were applied in the clean-up process of the sample. Extracts were collected and characterized by HPLC.

3.4.3. Pattering of GCB on the membrane surface

GCB was patterned on the Fusion 5 membrane (12 cm) using the LFA reagent dispenser (ClaremontBio, CA, USA). An aliquot of 400 µL of GCB (0.5 mg/mL) was taken by 1mL syringe and placed in the syringe pump which is connected to the LFA dispenser. Patterning was performed with the flow rate of 0.5 mL/min at voltage of 3V. The 4 mm thick deposited GCB lines were dried for 15 min at 37$_o$C. The pattering of GCB was done on the other side of membrane as well.

3.4.4 Preparation of conjugation pad and dipstick fabrication

Firstly, glass fibre were treated by plasma for 3 min followed by blocking with 2.5% of BSA (soaked into BSA solution for 15 min) and drying for 1h at 37$_o$C. Afterwards, the glass fibre were cut into pieces (12 mm x 2 mm) and soaked in 200 µL (0.0025%) of Europium beads (Eu beads) for 10 min at room temperature in the dark. Subsequently, the conjugated pads were dried at 37$_o$C for 1h.

2 mm-wide nitrocellulose strips were cut by a paper cutter. The LFA consists of 4 components: a sample clean-up pad for the removal of interferents which is overlapped (2 mm) with a conjugation pad containing deposited reporter molecules which, on the other side, overlaps with nitrocellulose strip (NC) used for a deposition of antibodies, a cellulose absorption pad that is attached to the nitrocellulose strip with an overlap of 2 mm to facilitate the flow of the solutions during the assay. The anti-PEG antibodies (2 µl; 1 mg/mL) are deposited manually on the NC placed 4 mm from the conjugation pad as a test line, followed by air drying at room temperature for 1 h. Subsequently, the strips were blocked with blocking buffer (PBS + 1% BSA; 60 µl) at 37 $_o$C for 1 h followed by washing step with PBS buffer and drying at 37 $_o$C for 1 h.

3.5 Characterization

3.5.1 Fluorescence Spectrometry

Fluorescence spectroscopy is a method used to determine the concentration of analyte (BoNT/A LC) in a sample based on the fluorescence properties of reporter molecule (fluorescence dye-SAPE). It has been applied in numerous biochemical research due to well understood fluorescence principals such as emission, quenching, resonance energy transfer, and rotational motion. Its application *in vitro* studies for molecular interactions is popular since it is negligibly invasive, extraordinary sensitive, selective, simple, ease-of-use, and reliable[14]. The quantitative and qualitative analysis of the sample can be done based on the information provided by emission spectrum. The fluorescence phenomenon is explained in Fig. 9 based on the electronic transition energy level diagram[14]. Briefly, the electronic state of molecule changes from the ground state to the excited state by absorbing a light of certain wavelength (e.g. 496 nm for the SAPE dye in this thesis). After excitation, the molecule is going through the vibrational relaxation process reaching the lowest vibrational level of the excited state, and finally the molecule reaches to the ground state by emitting the light (fluorescence emission; decay time) of a different wavelength (e.g. 578 nm for the SAPE dye).

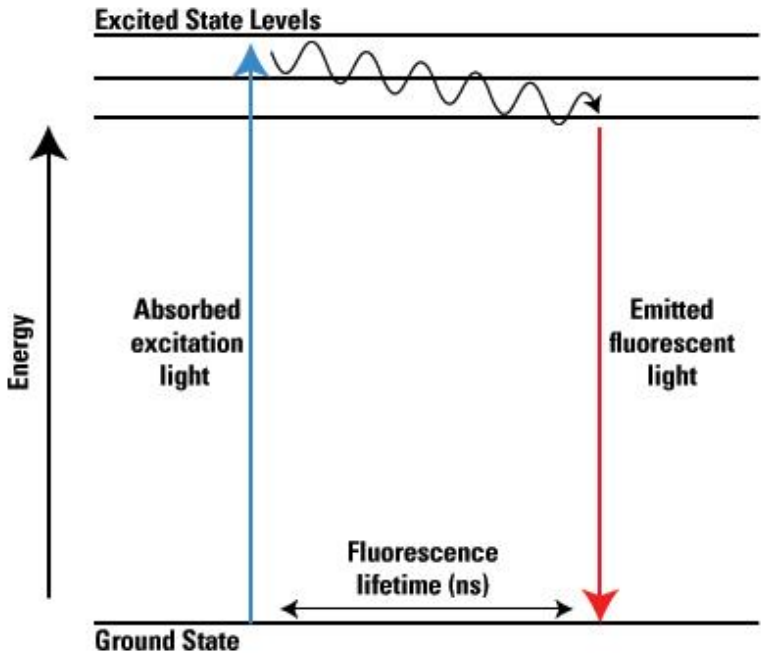

Figure 3.1. Jablonski diagram of fluorescence phenomenon[15].

3.5.2 MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF MS) is an ionization technique that has been widely utilized in multiple disciplines such as biochemistry, organic chemistry, microbiology, medicine, etc. for analysing biomolecules (peptides, proteins, DNA, biopolymers, carbohydrates, etc.), and organic molecules (e.g. polymers)[16]. The principal of MALDI-TOF MS is based on three steps (Fig. 10)[17]. First, a sample and suitable matrix material are mixed and subsequently applied on a metal plate. The sample-matrix complex is allowed to fully dry at RT prior introducing the mass spectrometer. Secondly, sample spots are irradiated with a pulsed laser beam, where the matrix molecules rapidly heat up by absorbing the laser energy and evaporating form the plate surface caring the analyte into the gas. During this step, analyte molecules are ionized by matrix molecules in protonation/deprotonation process. Thirdly, ionized sample molecules (different in size) are accelerated by an electric field towards TOF analyser, lighter ions (smaller mass-to-charge ratio; *m/z*) and more charged ions fly faster till they arrive earlier at the detector.

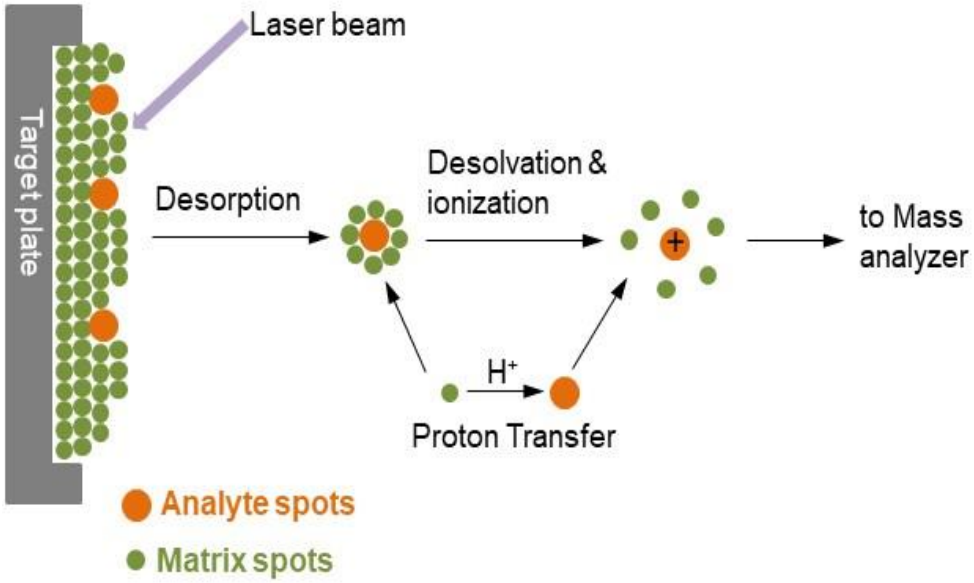

Figure 3.2. Principal of MALDI-TOF MS technique[17].

3.5.2.1 MALDI-TOF analysis of peptide digestion

In this study, MALDI-TOF analysis was carried to confirm digestion of substrate by enzyme using a MALDI Shimadzu Performance apparatus contained a nitrogen UV laser. For desorption and ionization of cleaved and un-cleaved substrate, CHCA (α-Cyano-4-hydroxycinnamic acid) was used as a matrix with a laser power of 85 mV, 100 profiles were collected (5 shots per profile) and adjusted for the best signal-to-noise ratio. Prior MALDI-TOF analysis, the digestion of substrate was preformed; 2 μM of substrate was exposed to BoNT/A LC at the concentration level of 20 nM for 3h at 37$_o$C. Subsequently, 1 μl of sample was applied on the metal plate and air-dried, and then 1 μl of matrix was deposited on the dried sample and air-dried as well. The sample-matrix spots were exposed to the laser (337 nm) and analysed in a positive mode.

3.5.2.2 MALDI-TOF analysis of clean-up and extraction protocol

MALDI-TOF analysis was carried using a MALDI Shimadzu Performance apparatus contained a nitrogen UV laser. For desorption and ionization of toxin, sinapic acid was used as a matrix with a laser power of 120 mV, 100 profiles were collected (5 shots per profile) and adjusted for the best signal-to-noise ratio. The ratio of matrix solution and samples was 3:1. For all probes, 1μL of the matrix-sample mixture was applied on the plate, air-dried and analyzed in a positive mode. The presence of the toxin was identified as a 50 kDa or 25 kDa peak (double charged chain).

3.5.3 Z-Potential

Zeta potential values give indirect correlation to the net surface charge of nanoparticles. It is mostly applied for colloidal stability studies and interactions between NPs and biological systems since the NP surface charge influences their physical properties (stability, adsorption of biomolecules, etc.) in the solution state. The principle of the method is shown in Fig. 11. In the solution, the negatively charged particles attract ions of opposite charge forming a positive charged layer called the Stern layer. The Stern layer, inner region, where

ions are strongly bound affects a formation of the diffuse outer layer (diffusive ion layer) in which ions are less firmly associated. Those two layers are called electrical double layers. Under applied electrical field, the particles move towards the electrode of opposite charge where the slipping plane, the boundary between the ions in the diffuse outer layer and the ions in the solution, is formed. The Z-potential is proportional to the electrostatic potential at the slipping plane. The velocity of particles is proportional to the magnitude of the charge.

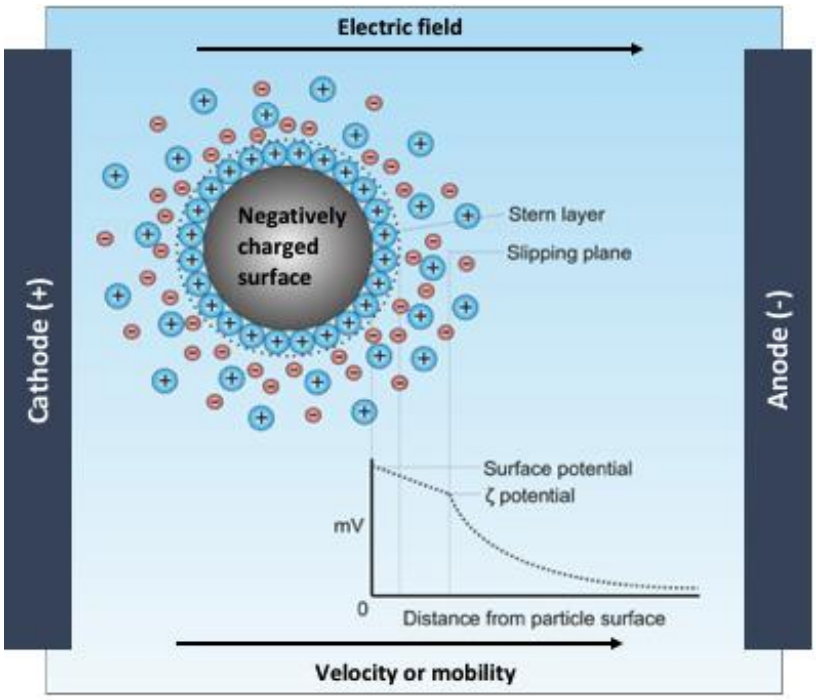

Figure 3.3. The principle of Z-potential measurement[18].

3.5.3.1 Characterization of magnetic bead-antiPEG antibody conjugation (MB-antiPEG) by Z-potential

In this study, the Z-potential was used to characterize successful immobilization of anti-PEG antibodies on the superparamagnetic bead surface. Briefly, the superparamagnetic beads (conjugated and unconjugated) were diluted in the HEPES buffer (500 times) and sonicated in an ice bath for 20 s. After sonication, approximately 1mL of the solution was

placed into capillary cells and analysed by Zetasizer Nano ZS, Malvern Instruments, UK.

3.5.4 The Bicinchoninic Acid Assay (BCA)

Bicinchoninic acid (BCA) assay is the most common used colorimetric assay for determining the total amount of protein in the sample (mg/mL). The principle of this assay is based on the reduction of Cu^{2+} to Cu^{+} by protein in the alkaline solution leading to the purple colour formation by bicinchoninic acid. The protein concentration presented in the sample is proportional to the concentration of Cu^{+}, as well as to the colour intensity. The absorbance of the purple complex BCA- Cu^{+} is measured at 562 nm and could be correlated to the concentration of protein based on the calibration curve. This assay is sensitive, simple, fast, and applicable assay over a broad working range of protein concentrations (20-2000 µg/mL).

3.5.4.1 Characterization MB-antiPEG by BCA

The total amount of anti-PEG antibodies coupled to the superparamagnetic beads surface was determined by the bicinchoninic acid (BCA) assay using the Pierce™ BCA protein assay kit (Thermo Scientific, USA) according to the manufacturer's protocol. Briefly, an aliquot of superparamagnetic beads coupled to anti-PEG antibodies (25 µl) and protein standards (25 µl) were transferred to the 96-well microplate and mixed with 200 µl of BCA working reagent. The plate was shacked and incubated at 37ºC for 30 min. Subsequently, the plate was cooled and the absorbance of the samples were measured at 562 nm using the plate reader. The total amount of anti-PEG antibodies bound to the beads surface was calculated against the standard curve.

3.5.5 High Performance Liquid Chromatography (HPLC)

HPLC is an analytical technique used to separate and identify each component in the sample. In this study reversed-phase (RP) chromatography was used, where the RP column (C-18) acts as hydrophobic stationary phase separating the components based on their

polarity. The polar compounds are eluted first.

3.5.5.1 HPLC analysis of extraction protocol

Carrot juice (300 µl) was spiked with substrate to obtain the final concentration of 100 nM. Subsequently, the clean-up of sample was performed with GCB (0.5mg/100 µl) and Q-sep (20 mg/100 µl) followed by centrifugation for 4 min at 4000 rpm. The supernatants were collected and further analyzed by HPLC to evaluate the recovery of substrate upon clean-up. Extracts characterization were performed on the Zorbax 300SB-C-18 reverse phase column (9.4 x 250 mm) and run at a flow rate of 1 mL/min with solvent A (0.1%TFA in 100% ACN) and solvent B (0.1%TFA in 100% in water) accompanied by gradient: 0-40 min, linear gradient 5-100% solvent A; 40-50 min, linear gradient 100-5% solvent A. Samples (200 µl) were injected via injector valve fitted with 500 µl injector loop. The system was maintained at room temperature. Chromatographic determination was based on measurements of absorbance at 220 nm. All data were processed in OriginPro software.

3.6 Overview of Methodologies

In summary, fluorescence spectroscopy has been used to characterize the assays optimisation and performance, MALDI-TOF MS has been used to investigate the cleavage of substrate by enzyme and presence of toxin after the extraction process, Z-potential has been used to confirm the immobilization of antibodies on the superparamagnetic bead surface, BCA assay has been used to determine the total amount of antibodies coupled to the superparamagnetic beads, and HPLC was used to characterized the performance of sorbents and membranes in removing the food interferences.

Reference

(1) Gijs, M. A. M. Magnetic Bead Handling On-Chip: New Opportunities for Analytical Applications. *Microfluidics and Nanofluidics* **2004**, *1* (1), 22–40.

(2) Chen, Y. T.; Kolhatkar, A. G.; Zenasni, O.; Xu, S.; Lee, T. R. Biosensing Using

Magnetic Particle Detection Techniques. *Sensors* **2017**, *17* (10), 6–8.

(3) Van Reenen, A.; De Jong, A. M.; Den Toonder, J. M. J.; Prins, M. W. J. Integrated Lab-on-Chip Biosensing Systems Based on Magnetic Particle Actuation-a Comprehensive Review. *Lab on a Chip* **2014**, *14* (12), 1966–1986.

(4) Jamshaid, T.; Neto, E. T. T.; Eissa, M. M.; Zine, N.; Kunita, M. H.; El-Salhi, A. E.; Elaissari, A. Magnetic Particles: From Preparation to Lab-on-a-Chip, Biosensors, Microsystems and Microfluidics Applications. *TrAC - Trends in Analytical Chemistry* **2016**, *79*, 344–362.

(5) Chen, Y. T.; Medhi, R.; Nekrashevich, I.; Litvinov, D.; Xu, S.; Lee, T. R. Specific Detection of Proteins Using Exceptionally Responsive Magnetic Particles. *Analytical Chemistry* **2018**, *90* (11), 6749–6756.

(6) Arol, A. I.; Aydogan, A. Recovery Enhancement of Magnetite Fines in Magnetic Separation. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2004**, *232* (2–3), 151–154.

(7) Wang, Y.; Xu, H.; Wei, M.; Gu, H.; Xu, Q.; Zhu, W. Study of Superparamagnetic Nanoparticles as Labels in the Quantitative Lateral Flow Immunoassay. *Materials Science and Engineering C* **2009**, *29* (3), 714–718.

(8) Guerrini, L.; Alvarez-Puebla, R. A.; Pazos-Perez, N. Surface Modifications of Nanoparticles for Stability in Biological Fluids. *Materials* **2018**, *11* (7), 1–28.

(9) Xianyu, Y.; Wang, Q.; Chen, Y. Magnetic Particles-Enabled Biosensors for Point-of-Care Testing. *TrAC - Trends in Analytical Chemistry* **2018**, *106*, 213–224.

(10) Pavan, S.; Berti, F. Short Peptides as Biosensor Transducers. *Analytical and Bioanalytical Chemistry* **2012**, *402* (10), 3055–3070.

(11) Gilmore, J. M.; Scheck, R. A.; Esser-Kahn, A. P.; Joshi, N. S.; Francis, M. B. N-Terminal Protein Modification through a Biomimetic Transamination Reaction. *Angewandte Chemie International Edition* **2006**, *45* (32), 5307–5311.

(12) Shock, E. L. Stability of Peptides in High-Temperature Aqueous Solutions. *Geochimica et Cosmochimica Acta* **1992**, *56* (9), 3481–3491.

(13) Kim, M.; Zahn, M.; Reporter, R.; Askar, Z.; Green, N.; Needham, M.; Rosen, H.; Kimura, A.; Terashita, D. Outbreak of Foodborne Botulism Associated With Prepackaged Pouches of Liquid Herbal Tea. *Open Forum Infectious Diseases* **2019**,

6 (2).

(14) Sci, K. N.-J. C. P.; 2014, U. Applications of Fluorescence Spectroscopy. *J. Chem. Pharm. Sci* **2014**, *974*, 2115.

(15) Fluorescence Microscopy | Center for Advanced Microscopy https://cam.facilities.northwestern.edu/588-2/fluorescence-microscopy/ (accessed Apr 9, 2019).

(16) Santos, I. C.; Hildenbrand, Z. L.; Schug, K. A. Applications of MALDI-TOF MS in Environmental Microbiology. *Analyst* **2016**, *141* (10), 2827–2837.

(17) MALDI-TOF Mass Spectrometry - Creative Proteomics https://www.creative-proteomics.com/technology/maldi-tof-mass-spectrometry.htm (accessed Apr 5, 2019).

(18) Zeta potential | Coriolis Pharma https://www.coriolis-pharma.com/contract-analytical-services/zeta-potential/ (accessed Apr 8, 2019).

Chapter 4

BoNT/A LC Detection Using Peptide Substrate and Magnetic Beads in Solution

This chapter describes detection of BoNT/A LC in complex food matrices using the functional fluorescence magnetic bead-based assay. The proposed assay utilizes a synthetic peptide designed to mimic the SNAP-25 protein (synaptosomal-associated protein 25) as substrate bound to a superparamagnetic bead and a fluorescent dye. The superparamagnetic beads enable efficient separation of the cleaved peptides from food matrices, thereby improving the reliability of responses. The proposed assay is robust and specific to the BoNT/A owing to the peptide design. Reduction in fluorescence signal is revealed upon the substrate cleavage by BoNT/A LC, yielding a LOD of 25 ng/mL in 6h than the gold standard-mouse bioassay (10 pg/mL in 4 days)- it offers rapid detection suitable for field applications.

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1.2 Introduction

The extremely toxic proteases such as botulinum neurotoxin poses a threat for health and food safety. The lethal dose for humans has been reported to be 1-2 ng/kg (intravenous), 10-12 ng/kg (inhalation) and 1µg/kg (oral)[1]. In most cases, improperly stored meat, fish, dairy products and vegetables were reported to cause production of the toxin and subsequent intoxication upon ingestion[2]. Foodborne botulism outbreaks recently occurred in California (2017) related to nacho cheese sauce, which caused one causality and affected 10 people[3]; in Ohio (2015), related to consumption of home-canned potato salad, caused one casualty and 29 confirmed cases of botulism, which is considered to be the largest botulism outbreak in the last 40 years[4], and in China (2013), associated with smoked ribs, which affected 12 people[5]. Therefore, preventing the occurrence and spread of botulism is of significant importance, especially considering the accessibility and ease of production of this target, which could lead to accidental or deliberate contamination of food supplies or/and bio-terroristic attacks similar to the anthrax incident in the US, 2001[6]. Due to the extreme neurotoxicity of BoNT/A and availability, high demand has been exerted on the development of on-site screening devices that enable rapid, facile, cost-effective identification of BoNTs serotypes in different matrices.

To this day, the mouse bioassay[7] is considered as the "gold standard" for determining the activity of toxins at concentrations in the order of pg/mL. Although the mouse bioassay is sensitive, it requires several days, trained personnel, sophisticated facilities and involvement of animals. Thus, alternative *in vitro* methods have been developed. The most frequently adopted affinity assay is the enzyme-linked immunosorbent assay (ELISA)[8], which possesses a good sensitivity. A couple of immunoassays are commercially available in the format of the lateral flow assay with a sensitivity/dynamic range of 10-100 ng/mL in complex matrices and with an assay time of 15 to 30 min[9]. Attempts to further decrease the limit of detection (LOD) require assays such as enzyme-linked coagulation assay (ELCA) and Digoxigenin-Enzyme Linked Immunosorbent Assay (DIG-ELISA) with sensitivities comparable to that of the mouse bioassay[10]. Sophisticated analytical techniques such as chromatography and mass spectrometry also have been reported[11–13] for

screening/detection purposes with varying sensitivities. However, most of these methods do not provide information on the enzymatic activity of the toxin, which is vital for assessing the toxicity of BoNTs and for precisely prescribing treatments. It is also known that the cross-reactivity has been a major limitation of antibody-based assays. Furthermore, as new BoNT serotypes are being discovered, the development of more specific and sensitive assays is required for identification of various serotypes.

Apart from the immunological methods mentioned above, functional assays also have been developed for evaluation of the BoNT activity and for specific detection of its serotypes. These assays typically utilize peptide substrates with a specific cleavage site for each serotype derived from one of SNARE complex proteins/peptides. The proteolytic activity of the toxin is subsequently evaluated via monitoring responses such as fluorescence signals, using FRET-based[14] or electro-chemiluminescense[15] assays, mass changes via SPR[16] and color changes due to gold nanoparticles aggregation/disaggregation phenomena[17]. Recently, magnetic bead-based assays have been utilized for food analysis due to the ease of isolation of molecules of interest[18]. A good sensitivity has been achieved with the bead-based assay utilizing fluorescent reporters in different formats such as a spin column[19], a microfluidic channel with the evaporation step at the detection port for amplification of the signal[20] and a microfluidic assay format combined with a LED-CCD[21]. Excellent sensitivity (0.5 fg/mL) and good performance in complex matrices, such as undiluted sera, also have been reported using ALISSA (Assay with a Large Immuno-sorbent Surface Area)[22]. However, most of these methodologies involve tedious extraction protocols and are therefore not suitable for on-site assaying in complex and diverse matrices. Therefore, in this study, we propose a facile protocol for a functional assay of BoNT/A (Fig. 4.1). Paramagnetic beads are utilized for the extraction of a cleaved peptide and for subsequent detection via fluorescence analysis. The proposed protocol also could be adopted for other proteinase assays, thereby enabling multiplexed detection of proteolytic enzymes, including botulinum serotypes.

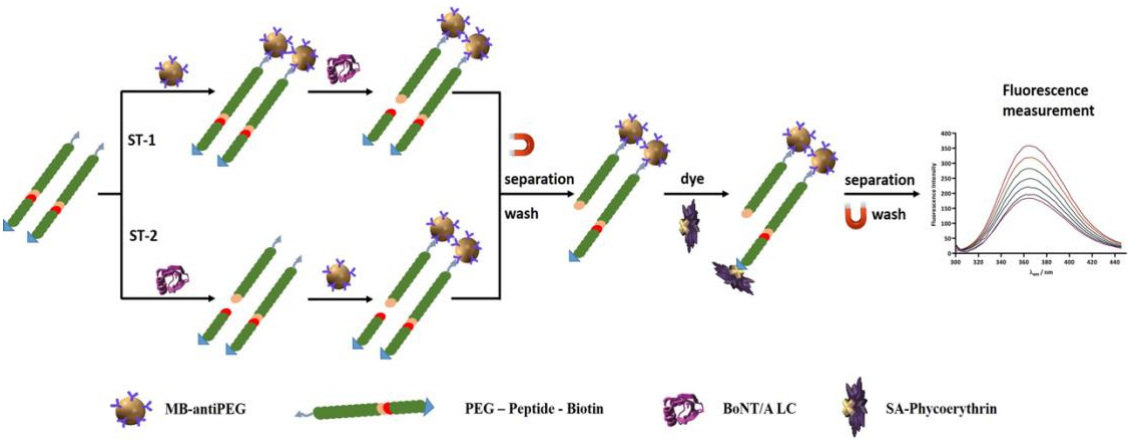

Figure 4.1. Schematic illustration of the proposed detection strategies. In Strategy 1 (ST-1) the peptides are bound to anti-PEG functionalized superparamagnetic beads prior exposure to BoNT/A LC. In Strategy 2 (ST-2) the free peptides are first exposed to BoNT/A LC followed by incubation with anti-PEG-functionalized superparamagnetic beads. Subsequently, superparamagnetic beads having intact and cleaved peptides on its surface are trapped with a magnet while other components (toxin and cleaved fragment of the peptide) are washed away. The next step is incubation with PE-labeled SA streptavidin followed by magnetic separation and washing to remove excess PE-labeled SA. Finally, fluorescence measurements are carried out using a plate reader.

4.2 Materials and reagents

Botulinum Toxin light chain A (recombinant) was purchased from List Biological Labs, Inc. USA. Botulinum Toxin light chain E (recombinant) was purchased from Creative Diagnostics, USA. Peptide sequence: Cys-PEG$_{11}$-SNKTRIDEANQRATKXK-Biotin was purchased from Mimotopes Pte Ltd, Australia. Streptavidin-R-phycoerythrin (SAPE) conjugate and superparamagnetic Dynabeads M-270 carboxylic acid were purchased from ThermoFisher Scientific, Singapore. Pierce BCA protein assay kit was purchased from ThermoFisher Scientific, USA. Anti-PEG antibody (Clone 09F02), mouse IgG3 was purchased from BioVision, Inc. (California, US). MES (2-(N-morpholino) ethanesulfonic acid), sulfo-NHS (N-hydrox- ysulfosuccinimide), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), bovine serum albumin (BSA), HEPES buffer, PBS tablets and skim milk powder for microbiology were purchased from Sigma-Aldrich (Singapore).

Protocols for superparamagnetic bead-antibody conjugation, ST-1 and ST-2 strategies, sample preparation protocol, and the protocol for specificity test are explained in the sections 3.2.1, 3.2.2, 3.2.3, 3.2.4, and 3.2.5.

MALDI-ToF characterization of the substrate cleavage, Z-potential measurements of immobilized antibodies, and BCA protein assay for determining the amount of immobilized antibodies are explained in the sections 3.5.2.1, 3.5.3.1, 3.5.4.1.

4.3. Results and Discussion

4.3.1 Substrate for BoNT/A LC

The peptide used in this study was derived from the C- terminus of SNAP25 protein (187-203), which has been reported to be an efficient substrate for BoNT/A [17 23]. The cleavage site Q-R is positioned in the middle of the peptide in order to facilitate the accessibility for BoNT/A. The peptide contains norleucine (X) instead of methionine (M) at the position 202, which prevents the oxidation of the peptide while maintaining a high catalytic rate constant, k_{cat} [24]. Furthermore, the peptide is modified by attaching a biotin at the C-terminus to enable interaction with streptavidin, while the N-terminus contains a 11-mer ethylene glycol PEG segment attached to cysteine. The PEG modified peptides were digested by BoNT/A LC and characterized by MALDI-TOF. As shown in Fig. 4.2a, the mass spectrum analysis reveals three peaks at *m/z*=943, m/z=1979 and m/z=2903 corresponding to C-terminal cleaved fragment (ATKXK-Biotin), N-terminal cleaved fragment (Cys-PEG11-SNKTRIDEANQR) and uncleaved peptide, respectively. The two fragment peaks at *m/z*=943 and m/z=1979 undoubtedly confirm that BoNT/A LC is capable of cleaving the modified peptide specifically at the Q-R position. However, as shown in Fig. 4.2b, not all the peptide was cleaved. This can be attributed to the reduced proteolytic activity towards the modified peptide (biotin and PEG modifications at both termini) or to the high concentration of the peptide adopted in these experiment (2 μM).

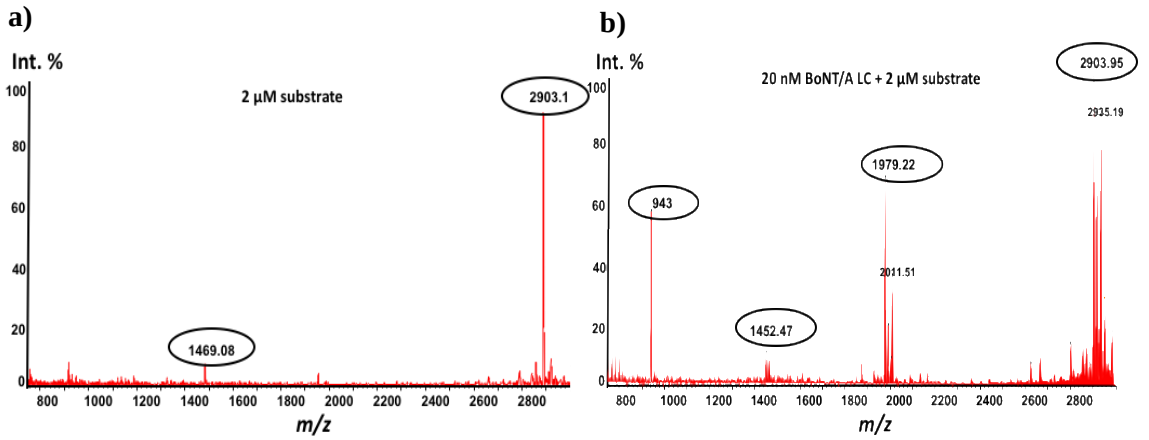

Figure 4.2. MALDI-TOF spectrum of uncleaved substrate (a) and of cleaved substrate with fragments C-terminus = 943, N-terminus = 1979 and full-length = 2903 (b).

4.3.2. Development and optimization of assay protocol

Non-porous and homogeneous (Fig. 4.3a) Dyna superparamagnetic beads (2.8 µm) were modified with anti-PEG antibodies. In order to evaluate the degree of immobilization, a BCA (bicinchoninic acid) protein assay was preformed according to the manufacturer's protocol and the absorbance measurements were scanned at λ=562 nm (Fig. 4.3b). From the calibration curve, it was calculated that 24.7 µg of anti-PEG antibodies was coupled per 3 mg beads, which is in agreement with the loading capacity provided by the manufacturer (5-10 µg/mg beads). With the obtained data for the antibody concentration immobilized on the surface, the number of antibodies per magnetic bead is calculated to be $4.97x10^5$, where the surface coverage (foot print area; FPA) [25] of antibody molecule (r=3.5 nm) on the bead surface is estimated as 38.5 nm^2, yielding a maximum attainable coverage of 77%. In order to ascertain successful immobilization, Z-potential was measured before and after labeling with antibodies. Due to the abundance –COOH groups on the surface, the superparamagnetic beads possess a negative surface charge of -45 mV, which is reduced to -32mV upon conjugation with antibodies (Fig. 4.3c). As indicated in Figure 4.3d, similar responses for ST-2 were obtained for magnetic bead antibody complexes evaluated over a period of 1 year and 3 months (from September 2017 as prepared to

December 2018), confirming their storage stability and the robustness of the proposed methodology.

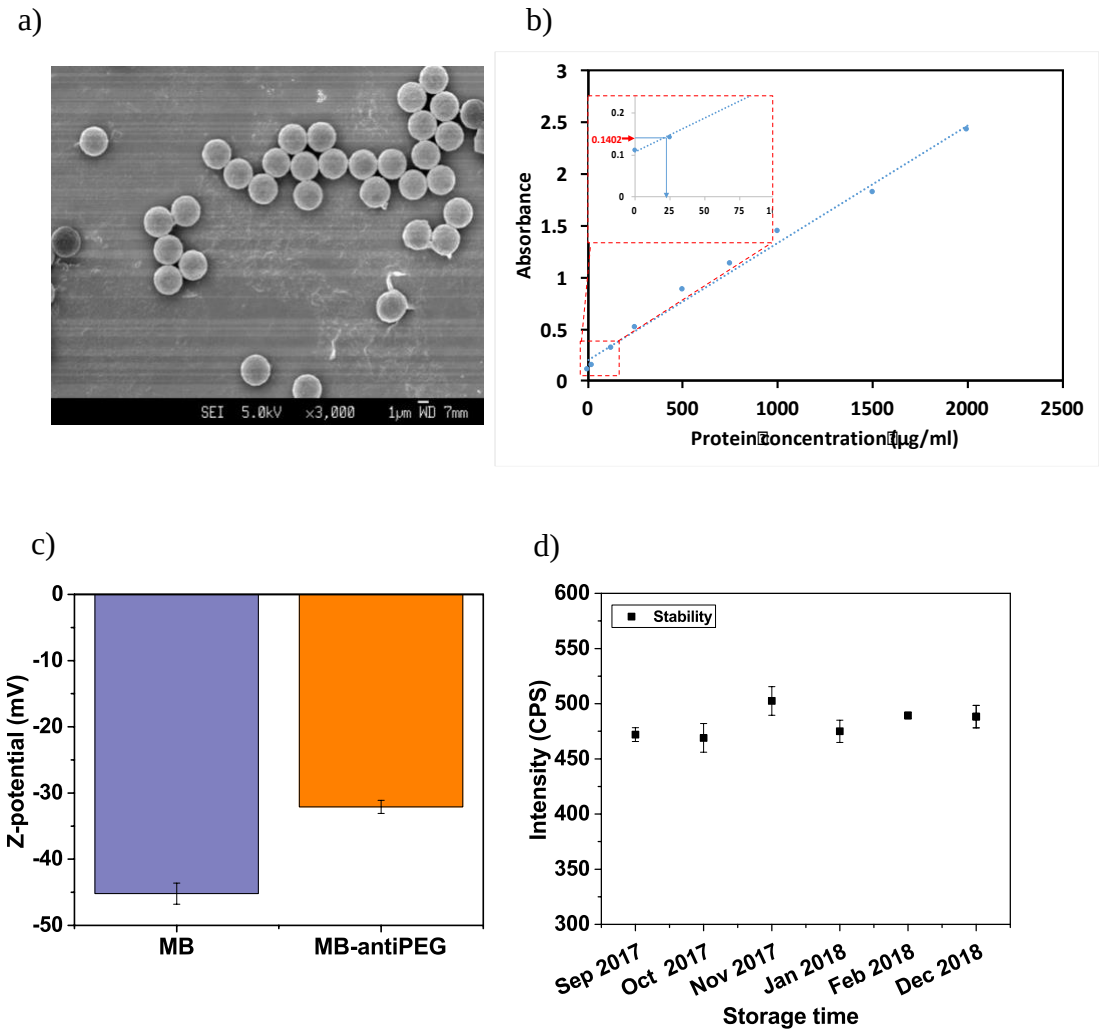

Figure 4.3. FESEM image of Dyna magnetic beads (a), magnetic beads-antiPEG antibody conjugate characterization by BCA (b), Z-potential of unconjugated and conjugated superparamagnetic beads to anti PEG-antibodies (c), and storage stability (d).

In order to characterize the functionalization protocol and to optimize the ratio of peptides to that of superparamagnetic beads, various concentrations of the peptide ranging from 50 nM to 1000 nM were incubated with a fixed concentration of anti-PEG functionalized magnetic beads (10_4 beads/assay) followed by exposure to SAPE (1µg/mL) (Fig. 4.4a). The observed difference of ~70% in the fluorescence intensity between the highest and the

lowest peptide concentration confirms the validity of functionalization protocol. Figure 4.4a illustrate that a maximum fluorescence intensity of 1200 CPS is achieved with a peptide concentration of 1000 nM suggesting that a densely packed layer of peptides is formed on the beads. The relatively small fluorescence intensity difference (<10%) between peptide concentrations of 500 and 1000 nM indicates saturation of antibody-functionalized beads surfaces with the peptide, see inset Fig. 4.4a. It is worthwhile emphasizing that the cleavage observed in ST-1 upon addition of BoNT/A LC to beads modified with different concentrations of peptide does not correlate with the degree of immobilization, Fig. 4.4b. For example, the cleavages are significantly higher for the particles modified with 100 and 300 nM of peptide compared to the concentration of 1000 nM, for which the cleavage is insignificant attributed to the steric hindrance inhibiting the accessibility of the cleavage site at a high degree of immobilization. In order to overcome the steric hindrance and the lack of sensitivity in ST-1, ST-2 was applied, which shows better response compare to ST-1 as observed from Fig. 4.4c. The cleavage percentage is presented as a normalized signal calculated by formula $1-(F/F_0)$, where F and F_0 are the fluorescence intensities at λ=578 nm measured after and before substrate digestion, respectively. As shown in Fig. 4.4c, a ~5-fold improvement in the degree of cleavage by BoNT/A LC is observed at 100 nM with respect to the peptide concentration of 1000 nM. Therefore, ST-2 strategy is adopted for the BoNT/A LC assay development with a peptide concentration of 100 nM.

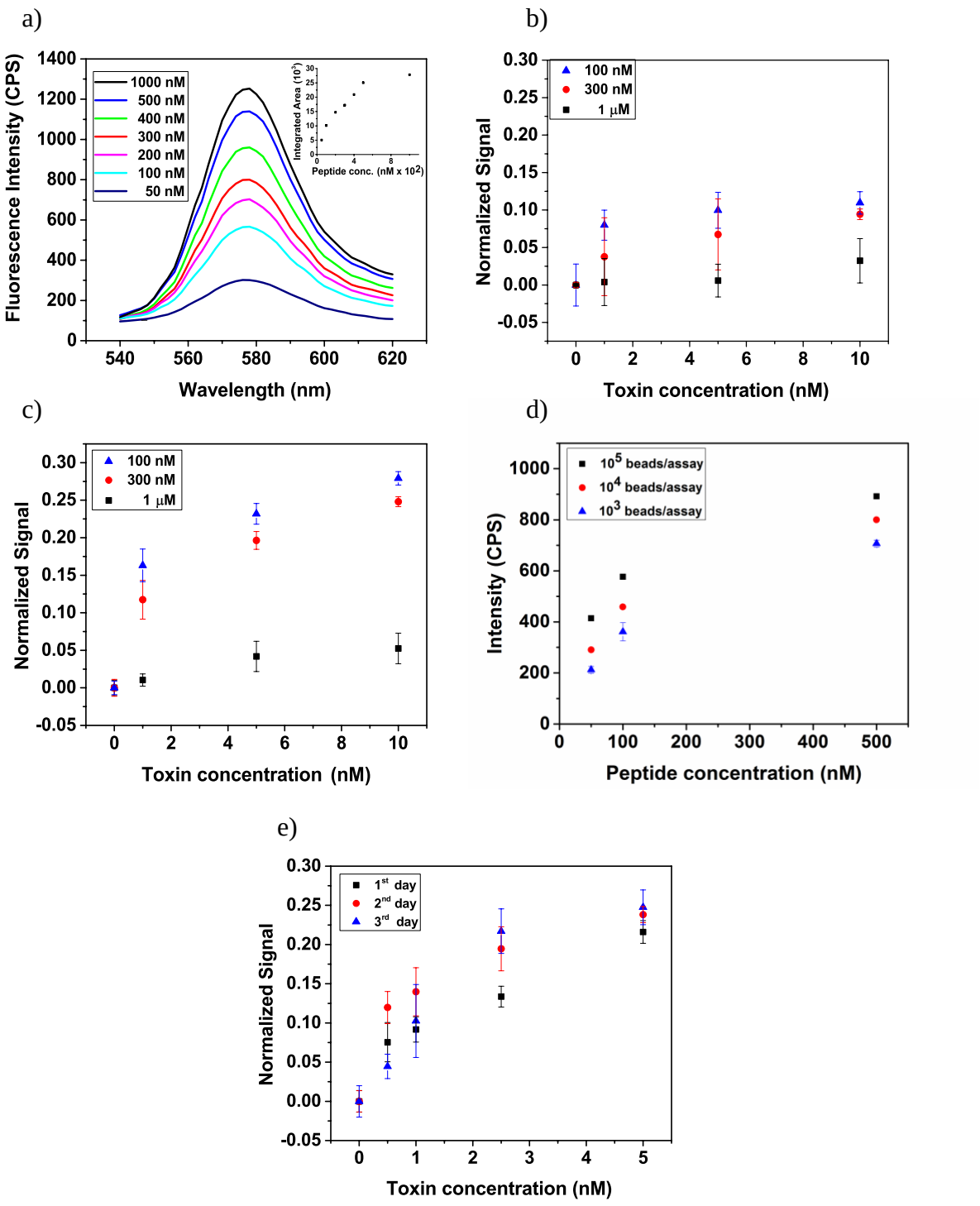

Figure 4.4. Evolution of fluorescence intensity upon functionalization of anti-PEG-superparamagnetic beads with different concentrations of peptide labeled with SAPE (inset shows integrated area from 550-610 nm (a). Normalized fluorescence signal for the peptide concentrations of 1 µM, 300 and 100 nM spiked with BoNT/A LC in buffer performed by ST-1 (b) and ST-2 (c),

respectively. Optimization of the number of beads per assay (10^5,10^4,10^3) were examined with varying peptide concentrations (d). Normalized fluorescence signal for the peptide concentration of 100 nM spiked with different concentrations of BoNT/A LC carried out in triplicates for 3 days according to the ST-2 (e).

Superparamagnetic beads possess a high surface to volume ratio (estimated to be SA/V= 492.6 cm^2 / 0.023 cm^3) enabling immobilization of a sufficient number of peptides, thereby providing excellent fluorescence sensitivities even while operating with a low number of beads. Therefore, the number of beads utilized per assay was optimized with respect to the fluorescence responses. Wells containing 10^3, 10^4 and 10^5 beads were tested with peptide concentrations of 50, 100, 500 nM while keeping the SAPE dye concentration constant (1µg/mL) as shown in Fig. 4.4d. A fluorescence response of 900 CPS is obtained for the peptide concentration of 500 nM and for 10^5 beads. The other two tested concentrations of the beads (10^4 and 10^3), for the same peptide concentration, yield less fluorescence intensity, 800 and 700 CPS, respectively. Figure 4.4d shows a 100 CPS difference in the fluorescence intensity between the tested number of beads per assay at a peptide concentration of 500 nM. This trend is also observed for peptide concentrations of 100 and 50 nM, confirming the consistency and the uniformity in degree of functionalization. However, a peptide concentration of 50 nM generated a fluorescence signal that is too low and thereby not favourable for the assay. Considering that the number of beads per assay does not significantly influence the response, and since the peptide concentration of 100 nM has been optimized, 10^4 beads/assay that yield around 450 CPS fluorescence intensity is chosen for further assay development.

4.3.3. Concentration dependent responses of the assay in buffer

The developed protocol is intended to exploit the proteolytic activity of BoNT/A for monitoring the toxin contamination in relevant matrices. In order to simplify the detection and to overcome tedious extraction of the toxin from complex matrices we employed the described superparamagnetic beads for extraction of peptides captured via a high affinity anti-PEG-PEG interaction. Therefore, the digested part of the peptide could be easily

isolated from matrices by utilizing a magnet, thereby improving the reliability of the responses. According to the ST-2 strategy, various concentrations of BoNT/A LC (0.3, 0.5, 1.0, 2.0, 2.5, 5.0 and 10.0 nM) were added to the peptide without the superparamagnetic beads and incubated for 5 h at 37 ^{o}C. As observed from Fig. 4.5a, the highest BoNT/A LC concentration could digest approximately 27% of peptide for the indicated period of time (as compared to the control, that is without BoNT/A LC). The LOD (Table 4.1) of the assay is estimated to be 0.5 nM (25 ng/mL), based on the 3σ rule, where σ is the standard deviation of the control (black dotted line, Fig. 4.5a). Experiments repeated in triplicates over 3 days yield similar assay responses, ascertaining reproducibility of the proposed methodology (Fig. 4.4e). The relatively low catalytic efficiency of BoNT/A LC (see 27% above) is likely due to the peptide termini modifications, wherein a biotin group at C-terminus could reduce the hydrolysis rate, as suggested by Dongxia Wang et al [26]. Furthermore, the utilized 17-mer peptide also might be too short for the successful recognition and digestion (k_{cat} =28 s^{-1}, k_{cat}/K_m= 0.019μM/sec) compared to the whole SNAP-25 protein (k_{cat}=76 s^{-1}, k_{cat}/K_m=2.3 μM/sec) [27]. Enhancement in assay performances could be achieved by overcoming the drawbacks associated with peptide design, for instance, by introducing smaller probes at the peptide's termini or by optimizing the amino acid sequence near the cleavage site, see below.

4.3.4. Concentration dependent responses of assay in carrot juice and milk

As the proposed protocol was developed for potential on-site applications, it was eventually verified with complex matrices such as carrot juice and milk. Figure 4.5b illustrates that the assay response is ~40% lower for carrot juice samples spiked with BoNT/A LC followed by serial dilutions in HEPES, as compared to that of responses obtained in buffer. This lower assay response is most probably due to the matrix interferences considering that no extensive clean up protocols were adopted. In order to understand the influence of the sample matrix on the assay response, additional experiments were performed. For instance, an additional 5-fold dilution of the sample matrix prior spiking with BoNT/A LC followed by serial dilutions with HEPES buffer improves the assay response by ~25% compared to that of BoNT/A LC directly spiked in non-diluted carrot juice, Fig. 4.5b. Since the

additional dilution does not completely eliminate the interference to the same extent, carrot juice was filtered through 0.45 µm and 0.22 µm filters prior to spiking BoNT/A LC followed by serial dilutions with HEPES buffer as mentioned above. Herein, the matrix interferences, such as solid particles, fibres, proteins, etc. were significantly removed leading to an improved peptide hydrolysis and fluorescence response by ~40% compared to that of BoNT/A LC spiked in non-diluted carrot juice. As observed from data and derived standard deviations, the assay performance is influenced by matrix interferents, probably in several ways such as constrained mobility of the toxin due to which the amount of the digested substrate can vary in replicates causing the standard deviation to be larger. Another possibility could be that substrates interact/or non-specifically bind with/to the matrix interferents (e.g. carbohydrates, pigments, other proteins), due to which the less number of substrates is exposed to the toxin for hydrolyzation, causing slight variations in assay responses of the replicates. Furthermore, the assay response obtained is on par with the response obtained in the buffer. After filtration and dilution of carrot juice the obtained LOD is 0.5 nM. As for assay in diluted milk samples, the response diminishes by ~80% with respect to the responses in buffer (Fig. 4.5c). It is worthwhile emphasizing that the responses do not follow the same trend as in the carrot juice, despite a dilution of 5 times prior spiking, confirming that the complexity of milk matrix significantly affects the cleavage and the response signal. The lowest concentration detected in diluted milk is 20 nM (1 µg/mL), see Table 4.1.

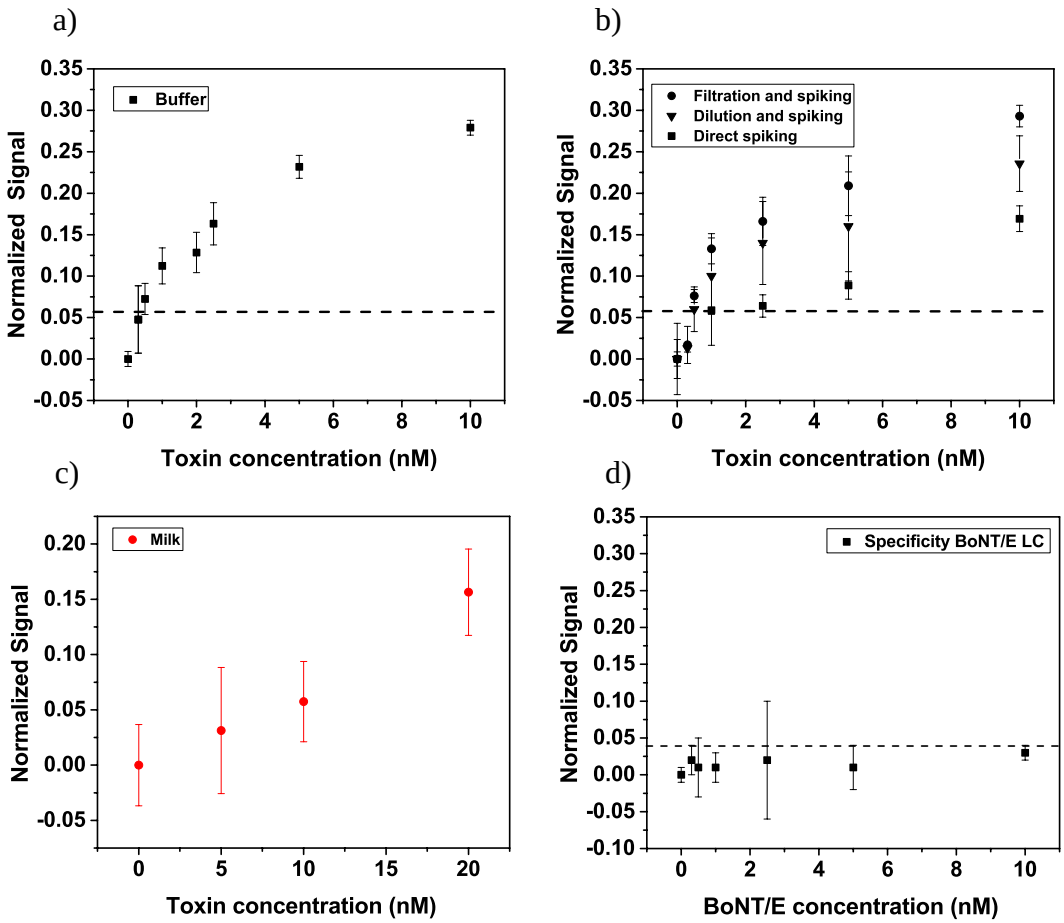

Figure 4.5. Normalized fluorescence signals for buffer (a), carrot juice (B), and milk (c) spiked with BoNT/A LC. Data shown were collected after 5 h at 37ₒC. The 3σ lines for LOD calculation are shown in black dotted lines (a and b). Normalized fluorescence signal for buffer spiked with BoNT/E LC, specificity test (d).

Table 4.1. Summary of LODs for the proposed protocol and the corresponding lethal doses in 200 and 100 mL of liquid (e.g. in milk supplement).

Sample	LOD (ng/mL)	Lethal dose for an adult (65kg) in 200 mL	Lethal dose for a baby (5kg) in 100 mL
Buffer	25 ng/mL	325 ng/mL	50 ng/mL
Carrot juice	50 ng/mL		
Diluted milk	1 µg/mL		

4.3.5. Specificity of proposed protocol

To ensure that developed protocol and peptide design are specific to BoNT/A, BoNT type E was tested. The choice of type E was based on the fact that the SNAP-25 can be cleaved by both types A and E in different positions, Q_{197}-R_{198} and R_{180}-I_{181}, respectively. In this study the C-terminus of SNAP25 protein (187-203) was used, designed in that way to exclude the cleavage site for the type E. Anyway, the peptide sequence (Cys-PEG_{11}-SNKTRIDEANQRATKXK-Biotin) has a region containing a similar scissile bond (R-I) at positions 191-192, herein, examined to be cleaved by type E LC. As speculated elsewhere the type E is capable of cleaving only $_{178}$IDRIME$_{183}$ due to the presence of Met_{182} and Asp_{179} amino acids, which helps in docking and aligning the substrate as well as stabilizing the substrate-BoNT/E complex[28]. In agreement to this, BoNT/E LC (at different concentrations) was unable to cleave the substrate as shown in Fig. 4.5d (threshold is presented with the black dashed line), confirming the specificity of the proposed assay for BoNT/A.

4.3.6 Comparison of BoNT assays

Table 4.2 summarizes the performance of some of the reported BoNT assays including their advantages and disadvantages, and the samples matrices in which the assays were performed. These assays provide an insight in to the unique challenges in the detection process. In summary, the assay in complex food matrices remains challenging due to the pH variations and interferences such as fat, protein, salt, presence of other proteases, thereby reducing the assay sensitivity and specificity. The mouse bioassay has been used successfully in various food matrices. However, the assay is time consuming, tedious and expensive with the limited number of laboratories worldwide able to perform the test. Compared to the mouse bioassay, ELISA is rapid and facile, however, tedious extraction protocols are needed for reducing the influence of interfering components on the sensitivity and specificity. The food matrix is also affecting the colorimetric LSPR and FRET-based assays, where food pigments might interfere with the response thus diminishing the sensitivity. Furthermore, pH and ionic strength are known to influence the fluorescence

intensity of organic dyes (environmentally sensitive) as well as the aggregation behaviour of plasmonic nanoparticles, thus compromising the overall performance of such assays. Electrochemiluminescence is one promising alternative for rapid detection of BoNT. However, the disadvantage related to the electrode fouling by reagents and interfering matrix components still diminishes the sensitivity. Herein, the proposed methodology, with a simple dilution or filtration step, possesses huge potential to exclude tedious pre-treatment protocols associated with matrix clean-up for on-site monitoring. Moreover, the proposed methodology is cost effective, requiring very less reagents, which is crucial for on-site applications. Furthermore, the proposed approach possesses the potential for multiplexed detection of proteases, for instance, by attaching fluorophores with similar excitation but distinctly different emission wavelength, to substrates that are specific to various proteases for yielding distinguishable signal responses.

Table 4.2 Performance of BoNT assays.

Assay	LOD	Sample (Assay time)	Advantages	Disadvantages
Mouse bioassay[29]	10 pg/mL	food, serum, stool (2-4 days)	sensitive and specific detects the active form of the toxin	expensive requires trained personnel and advanced infrastructure possess ethical dilemma
ELISA[30]	10-10^4 MLD_{50}	inoculated canned green beans and mushrooms (2 days)	sensitive it is possible to measure many samples at the same time	cross reactivity with other interfering molecules instability of antibodies batch to batch variations of antibodies not able to distinguish between active and inactive form of the toxin tedious sample pre-treatment protocols needed
DIG-ELISA[31]	60 pg/mL – 2 ng /mL	buffer broccoli, orange juice, bottled water, cola soft drinks, vanilla extract, oregano, potato salad, apple juice, meat products, and dairy foods (>5 h)		
ELISA-ELICA[32]	5-10 pg/mL	bacterial culture (>19 h)		

FRET-based assay 33343536	17.5 ng/mL, 1 fg/mL 100 fg/mL	buffer (2-3 h) buffer (30 min) apple juice, skimmed milk (30 min)	sensitive and specific detects the active form of the toxin stability is achieved with edc/nhs coupling (chemisorption)	SNAP-25 physisorption is unstable donor reabsorbs emission photo-bleaching over time detects domain for receptor binding (HCR)
	0.2 ng/mL	buffer (3 h)		
	20-40 pM 37.5 pM 57.2 pM	buffer (5 min) milk serum		
LSPR-based assay 17,37	5-250 ng/mL	buffer (3-4 h)	highly sensitive detects the active form of the toxin low sample volume colorimetric detection	sensitive to matrix interferences
	50 pg/mL	buffer (2-3 h)		
Electrochemi-luminiscence 38	1-2 ng/mL	buffer, purified toxin (2.5 h)	sensitive inexpensive	reproducibility problems requires sensitive device to read the signal sample pre-treatment required
Lateral flow assay39,40	0.18-0.3 ng/mL	buffer, milk, apple juice, orange juice (25 min)	sensitive easy to perform low sample volume	not able to distinguish between active and inactive form of the toxin sample pretreatment is required
	5-100 ng/mL	buffer, apple juice, orange juice,whole/deffated milk (10-15 min)		
Functional magnetic-bead based assay (this study)	25-50 ng/mL	buffer, carrot juice, milk (6 h)	detects the active form of the toxin easy to perform reproducible and specific	sensitive to matrix interferences in milk sample

Considering the extreme toxicity of BoNT, detection of trace amounts of toxin is of critical importance. In many cases, such sensitivity is achieved by affinity-based immunoassays that also provide rapid responses. However, these affinity-based assays yield responses for both the active and inactive forms of the toxin, potentially leading to false-positive results. Furthermore, different serotypes might display varying affinities towards the capturing antibody leading to false positive/negative results 41. Herein, a functional assay is proposed,

that yields responses only for the active form of the toxin. Although the proposed assay is more accurate in terms of detecting the active population of the protease it typically compromises on sensitivity as compared to antibody-based assays. The lethal dose of the toxin of 1µg/kg refers to 65 µg of toxin for a person with a body weight of 65 kg, which corresponds to 325 ng/mL for a sample volume of 200 mL. The obtained responses illustrate that the proposed methodology yields a LOD lower than the lethal dose both in buffer and carrot juice. Moreover, it should be noted that recombinant toxoids are known to be less enzymatically active compared to that of the wild type due to the improper folding in *E.coli* [42]. Thus, the LOD can be enhanced by employing the native toxin as compared to that of the light chain tested in this study, which provides an avenue for improving the sensitivity of the proposed methodology. Furthermore, optimization of the amino acids in the peptide sequence also can enhance sensitivity and reduce the assay time. For instance, it has been reported that replacing lysine residues (K189, K201) with arginine residues (Q) enhances hydrolysis as well as replacing glutamic acid (E194) with arginine (R) leads to a better cleavage[24,43]. Thus, identifying preferred amino acid residues in the peptide sequence that will boost up the protease activity is of critical importance for assay development. For instance, a recent report on substrate optimization for OmpT (a membrane bound protease) using rationally designed peptide arrays and SAMDI-MS (*S*elf-*A*ssembled *M*onolayers for matrix-assisted Laser *D*esorption/*I*onization mass spectrometry) yielded a peptide sequence with a 400-fold better catalytic efficiency than that of the native sequence[44]. A similar approach can be adopted for designing a peptide sequence for the BoNT/A LC assay and for improving the LOD and assay time of the proposed methodology, which constitutes our future studies. In summary, Figure 4.5 ascertains that the proposed protocol utilizing superparamagnetic beads and a simple dilution step minimizes the influence of the sample matrix on the assay performance. The false positives/negatives are diminished by washing away the potential interferences from the sample matrix, including excess dye and digested part of the substrate owing to the facile separation by superparamagnetic beads with a magnet. Moreover, the use of robust peptides and assay protocol (ST-2) could serve as an alternative approach for designing facile diagnostic tools for on-site screening of food samples.

4.4 Conclusion

This chapter investigates the first hypothesis by employing the synthetic peptide as a recognition molecule to detect the activity of the toxin rather than its total concentration, which is crucial for the evaluation of the toxicity levels in contaminated food. Furthermore, determining the active versus inactive form of the toxin is critical for the precise diagnosis in toxicosis. Additionally, the peptide used in this assay ensures specificity for the serotype A as it precisely cleaves the peptide at the position Q–R mimicking the actual substrate (SNAP 25). The magnetic bead-based methodology is adopted to facilitate detection in complex matrices by washing away the sample matrix as well as excess reporter dye, thereby improving the reliability of the responses. The reported sensitivity and assay time are dependent only on the interaction between the peptide and BoNT/A LC as observed with the adopted pre-cleavage strategy. Therefore, the assay sensitivity can be improved by optimizing the peptide sequence and by exploring the other modifications of the peptide's termini to expedite digestion. The proposed protocol offers a facile and appealing alternative for the detection of BoNT serotypes, with a possibility of multiplexed detection of other proteolytic enzymes by rationally designing highly potent peptide substrates. Apart from food screening, the proposed methodology is also applicable for environmental monitoring and national security applications.

References

(1) Rossetto, O.; Pirazzini, M.; Montecucco, C. Botulinum Neurotoxins: Genetic, Structural and Mechanistic Insights. *Nature Reviews Microbiology* **2014**, *12* (8), 535–549.

(2) Cheng, L. W.; Land, K. M.; Tam, C.; Brandon, D. L.; Stanker, L. H. Technologies for Detecting Botulinum Neurotoxins in Biological and Environmental Matrices. In *Significance, Prevention and Control of Food Related Diseases*; InTech, 2016.

(3) CDPH Testing Confirms Botulism Linked to Nacho Cheese Sauce.

(4) Courtney-Long, E. A.; Carroll, D. D.; Zhang, Q. C.; Stevens, A. C.; GriffinBlake, S.; Armour, B. S.; Campbell, V. A. *25th Anniversary of the Americans with*

Disabilities Act — July 2015 Prevalence of Disability and Disability Type Among Adults — United States; 2015; Vol. 64.

(5) Feng, L.; Chen, X.; Liu, S.; Zhou, Z.; Yang, R. Two-Family Outbreak of Botulism Associated with the Consumption of Smoked Ribs in Sichuan Province, China. *International Journal of Infectious Diseases* **2015**, *30*, 74–77.

(6) A History of Anthrax | Anthrax | CDC.

(7) Wictome, M.; Newton, K. A.; Jameson, K.; Dunnigan, P.; Clarke, S.; Gaze, J.; Tauk, A.; Foster, K. A.; Shone, C. C. Development of in Vitro Assays for the Detection of Botulinum Toxins in Foods. *FEMS immunology and medical microbiology* **1999**, *24* (3), 319–323.

(8) Grate, J. W.; Ozanich, R. M.; Warner, M. G.; Bruckner-Lea, C. J.; Marks, J. D. Advances in Assays and Analytical Approaches for Botulinum-Toxin Detection. *TrAC Trends in Analytical Chemistry* **2010**, *29* (10), 1137–1156.

(9) Sharma, S. K.; Eblen, B. S.; Bull, R. L.; Burr, D. H.; Whiting, R. C.; Donald, H.; Whiting, R. C.; Burr, D. H. Evaluation of Lateral-Flow Clostridium Botulinum Neurotoxin Detection Kits for Food Analysis. *Applied and environmental microbiology* **2005**, *71* (7), 3935–3941.

(10) Shone, C.; Ferreira, J.; Boyer, A.; Cirino, N.; Egan, C.; Evans, E.; Kools, J.; Sharma, S. The 5th International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Neurotoxins. Workshop Review: Assays and Detection. *Neurotoxicity research* **2006**, *9* (2–3), 205–216.

(11) Barr, J. R.; Moura, H.; Boyer, A. E.; Woolfitt, A. R.; Kalb, S. R.; Pavlopoulos, A.; McWilliams, L. G.; Schmidt, J. G.; Martinez, R. A.; Ashley, D. L. Botulinum Neurotoxin Detection and Differentiation by Mass Spectrometry. *Emerging Infectious Diseases* **2005**, *11* (10), 1578–1583.

(12) Tsukamoto, K.; Kohda, T.; Mukamoto, M.; Takeuchi, K.; Ihara, H.; Saito, M.; Kozaki, S. Binding of Clostridium Botulinum Type C and D Neurotoxins to Ganglioside and Phospholipid NOVEL INSIGHTS INTO THE RECEPTOR FOR CLOSTRIDIAL NEUROTOXINS *. *Journal of Biological Chemistry* **2005**, *280* (42), 35164–35171.

(13) Kalb, S. R.; Moura, H.; Boyer, A. E.; McWilliams, L. G.; Pirkle, J. L.; Barr, J. R.

The Use of Endopep–MS for the Detection of Botulinum Toxins A, B, E, and F in Serum and Stool Samples. *Analytical Biochemistry* **2006**, *351* (1), 84–92.

(14) Shi, J.; Guo, J.; Bai, G.; Chan, C.; Liu, X.; Ye, W.; Hao, J.; Chen, S.; Yang, M. A Graphene Oxide Based Fluorescence Resonance Energy Transfer (FRET) Biosensor for Ultrasensitive Detection of Botulinum Neurotoxin A (BoNT / A) Enzymatic Activity. *Biosensors and Bioelectronics* **2015**, *65*, 238–244.

(15) Rivera, V. R.; Gamez, F. J.; Keener, W. K.; White, J. A.; Poli, M. A. Rapid Detection of Clostridium Botulinum Toxins A, B, E, and F in Clinical Samples, Selected Food Matrices, and Buffer Using Paramagnetic Bead-Based Electrochemiluminescence Detection. *Analytical Biochemistry* **2006**, *353* (2), 248–256.

(16) Lévêque, C.; Ferracci, G.; Maulet, Y.; Mazuet, C.; Popoff, M. R.; Blanchard, M.-P.; Seagar, M.; El Far, O. An Optical Biosensor Assay for Rapid Dual Detection of Botulinum Neurotoxins A and E. *Scientific Reports* **2016**, *5* (1), 17953.

(17) Liu, X.; Wang, Y.; Chen, P.; Wang, Y.; Zhang, J.; Aili, D.; Liedberg, B. Biofunctionalized Gold Nanoparticles for Colorimetric Sensing of Botulinum Neurotoxin A Light Chain. *Analytical Chemistry* **2014**, *86* (5), 2345–2352.

(18) van Oordt, T.; Stevens, G. B.; Vashist, S. K.; Zengerle, R.; von Stetten, F. Rapid and Highly Sensitive Luciferase Reporter Assay for the Automated Detection of Botulinum Toxin in the Centrifugal Microfluidic LabDisk Platform. *RSC Advances* **2013**, *3* (44), 22046.

(19) Brunt, J.; Webb, M. D.; Peck, M. W. Rapid Affinity Immunochromatography Column-Based Tests for Sensitive Detection of Clostridium Botulinum Neurotoxins and Escherichia Coli O157. *Applied and Environmental Microbiology* **2010**, *76* (13), 4143–4150.

(20) Frisk, M. L.; Berthier, E.; Tepp, W. H.; Johnson, E. A.; Beebe, D. J. Bead-Based Microfluidic Toxin Sensor Integrating Evaporative Signal Amplification. *Lab on a Chip* **2008**, *8* (11), 1793.

(21) Rajaseger, G.; Saravanan, P.; Lee, V. J.; Novem, V.; Yap, E. P.; Moochhala, S.; Lee, L. H.; Gopalakrishnakone, P. Fieldable Assay For Botulinum Neurotoxins. *J Med CBR Def* **2010**, *8* (22), 1–21.

(22) Bagramyan, K.; Barash, J. R.; Arnon, S. S.; Kalkum, M. Attomolar Detection of

Botulinum Toxin Type A in Complex Biological Matrices. *PloS one* **2008**, *3* (4), e2041.

(23) Wang, Y.; Liu, X.; Zhang, J.; Aili, D.; Liedberg, B. Time-Resolved Botulinum Neurotoxin A Activity Monitored Using Peptide-Functionalized Au Nanoparticle Energy Transfer Sensors. *Chemical Science* **2014**, *5* (7), 2651–2656.

(24) Schmidt, J. J.; Bostian, K. A. Endoproteinase Activity of Type A Botulinum Neurotoxin: Substrate Requirements and Activation by Serum Albumin. *Journal of protein chemistry* **1997**, *16* (1), 19–26.

(25) Oh, E.; Susumu, K.; Blanco-Canosa, J. B.; Medintz, I. L.; Dawson, P. E.; Mattoussi, H. Preparation of Stable Maleimide-Functionalized Au Nanoparticles and Their Use in Counting Surface Ligands. *Small* **2010**, *6* (12), 1273–1278.

(26) Wang, D.; Baudys, J.; Ye, Y.; Rees, J. C.; Barr, J. R.; Pirkle, J. L.; Kalb, S. R. Improved Detection of Botulinum Neurotoxin Serotype A by Endopep–MS through Peptide Substrate Modification. *Analytical Biochemistry* **2013**, *432* (2), 115–123.

(27) Mizanur, R. M.; Stafford, R. G.; Ahmed, S. A. Cleavage of SNAP25 and Its Shorter Versions by the Protease Domain of Serotype A Botulinum Neurotoxin. *PLoS ONE* **2014**, *9* (4), e95188.

(28) Agarwal, R.; Swaminathan, S. SNAP-25 Substrate Peptide (Residues 180-183) Binds to but Bypasses Cleavage by Catalytically Active Clostridium Botulinum Neurotoxin E. *Journal of Biological Chemistry* **2008**, *283* (38), 25944–25951.

(29) Maslanka, S. E.; Solomon, H. M.; Sharma, S.; Johnson, E. A. Clostridium Botulinum and Its Toxins. In *Compendium of Methods for the Microbiological Examination of Foods*; American Public Health Association, 2015.

(30) Rodriguez, A.; Dezfulian, M. Rapid Identification OfClostridium Botulinum and Botulinal Toxin in Food. *Folia Microbiologica* **1997**, *42* (2), 149–151.

(31) Sharma, S. K.; Ferreira, J. L.; Eblen, B. S.; Whiting, R. C. Detection of Type A, B, E, and F Clostridium Botulinum Neurotoxins in Foods by Using an Amplified Enzyme-Linked Immunosorbent Assay with Digoxigenin-Labeled Antibodies. *Applied and Environmental Microbiology* **2006**, *72* (2), 1231–1238.

(32) Doellgast, G. J.; Triscott, M. X.; Beard, G. A.; Bottoms, J. D.; Cheng, T.; Roh, B. H.; Roman, M. G.; Hall, P. A.; Brown, J. E. Sensitive Enzyme-Linked

Immunosorbent Assay for Detection of Clostridium Botulinum Neurotoxins A, B, and E Using Signal Amplification via Enzyme-Linked Coagulation Assay. *Journal of clinical microbiology* **1993**, *31* (9), 2402–2409.

(33) Sapsford, K. E.; Granek, J.; Deschamps, J. R.; Boeneman, K.; Blanco-Canosa, J. B.; Dawson, P. E.; Susumu, K.; Stewart, M. H.; Medintz, I. L. Monitoring Botulinum Neurotoxin A Activity with Peptide-Functionalized Quantum Dot Resonance Energy Transfer Sensors. *ACS Nano* **2011**, *5* (4), 2687–2699.

(34) Shi, J.; Guo, J.; Bai, G.; Chan, C.; Liu, X.; Ye, W.; Hao, J.; Chen, S.; Yang, M. A Graphene Oxide Based Fluorescence Resonance Energy Transfer (FRET) Biosensor for Ultrasensitive Detection of Botulinum Neurotoxin A (BoNT/A) Enzymatic Activity. *Biosensors and Bioelectronics* **2015**, *65*, 238–244.

(35) Wang, Y.; Fry, H. C.; Skinner, G. E.; Schill, K. M.; Duncan, T. V. Detection and Quantification of Biologically Active Botulinum Neurotoxin Serotypes A and B Using a Förster Resonance Energy Transfer-Based Quantum Dot Nanobiosensor. *ACS Applied Materials & Interfaces* **2017**, *9* (37), 31446–31457.

(36) Lee, J.; Brennan, M. B.; Wilton, R.; Rowland, C. E.; Rozhkova, E. A.; Forrester, S.; Hannah, D. C.; Carlson, J.; Shevchenko, E. V.; Schabacker, D. S.; et al. Fast, Ratiometric FRET from Quantum Dot Conjugated Stabilized Single Chain Variable Fragments for Quantitative Botulinum Neurotoxin Sensing. *Nano Letters* **2015**, *15* (10), 7161–7167.

(37) Wang, Y.; Liu, X.; Zhang, J.; Aili, D.; Liedberg, B. Time-Resolved Botulinum Neurotoxin A Activity Monitored Using Peptide-Functionalized Au Nanoparticle Energy Transfer Sensors. *Chem. Sci.* **2014**, *5* (7), 2651–2656.

(38) Guglielmo-Viret, V.; Attrée, O.; Blanco-Gros, V.; Thullier, P. Comparison of Electrochemiluminescence Assay and ELISA for the Detection of Clostridium Botulinum Type B Neurotoxin. *Journal of Immunological Methods* **2005**, *301* (1–2), 164–172.

(39) Orlov, A. V.; Znoyko, S. L.; Cherkasov, V. R.; Nikitin, M. P.; Nikitin, P. I. Multiplex Biosensing Based on Highly Sensitive Magnetic Nanolabel Quantification: Rapid Detection of Botulinum Neurotoxins A, B, and E in Liquids. *Analytical Chemistry* **2016**, *88* (21), 10419–10426.

(40) Ching, K. H.; Lin, A.; McGarvey, J. A.; Stanker, L. H.; Hnasko, R. Rapid and Selective Detection of Botulinum Neurotoxin Serotype-A and -B with a Single Immunochromatographic Test Strip. *Journal of Immunological Methods* **2012**, *380* (1–2), 23–29.

(41) Lindström, M.; Korkeala, H. Laboratory Diagnostics of Botulism. *Clinical microbiology reviews* **2006**, *19* (2), 298–314.

(42) Yang, W.; Lindo, P.; Riding, S.; Chang, T. W.; Cai, S.; Van, T.; Kukreja, R.; Zhou, Y.; Vasa, K.; Singh, B. R. Expression, Purification and Comparative Characterisation of Enzymatically Deactivated Recombinant Botulinum Neurotoxin Type A. *The Botulinum J.* **2008**, *1* (2), 219–241.

(43) Schmidt, J. J.; Bostian, K. A. Assay for the Proteolytic Activity of Serotype a from Clostridium Botulinum, 1999.

(44) Wood, S. E.; Sinsinbar, G.; Gudlur, S.; Nallani, M.; Huang, C.-F.; Liedberg, B.; Mrksich, M. A Bottom-Up Proteomic Approach to Identify Substrate Specificity of Outer-Membrane Protease OmpT. *Angewandte Chemie International Edition* **2017**, *56* (52), 16531–16535.

Chapter 5

Paper-based visual detection of BoNT/A

This chapter explains the possibility of transferring the solution based-assay for the detection of BoNT/A (explained in chapter 4) to a paper-based dipstick format. The developed assay is simple, specific, and for on-site detection utilizing the peptide substrate, design specifically for the botulinum type A, and magnetic-bead based methodology for fasil matrix clean-up. The proposed functional LFA is targeting just active forms of BoNT/A with yielded LOD of 2.5 nM (125 ng/mL). Further improvement in LOD and visual detection was obtained after amplification step (1 nM; 50 ng/mL), and by increasing the digestion time (0.1 nM; 5 ng/mL), supported by RGB (ΔE) analysis. The proposed approach could be extended for detection of BoNT serotypes and other proteases in food matrices, upon utilizing appropriate substrates with specific cleavage sites.

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5.1 Introduction

Early detection of toxic proteases and biomarkers is of critical importance in clinical diagnosis, food safety and environmental monitoring in order to prevent outbreaks, especially in resource-limited settings. Most of these methodologies reported so far are time consuming, labour intensive and not suitable for on-site detection as they require sophisticated instrumentations for analysis. Therefore, significant efforts have been devoted to the development of assays/devices for on-site monitoring. For instance, lateral flow assays (LFAs) have been explored owing to their rapid responses, simplicity to use and affordability[1].

LFAs have been considered potentially useful for detection of viruses[2], bacteria[3], pesticides[4], hormones[5], toxins[6], etc. Most of the commercially available and reported LFAs are affinity-based assays[7–10], involving antibodies that capture and recognize targets based on their structure/epitopes rather than their functionality/activity. Since both the active and inactive forms are captured by the antibodies, vital information on toxicity of proteases cannot be deduced, thereby inhibiting a precise analysis. Efforts have been directed towards developing LFA devices that yield responses only for active forms of proteases using gold nanoparticles as reporter molecules for visual detection[11]. However, assaying in complex food matrices requires tedious lab based clean-up protocols that may not be suitable for on-site applications.

In this study we developed functional lateral flow assay in the dipstick format for on-site detection of BoNT/A (proteases) based on magnetic beads as reporters. As in the previous study, here as well, the magnetic beads serve as facilitators for sample matrix clean-up, owing to their efficient magnetic separation properties. The proposed assay utilizes a synthetic peptide (truncated C-terminus of the SNAP-25 protein (synaptosomal-associated protein 25)) for BoNT/A LC detection in food matrix.

A dipstick test comprises of a detection zone with capturing antiPEG antibodies and an absorption pad. Briefly, the principle of the dipstick assay is based on specific cleavage of

a peptide substrate and PEG–antiPEG interaction to form brownish test lines as shown in Fig. 5.1. After cleavage of the substrate by BoNT/LC A, SA-MBs are added to the sample solution to separate both cleaved and un-cleaved substrates (without and with PEG, respectively) from the sample matrix. Separation of complexes SA-MBs-substrate (cleaved and un-cleaved) is performed using the magnet. Afterwards, the washing steps are carried out to ensure the removal sample interferents removal and the isolation of complexes SA-MBs-substrate is done by magnetic separation. Upon immersion of LFA dipstick into isolated SA-MBs with cleaved and un-cleaved substrates, they migrate as complexes to the test line via capillary forces. The PEGylated terminus of the un-cleaved substrate is subsequently captured by the immobilized anti-PEG antibodies leading to accumulation of SA-MBs on the test line and formation of brownish band that is readily visualized by naked-eye. SA-MBs bound with cleaved substrate do not adsorb on the test line due to the removal of PEG during the separation process. Therefore, higher concentrations of BoNT/A LC in the sample matrix yield extensive cleavage of the substrate, resulting in a test line of low contrast (light brownish color), whereas high contrast is observed in a sample without BoNT/LC A (control; dark brownish color), which is utilized as a reference in the assay. Thus, the response is inversely proportional to the concentration of active BoNT/A LC.

Unlike typical LFAs that yield responses for both active and inactive proteases, the proposed approach yield responses only for active form of BoNT/A LC. Moreover, utilization of magnetic beads substitutes tedious clean-up procedures of intricate samples yielding a high-throughput and cost-effective on-site protease assay platform. The similar set of magnetic beads were used for amplification to increase the contrast of the test line.

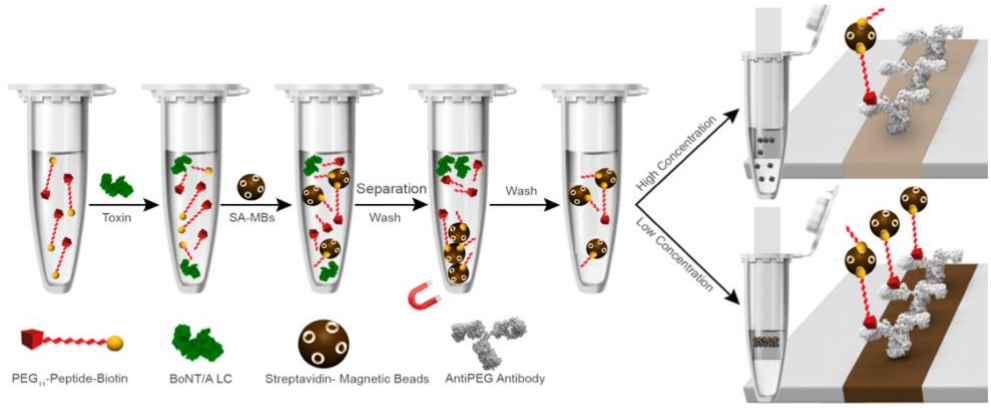


Figure 5.1. Schematic of the dipstick for protease assaying in food matrices, illustrated using BoNT/A LC as a model system.

5.2 Materials and reagents

Botulinum Toxin light chain A (BoNT/A LC, recombinant) was obtained from Toxogen GmbH, Germany. Peptide sequence: Cys-PEG11-SNKTRIDEANQRATKXK-Biotin was purchased from Mimotopes Pte Ltd, Australia. Streptavidin-magnetic beads (200 nm) and Botulinum Toxin light chain E (BoNT/E LC) were purchased from Creative Diagnostics, USA. Maleimide-magnetic beads (150 nm) were purchased from Ocean Nanotech, San Diego, USA. Anti-PEG antibody (Clone 09F02) was purchased from BioVision, Inc. (California, US). Nitrocellulose (HF135MC100) and cellulose membranes were obtained from EMD Millipore, US. Bovine serum albumin (BSA), HEPES buffer and PBS tablets were purchased from Sigma-Aldrich (Singapore). Carrot juice was obtained from a local market.

Protocols for biosensing using the dipstick, fabrication of dipstick test, RGB analysis, optimization of signal amplification and specificity test are described in sections 3.3, 3.3.1, 3.3.2, 3.3.3, and 3.3.4 respectively.

5.3 Results and discussion

5.3.1 Optimization of the assay performance

A series of experiments relying on different concentrations of substrate and SA-MBs were undertaken to optimize the assay performance. As illustrated in Fig. 5.2a, the desired colour intensity of the test line for visual detection was determined based on evaluation of responses for different concentrations of substrate (100, 50 and 10 nM) in combination with different SA-MB concentrations (10, 20 and 30 µg/mL). Figure 5.2a illustrates that visible brownish bands along the test line (the colour of accumulated SA-MBs) are obtained for all the concentrations of substrate and SA-MB. The most distinct band is recorded for the substrate concentration of 100 nM with the SA-MB concentrations of 30 µg/mL. The test line intensity gradually decreases by reducing both the SA-MBs and substrate concentrations. Although higher concentrations of both SA-MB and substrate yield bands with better contrast, it is desirable to optimize the substrate concentration, considering that a densely packed layer of peptides may have a negative impact on the hydrolysis due to steric hindrance. Along this line, the series of experiments were performed to optimize the substrate concentration according to the assay response (Fig. 5.2b). Herein, the concentrations of peptide (10, 50, 100 nM) were incubated with different concentrations of BoNT/A LC (0, 1, 2.5, 5, 10 nM) for 5 h at 37 ^{o}C, with the fixed concentration of SA-MB (20 µg/mL). It could be observed that the colour intensity of the test line reduces with increasing BoNT/A LC concentrations for the lowest peptide concentration used (10 nM). On the other hand, by increasing the peptide concentration (50 and 100 nM), the reduction in the colour intensity of the test line is less obvious. Most probably higher coverages of substrate reduces the sensitivity and could prolong the assay time. Additionally, reducing the amount of reagents such as peptide and SA-MB lowers the overall cost of the assay. Thus, a substrate concentration of 10 nM and a SA-MBs concentration of 20 µg/mL, that yielded a sufficiently sharp band (highlighted in red dashed box, Fig. 5.2a) and show the best assay response (highlighted in red dotted box, Fig. 5.2b) were utilized for assay development.

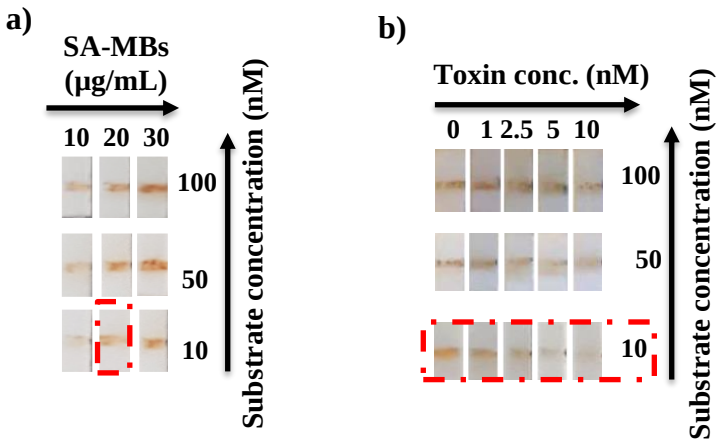


Figure 5.2. Optimization of the substrate and SA-MBs concentrations (a), and optimization of the substrate concentration based on the assay responses (b).

5.3.2 Concentration dependent responses of assay in buffer and carrot juice

Herein, we explored the performance of the dipstick assay in the buffer and carrot juice spiked with different BoNT/A LC concentrations (1, 2.5, 5, 10 nM) using the optimized assay conditions (Fig. 5.3). Upon immersion of the dipstick in the buffer for 15 min, a significant colour change of the test line as compared to the control line (0 nM BoNT/A LC) is observed as shown in Fig. 5.3a (inset) for the highest test concentration of 10 nM. For the BoNT/A LC concentrations of 5 nM and 2.5 nM in buffer, reduction in colour intensities is observed by naked eye compared to the intensity of the control line. Although the visual intensity of the band for the lowest test concentration of 1 nM is less distinguishable from the control line, differences in colour intensities are still readily observed. In order to ascertain and quantify the difference in colour intensities between test and control samples, RGB analysis was carried out using ImageJ software. Based on the RGB analysis, the cleavage percentage was calculated as $1-(L_c/L)$, where L_c and L are the luminance mean values for the control and test samples, respectively. The luminance values (Fig. 5.3a) were calculated using a weighted average of red, green and blue components using the eye sensitivity function $(L=0.299R+0.587G+0.114B)$[12], where the changes in G-green (e.g. in buffer, values change from 17.7 to 5.6 for 0 nM and 10 nM, respectively) and B-blue (e.g. from 28.8 to 8.9 for 0 nM and 10 nM, respectively) values are observed upon the addition of BoNT/A LC (Fig. 5.3b). According to the luminance

results obtained for test lines in the buffer, approximately 70%, 60%, 35% and 13 % of the substrate is digested by 10 nM, 5 nM, 2.5 nM and 1 nM, respectively, as compared to control samples (Fig. 5.3a). The limit of detection (LOD) of the assay is estimated to be 1 nM (50 ng/mL) in buffer using 3σ approach, where σ is the standard deviation of the control (black dashed line, Fig. 5.3a). In order to ensure reproducibility, three independent assays were conducted. Furthermore, in order to confirm that the colour difference between control and test lines are sufficiently distinct to be visually differentiated by human eye, ΔE values were calculated according to the International Commission of Illumination (CIE) 1976 algorithm[13] (a metric for understanding how the human eye perceives colour difference). The ΔE value typically ranges from 0 to 100 with the experimentally established perceptibility threshold value of 2. Thus, values below 2 are not considered to be perceptible by the human eye. Figure 5.3c illustrates the values of ΔE are between 2.7 to 13.4 for the tested BoNT/A LC concentrations (from 1 nM to 10 nM), suggesting that it is feasible to differentiate the colour intensities of the test lines from the control line by the naked eye.

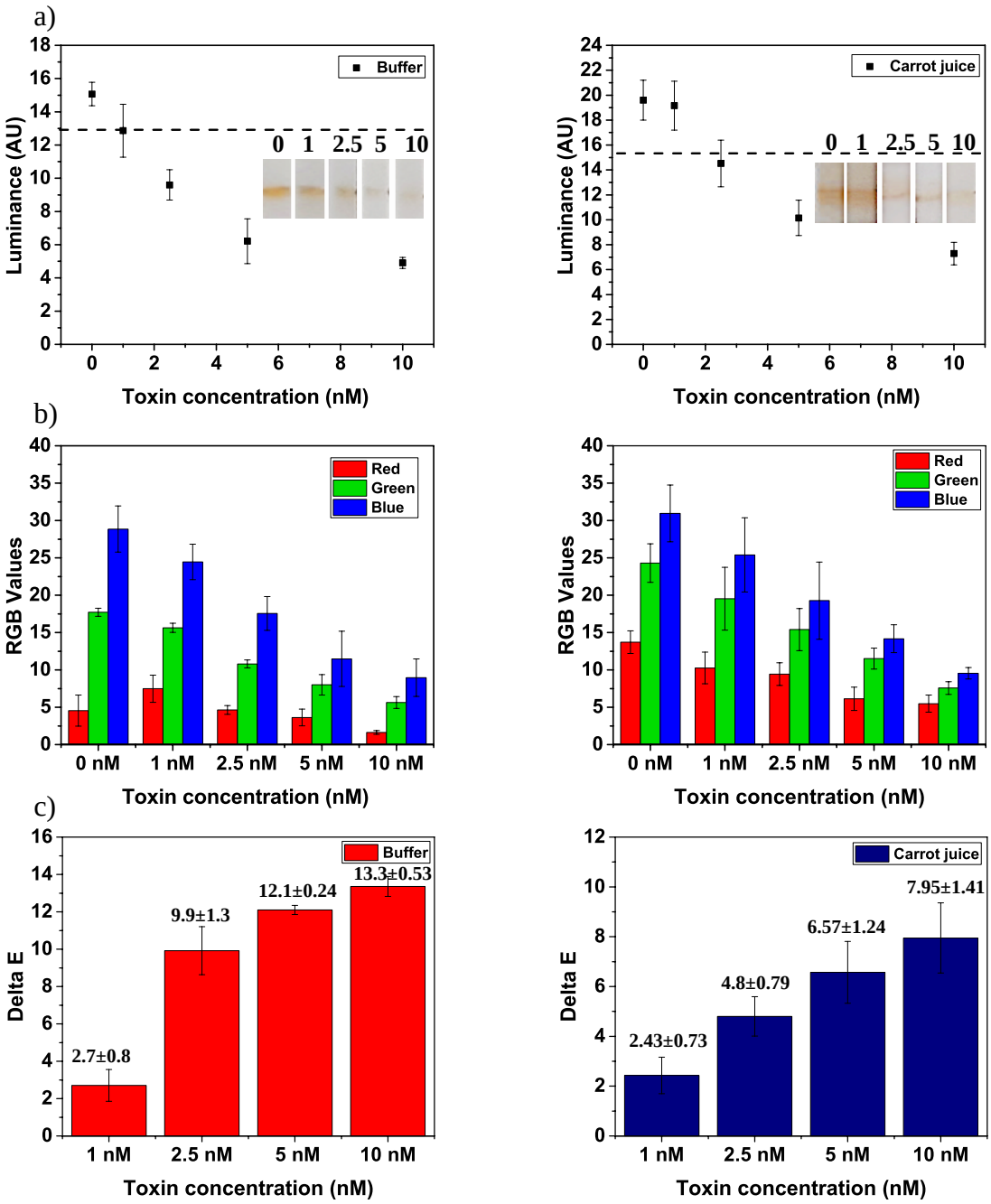


Figure 5.3. Visual detection of BoNT/A LC by dipstick in the buffer and carrot juice ((insets in (a)) and the corresponding luminance values of the test lines at varying concentrations of BoNT/A LC (a), mean RGB values of test lines for responses in buffer and carrot juice (b), and mean ΔE values of the test lines for responses in buffer and carrot juice (c).

Since the assay is intended for on-site analysis of food matrices, the proposed dipstick approach was subsequently evaluated for assaying BoNT/A LC in carrot juice. The spiked carrot juice with BoNT/A LC was tested according to the protocol mentioned above. A reduction in the colour intensities of the test lines is noticed for the concentrations of 10 nM, 5 nM and 2.5 nM and they are observable by naked eye, while for the lowest concentration of 1 nM the reduction is less noticeable (Fig. 5.3b, inset). However, a concentration dependent response is confirmed as illustrated in Fig. 5.3b based on the luminance values, which concurs with the responses in buffer. The substrate appears to be less digested (63%, 48%, 25 % and 2%, respectively) as compared to that in buffer, which could be attributed to the interferences from the carrot juice sample matrix. Accordingly, the LOD evaluated based on 3σ approach is higher compared to that of LOD obtained in buffer (2.5 nM; 125 ng/mL). Although, ΔE values (from 2.4 to 7.9) obtained in carrot juice are lower compared to that of values obtained in buffer, they still enable naked eye differentiation of colour intensities for BoNT/A LC concentrations above 2.5 nM (Fig. 5.3c).

5.3.3 Signal enhancement by employing similar magnetic beads

It is well known that the visual detection of LFA is constrained by the signal from labels prompting approaches for signal amplification methodologies[14]. In this study, we have attempted to improve the visual contrast of the SA-MB reporters accumulated on the test line by attaching a larger amount of MBs to the unoccupied streptavidin on the reporter SA-MB surface. In this way, SA-MBs as reporters carrying more magnetic beads (enhancers) to the test line making it more visible for the naked eye detection. In this study MAL-MBs, used for signal amplification, are coupled to SA-MBs (reporters) via biotin-streptavidin interaction using the linker as illustrated in Fig. 5.4.

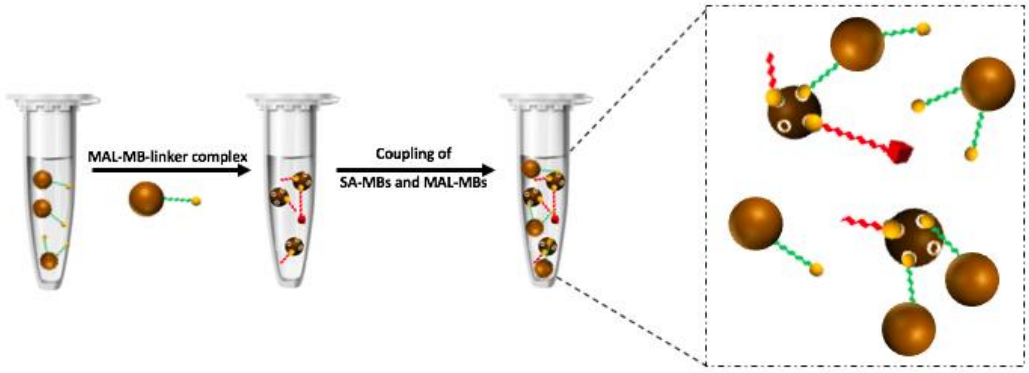


Figure 5.4. Illustration of coupling of MAL-MB-linker complex (enhancer) to SA-MBs (reporter) for the amplification step.

Signal enhancement was optimized via several experiments, where different linker/peptide concentrations (100 µL; 10 nM, 50 nM, 100 nM) were mixed with different MAL-MBs concentrations (3 µL; 10 µg/mL, 20 µg/mL, 30 µg/mL, 50 µg/mL, 100 µg/mL), and subsequently, the MAL-MB-linker complexes were added to system for the signal amplification (Figs. 5.4 and 5.5). Most noticeable improvement in the colour intensity of the test line is obtained by higher concentration of linker/peptide (100 nM and 50 nM) and MAL-MBs (50 µg/mL and 100 µg/mL). Further, the intensity of the test line slightly decreases by reducing the concentration of both reagents. Finally, the lowest linker/peptide concentration (10 nM) and MAL-MBs (20 µg/mL and 10 µg/mL) did not yield bands with significantly improved contrast compared to the control experiment. Among all combination examined in this study, the volumes/concentrations of MAL-MBs (3 µL, 30 µg/mL) and the concentration of linker (100 µL; 100 nM) were chosen as optimized for further experiments.

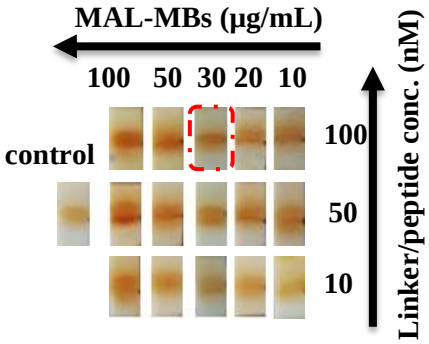


Figure 5.5. Optimization of signal amplification process

In order to ascertain that amplification of signal (increased color intensity of the test line) is due to the coupling of MAL-MBs to SA-MBs, rather than from non-specific binding to the NC membrane or SA-MBs, control experiments are performed (Fig. 5.6). Herein, MAL-MBs without the linker (which is required for coupling enhancer and reporter) (strip number 2), and MAL-MBs coupled to a non-biotinylated linker via cysteine residue (cannot react with the streptavidin on reporter molecules due to the lack of biotin molecule) (strip number 3) were added in the amplification step according to the protocol (see section 3.3.3). It can be visually observed from Fig. 5.6 that there is no signal enhancement compared to the control strip 1 (without MAL-MBs; without amplification step), which is further supported by luminance values (17-19 AU). These observation confirm that the amplification of signal of the test line is due to the coupling of MAL-MBs to SA-MBs via biotinylated linker.

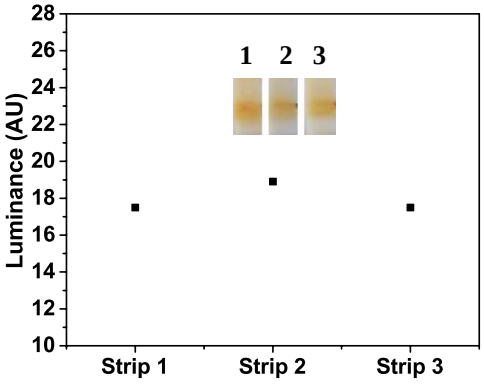


Figure 5.6. Visual detection of the test line without amplification (1), with MAL-MBs without

linker/peptide (2), and with MAL-MBs coupled to a non-biotinylated linker containing cysteine residue (3).

Figure 5.7 represents the assay performance in buffer and carrot juice with the amplification step according to the procedure described in the experimental section. As observed from Fig. 5.7a, the intensities of test line bands obtained in buffer and carrot juice appear darker by visual observation compared to the bands obtained in buffer and carrot juice without signal amplification (insets in (Fig. 5.7a)). Figure 5.7a illustrates that higher luminance values (e.g. 48 and 68 for the control test lines in the buffer and carrot juice, respectively; Fig. 5.7a) are obtained compared to those without amplification (e.g. 15 and 19 for the control test lines in the buffer and carrot juice, respectively; Fig. 5.3a). Additionally, RGB values (green and blue, Fig. 5.7b) show the same descending trend as observed in experiments before amplification step (Fig. 5.3b). Furthermore higher ΔE values upon amplification for responses both in buffer (from 9.7 to 46.9) and carrot juice (from 10.3 to 34.6) ascertain better differentiation of colour intensities of test line by the naked eye (Fig. 5.7c). Additionally, the LOD is improved in buffer as well as in carrot juice, where BoNT/A LC can be detected at the concentration level of 1 nM (50 ng/mL) in carrot juice (inset, Fig. 5.7a). Although the complexity of the assay is increasing with amplification, the improved contrasts of the test bands (as measured by luminance) facilitate visual detection (Fig. 5.7a). The improvement in visual detection, by a factor of ~5 based on ΔE values (e.g. from 2.4 to 10.3 before and after amplification, respectively in carrot juice for 0 nM of BoNT/A LC), led to better LOD of the dipstick assay (1 nM; 50 ng/mL). The assay response could be further improved by optimizing the substrate sequence, considering that a 17-mer substrate utilized in this study might be short for the effective recognition and digestion by BoNT/A LC[15,16] as compared to SNAP-25 sequence. Moreover, the improvement in assay performance related to the substrate design could be achieved by optimizing conjugation molecules (e.g. PEG chain length) to avoid steric hindrance introduced by the terminal modifications, thus providing a better accessibility to the cleavage site, thereby enabling better hydrolysis. An optimized amplification strategy in terms of reduced smearing of the test line that yield better contrast would also contribute to improve visual perception and LOD.

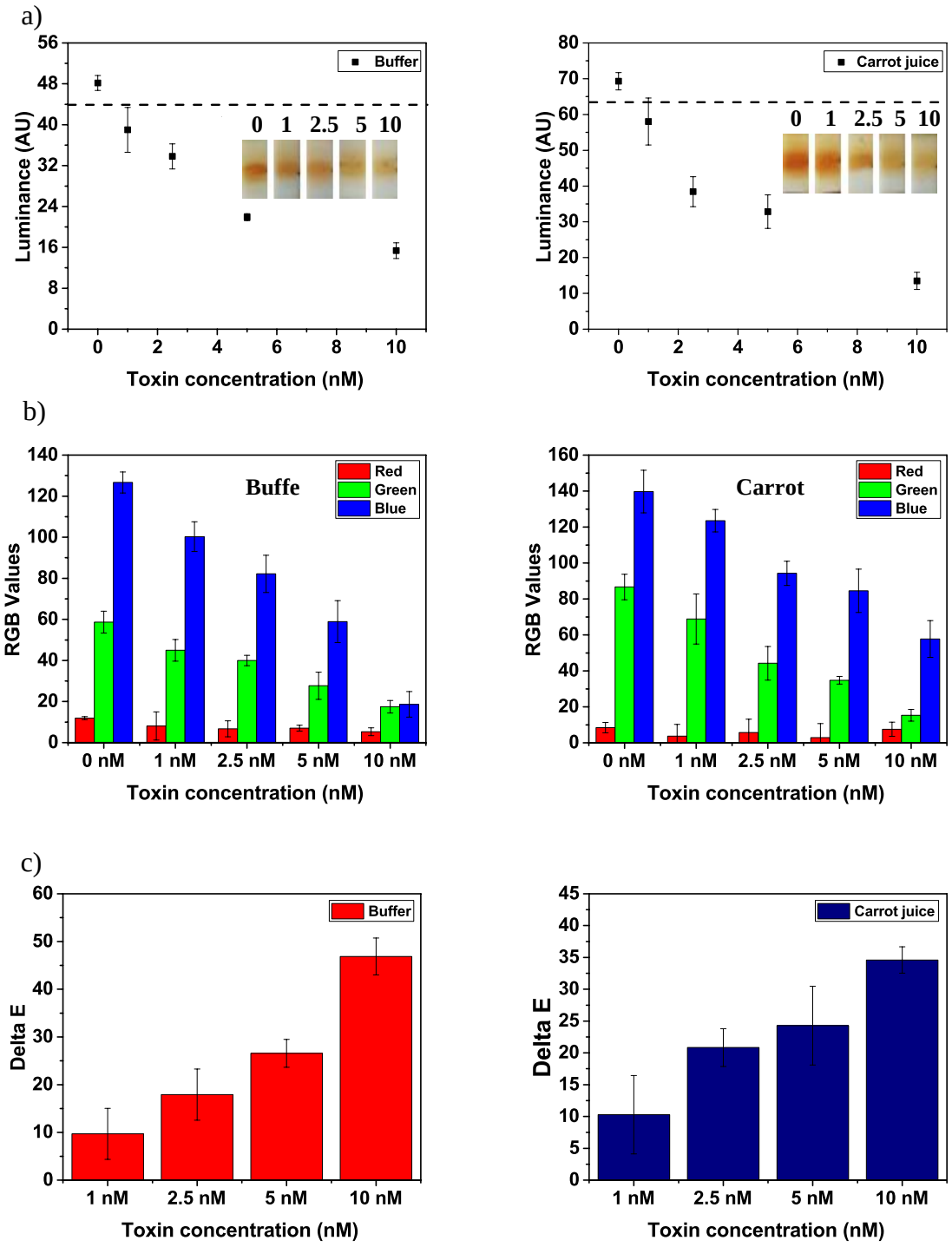


Figure 5.7. Visual detection of BoNT/A LC by dipstick upon signal amplification in buffer and carrot juice ((insets in (a)) and corresponding luminance values of the test lines at varying

concentrations of BoNT/A LC (a), mean RGB values of test lines for responses in buffer and carrot juice, and mean ΔE values of the test lines for responses in buffer and carrot juice (b).

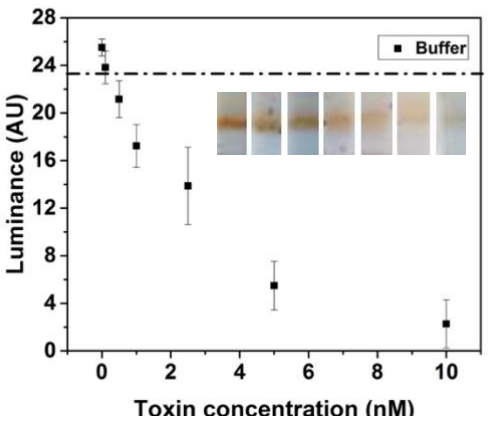
5.3.4 Signal enhancement by increasing digestion time

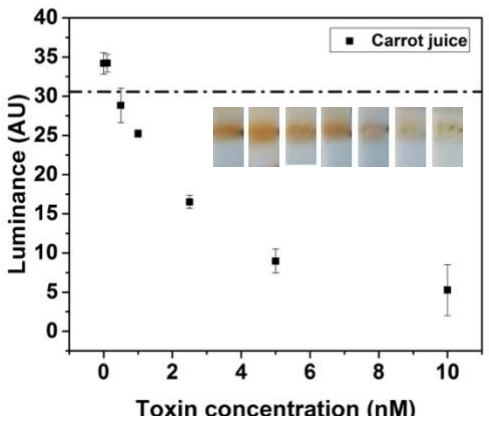
An alternative approach for improving the LOD of assay is to increase the digestion time. Figure 5.8a (luminance and ΔE values) illustrates that BoNT/A LC could be detected down to 0.5 nM in both buffer and carrot juice, upon increasing the digestion time, for instance, from 5 h to 12 h. The synergistic effect of the prolonged digestion and amplification further improved the LOD by an order of magnitude (0.1 nM; 5 ng/mL) as shown in Fig. 5.8b. Although the LOD can be improved by an order of magnitude upon amplification and by increasing the digestion time, it is to be emphasized that the proposed assay yield a LOD below the oral toxicity without amplification or prolonged digestion. The assay response could be further improved by optimizing the substrate sequence, considering that a 17-mer substrate utilized in this study might be short for the effective recognition and digestion by BoNT/A LC[15,16] as compared to SNAP-25 sequence. Moreover, the improvement in assay performance related to the substrate design could be achieved by optimizing conjugation molecules (e.g. PEG chain length) to avoid steric hindrance introduced by the terminal modifications, thus providing a better accessibility to the cleavage site, and enabling better hydrolysis. An optimized amplification strategy in terms of reduced smearing of the test line that yield better contrast would also contribute to improve visual perception and LOD.

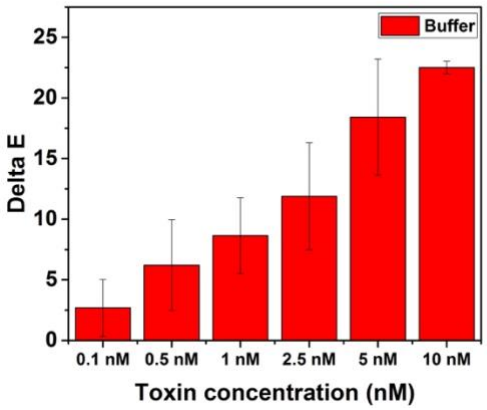
5.3.5 Specificity test

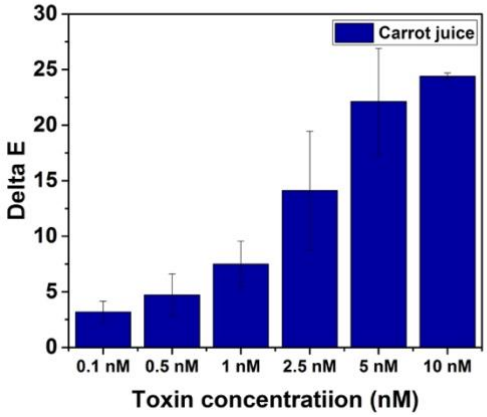
In order to evaluate the specificity of this assay, response to BoNT/E LC was evaluated according to the assay protocol. Different concentrations of BoNT/E LC were tested before (ranging from 2.5 nM to 10 nM) and after (ranging from 0.5 nM to 10 nM) amplification as shown in Fig. 5.9. It can be observed (both before and after amplification) visually as well as from luminance values that substrate is not cleaved by BoNT/E LC. Therefore, the proposed dipstick assay is specific for detection of BoNT/A. Detailed explanation on the unsuccessful BoNT/E LC cleavage is provided in Chapter 4.

a)

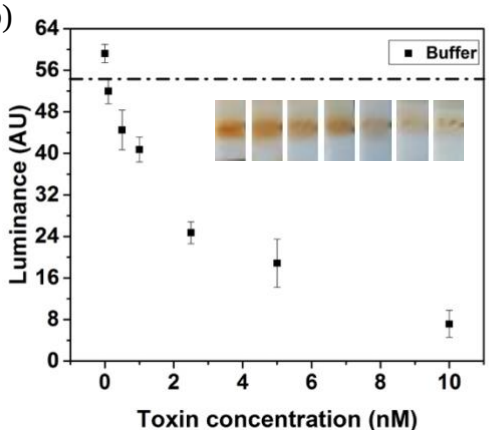

Buffer
Luminance (AU)
Toxin concentration (nM)

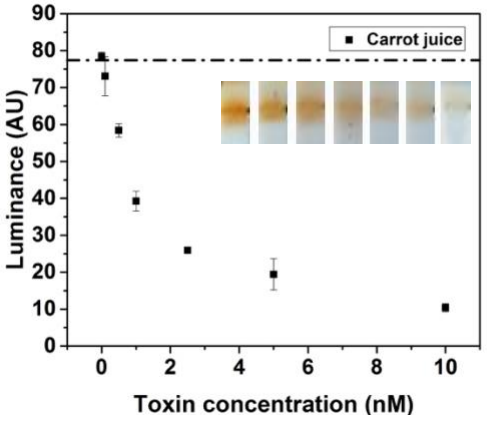

Carrot juice
Luminance (AU)
Toxin concentration (nM)

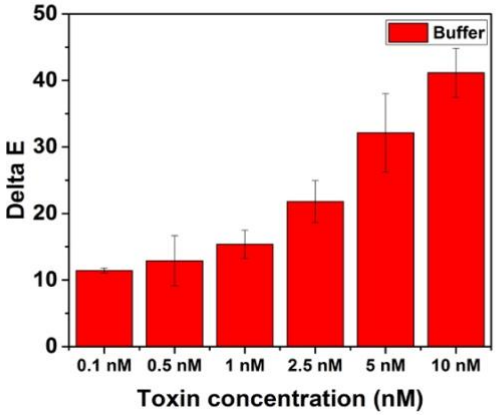

Buffer
Delta E
0.1 nM 0.5 nM 1 nM 2.5 nM 5 nM 10 nM
Toxin concentration (nM)


Carrot juice
Delta E
0.1 nM 0.5 nM 1 nM 2.5 nM 5 nM 10 nM
Toxin concentratiion (nM)

b)


Buffer
Luminance (AU)
Toxin concentration (nM)


Carrot juice
Luminance (AU)
Toxin concentration (nM)


Buffer
Delta E
0.1 nM 0.5 nM 1 nM 2.5 nM 5 nM 10 nM
Toxin concentration (nM)

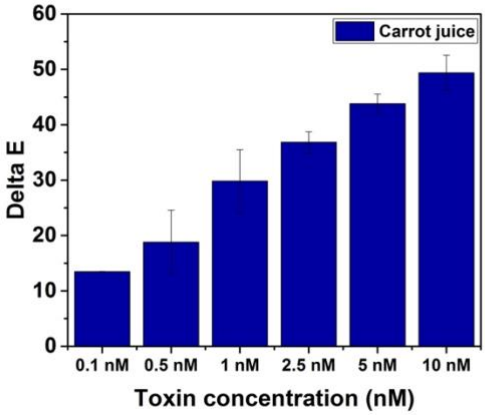

Carrot juice
Delta E
0.1 nM 0.5 nM 1 nM 2.5 nM 5 nM 10 nM
Toxin concentration (nM)

Figure 5.8. Assay performance characterized by luminance and ΔE values after 12 h of digestion before amplification (a) and after amplification step (b).

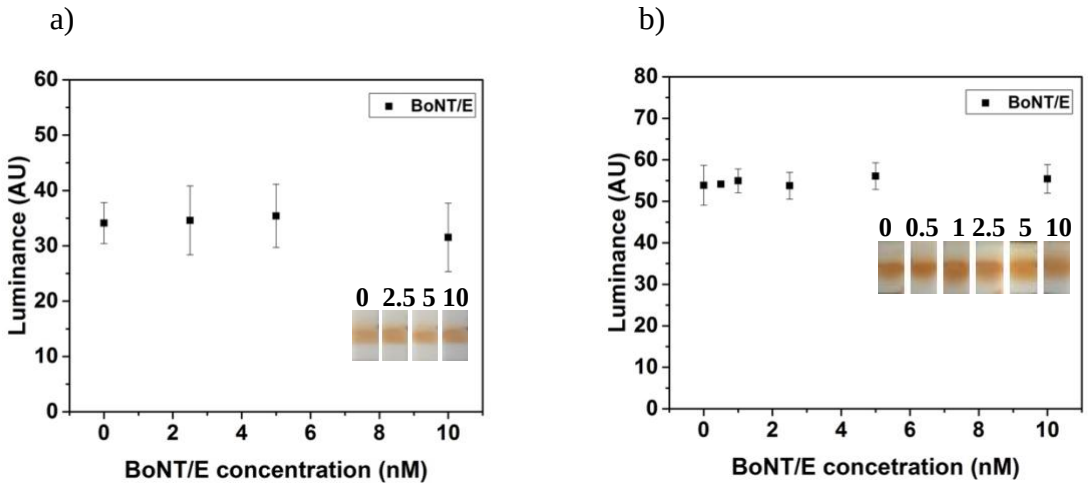


Figure 5.9. Assay performed with BoNT/E LC before (a) and after amplification (b).

5.4 Conclusion

This work demonstrate the feasibility of translating the solution-based detection (presented in chapter 4) to the simple, naked-eye detection on the paper. Herein, a functional assay in a LFA dipstick format is demonstrated for specific BoNT/A detection in carrot juice. The assay utilizes magnetic beads as reporters as well as main facilitators for sample clean-up. The developed assay yields responses only for the active form of proteases, without using expensive instruments, trained personnel, and long and tedious clean-up protocols. The lowest concentration of BoNT/A LC detected by the assay is 1 nM (50 ng/ mL), which is further improved by signal amplification, especially in carrot juice. Additionally, the LOD can be improved by an order of magnitude by combining the amplification step and extended digestion. The proposed assay possesses a great potential for detection of other BoNT serotypes and other proteases upon utilizing appropriate substrates with specific cleavage sites as well. Finally, the assay can yield potentially a multiplexed detection of proteases in resource-limited settings.

References

(1) Parolo, C.; de la Escosura-Muñiz, A.; Merkoçi, A. Enhanced Lateral Flow Immunoassay Using Gold Nanoparticles Loaded with Enzymes. *Biosensors and Bioelectronics* **2013**, *40* (1), 412–416.

(2) Le, T. T.; Chang, P.; Benton, D. J.; McCauley, J. W.; Iqbal, M.; Cass, A. E. G. Dual Recognition Element Lateral Flow Assay Toward Multiplex Strain Specific Influenza Virus Detection. *Analytical Chemistry* **2017**, *89* (12), 6781–6786.

(3) Zhao, Y.; Wang, H.; Zhang, P.; Sun, C.; Wang, X.; Wang, X.; Yang, R.; Wang, C.; Zhou, L. Rapid Multiplex Detection of 10 Foodborne Pathogens with an Up-Converting Phosphor Technology-Based 10-Channel Lateral Flow Assay. *Scientific Reports* **2016**, *6* (1), 21342.

(4) Kolosova, A. Y.; De Saeger, S.; Sibanda, L.; Verheijen, R.; Van Peteghem, C. Development of a Colloidal Gold-Based Lateral-Flow Immunoassay for the Rapid Simultaneous Detection of Zearalenone and Deoxynivalenol. *Analytical and Bioanalytical Chemistry* **2007**, *389* (7–8), 2103–2107.

(5) Posthuma-Trumpie, G. A.; Korf, J.; van Amerongen, A. Lateral Flow (Immuno)Assay: Its Strengths, Weaknesses, Opportunities and Threats. A Literature Survey. *Analytical and Bioanalytical Chemistry* **2009**, *393* (2), 569–582.

(6) Sharma, S. K.; Eblen, B. S.; Bull, R. L.; Burr, D. H.; Whiting, R. C. Evaluation of Lateral-Flow Clostridium Botulinum Neurotoxin Detection Kits for Food Analysis. *Applied and Environmental Microbiology* **2005**, *71* (7), 3935–3941.

(7) Wang, D.-B.; Tian, B.; Zhang, Z.-P.; Wang, X.-Y.; Fleming, J.; Bi, L.-J.; Yang, R.-F.; Zhang, X.-E. Detection of Bacillus Anthracis Spores by Super-Paramagnetic Lateral-Flow Immunoassays Based on "Road Closure." *Biosensors and Bioelectronics* **2015**, *67*, 608–614.

(8) Liu, D.; Huang, Y.; Wang, S.; Liu, K.; Chen, M.; Xiong, Y.; Yang, W.; Lai, W. A Modified Lateral Flow Immunoassay for the Detection of Trace Aflatoxin M1 Based on Immunomagnetic Nanobeads with Different Antibody Concentrations. *Food Control* **2015**, *51*, 218–224.

(9) Xia, S.; Yu, Z.; Liu, D.; Xu, C.; Lai, W. Developing a Novel

Immunochromatographic Test Strip with Gold Magnetic Bifunctional Nanobeads (GMBN) for Efficient Detection of Salmonella Choleraesuis in Milk. *Food Control* **2016**, *59*, 507–512.

(10) Huang, Z.; Peng, J.; Han, J.; Zhang, G.; Huang, Y.; Duan, M.; Liu, D.; Xiong, Y.; Xia, S.; Lai, W. A Novel Method Based on Fluorescent Magnetic Nanobeads for Rapid Detection of Escherichia Coli O157:H7. *Food Chemistry* **2019**, *276*, 333–341.

(11) Liu, J.; Gao, S.; Kang, L.; Ji, B.; Xin, W.; Kang, J.; Li, P.; Gao, J.; Wang, H.; Wang, J.; et al. An Ultrasensitive Gold Nanoparticle-Based Lateral Flow Test for the Detection of Active Botulinum Neurotoxin Type A. *Nanoscale Research Letters* **2017**, *12* (1), 227.

(12) Vos, J. J. Colorimetric and Photometric Properties of a 2 Fundamental Observer. *Color Res. Appl.* **1978**, *3*, 125–128.

(13) Hill, B.; Roger, T.; Vorhagen, F. W. Comparative Analysis of the Quantization of Color Spaces on the Basis of the CIELAB Color-Difference Formula. *ACM Trans. Graph.* **1997**, *16* (2), 109–154.

(14) Gong, X.; Cai, J.; Zhang, B.; Zhao, Q.; Piao, J.; Peng, W. A Review of Fluorescent Signal-Based Lateral Flow Immunochromatographic Strips. *Journal of Materials Chemistry B* **2017**, *5*, 5079–5091.

(15) Mizanur, R. M.; Stafford, R. G.; Ahmed, S. A. Cleavage of SNAP25 and Its Shorter Versions by the Protease Domain of Serotype A Botulinum Neurotoxin. *PLoS ONE* **2014**, *9* (4), e95188.

(16) Wood, S. E.; Sinsinbar, G.; Gudlur, S.; Nallani, M.; Huang, C.-F.; Liedberg, B.; Mrksich, M. A Bottom-Up Proteomic Approach to Identify Substrate Specificity of Outer-Membrane Protease OmpT. *Angewandte Chemie International Edition* **2017**, *56* (52), 16531–16535.

Chapter 6

Sorbents incorporated membrane based clean-up

This chapter focuses on development of the state of the art dipstick assay capable of removing the matrix interferents while delivering the specific and sensitive detection. The potential of sorbents (graphitized carbon black) incorporated dipstick was evaluated for retention of matrix interferents. Here, the sample matrix containing substrate flows vertically through the GCB deposited clean-up sample pad of the dipstick. The matrix interferents are captured on the sample pad, while the substrate continues to flow through the conjugation pad and subsequently is captured on the test line producing a pinkish band, which was not visible in the absence of GCB incorporated sample. The dipstick assay revealed a LOD of 0.1 nM (5 ng/mL) of BoNT/A LC, both in buffer and carrot juice, within 20 min. The reported membrane-based clean-up integrated dipstick assay has a potential to be applied across wide range of food matrices with the proper choice of sorbents, and in that way exclude tedious sample pre-treatment protocols.

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6.1 Introduction

Delivering of highly sensitive and specific dipstick/lateral flow assay for the on-site food testing is a challenging task, mostly due to the complexity of sample matrices that affect the overall assay performance. Therefore, removing the interferents from the sample matrix would significantly improve the assay response. Food samples typically undergo pre-treatment processes where the most commonly adopted techniques in food analysis are liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid phase micro-extraction, and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique[1,2]. Most of these methods require laboratory facilities and a range of reagents and tools for removal of interferents and extraction of analytes of interest prior analysis, which further prolong detection time and limit on-site analysis. Apart from these techniques, treatment with sorbent materials (zirconium dioxide-based sorbents, diatomaceous earth, aluminia, florisil, PSA, GCB, C-18, Q-sep)[3–7] has been reported to be very effective in removal of interferences such as pigments, fatty acids, carbohydrates, proteins, etc., from various food matrices. However, sorbents have been utilized in powder forms for treatment of liquid samples followed by laboratory based pre-treatment protocols such as centrifugation or filtration prior further analysis. Efforts have been devoted to utilize magnetic beads[8–11] for facile separation of analytes from the sample matrix, avoiding the centrifugation step and sophisticated instruments. However, the magnetic bead-based approach is performed as an external/additional step followed by washing off the matrix interferents. This process involves several rinsing steps, which may significantly compromise the sensitivity in on-site assaying, especially when performed by the end users. Membrane-based sample pre-treatment[12] have also been attempted by utilizing membranes with different morphology (porous and nonporous), shapes (planar, tubes and sheets), surface areas, physical and chemical properties as clean-up pads in LFAs for extraction and enrichment of analytes[13]. However, they might not be effective in the sample pre-treatment process as membranes with small pore sizes are prone to fouling/clogging if the size of interferent molecules are the same or/and smaller than that of the pores, thereby adversely affecting the flow rate. On the other hand, membranes with wider pore size distribution may result in poor separation performance. Furthermore, adsorption is an irreversible physiochemical

interaction between molecules and the membrane surface, resulting in the formation of molecule layers, which further obstruct the sample flow through the membrane by changing the pore size density.

Food sample pre-treatment protocols are typically performed as additional (external) steps, which account for 70-90% of overall assaying time. Besides, clean-up of food samples of complex nature remains challenging task, and cannot be generalized due to the huge variation in the content and the diversity of the various food matrices. Thus, different agents, sorbents and their combinations need to be optimized for each category of food for on-site analysis using LFA. Herein, I attempt to address this issue by delivering an on-site membrane-based clean-up protocol using a dipstick incorporated with sorbents as active compounds, which play a major role in retention of interferents while improving the assay performance. Graphitized carbon black (GCB) is evaluated as the sorbent material for membrane-based clean-up to yield a sensitive dipstick assay for on-site analysis of toxic substances in food matrices (Fig. 6.1).

Carrot juice is used as the sample matrix for evaluation of GCB-incorporated membrane-based clean-up, while Botulinum Neurotoxin A protease is used as a model target, due to its extreme toxicity (1ng/kg - 1µg/kg)[14]. Currently, most of the LFAs/dipsticks for assaying of BoNT/A protease and other proteases in food matrices are affinity-based[15–18], where the main drawbacks are the inability to differentiate between active and inactive forms of toxins due to the affinity-based approach and the potential false positives due to the matrix interferents. The proposed assay yields responses only for active form of proteases accomplished by employing a peptide substrate that is specifically cleaved only by active BoNT/A, thereby enabling precise detection, unlike the reported affinity-based assays. Additionally, tedious laboratory-based sample pre-treatments are substituted with the proposed sorbent incorporation approach, thereby diminishing the false positives. The proposed dipstick configuration results in a shorter turnaround time for assaying and yields LOD at nanomolar levels. The proposed assay could be customized for assaying of various analytes in different food matrices by utilizing appropriate substrates and sorbents incorporated within the membrane-based clean-up pad for retention of wide range of

interferents. To the best of my knowledge, this is the first demonstration of a sorbent incorporated membrane based clean-up approach for development of a specific and sensitive dipstick/LFA.

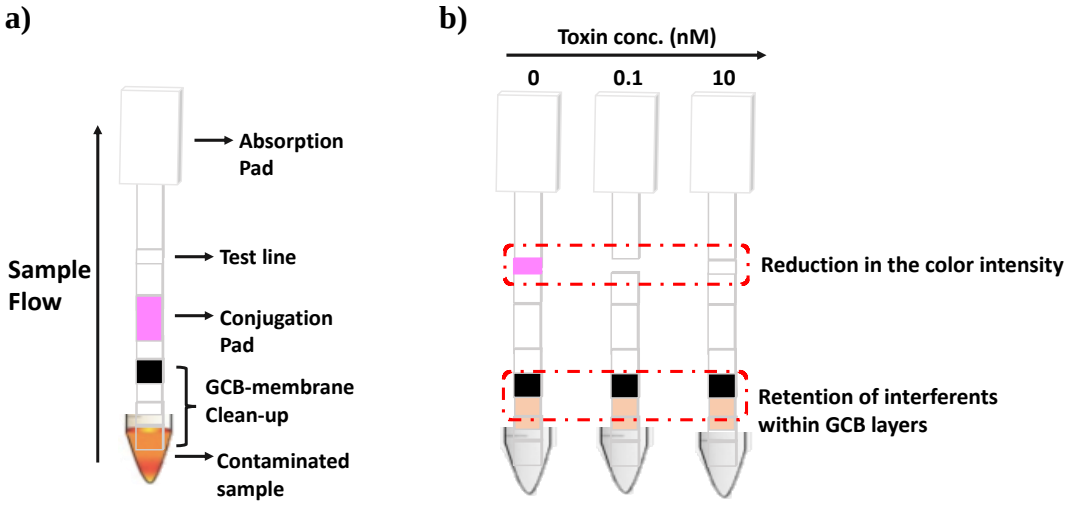


Figure 6.1. Schematic illustration of the GCB-membrane based clean-up within dipstick assay (a). Expected performance of dipstick assay in the contaminated sample matrix (carrot juice) for increasing concentration of the toxin (b).

6.2 Materials and reagents

Botulinum Toxin light chain A (recombinant) was purchased from List Biological Labs, Inc. USA. Botulinum Toxin light chain E (recombinant) was purchased from Creative Diagnostics, USA. Peptide sequence: PEG$_{11}$-SNKTRIDEANQRATKXK-Biotin was purchased from Mimotopes Pte Ltd, Australia. Anti-PEG antibody (Clone 09F02), mouse IgG3 was purchased from BioVision, Inc. (California, US). Europium-streptavidin (SA-Eu) conjugates were purchased from Innova Biosciences (UK). Graphitized carbon black (GCB), bovine serum albumin (BSA), HEPES buffer, and sinapic acid were purchased from Sigma-Aldrich (Singapore). Nitrocellulose (HF135MC100), cellulose and Fusion 5 membranes were obtained from EMD Millipore, US. Carrot juice was obtained from a local market.

Protocols for the solution-based clean-up, membrane-based clean-up, GCB pattering on the membrane, preparation of conjugation pad and fabrication of dipstick test are describe in sections 3.4.1, 3.4.2, 3.4.3, 3.4.4, respectively. Characterizations of extracts after clean-up by MALDI-ToF and HPLC are described in sections 3.5.2.2 and 3.5.5.1, respectively.

6.3 Results and Discussion

6.3.1 Principle of Assay

The test samples are incubated with Cys-PEG$_{11}$-SNKTRIDEANQRATKXK-Biotin, a peptide that contains a specific cleavage site for active forms of BoNT/A LC, prior analysis with the dipstick test. The principle of the proposed assay is based on capturing the sample interferents (pigments, colorants, non-polar interferences) by GCB patterned on the sample pad whilst the sample liquid, containing cleaved and un-cleaved peptides, is driven by capillary forces towards the conjugation pad containing SA-Eu beads (reporter molecule, pinkish color). Here, biotinylated peptides are labelled followed by their migration to the test line via capillary forces. The PEGylated peptides (both cleaved and un-cleaved) binds on the test line modified with antiPEG antibodies, forming pinkish test lines (accumulation of SA-Eu beads; fluorescence detection) that are visible to naked eye. It is to be emphasized that the cleaved peptide fragments do not absorb on the test line as they do not contain the PEG terminal groups for binding to the anti-PEG antibodies on the test line. Therefore, in the absence of BoNT/A LC the dipstick assay will show a maximum color intensity of the test line band. On the other hand, the intensity of test line band decreases with increasing BoNT/A LC concentration. Therefore, the reduction of test line's color intensity is inversely proportional to the BoNT/A LC concentration.

6.3.2 Efficiency of Sorbents in Solution-based Clean-up Process

In this study, carrot juice was used as a sample for evaluating the efficiency of sorbents materials such as GCB, Q-sep (commercially available powder; a mixture of sorbents such as PSA/GCB/$MgSO_4$ and C-18), PSA, and $MgSO_4$ in removing interferents. Firstly,

individual sorbents and their combinations were studied in solution state in the presence of acetonitrile (BoNT/A LC spiked carrot juice) for evaluation of carrot juice clean-up (Fig. 6.2a). After addition of sorbents and subsequent centrifugation step, supernatants were observed visually, where a colorless solution that confirms the removal of the pigment, was obtained by using GCB in combination with $MgSO_4$ (vial 6) and also with Q-sep sorbents (vial 8). Additionally, GCB (vial 2), GCB in combination with PSA (vial 4), and GCB in combination with PSA and $MgSO_4$ (vial 7) showed a potential in retaining the pigments. However, the sorbent PSA was excluded from further studies since it negatively affects/dissolves NC membranes, which would constrain the development of dipstick assay. Finally, PSA (vial 1), $MgSO_4$ (vial 3) and their combination (vial 5) did not yield any removal of pigments as observed visually, thus they were excluded from further studies as well. A good removal of pigments in the vial 6 is observed as compared to the vial 2 due to the ability of $MgSO_4$ in the presence of acetonitrile to absorb water and induce a phase separation (organic/aqueous). In the absent of acetonitrile, GCB and its combination with $MgSO_4$ did not show difference in removal of pigments, as observed visually (Fig. 6.2b). Therefore, in further development of dipstick assay and choice of sorbents only GCB (vial 2) and Q-sep (vial 8) were used. Subsequently, MALDI-ToF characterization of vial 2 and 8 (Fig. 6.3 b and c) were performed to ascertain successful clean-up by a comparison with the MALDI-ToF spectra of carrot juice before clean-up (Fig. 6.3a). Q-sep showed a better clean-up efficacy concluded by the reduction/absence of peaks between *m/*z of 24000 and 52000 as compared to the GCB clean-up. This could be attributed to the fact that Q-Sep contains a mixture of other sorbents including GCB, thereby possessing better capacity of absorbing/removing different compounds from the sample. However, some of matrix interferents remained after clean-up which can be concluded from the presence of peaks at *m/z* of ~33 000, 34 000, 35 000, 43 000 and 50 000. Additionally, the presence of toxin in the sample matrix after clean-up is revealed as a 25 kDa peak, which could not be observed prior the sample clean-up due to the presence of matrix interferents. In order to ensure that sorbents, GCB and Q-sep, will not retain peptides/substrates that are required for generating the test line, while retaining the interferants, the recovery percentage is estimated based on the HPLC data (Table 6.1, Fig. 6.4). It can be inferred from results that

~90% of substrate still remains in extracts after clean-up, further providing the opportunity to work with low substrate concentrations considering the minimal losses.

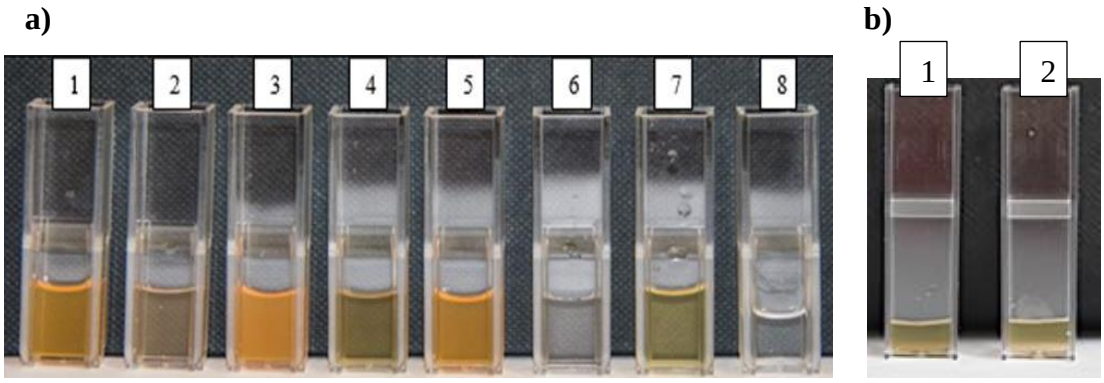


Figure 6.2. Carrot juice treated with 8 different sorbent combinations: 1) PSA, 2) GCB, 3) MgSO4, 4) PSA + GCB, 5) PSA + MgSO4, 6) GCB + MgSO4, 7) PSA +GCB + MgSO4, and 8) Q-sep, respectively (a). Clean-up of carrot juice with GCB (1) and GCB + $MgSO_4$ (2) in the absence of acetonitrile, respectively (b).

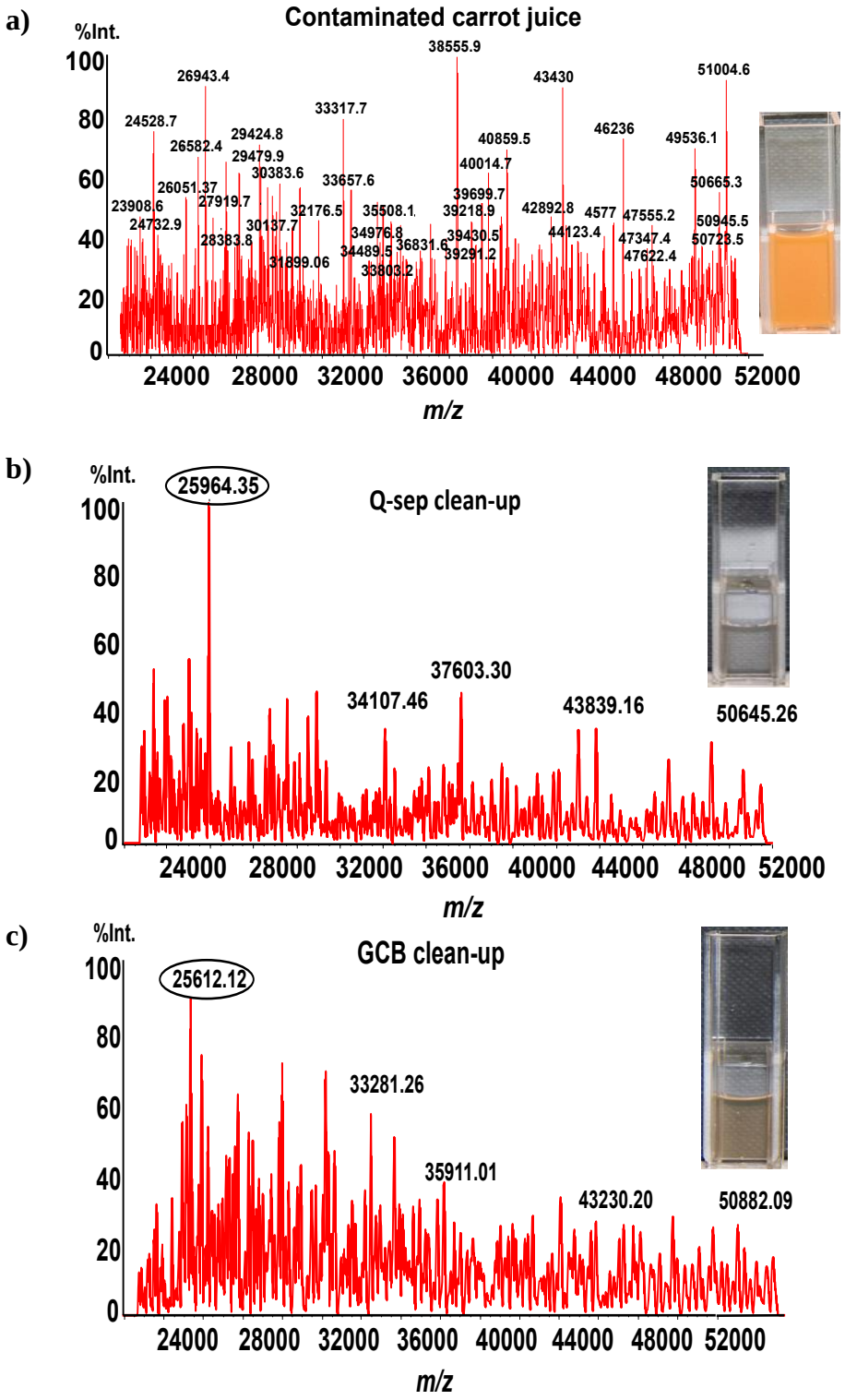


Figure 6.3. MALDI ToF analysis of carrot juice before clean-up (a) and after clean-up with Q-sep (b) and GCB (c).

Table 6.1. Clean-up process performed in solution and recovery calculated from HPLC data.

Extraction of peptide by	Area under the peak	Recovery (%)
GCB	29 481	91.9
Q-sep	29 114	90.8
Buffer	32 074	-

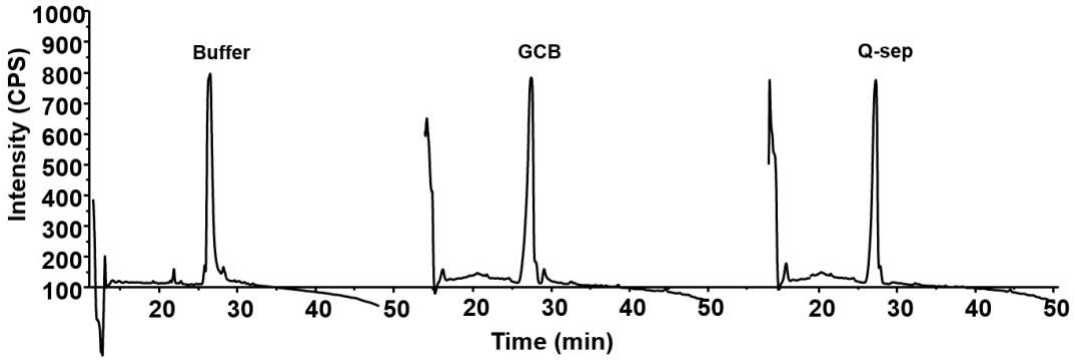


Figure 6.4. HPLC analysis of substrate recovery after clean-up with GCB and Q-sep sorbents, respectively.

6.3.3 Efficiency of GCB-incorporated Membrane in Eliminating the Matrix Effects

Subsequently, GCB and Q-sep sorbents were examined in membrane-based clean-up. Here, the deposition of GCB by dispenser was feasible due to its solubility in 0.1% SDS solution, unlike Q-sep sorbent, in which their insoluble composition restrains deposition on the membrane by dispenser. Therefore, in exploring the membrane based clean-up, GCB was used as the chosen sorbent. GCB was patterned on the Fusion 5 membranes in a zebra-configuration and subsequently evaluated as a sorbent for removal of pigments from carrot juice. It can be noticed from Fig. 6.5a that pigments from carrot juice are more efficiently retained with increasing the number (strip 3, Fig.6.5a) and the width (3 mm) of GCB layers (strip 4, Fig. 6.5a) compared to the control strip (strip 1, without clean-up pad) and the strip 2 with one GCB layer. However, in order to maximize the retention of pigments, GCB was

patterned on the other side of Fusion 5 membrane (strip 5, Fig. 6.5a). As observed visually, less pigments are retained at an overlap of conjugation pad-NC membrane as well as on the NC membrane itself (less orangish) compared to other strips. Subsequently, the influence of the GCB incorporated membrane-based clean-up on the dipstick signal response was evaluated with carrot juice (Fig. 6.5b, strip number 1 (1') and 2 (2')) and 10x diluted carrot juice (Fig. 6.5b, strip number 3 (3')). Without clean-up (strip 1, Fig. 6.5b), pigments bind nonspecifically to anti-PEG antibodies immobilized on the test line interfering with the assay response (strip 1', Fig. 6.5b). However, integrating the sample clean-up pad within dipstick assay helped in preventing the non-specific binding to the test line (Fig. 6.5b, strip 2 and 3), and subsequently yielded a better visual response both in carrot juice and 10x diluted carrot juice (Fig. 6.5b, strip 2' and 3').

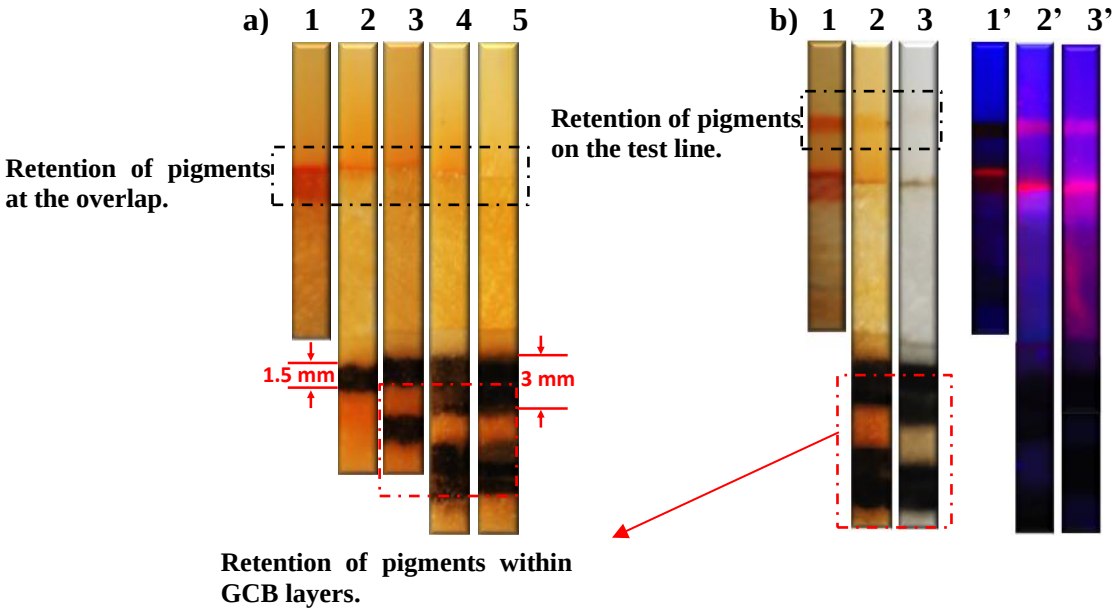


Figure 6.5. Efficiency of GCB incorporated Fusion 5 membrane in retaining the pigments from carrot juice (strips: 1- no GCB; 2-one GCB line; 3- two GCB lines; 4- two GCB lines with incrased width; and 5- GCB patterned on both sides of Fusion 5 membrane) (a). The fluorescence and visual responses of test lines without clean- up (strip 1 and 1') and with clean-up (strips: 2,2'- non diluted carrot juice; 3, 3'- 10x diluted carrot juice) (b).

6.3.4 Optimization of Releasing of Eu-beads From the Conjugation Pad

The visibility of test lines depends on types of reporter molecules as well as concentration used for generating the visual response. However, the release of reporter molecules from the conjugation pad affects the response signal as well. In order to obtain the best release of SA-Eu beads from the conjugation pad (glass fiber; GF), two approaches were adopted. First approach is based on the blocking of GF with different concentrations of BSA as shown in Fig. 6.6. A very poor release of SA-Eu beads was noticed when the GF was not blocked with BSA, compared to the GF before the release of SA-Eu beads. It can be noticed, that a better release was achieved by blocking GF with BSA (1%, 2.5%) prior the deposition of reporter molecules. In order to maximize discharge of SA-Eu beads from GF, the second approach was performed. Here, the plasma treatment was used to increase the surface hydrophilicity of GF and subsequently to facilitate the better flow of liquid sample as well as the release of SA-Eu beads. Thus, plasma treated GF (0% BSA) showed better release of SA-Eu beads compared to GF without plasma treatment (0% BSA). However, the maximum discharge of SA-Eu beads was accomplished by the synergistic effect of plasma treatment and BSA blockage (2.5%), observed visually and confirmed by SEM analysis. As illustrated in Fig. 6.7, almost 100% of SA-Eu beads (observed as whitish spots on fibers) are released (b) compared to the control (a). Thus, this optimized condition is further used in subsequent experiments.

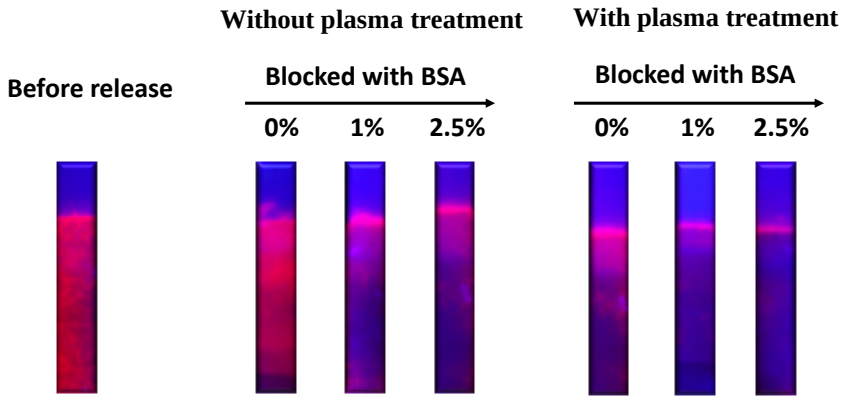


Figure 6.6. Optimization of SA-Eu beads release from the conjugation pad using the BSA and plasma treatment.

a) GF before SA-Eu beads release **b) GF after SA-Eu beads release**

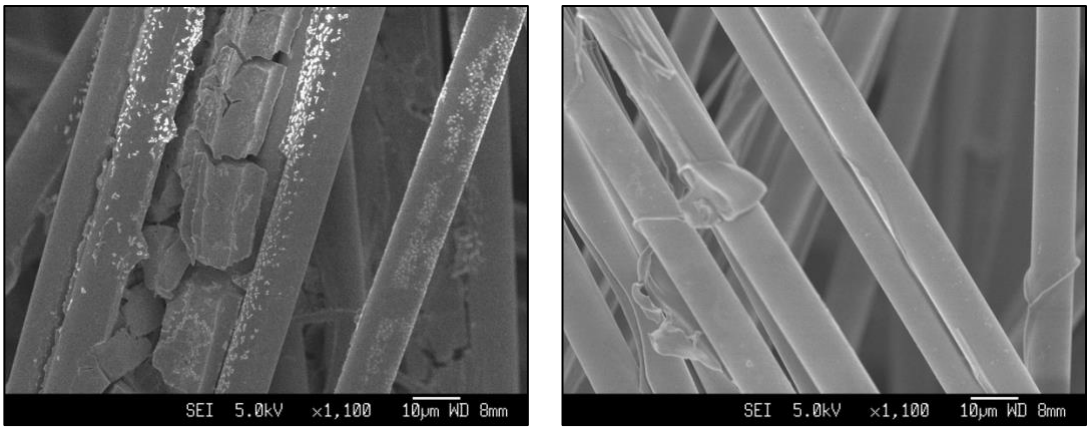


Figure 6.7. SEM images of GF containing SA-Eu beads before (a) and after release (b).

6.3.5 Optimization of Assay Performance

To optimize the desired colour intensity of the test line for the visual inspection, different concentrations of substrate (10, 100, and 1000 nM) in combination with different concentrations of antiPEG antibodies (0.25, 0.5, and 1 mg/mL) at fixed SA-Eu beads concertation (0.00125%) were examined, Fig. 6.8a. As illustrated in Fig. 6.8a, the distinct pinkish test bands (accumulation of SA-Eu beads) are obtained for all substrate concentrations at antiPEG antibody concentration of 1 mg/mL. On the other hand, the test line intensity decreases by reducing the antibody concentration (0.5 mg/mL) for the highest substrate concentration used (1000 nM), while the test lines are not generated or less visible for the rest of examined combinations. However, reducing the substrate concentration can boost up the sensitivity and reduce the digestion time considering that higher concentration may negatively affect hydrolysis further concealing the toxin presence. Thus, a substrate concertation of 10 nM that generated a sufficient visible band (1 mg/mL of antibody) was utilized for assay development (Fig. 6.8a, red dashed box). Further, the assay response was optimized based on the BoNT/A LC ability to cleave the substrate as a function of temperature (Fig. 6.8b). Here, the digestion was performed at room temperature (RT) and 37$_o$C for 5 h, and subsequently responses were evaluated by dipstick assay. As inferred

from the obtained results, BoNT/A LC shows a better activity at 37 ₀C, where ~73% and 92% of presented substrates are cleaved by 5 nM and 10 nM, with the pinkish test lines almost disappeared compared to the digestion at RT. For the digestion at RT, pinkish test lines are still visible but less intense (~34% and ~50% of the cleaved substrates for 5 nM and 10 nM of BoNT/A LC, respectively) compared to the control test line (in the absence of BoNT/A LC, 0 nM).

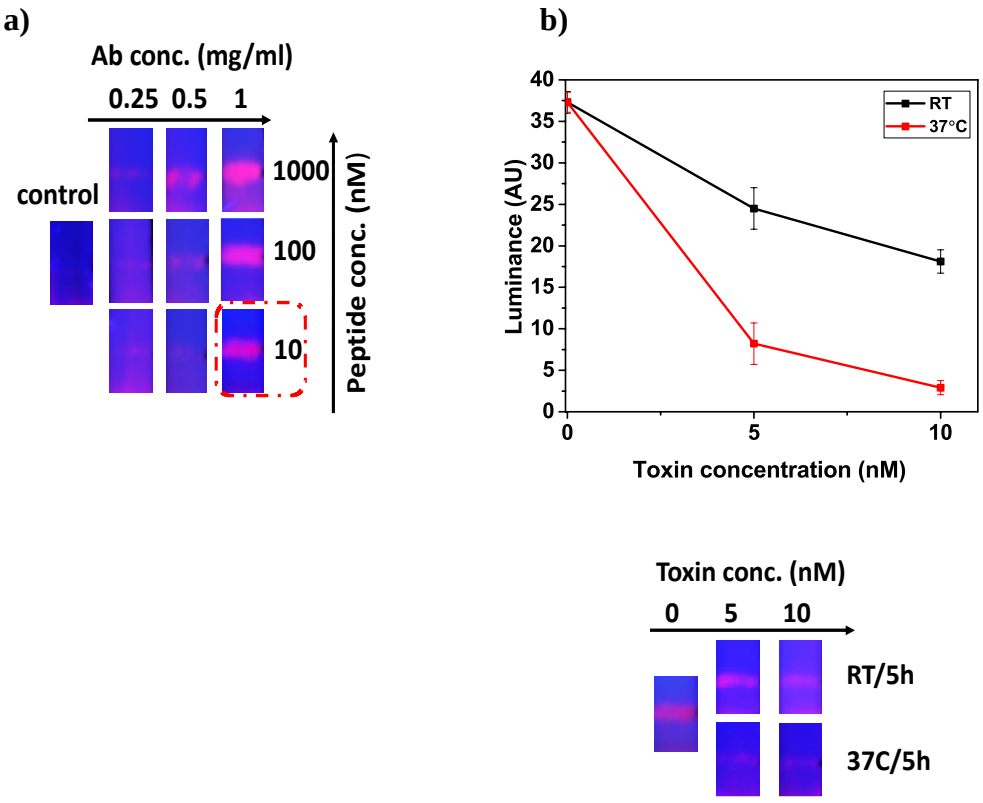


Figure 6.8. Optimization of peptide and antibody concentration for the dipstick test (a). Optimization of assay response based on the BoNT/A LC digestion ability as a function of temperature (RT and 37₀C) evaluated by luminance values and visual response (b).

6.3.6 Concentration Dependent Responses in Buffer and Carrot juice

The performance of optimized GCB incorporated dipstick assay was explored in the buffer and carrot juice spiked with different BoNT/A LC concentrations (0.1, 0.5, 1, 5, 10 nM).

Upon 5 h digestion, the dipstick is immersed in the sample for 20 min, where the change in the colour intensity of test lines is observed (Fig. 6.9a, inset). The best assay response with fully disappearing test lines is noticed for the BoNT/A LC test concentrations of 10 nM and 5 nM compared to the intensity of control line (0 nM), both in buffer and carrot juice. For all the BoNT/A LC concentrations tested (1, 0.5, and 0.1 nM), the reduction in colour intensity of test lines can be easily observed compared to the control line. In order to quantitatively evaluate the concentration dependent responses and ascertain the differences in the colour intensity of test lines, RGB analysis was performed using ImageJ software. Luminance values were calculated to estimate the percentage of cleavage, represented by $1-(L/L_c)$, where L and L_c are the luminance mean values for test samples and the control sample, respectively (Fig. 6.9a). Luminance results reveal better digestion in buffer, where approximately 78%, 65%, 58%, 55% and 33% of substrate is digested by 10, 5, 1, 0.5 and 0.1 nM, respectively, as compared to the digestion in carrot juice (73%, 63%, 57%, 35% and 11%, respectively). The limit of detection (LOD) in samples (buffer and carrot juice) is estimated to be 0.1 nM (5 ng/mL), based on 3σ approach, where σ is the standard deviation of the control (blacked dashed line, Fig. 6.9a). Furthermore, to confirm that colour differences between test lines and control line are visually distinguishable by human eye, ΔE values (typically ranges from 0 to 100) were calculated, which represent a metric for understanding how the human eye perceives colour difference. Figure 6.9b illustrates that the ΔE values ranges from 20 to 62 in the buffer, and from 12.6 to 43.6 in carrot juice for concentrations of 0.1 nM to 10 nM, respectively. Since the established threshold value of ΔE for naked eye differentiation of colours is 2, the obtained range of ΔE values suggests that colour intensities of the test lines can be differentiated visually by the naked eye.

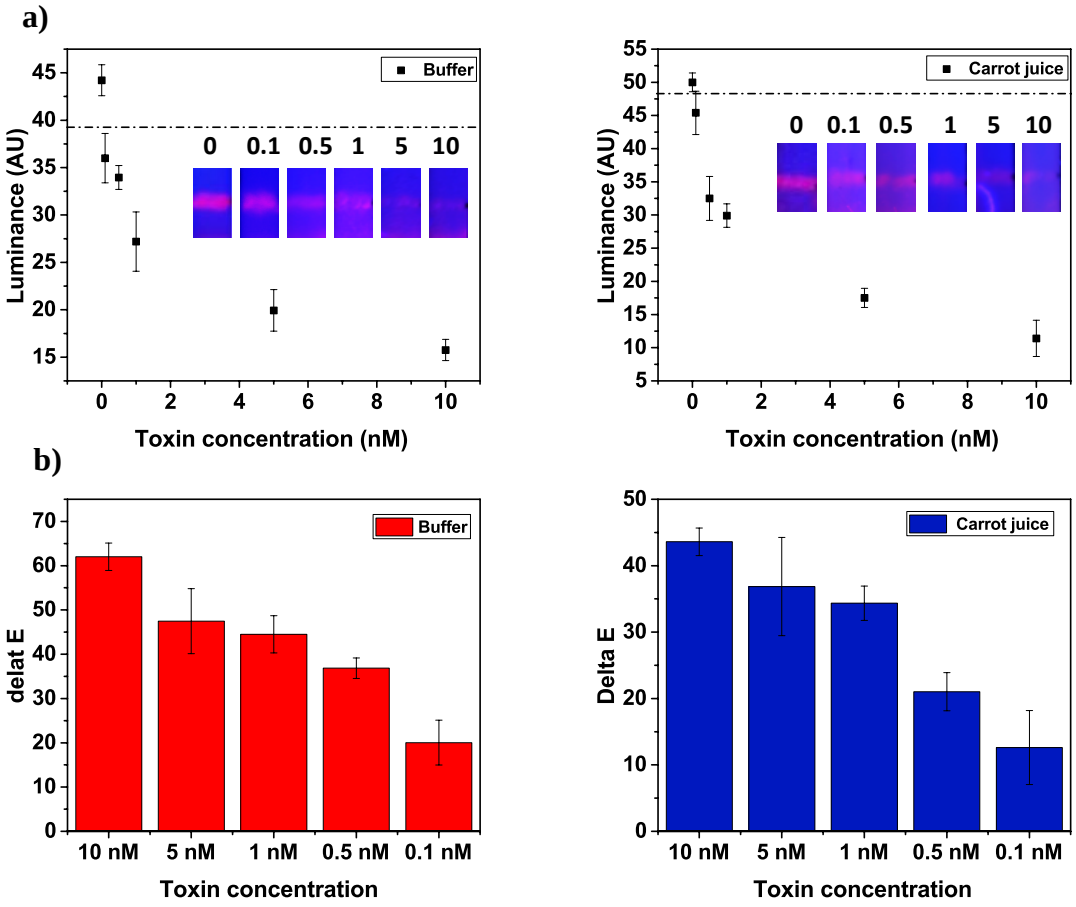


Figure 6.9. Assay performance in buffer and carrot juice characterized by luminance values and visually-insets (a), and by delta E values (b), respectively.

6.3.7 Specificity test

To ensure that developed dipstick test and peptide design are specific to BoNT/A, BoNT type E was also tested. The choice of type E was based on the fact that the SNAP-25 can be cleaved by both types A and E in different positions, Q_{197}-R_{198} and R_{180}-I_{181}, respectively. In this study the C-terminus of SNAP25 protein (187-203) was used, designed in that way to exclude the cleavage site for the type E. Anyway, the peptide sequence (Cys-PEG_{11}-SNKTRIDEANQRATKXK-Biotin) has a region containing a similar scissile bond (R-I) at positions 191-192, herein, examined to be cleaved by BoNT/E LC. As speculated

elsewhere the type E is capable of cleaving only $_{178}$IDRIME$_{183}$ due to the presence of Met$_{182}$ and Asp$_{179}$ amino acids, which helps in docking and aligning the substrate as well as stabilizing the substrate-BoNT/E complex[19]. In agreement to this, BoNT/E LC (at different concentrations) was unable to cleave the substrate since there is no reduction in luminance values as shown in Fig. 6.10 (threshold is presented with the black dashed line), confirming the specificity of the proposed assay for BoNT/A.

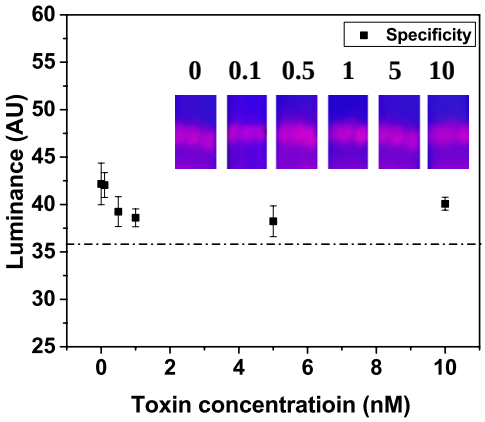


Figure 6.10. Assay performance in buffer spiked with BoNT/E LC.

6.4 Conclusion

This study demonstrates a facile approach for on-site food analysis by merging state-of-the-art sorbent incorporated membrane-based sample pre-treatment and detection of a model target, BoNT/A protease, within a dipstick assay. The effect of GCB incorporated membrane on retention of interferents from carrot juice and dipstick assay responses were optimized and studied. Assaying of carrot juice without clean-up was not possible since the sample matrix interfered with the responses. However, GCB layers incorporated membrane retained the interfering compounds (e.g. orangish pigments observed visually), while protecting the test line from the non-specific binding and revealing the pinkish test line observable by the naked eye. The dipstick assay was tested against BoNT/A LC protease, which yielded an LOD of 0.1 nM (5 ng/mL) both in buffer and carrot juice, thereby enabling detection of target analytes at analytically relevant concentration levels. The proposed dipstick assay with integrated membrane-based clean-up represents an attractive alternative to the existing LFA/dipstick for the facile on-site detection in complex

food matrices with no requirements for tedious and separate sample pre-treatment protocols. Additionally, this proposed methodology can be generalized and applied for assaying a wide range of food matrices with appropriate combinations of sorbents, and can be used to detect other relevant analytes.

References

(1) Bueno, D.; Istamboulie, G.; Muñoz, R.; Marty, J. L. Determination of Mycotoxins in Food: A Review of Bioanalytical to Analytical Methods. *Applied Spectroscopy Reviews* **2015**, *50* (9), 728–774.

(2) González-Curbelo, M.; Socas-Rodríguez, B.; Herrera-Herrera, A. V.; González-Sálamo, J.; Hernández-Borges, J.; Rodríguez-Delgado, M. Evolution and Applications of the QuEChERS Method. *TrAC - Trends in Analytical Chemistry* **2015**, *71*, 169–185.

(3) Rahmani, A.; Jinap, S.; Soleimany, F. Qualitative and Quantitative Analysis of Mycotoxins. *Comprehensive Reviews in Food Science and Food Safety* **2009**, *8* (3), 202–251.

(4) Rejczak, T.; Tuzimski, T. Method Development for Sulfonylurea Herbicides Analysis in Rapeseed Oil Samples by HPLC–DAD: Comparison of Zirconium-Based Sorbents and EMR-Lipid for Clean-up of QuEChERS Extract. *Food Analytical Methods* **2017**, *10* (11), 3666–3679.

(5) Ötles, S.; Kartal, C. Solid-Phase Extraction (SPE): Principles and Applications in Food Samples. *Acta Scientiarum Polonorum Technologia Alimentaria* **2016**, *15* (1), 5–15.

(6) Cabrera, L. da C.; Caldas, S. S.; Prestes, O. D.; Primel, E. G.; Zanella, R. Evaluation of Alternative Sorbents for Dispersive Solid-Phase Extraction Clean-up in the QuEChERS Method for the Determination of Pesticide Residues in Rice by Liquid Chromatography with Tandem Mass Spectrometry. *Journal of Separation Science* **2016**, *39* (10), 1945–1954.

(7) P. Parrilla Vazquez, C. Ferrer, M.J. Martínez Bueno, A. R. F.-A. Pesticide Residues in Spices and Herbs: Sample Preparation Methods and Determination by

Chromatographic Techniques. *TrAC Trends in Analytical Chemistry* **2019**, *115*, 13–22.

(8) Klisara, N.; Peters, J.; Haasnoot, W.; Nielen, M. W. F.; Palaniappan, A.; Liedberg, B. Functional Fluorescence Assay of Botulinum Neurotoxin A in Complex Matrices Using Magnetic Beads. *Sensors and Actuators B: Chemical* **2019**, *281*, 912–919.

(9) Peters, J.; Cardall, A.; Haasnoot, W.; Nielen, M. W. F. 6-Plex Microsphere Immunoassay with Imaging Planar Array Detection for Mycotoxins in Barley †. **2014**.

(10) Čadková, M.; Metelka, R.; Holubová, L.; Horák, D.; Dvořáková, V.; Bílková, Z.; Korecká, L. Magnetic Beads-Based Electrochemical Immunosensor for Monitoring Allergenic Food Proteins. *Analytical Biochemistry* **2015**, *484*, 4–8.

(11) Lu, C.; Tang, Z.; Liu, C.; Kang, L.; Sun, F. Magnetic-Nanobead-Based Competitive Enzyme-Linked Aptamer Assay for the Analysis of Oxytetracycline in Food. *Analytical and Bioanalytical Chemistry* **2015**, *407* (14), 4155–4163.

(12) Jönsson, J. Å.; Mathiasson, L. Membrane-Based Techniques for Sample Enrichment. *Journal of Chromatography A* **2000**, *902* (1), 205–225.

(13) Gorecki, T.; Namiesnik, J. Passive Sampling. *TrAC Trends in Analytical Chemistry* **2002**, *21* (4), 276–291.

(14) Shukla, H. D.; Sharma, S. K. Clostridium Botulinum : A Bug with Beauty and Weapon. *Critical Reviews in Microbiology* **2005**, *31* (1), 11–18.

(15) Gessler, F.; Pagel-Wieder, S.; Avondet, M.-A.; Böhnel, H. Evaluation of Lateral Flow Assays for the Detection of Botulinum Neurotoxin Type A and Their Application in Laboratory Diagnosis of Botulism. *Diagnostic Microbiology and Infectious Disease* **2007**, *57* (3), 243–249.

(16) Sharma, S. K.; Eblen, B. S.; Bull, R. L.; Burr, D. H.; Whiting, R. C.; Donald, H.; Whiting, R. C.; Burr, D. H. Evaluation of Lateral-Flow Clostridium Botulinum Neurotoxin Detection Kits for Food Analysis. *Applied and environmental microbiology* **2005**, *71* (7), 3935–3941.

(17) Tripathi, P.; Upadhyay, N.; Nara, S. Recent Advancements in Lateral Flow Immunoassays: A Journey for Toxin Detection in Food. *Critical Reviews in Food Science and Nutrition* **2018**, *58* (10), 1715–1734.

(18) Orlov, A. V.; Znoyko, S. L.; Cherkasov, V. R.; Nikitin, M. P.; Nikitin, P. I. Multiplex Biosensing Based on Highly Sensitive Magnetic Nanolabel Quantification: Rapid Detection of Botulinum Neurotoxins A, B, and E in Liquids. *Analytical Chemistry* **2016**, *88* (21), 10419–10426.

(19) Agarwal, R.; Swaminathan, S. SNAP-25 Substrate Peptide (Residues 180-183) Binds to but Bypasses Cleavage by Catalytically Active Clostridium Botulinum Neurotoxin E. *Journal of Biological Chemistry* **2008**, *283* (38), 25944–25951.

Chapter 7

Membrane-based clean-up for solid and canned food matrices

This chapter describes preliminary results for testing of solid and canned complex food matrices with the developed dipstick assay and sorbent incorporated membrane-based clean-up (Chapter 6). In this study sausage and canned beans were examined as potential source of food-borne botulism. Besides GCB sorbent used for the retention of matrix interferents, Q-sep sorbent is utilized as well within dipstick assay. Based on the assay performance and preliminary results, the dipstick assay with developed clean-up GCB pad and Q-sep bulb/tip has a potential to be applied for testing of solid and canned food matrices.

7.1 Introduction

As discussed in chapter 6, the main limitations of LFA/dipstick assays are the poor response and overall performance due to the various matrix interferents[1–3]. In this chapter, the emphasis is placed on testing the solid and canned food matrices with the developed dipstick test in chapter 6. However, different food matrices contain different compounds that could interfere in different ways with the assay performance (e.g. response, flow of the liquid sample, binding to recognition molecules/reporter molecules/analyte, etc.), thus requiring different sorbents for their removal. Therefore, in this study, besides the GCB sorbent, Q-sep sorbent is examined as well.

7.2 Materials and reagents

Botulinum Toxin light chain A (recombinant) was purchased from List Biological Labs, Inc. USA. Peptide sequence: PEG$_{11}$-SNKTRIDEANQRATKXK-Biotin was purchased from Mimotopes Pte Ltd, Australia. Anti-PEG antibody (Clone 09F02), mouse IgG3 was purchased from BioVision, Inc. (California, US). Europium-streptavidin (SA-Eu) conjugates were purchased from Innova Biosciences (UK). Graphitized carbon black (GCB), bovine serum albumin (BSA), and HEPES buffer were purchased from Sigma-Aldrich (Singapore). Q-sep QuEChERS tubes for extraction clean-up were purchased from RESTEK. Nitrocellulose (HF135MC100), cellulose and Fusion 5 membranes were obtained from EMD Millipore, US. Carrot juice, sausage and canned beans were obtained from a local supermarket.

7.3 Results and discussion

7.3.1 Performance of dipstick assay with canned and solid food

In this study, the performance of the GCB clean-up sample pad integrated within the dipstick assay was examined with the solution from canned beans (1), canned beans (2) and sausage (3), as shown in Fig. 7.1. For assaying with sausage, two different sample spiking strategies were performed. In the first spiking, a surface of sausage (50 mg) was

contaminated with different BoNT/A LC concentrations (0, 10, 100, 500, 1000, and 2000 nM) followed by extraction with the working buffer (100 µL; HEPES + 0.1% BSA) for 30 min with the constant shaking, and subsequently the substrate was added to the supernatant for digestion (5h/37ºC). In the second spiking, BoNT/A LC was injected in the sausage followed by extraction and digestion steps in the same way as described in the first spiking. Upon digestion, the samples were evaluated with the dipstick assay. The poor digestion of substrate in the sausage sample with the surface contamination and injection (Fig. 7.2a) is most probably due to the poor extraction because of binding of the toxin to the sausage solid matrix and other interferents as well. Therefore, concentration dependent responses are not obtained for the lower BoNT/A LC concentrations (10, 100, 500 nM), whereas the highest BoNT/A LC concentrations of 1000 nM and 2000 nM can be detected as suggested by luminance and ΔE values. In order to evaluate how different extraction protocols affect the assay performance and the cleavage of substrate, a study involving the supernatant was performed. Here, the sausage was cut in small pieces and that were subsequently soaked in the working buffer for 30 min under constant shaking (allowing the extraction of matrix interferents in the surrounding solution). Spiking the supernatant with different BoNT/A LC concentrations (final concentrations of 0.1, 1, 10 and 100 nM) was followed by digestion. It can be observed from luminance values and visually (Fig. 7.2c, inset) that concentration dependent responses are obtained suggesting a good digestion of substrate by BoNT/A LC, further supporting the assumption that the poor digestion in sausage samples mentioned above (Fig. 7.2 a,b) was due to BoNT/A LC binding to the sausage solid matrix. On the other hand, it can be observed visually as well as from luminance values (43 and 48 for control lines of sausage samples with surface contamination and injection, respectively; Fig.7.2 a and b) that control test lines lacking the color intensity compared to the control line obtained in the supernatant study (luminance values of 68; Fig. 2c). This might be due to the loss of substrate that could interact with matrix interferents and subsequently retained within GCB layers. Therefore, further optimization of the sample extraction are required for the proper assay performance. For instance, one possible solution could involve addition of surfactants in working/extraction buffer to minimize the interaction with the sample matrix, extension of extraction time or increasing the substrate concentrations in order to obtain better visible test lines.

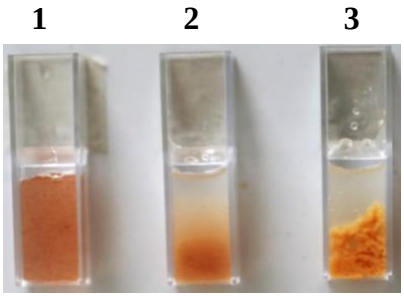
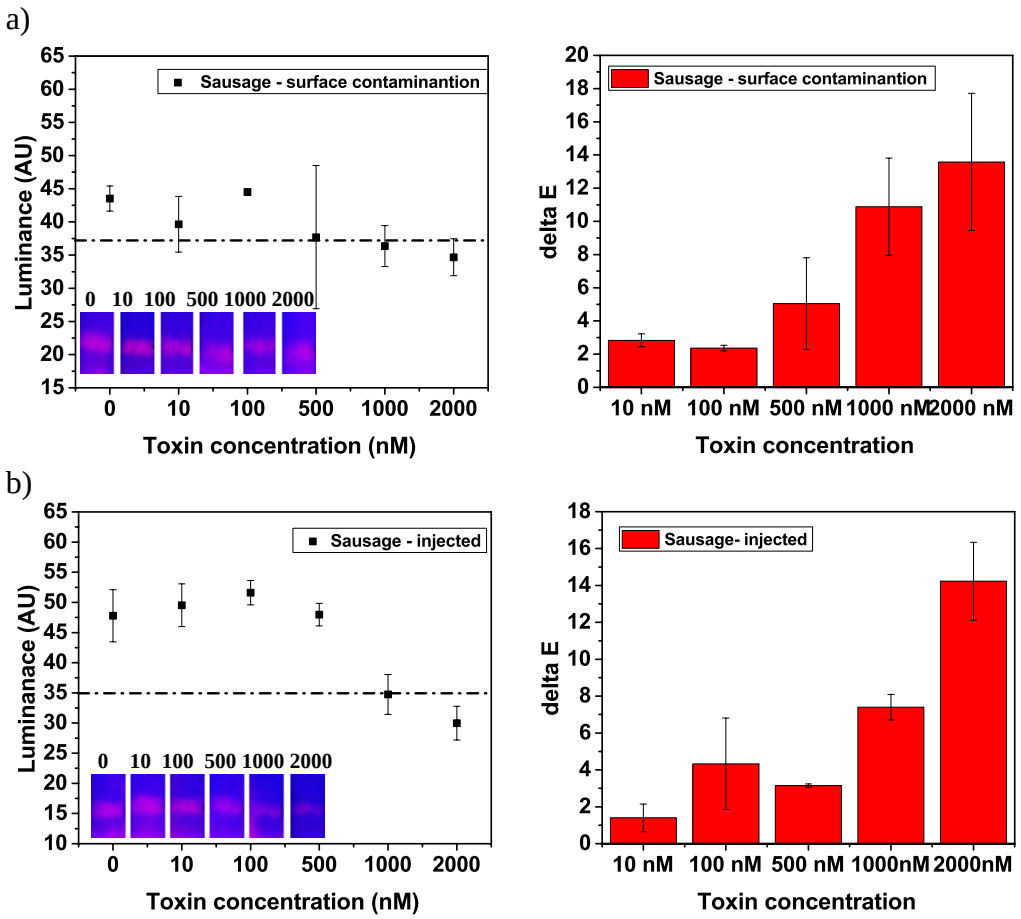


Figure 7.1. Food samples used in the assay testing: liquid of canned beans, mashed canned beans (2), and sausage (3).



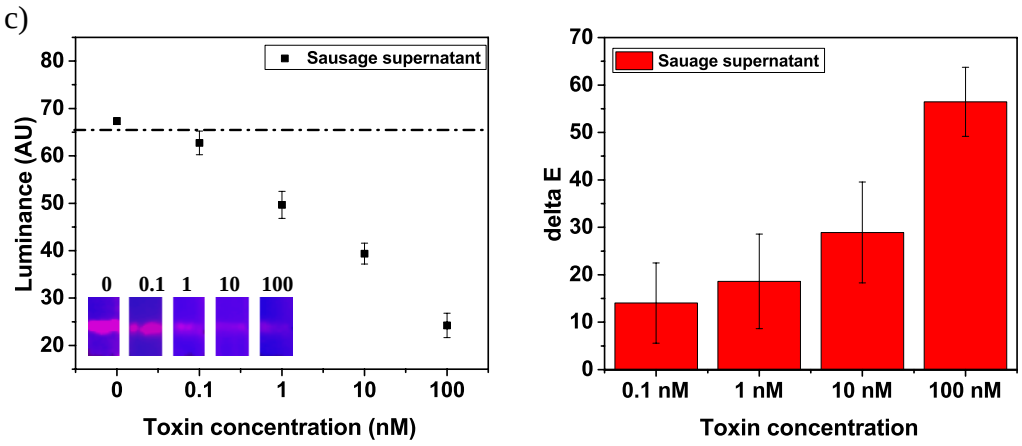


Figure 7.2. Dipstick assay performance in sausage samples with surface contamination (a), injection (b), and supernatant contamination (c) characterized by luminance and ΔE values.

In the case of assaying with canned beans, two samples were prepared. First sample was prepared by meshing a bean (20 mg) into paste followed by addition of working buffer (100 µL; vortexing) and by spiking of BoNT/A LC (0, 0.1, 1, 10, 100 nM). The supernatant was further incubated with substrate for digestion. Here, the concentration dependant responses are obtained as depicted in Fig. 7.3 (confirmed by luminance and ΔE values), further revealing that the matrix interferents from beans are less reactive towards the toxin enabling the detection without any obstacles.

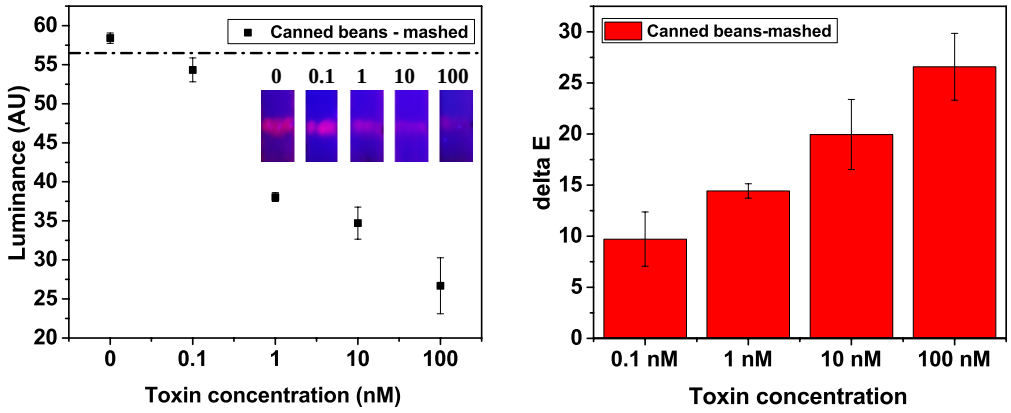


Figure 7.3 Assay performance in canned beans (supernatant) spiked with different concentrations of BoNT/A LC characterized by luminance and ΔE values, respectively.

The second sample was solution from the canned beans (Fig. 7.1). Here, the problems with the dipstick assay performance was associated with the flow of sample liquid due to the viscosity of beans solution. Even after dilution of 10x (Fig. 7.4) the wicking was too slow requiring approx. 55 min to reach the adsorption pad and generate the test bands. Apart from this issue, it can be observed that Eu-beads non-specifically bind along the NC membrane constraining the visual evaluation of results. This is most probably due to the non-efficient retention of matrix interferents by GCB, that move along the membranes and interact with Eu-beads further resulting in non-specific binding. In order to overcome this issue, Q-sep with better capacity in retaining the matrix interference (as discussed in the chapter 6) can be examined as well as optimization of sample dilution.

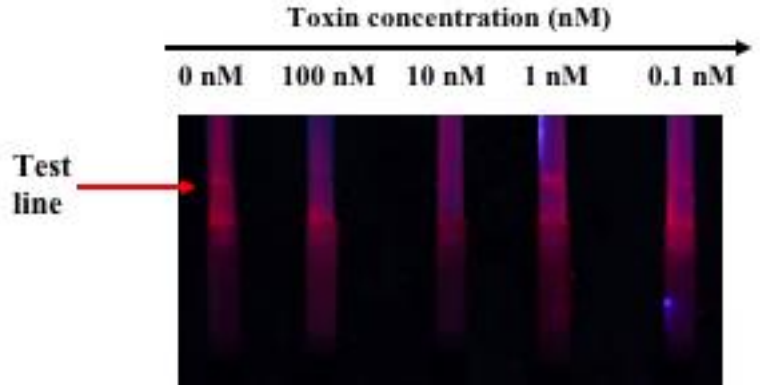


Figure 7.4. Assay performance in 10x diluted solution from canned beans spiked with different concentrations of BoNT/A LC.

7.3.2 Evaluation of dipstick assay performance with Q-sep sorbent

Q-sep sorbent efficiency in retaining the matrix interferents (in the solution) was discussed in the chapter 6. However, printing Q-sep on the Fusion 5 membranes was not possible due to its composition, unlike GCB sorbent. Therefore, in order to use Q-sep within dipstick test, the clean-up bulb/tip was fabricated consisting of plastic holder for Fusion 5 membranes and Q-sep sorbent, as shown in Fig. 7.5. Here, Q-sep sorbent was stacked in between the Fusion 5 membranes to facilitate the sample flow through the clean-up bulb/tip.

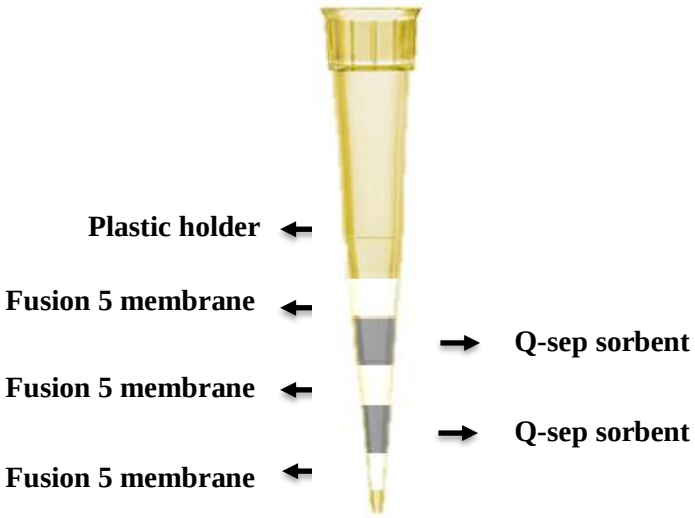


Figure 7.5. Schematic illustration of the plastic bulb/tip for the clean-up consisting of Q-sep sorbent stacked between Fusion 5 membranes and supported by the plastic holder.

Firstly, different amount of Q-sep sorbents was examined for the efficient retention of pigments from carrot juice (Fig.7.6 a), where the best clean-up was accomplished by 20 mg of Q-sep sorbent which is in correspondence with the solution-based clean-up (chapter 6). Subsequently, the assay response was evaluated with carrot juice and 10x diluted carrot juice (Fig. 7.6b), where the test lines are obtained confirming a good retention of pigments. Since, the GCB incorporated dipstick assay demonstrated a good performance in carrot juice (chapter 6), the Q-sep clean-up bulb/tip would be more necessary for other problematic food samples such as canned beans solution. Thus, in order to evaluate if Q-sep sorbent could be a better asset in the clean-up of canned food where the GCB incorporated dipstick assay did not work properly, the beans solution was examined due to the non-specific binding of Eu-beads which constrained the evaluation of responses as shown in Fig. 7.4. In this study, with the Q-sep clean-up bulb/tip, it can be observed from the Fig. 7.7 that there is very less amount of Eu-beads that bind non-specifically to the NC membrane. Therefore, it can be inferred that Q-sep sorbent retaining more matrix interferents compared to the GCB sorbent, further revealing the concentration dependent responses as can be observed visually and confirmed by luminance values (Fig. 7.7).

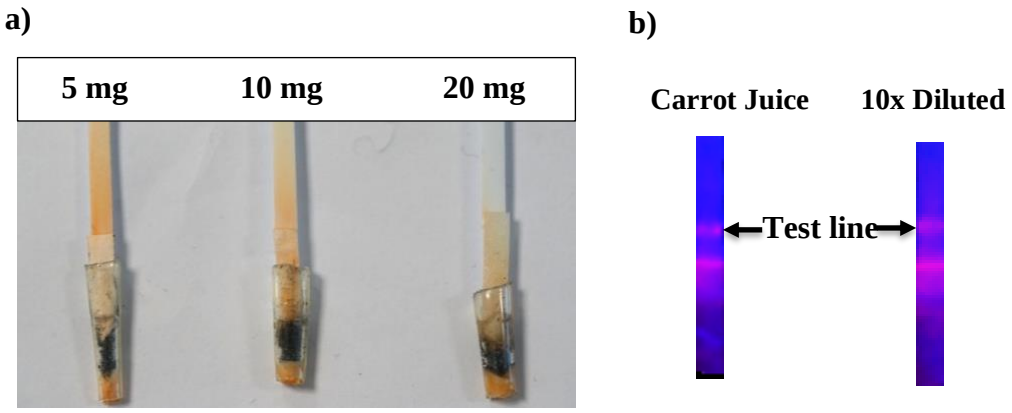


Figure 7.6. Evaluation of clean-up bulb/tip with Q-sep sorbent amount of 5, 10, and 20 mg for the clean-up efficiency of carrot juice (a). Assay response in carrot juice and 10x diluted carrot juice, respectively, using the clean-up bulb/tip.

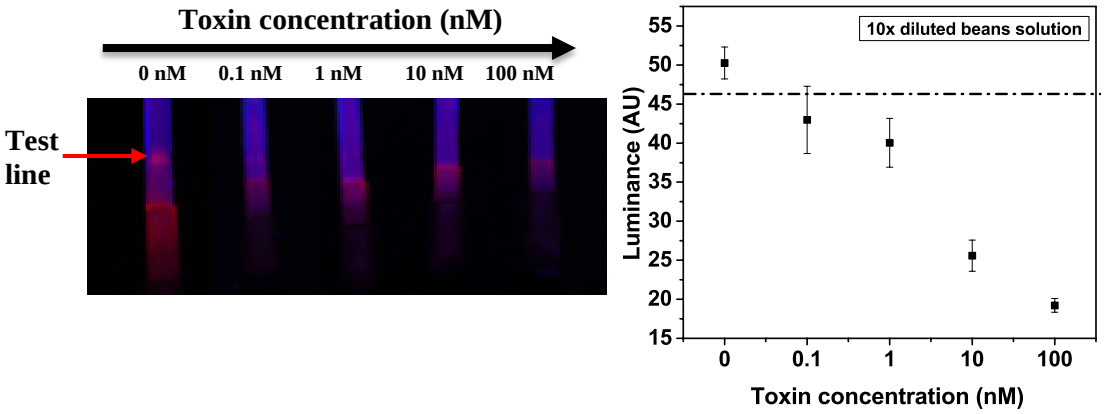


Figure 7.7. Assay performance in 10x diluted solution from canned beans spiked with different concentrations of BoNT/A LC using the clean-up bulb/tip for the matrix interferents retention

7.4 Conclusion

In this chapter, the performance of dipstick assay integrated with GCB clean-up sample pad was examined with solid (sausage) and canned food (beans). The assay performance in sausage was dependent on the sample extraction of the spiked toxin which is confirmed by supernatant study where the concentration dependent responses were obtained.

Additionally, assaying in mashed canned beans was without any obstacles, further revealing the detection limit of 0.1 nM (5 ng/mL). However, the viscosity and content of the beans solution affected the assay response. This is most probably due to the insufficient retention of matrix interferents by GCB layers, which is overcome by fabricating the clean-up bulb/tip with Q-sep sorbent that showed a better capacity in retaining the matrix interferents, thus improving the assay performance. With further improvements in the sample preparation/extraction procedures, the GCB and Q-sep bulb/tip clean-up integrated within dipstick assay possess a potential to be tested with diverse food matrices.

References

(1) Posthuma-Trumpie, G. A.; Korf, J.; van Amerongen, A. Lateral Flow (Immuno)Assay: Its Strengths, Weaknesses, Opportunities and Threats. A Literature Survey. *Analytical and Bioanalytical Chemistry* **2009**, *393* (2), 569–582.

(2) Sajid, M.; Kawde, A.-N.; Daud, M. Designs, Formats and Applications of Lateral Flow Assay: A Literature Review. *Journal of Saudi Chemical Society* **2015**, *19* (6), 689–705.

(3) Carrell, C.; Kava, A.; Nguyen, M.; Menger, R.; Munshi, Z.; Call, Z.; Nussbaum, M.; Henry, C. Beyond the Lateral Flow Assay: A Review of Paper-Based Microfluidics. *Microelectronic Engineering* **2019**, *206*, 45–54.

Chapter 8

Discussion and Future Work

In this chapter, a brief summary of discussed projects is presented, including the major outcomes. Hypotheses raised for this research are clarified based on the results presented in this thesis. At the end of the chapter, the future work/directions to improve the assays performance including synthesis of better substrate, more precise deposition of antibodies, possible multiple detection of various proteases are discussed.

8.1 Research Summary

This thesis work was focused on developing and delivering sensing platforms for on-site food safety monitoring to address the presence of active toxic proteases (in this study BoNT/A) with high sensitivity and specificity in order to provide an early detection and prevention of foodborne related diseases. Food testing capable of overcoming threads related to food contaminations is a challenging task, mostly due to the complexity of sample matrices that affect the overall assay performance. Therefore, in this thesis, approaches to overcome time consuming and labour intensive sample pre-treatments were explored. The sensing strategy of active BoNT/A (the only form related to foodborne diseases) was based on cleaving the specifically designed substrate which ensures a good specificity further enabling more precise detection compared to the conventional antibody-based immunoassays.

In the chapter 4, the peptide (H2N-Cys-PEG11-SNKTRIDEANQ-RATK-Nle-K(Biotin)-COOH) is successfully utilized in a MB-fluorescence based assay. Besides easy manipulation and handling in food matrices, intended to minimize the interferences associated with sample matrices, superparamagnetic beads with a high surface to volume ratio enabled immobilization of a sufficient number of peptides, and provided good sensitivity during operating with less number of beads. The obtained results revealed a LOD of 0.5-1 nM (25 – 50 ng/mL) of active BoNT/A in buffer and carrot juice, which is below the lethal dose (1 μg/kg) supporting the first hypothesis of the thesis. Facile assay protocol, robustness, cost-effectiveness, low sample volume requirements makes the proposed protocol competitive in comparison with conventional immunological assays for detection of proteases in food matrices.

In the chapter 5, the functional fluorescence magnetic bead-based assay in solution (explained in chapter 4) was transferred to the dipstick assay. Assay responses (reduction in the color intensity of test bands) for the BoNT/A presence were validated with the naked eye and quantitatively confirmed with RGB (ΔE) analysis. The sensitivity of proposed approach (2.5 nM; 125 ng/mL) was improved by implementing the simple amplification

step, which further enabled the detection at a concentration level of 1 nM (50 ng/mL). Besides, the amplification step improved the contrast of test lines allowing a better visual perception and easier naked-eye detection. This is the first study reported on the use of magnetic beads in a dipstick format to detect activity of BoNT/A LC in food matrices. The proposed functional dipstick targets only the active form, and thereby enabling a more precise analysis for preventing potential false-positives unlike typical affinity-based LFA assay. Also, it can be potentially used to substitute the gold standard functional-based assay – the mouse bioassay[1]. This sensing approach simplified the MB-based assay from chapter 4 by excluding the use of sophisticated instrument making the dipstick assay more applicable for on-site assaying, which supports the second hypothesis. Furthermore, the proposed dipstick approach does not require trained personnel, long and tedious clean-up protocols, while being facile, robust, cost-effective and with low sample volume requirements.

In the chapter 6, the functional dipstick assay for on-site analysis (chapter 5) was simplified in terms of assay performance, sample clean-up, and the assay sensitivity was improved. Here, GCB incorporated membrane (FUSION 5) for the retention of matrix interferents was fabricated and subsequently integrated within the dipstick assay. Experimental results indicate that GCB layers were efficient in retaining the matrix interferents e.g. pigments, which was observed visually. Moreover, the assay performance was poor without sorbent incorporated membrane-based sample pad, where the test lines were not visible since the sample matrix interfered with the signal response. After successful removal of sample matrix interferent, the dipstick assay revealed an order of magnitude better LOD (0.1 nM; 5 ng/mL) compared to the magnetic bead-based dipstick assay (chapter 4), which supports the third hypothesis. Additionally, the proposed dipstick assay is merging extraction, clean-up and pre-concentration step with sensitive fluorescence detection, thus simplifying the previous protocol by excluding the external conjugation step, the use of magnet and washing steps. The proposed dipstick assay with integrated membrane-based clean-up represents an attractive alternative to the existing dipstick assays for the facile on-site detection in complex food matrices with no requirements for tedious and separate sample pre-treatment protocols.

In the chapter 7, the functional dipstick assay for on-site analysis (developed in the chapter 6) was tested with solid (sausage) and canned food (beans) as a potential source of botulism. Problems with assaying in the sausage samples were due to the poor extraction of BoNT/A LC, which is confirmed with the supernatant study where the concentration dependant responses are revealed. Additionally, the assay performance in beans was without encountering any issues, unlike assaying in beans solution. Here, the assay performance was poor due to the sample viscosity as well as poor retention of matrix interferents by GCB layers. This issue was solved by fabricating the clean-up bulb/tip with Q-sep sorbent (stacked between Fusion 5 membranes) that retain interferents more efficiently. Finally, the developed functional dipstick assay can be potentially used across a wide range of food matrices with a proper choice of sorbents and extraction protocols.

In conclusion, all developed assays possess a good sensitivity meeting the requirements for analytically relevant levels, and excellent specificity for the targeted protease BoNT/A due to the utilization of the specifically designed peptide substrate. The summary of main features of developed assays is presented in Table 8.1. Proposed methodologies can be generalized for other botulinum serotypes (B, C, D, E, F and G) and proteases such as tetanus and anthrax by rationally designing peptide substrates. Besides on-site screening of food samples, proposed methodologies can be applicable for the environmental, national security monitoring as well as for biomedical application. However, further improvements in assays performance in terms of reducing the total assay time and LODs in order to achieve a better sensitivity as well as a potential multiplexing detection are discussed in Future Outlook below.

Table 8.1. Performances of developed assays in the thesis.

Assay	LOD	Time	Readout	Easy-to-use
MB-fluorescence-based assay in solution	0.5-1 nM	6 h	Instrument	● ○ ○
Functional MB dipstick assay	1 nM	~5h	Naked-eye	● ● ○
Sorbent incorporated dipstick assay	0.1 nM	5h	Naked-eye	● ● ●

8.2 Future Outlook

As discussed, currently the total time required to perform assays is ~5-6h, mostly due to the long digestion step. The longer digestion time was required in order to reach the LOD below that of oral toxicity. This slow cleavage process might be due to the peptide substrate or BoNT/A form used in this study. In order to reduce the digestion time while improving the assay sensitivity, the optimization of new peptide sequences for BoNT/A detection is needed. By identifying and optimizing preferred amino acids residues in the peptide sequence can boost up the BoNT/A activity which is of crucial importance for the assay development. It has been reported elsewhere that substituting certain amino acids such as lysine residues (K189, K201) with arginine residues (Q) and/or glutamic acid (E194) with arginine (R) showed a better hydrolysis of the scissile bond[2,3]. The optimization of peptide sequence can be done by rationally designing the peptide arrays by SAMDI-MS technique (*S*elf-*A*ssembled *M*onolayers for matrix-assisted Laser *D*esorption/*I*onization mass spectrometry)[4]. Briefly, SAMDI-MS is a label-free high through screening technique that uses the self-assembled monolayers of alkane thiolates having the maleimide group in order to immobilize various molecules with cysteine in an array format on the SAM surface. The non-specific adsorption of proteins to the gold surface is prevented by tri(ethylene glycol) groups, further ensuring proper analysis. After modification of alkane thiolates by molecules, the technique detects the increase in the mass of alkane thiolates itself. This technique provides the protease activity screening for a huge number of different peptides in the short period of time using the mentioned high throughput assay format (Fig. 8.1). The technique generates a heat map that provides information about peptides sequences that are cleaved at a high catalytic rate (efficiency) by BoNT/A. With optimized peptide sequence it can be feasible to detect BoNT/A in more complex food matrices such as milk which was not possible with the current one as shown in the chapter 4.

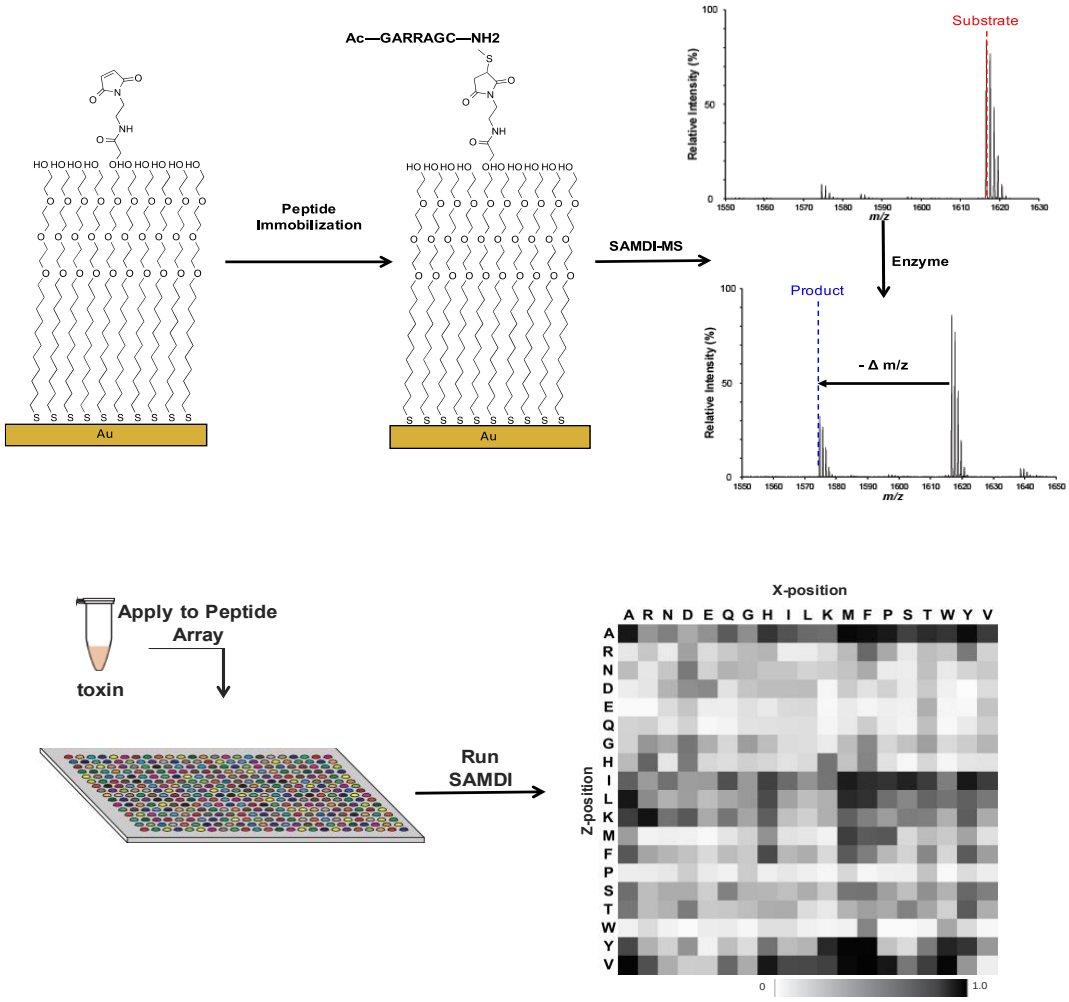


Figure 8.1. Protease activity detection by SAMDI-MS technique and a heat map.

Additionally, the overall assay time can be reduced by excluding some of conjugation steps. For instance, in ST-2 strategy (chapter 4), the labelling with SAPE dye as an additional step of 30 min can be eliminated by synthesizing the peptide containing small dye molecule in order to avoid the steric hindrance during the cleavage process. Apart from this, the multiplexing is feasible by attaching fluorophores, having similar excitation, but distinctly different emission wavelength to substrates that are specific to various proteases for yielding distinguishable signal responses.

In the chapter 5, the dipstick assay showed a good LOD and an obvious change of color intensity of test lines, but as it has been mentioned broadening/smearing might adversely affect the assay performance. The smearing of test bands might be attributed to the manual deposition of antibodies on the test line, which can be overcome by using the LFA dispenser. Additionally, the amplification step can be simplified by using more intense contrast-rich labels embedded within the magnetic bead polystyrene layer, which could contribute to the better LOD.

Also, fabrication of multiplexed dipstick test for the simultaneous detection of various proteases would contribute to faster screen through many samples in a short period of time. For instance, in chapter 4, this is possible by adding more test lines with immobilized antibodies for capturing the corresponding terminus of substrates that are specific to various proteases. On the other hand, in chapter 5, besides adding more test lines for capturing the corresponding substrates, different fluorescent reporter molecules can be attached. In chapter 5, besides adding more test lines for capturing the corresponding substrates, different fluorescent reporter molecules can be utilized. This will give different colorimetric responses for test lines according to tested proteases.

Finally, testing the assays with real contaminated samples (native toxin) is needed to find out the assay performance. The obtained responses from real samples would be then compared with current functional-based assays and the benchmark – mouse bioassay. The aim of such a study will be to examine the false positives/negatives in order to evaluate the real application for the verification laboratories and for on-site analysis.

References

(1) Os, A. T.; Uce, R.; Cher, R. Methods for Detecting Botulinum Toxin with Applicability to Screening Foods. *Concise Reviews / Hypotheses in Food Science* **2005**, *70* (8), 121–130.

(2) Rosen, O.; Feldberg, L.; Dor, E.; Gura, S.; Zichel, R. Optimization of SNAP-25-Derived Peptide Substrate for Improved Detection of Botulinum A in the Endopep-

MS Assay. *Analytical Biochemistry* **2017**, *528* (April), 34–37.

(3) Schmidt, J. J.; Bostian, K. A. Endoproteinase Activity of Type A Botulinum Neurotoxin: Substrate Requirements and Activation by Serum Albumin. *Journal of protein chemistry* **1997**, *16* (1), 19–26.

(4) Mrksich, M. Mass Spectrometry of Self-Assembled Monolayers: A New Tool for Molecular Surface Science. *ACS Nano* **2008**, *2* (1), 7–18.

Research Publications

1. Klisara, Nevena, et al. "Functional fluorescence assay of botulinum neurotoxin A in complex matrices using magnetic beads." *Sensors and Actuators B: Chemical* 281 (2019): 912-919.
2. Klisara, Nevena, et al. "Towards on-site visual detection of proteases in food matrices." *Analytica Chimica Acta* (2019).
3. Klisara, Nevena, et al. "Sorbent Incorporated Dipstick for Direct Assaying of Proteases." *Analytical and Bioanalytical Chemistry* (2020).