TOWARDS BIOSENSORS IN FOOD PACKAGING

IMMOBILIZATION AND CHARACTERIZATION OF FLEXIBLE DNAzyme-BASED BIOSENSORS FOR ON-THE-SHELF FOOD MONITORING

By

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Lay Abstract

Microbial pathogens can grow in food following packaging and preceding consumption. Current biosensors are not efficient for post-packaging real-time food monitoring without separating the sample from the stock. Packaged food such as meat and juice are directly in touch with the surface of their containers or covers. Therefore, real-time sensing mechanisms, installed inside the food packaging, tracing the presence of pathogens, are much useful to ensure the food safety. Here we report on developing thin, transparent, flexible and durable sensing surfaces using DNA biosensors, which generate a fluorescence signal in the presence of a target bacterium in food or water samples. The covalently-attached DNA probes can detect as low as 10^3 CFU/mL of *Escherichia coli* in meat, sliced apple and apple juice. The fabricated sensing surfaces remained stable up to several days under varying pH conditions (pH 5 to 9). In addition to pathogen monitoring in packaged food or drinking bottles, these surfaces are promising for a variety of other applications in health care settings, environmental monitoring, and biomaterials like wound dressing.

Abstract

While the Canadian food supply is among the healthiest in the world, almost 4 million (1 in 8) Canadians are affected by food-borne illnesses, resulting in 11,600 hospitalizations and 238 deaths per year. Microbial pathogens are one of the major causes of foodborne sicknesses that can grow in food before or following packaging. Food distribution is an important part of the food processing chain, in which food supplies are at a higher risk of contamination due to lack of proper monitoring. Among myriad of research around biosensors, current devices focusing on packaged food monitoring, such as leakage indicators or time temperature sensors are not efficient for real-time food monitoring without separating the sample from the stock. Packaged food such as meat and juice are directly in touch with the surface of their containers or covers. Therefore, real-time sensing mechanisms, installed inside the food packaging and capable of tracing the presence of pathogens, are of great interest to ensure food safety. This work involves developing thin, transparent, flexible and durable sensing surfaces using DNA biosensors, which report the presence of a target bacterium in food or water samples by generating a fluorescence signal that can be detected by simple fluorescence detecting devices. The covalently-attached DNA probes generate the signal upon contact with the target bacteria with as low as 10^3 CFU/mL of *Escherichia coli* in meat and apple juice. The fabricated sensing surfaces remained stable up to several days under varying pH conditions (pH 5 to 9). In addition to detecting pathogens on packaged food or drinking bottles, these surfaces have the potential to be used for a variety of other applications in health care settings, environmental monitoring, food production chain, and biomaterials like wound dressing.

In Memory of My Dearest Cousin

Reyhaneh

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Abbreviations

AX	*Achromobacter Xylosoxidans*
BS	*Bacillus Subtilis*
CEM	Crude Extracellular Mixtures
CIM	Crude Intracellular Mixtures
COP	Cyclo Olefin Copolymer
DNA	Deoxyribonucleic Acid
DNAzyme	Deoxyribozyme
E. coli	*Escherichia Coli*
LOD	Limit of Detection
MW	Molecular Weight
PBS	Phosphate Buffer Solution
PA	*Pediococcus Acidilactici*
RB	Reaction Buffer
RFD	RNA-cleaving Fluorescent DNAzyme
SDS	Sodium Dodecyl Sulfate
TSB	Tryptic Soy Broth
XPS	X-ray Photoelectron Spectroscopy
YR	*Yersinia Ruckeri*

1. Introduction

While the Canadian food supply is among the healthiest in the world, almost 4 million (1 in 8) Canadians are affected by food-borne illnesses, resulting in 11,600 hospitalizations and 238 deaths per year (Bélanger, Tanguay, Hamel, & Phypers, 2015). Due to the various storage conditions of food supplies during their shelf lives, expiration dates cannot accurately detect food health at the time of usage. On the other hand, conservative expiration dates lead to the mass wasting of on-the-shelf food that were otherwise still in good condition. Therefore, food quality needs to be monitored as accurately as possible during shelf life.

A few successful applications of sensors in food packaging are fruit freshness indicators, time temperature sensors, fish spoilage sensors and leakage indicators. Figure 1.1 shows some examples of final applications of these sensors. The significance of food health monitoring underlines the need to improve the reliability of current methods such as available sensors for food packaging. Biosensors have the potential to provide high accuracy, processing speeds, and specificity. With recent advances in developing innovative biosensing platforms, viable products have been introduced for real-time monitoring, such as food processing, quality control, and the detection of specific elements or contaminants (Mutlu, 2016; Thakur & Ragavan, 2013; Viswanathan, Radecka, & Radecki, 2009).

In this work, we focused on developing specific, sensitive, reusable and stable biosensors for real-time, and hands-free monitoring for packaged food. This chapter discusses recent advances in biosensing for food monitoring and introduces DNAzyme-based sensors as

reliable probes in biosensing devices for bacterial detection. In the second chapter, we focus on developing and optimizing surfaces suitable for designing biosensors in food packaging. In addition, we demonstrate possibility of developing reloadable biosensors that can be re-used multiple times for detecting different target bacteria. In the third chapter, we introduce thin, flexible and transparent DNAzyme-based biosensors for detecting bacteria in food packaging. These physical characteristics, combined with the high stability and specificity of the biosensors, could provide food suppliers or consumers with the ability to perform real-time health monitoring of packaged food.

1.1. Importance of monitoring food contamination

Food contaminants

According to the World Health Organization's (WHO) 2015 report, food supplies can be contaminated with 31 infectious agents or chemicals (Kirk, Angulo, Havelaar, & Black, 2017). Food contaminants are a wide range of bacteria, viruses, parasites, prions, toxins and chemicals (Dougherty et al., 2000). Biological contamination is when biological hazards (biohazards) contaminate food. This is a common cause of food poisoning and food spoilage. Among all biohazards, harmful bacteria (also called pathogens) are the main source of foodborne diseases (Scallan et al., 2011), and may occur during any of the steps in the farm-to-table period causing foodborne illnesses (Yang, Lin, Aljuffali, & Fang, 2017). Bacteria are small microorganisms that replicate very quickly. If one single-cell bacterium enters a food supply, it can multiply and make the food prone to cause foodborne illnesses in just a few hours (Zwietering, De Koos, Hasenack, De Witt, & Van't Riet, 1991). Hence, fast, specific and accurate detection of bacteria is crucial in food health monitoring.

Post-processing food contamination

The food production chain (food system) consists of several processes, usually starting from the farm or fishery and ending at the consumers' dining tables. The food production chain includes 4 major categories (Control & Prevention, 2015):

- Production (farm or fishery)
- Processing (preparations, packaging)
- Distribution (transportation)
- Storage (retail)

Although contaminations can occur at any point along the food production chain (Roday, 1998), distribution and storage are two critical steps in which food products are at risk of contamination (BRACKETT, 1992; Bryan, 1990; Food & Administration, 2010; Kennedy et al., 2005; Lianou & Sofos, 2007). This is because of:

- unsuitable distribution (or inappropriate transportation)
- Incorrect refrigeration (or temperature control) of food products
- Lack of monitoring systems to provide proper hazards identification
- High chances of contamination while bringing the food supplies to the shelves
- The shelf storage period and potential contacts of the food with consumers or workers

As discussed above, the lack of monitoring systems in stored food both in distribution and shelf storage, may prevent on-time food recall and cause foodborne illnesses once spoiled food is distributed to consumers. Therefore, the development of monitoring

systems suitable for the storage period is fundamental for the future of food health identification technology.

1.2. Monitoring contamination in packaged food

Food contamination detection methods can be categorized as slow (such as culture and colony counting methods (Hill, Payne, & Aulisio, 1983) and immunology-based methods (Lazcka, Del Campo, & Munoz, 2007)) and rapid (culture independent methods (Y. Xu et al., 2015) such as time temperature sensors (Ahvenainen, 2003) and bacteria detecting biosensors (Han, Bae, Magda, & Baek, 2001)). With respect to on-the-shelf food monitoring needs, conventional methods are not acceptable to be used since they are not integrated in food packaging and require several sample handling steps. Biosensors are the new generation of rapid detection methods that combine a bioreceptor (or biochemical recognition element) with a transducer (or detector) to capture and report the presence of a specific target (Han et al., 2001). Biosensors are being increasingly used for medical applications and environmental tests. Biosensors have shown great potential for microbial pathogen detection in the food production chain (Rasooly & Herold, 2006) and are continuously leading to reliable and promising advances in food pathogen detection (Lazcka et al., 2007; Mutlu, 2016; Srinivasan, Umesh, Murali, Asokan, & Siva Gorthi, 2017; Thakur & Ragavan, 2013). Even so, there are still many challenges, such as biosensors' dependency on large accessories or electronic supports, sample handing and lack of stability; this leads to many opportunities to improve current technologies and make them practical and reliable choices (Nugen & Baeumner, 2008; Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010). The ideal characteristics for the development of

biosensors in resource limited settings are defined by the World Health Organization, as: affordability (feasible to be used in a monitoring system), high sensitivity (able to detect the lowest amount of pathogens capable of causing illness), user friendliness, rapidity (fast response), equipment-free (no need for high end facilities), and deliverability (portable or hand-held) (Wu & Zaman, 2012).

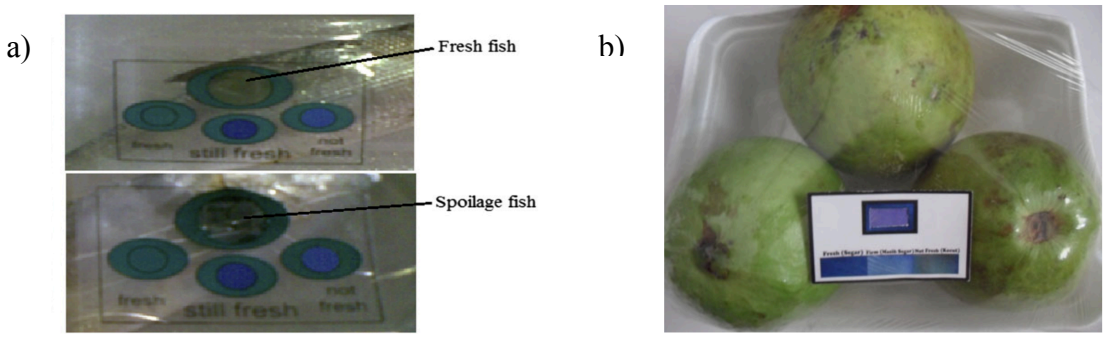

Figure 1.1 Current food packaging monitoring applications. a) Fish spoilage indicator installed inside fish packaging. The middle section changes its color in case of product spoilage. b) Freshness indicator for Guava's packaging. Depending on ripeness of the fruit, the sensor shows different colors.

Biosensors in food packaging

Recent advances in food processing technology have resulted in an increasing utilization of biosensors in food preparations and analytical measurements related to food processing (Mello & Kubota, 2002; Patel, 2002; Prodromidis & Karayannis, 2002). Considering the recent improvements in biosensors over the last decade, current technologies need to be enhanced in three major criteria so that biosensors are suitable for food packaging purposes (Vanderroost, Ragaert, Devlieghere, & De Meulenaer, 2014):

- **Self-reliance:** Self-reliance of the sensors makes them independent from other devices, accessories or complicated steps (ideally, hands-free applications).

- **Stability**: Stability helps the sensors to endure their shelf life and prevents the bioreceptor from being released in to the food source.
- **Reloadability:** Reloadability makes replacing bioreceptors easy and having biosensors with different functionalities possible.

Deoxyribozymes (DNAzyme) as bacterial detection probes

Synthetic catalytic DNA molecules (DNAzymes) are synthetic single-stranded DNA molecules that have a catalytic ability or capable of performing a specific reaction (Breaker, 1997; Breaker & Joyce, 1994). The first generation of developed DNAzymes were able to detect metal ions such as pb^{2+} with high specificity (Lan, Furuya, & Lu, 2010). Among different DNAzyme types, the RNA-cleaving variety have become useful for developing detection methods for a wide variety of targets (Schubert et al., 2003; D. Y. Wang & Sen, 2001). Recently, RNA-cleaving fluorescent DNAzymes (RFD) were generated by *in vitro* selection for specific bacteria and optimized for real-time bacterial detection purposes (Sergio D Aguirre, Ali, Kanda, & Li, 2012; Li, 2011; Zhang, Feng, Chang, Tram, & Li, 2016). These DNAzymes cleave a fluorogenic DNA substrate at a single ribonucleotide embedded in the substrate. The cleavage section is contained by a fluorophore molecule and a quencher, thus the substrate before cleavage reaction possesses minimal fluorescence signal (meaning no bacteria is in contact with DNAzyme). When the substrate is cleaved by the DNAzyme in the presence of the target bacterium, the fluorophore and the quencher separates away from each other, which leads to a significant increase in fluorescence intensity. High sensitivity and selectivity of these DNAzyme probes combined with their facile real-time behavior in bacterial detection (S. D. Aguirre, Ali, Salena, & Li, 2013) and

higher stability make them an ideal candidate for contamination monitoring in food packaging (Gong et al., 2015). DNAzymes were previously optimized in liquid phase as pathogen-sensing agents on magnetic beads (H. Zhang et al., 2016), metal organic frameworks (MOF) (Chen et al., 2017), gold nanoparticles (J. Liu & Lu, 2004; Yin, Zuo, Huo, Zhong, & Ye, 2010), carbon nanotubes (Lu & Liu, 2006), and with liquid crystals (Liao et al., 2016). **Figure 1.2** provides examples of DNAzyme immobilized on different surfaces. However, so far there has not been a report to attached DNAzymes to surfaces in a suitable manner for food packaging applications. In addition, these DNAzyme sensors were only shown to respond to the crude extracellular mixtures (CEM) (Ali, Aguirre, Lazim, & Li, 2011) and crude intracellular mixtures (CIM) (S. D. Aguirre et al., 2013) of specific bacteria; however, their ability to detect live bacteria has not been demonstrated so far.

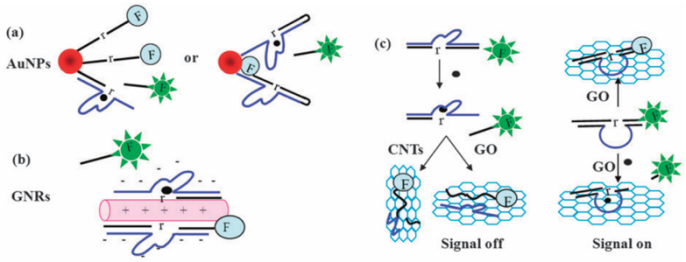

Figure 1.2 DNAzyme-based fluorescent biosensors. DNAzyme probes are attached to **a)** gold nanoparticles (AuNPs), **b)** gold nanorods (GNRs), **c)** carbon nanotubes (CNTs) adapted from Ref. (Gong et al., 2015)

1.3. Immobilization of bioreceptors on sensing interfaces

Immobilization can be defined as the attachment of molecules to a surface, resulting in reduction or loss of mobility (Nimse, Song, Sonawane, Sayyed, & Kim, 2014). One major requirement for a biosensor is that the bioreceptor molecule has to be immobilized in the

biosensor system (Prieto-Simon, Campas, & Marty, 2008; Sassolas, Blum, & Leca-Bouvier, 2012). The probe may be immobilized by entrapment (immobilization in matrices), adsorption (onto solid supports such as MOFs), cross-linking (covalently binding the biomolecule with other biomaterials such as glutaraldehyde), covalent immobilization (covalently coupling the biomolecule to a functionalized structure), affinity (biomolecule is specifically oriented by having an activated support and a specific segment of the biomolecule protein sequence) (Sassolas et al., 2012).

Surface immobilization of biomolecules for food packaging

Considering that most of the aforementioned immobilization methods do not show adequate stability under different environmental conditions such as ionic strength, pH, humidity and temperature, they may cause desorption of the biomolecules to the food source. Sensing molecules should be properly bound to the surface; therefore, covalent coupling is the most promising method to immobilize biomolecules for food packaging purposes (Williams & Blanch, 1994). Generally, the choice of a suitable immobilization strategy is determined by the physicochemical properties of both surface and biomolecule probes. However, in specific applications such as food packaging, many of the current methods turn out to be not appropriate in either stability or require physical characteristics for packaging.

Several methods have been developed for fabricating biomolecular patterns, particularly, DNA patterns, including contact and noncontact printing of DNA onto substrates, and in situ synthesis of microarrays using electrochemistry (Egeland & Southern, 2005) and photolithography (Barbulovic-Nad et al., 2006). On the other hand, there are several

recommended chemistries to functionalize the surfaces and immobilize DNA through them. The most well-known functional groups for covalent immobilization of biomolecules are the following:

- Aldehyde
- Epoxy
- Amine
- Carboxyl
- *N*-Hydroxysuccinimide **(**NHS)

Choosing the appropriate functional group requires an in-depth understanding of the physical and chemical interactions involved (Gibbs & Kennebunk, 2001). Therefore, there is a need to investigate and optimize the most suitable chemistry among these functional groups for developing stable biosensors for food packaging.

1.4. Objectives and thesis outline

The main objective of this work is to develop flexible biosensors suitable for food packaging. In particular, these devices will perform real-time and easy-to-use bacteria monitoring without the need for sample handling, accessories and complex procedures. More detailed objectives are the following:

- To investigate critical parameters in order to choose the best surface chemistry among several options based on physical characteristics, stability and reusability (chapter 2)
- To test the reusability of the developed substrates for several repeated detection steps (Chapter 2)

- To demonstrate stability and performance of the chosen substrate and chemistry (Chapter 2 and 3)
- To develop the biosensors on thin, flexible and transparent polymer substrates (Chapters 3)
- To introduce real-time bacteria monitoring systems that can report the presence of bacteria shortly after it is introduced (Chapter 3)

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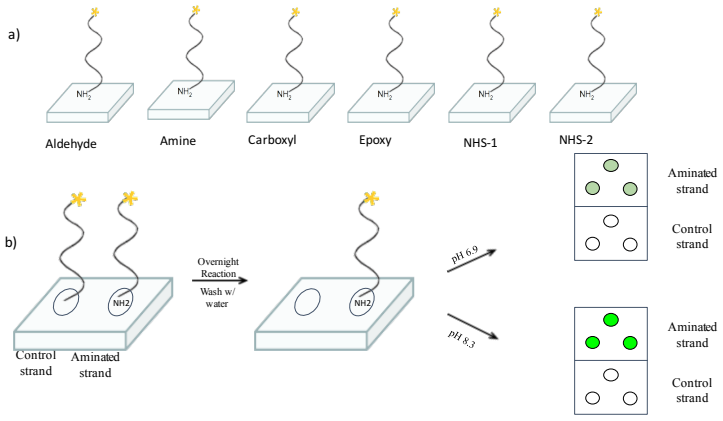
Chapter 2

Investigation of functionalized surfaces to develop stable and reloadable biosensors for food packaging



In chapter 2, all the experiments were conducted by myself and Hsuan-Ming Su who worked with me as undergraduate student. My advisors (Prof. Filipe and Prof. Didar) gave me many helpful suggestions in both experiments and data analysis. Dr. Ali Monsur helped me with data analysis. I wrote the first draft of the paper with help of Hsuan-Ming Su. Prof. Didar, Dr. Monsur and Prof. Filipe helped me in revising the draft to final version.

2. Investigation of functionalized surfaces to develop stable and reloadable biosensors for food packaging

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Abstract

Real-time monitoring of food quality is a trending topic in response to the high prevalence of food contamination due to poor storage of fresh food products. Despite the development of biosensors in the food packaging industry, certain characteristics such as stability, specificity, real-time sample free monitoring, and reusability have not yet been properly addressed; these are important qualities needed in an effective biosensor for monitoring food contamination. In this work, we performed a comparative study on several plastic and glass based substrates with different surface chemistries to address the viability of these sensors in detecting food-borne pathogens. We conducted various experiments on these substrates to further evaluate their characteristics and effectiveness in food packaging applications. Through our investigation on the durability and reproducibility of different substrates and chemistries, we concluded that epoxy-coated cyclo olefin copolymer (COP) films are the best candidates for the creation of bio-sensing wraps in food packaging.

Multiple rounds (up to 8) of hybridization and de-hybridization experiments on a DNA-treated surface showed stable fluorescence intensities over time, demonstrating the reusability of the developed biosensors.

Introduction.

Food contamination represents one of the most prevalent biosafety hazards in the world, resulting in over 600 million illnesses and 420,000 deaths every year (Organization, 2015). Although the responsibility of producing safe consumables lies within the mandate of the food and packaging industry, food sources can become contaminated in the distribution and storage process due to poor handling, improper refrigeration and lack of monitoring (BRACKETT, 1992; Bryan, 1990; Food & Administration, 2010; Kennedy et al., 2005; Lazcka et al., 2007; Lianou & Sofos, 2007). This highlights the need for real-time monitoring of food safety during the critical time period between packaging and consumption. While the unsafe food handling processes associated with the packaging systems remain an area of continual development, biosensors are currently the most promising technologies in detecting contamination within food packaging (Brockgreitens & Abbas, 2016).

Among the myriad of biosensors currently in development, surface-based biosensors have shown promising results in food packaging, pharmaceutical chemistry, and environmental analysis (Baeumner, 2003; Bejjani & Shaffer, 2006; Lee, Harbers, Grainger, Gamble, & Castner, 2007; Scott, 1998). Choosing the appropriate surface and biomolecule requires an in-depth understanding of the physical and chemical interactions involved (Gibbs &

Kennebunk, 2001). The need for several operations, such as packaging, storage and re-usability require that the biosensors have long-term storage stability and high reproducibility. These biosensors usually have specific types of biomolecules that must remain bonded to the surface and maintain their structure, function, and biological activity after immobilization. Although efforts have been made to develop successful immobilization strategies in order to assure greater sensitivity and selectivity (Sassolas et al., 2012), stability still remains a concern that needs to be addressed.

While the research on DNA-based biosensors has mostly been performed on glass substrates, other biosensors have also been developed using non-glass substrates like polymers, which have different physical and chemical properties(Karamessini, Poyer, Charles, & Lutz, 2017; Y. Liu & Rauch, 2003; Pu, Oyesanya, Thompson, Liu, & Alvarez, 2007). The importance of a substrate's physical properties in food packaging has inspired us to perform this study on both glass- and polymer-based surfaces. We chose five different chemistries that are considered suitable for covalent DNA immobilization and created our DNA microarrays on both glass and plastic substrates.

In this work, complementary surface characterization techniques, including X-ray photoelectron spectroscopy (XPS), fluorescence scanning, and hydrophobicity (contact angle measurements) were used to study DNA immobilization efficiency and its effect on the physical properties of each surface. The combination of these results with stability testing has led us to consider one substrate as the strongest candidate. We were then able to compare the hybridization efficiency of the amine-terminated single-stranded DNA (ssDNA) probes on the selected substrate for 8 rounds of hybridization and dehybridization.

We demonstrated that thin, flexible, and transparent epoxy-coated COP films show other favorable and important aspects for food packaging biosensors in terms of stability and efficiency. Amongst all of the selected surfaces and chemistries, epoxy coated COP films showed considerable stability through the hybridization steps, which makes them great candidates for the creation of reusable biosensors assays.

Results and discussion

Investigating concentration of immobilized DNA probes on different chemistries

Amine-terminated DNA probes were printed onto the functionalized surfaces along the control strands, which did not contain a terminal amine group. Printing was done with an inkjet printer with droplet sizes of 450 picoliters. Details of the printing procedures are provided in materials section. Fluorescence intensities across the substrates were measured and quantified using a fluorescence microscope and a fluorescence scanner in order to determine the most effective chemistry for immobilizing amine-terminated DNA. **Figure 1.2a,b** shows images of each substrate before and after rinsing with water. As shown across all chemistries, the amine terminated DNA has a significantly higher binding affinity to the functionalized surfaces than the control DNA strand. **Figure 2.1c** shows the average fluorescence intensity of the immobilized DNA on each substrate. The results have been categorized according to the type of substrate material; the epoxy and carboxyl surfaces (red bar plot) were plastic-based, while NHS, amine, and aldehyde (blue) were made of glass. To better present the florescence imaging results, we chose to calculate the relative fluorescence as the ratio of the immobilized amine terminated DNA signal to the

control (DNA with no amine groups). As shown in **Figure 2.1c** the epoxy-functionalized substrates emitted the highest relative fluorescence signal (13 times higher than that of the background), suggesting that epoxy is the most effective functional group for immobilizing amine-terminated DNA. In contrast, DNA immobilized onto carboxyl-functionalized slides showed the lowest relative fluorescence.

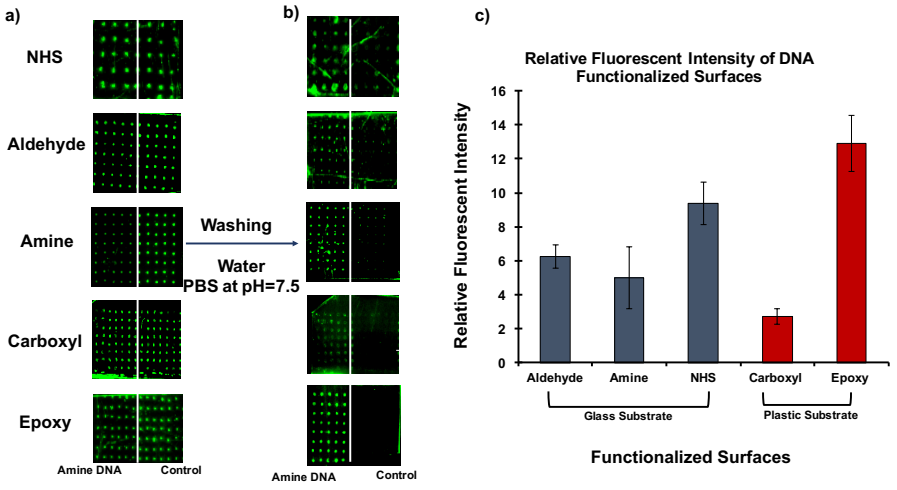

Figure 2.1 Covalent attachment of the probes to the selected surfaces. **a)** Representative fluorescence images of DNA-printed surfaces. (The distance between each two printed are in 100 *μm).* **b)** Representative fluorescence images of DNA-printed surfaces after 12 hours of incubation and washing. **c)** Relative fluorescence intensities of the surfaces after the washing step, comparing the intensity in covalent and non-covalent attachments. Red bars refer to plastic substrates and blue bars to glass substrates.

Surface characterization of the functionalized surfaces with DNA probes

Depending on the substrate's material and its surface coating chemistry, a sensor's hydrophobicity may differ. Hydrophobicity can directly affect the DNA probe density in covalent attachment protocols. In addition, DNA probe concentration and surface hydration can conversely change the properties of the surfaces. Therefore, we measured contact angles of the developed surfaces to investigate their hydrophobicity. Furthermore, to

confirm covalent attachment of the probes, we used X-ray photoelectron spectroscopy (XPS) to investigate the chemical composition of the functionalized surfaces.

Contact angle measurements using water droplets were performed on the functionalized slides before and after DNA immobilization in order to identify and compare the differences in hydrophobicity of the surfaces **Figure 2.2a**. These results describe that epoxy-functionalized substrates are the most hydrophobic, while carboxyl substrates are the most hydrophilic. DNA has been previously shown to decrease the contact angle after surface immobilization due to the hydrophilic hydroxyl groups on its ribose and phosphate backbone (Chrisey, Lee, & O'Ferrall, 1996) and our findings confirm this (Liechti, Schnapp, & Swadener, 1997; Metwalli, Haines, Becker, Conzone, & Pantano, 2006). Other side reactions, such as hydrolysis, can escalate the effect of DNA immobilization on

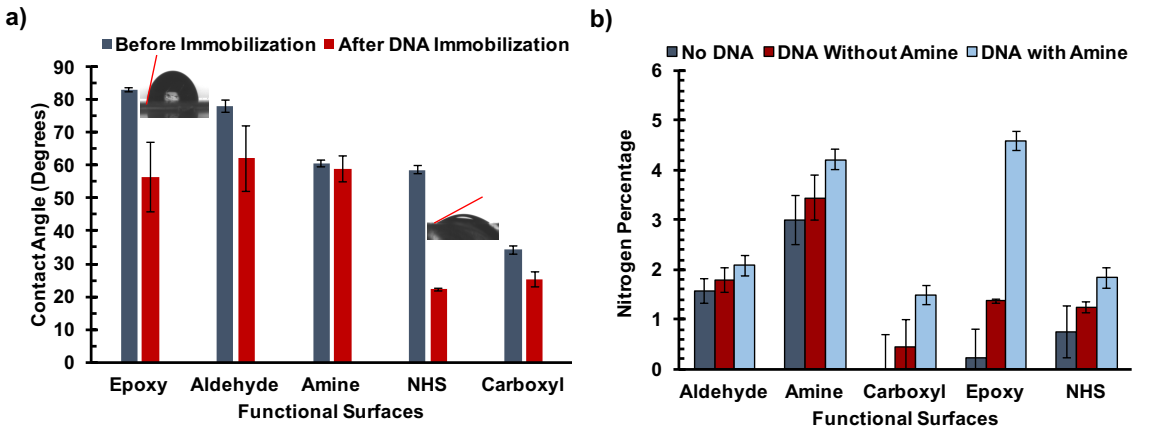

Figure 2.2 Surface Characterization of the functionalized substrates. **a)** Contact angle measurements of the surfaces modified with DNA. Contact angle of the surfaces were measured before and after DNA treatment. Epoxy surfaces showed the highest hydrophobicity and carboxyl slides showed the highest hydrophilicity. **b)** XPS results for nitrogen element on amine-DNA and control DNA treated surfaces. Nitrogen increased after covalent attachment, indicating the presence of DNA on the surfaces. Results showed that epoxy surfaces have the largest capacity to accommodate the highest concentration of DNA probes on them.

hydrophobicity on some surfaces (Hermanson, 2008; Wong, 1991) mostly on NHS and carboxyl surfaces.

Since nitrogen is unique to DNA in most of the surfaces due to its nitrogenous bases, its surface composition percentage can therefore be an indicator for the relative presence of DNA. For each substrate, measurements were taken from areas covered with amine-terminated DNA, control DNA (without terminal functional group), and only the surface without DNA immobilization. The results are summarized **Figure 2.2b.** A consistent trend across all substrates was found. Areas with amine-terminated DNA showed the highest nitrogen composition, followed by areas with control DNA, with the areas without any DNA showing the least amount of nitrogen. Reported nitrogen percentages are evident of the presence of this element at the site of immobilization alongside nitrogenous bases on DNA with terminal amine. Different functionalized surfaces also showed varying percent composition of nitrogen, with amine being the highest due to the presence of nitrogen in its structure. Therefore, in order to conduct a precise calculation of the DNA covalently attached to the surfaces, changes in the nitrogen percentage must be monitored. As depicted in **Figure 2.2b**, nitrogen has the highest increase in epoxy based substrates compared to the control surfaces presenting epoxy as the best candidate for covalent DNA immobilization.

Stability assay under varying pH

Immobilization chemistry, printing buffer, pH, probe concentration, incubation temperature, and reaction time are all factors that may influence the fabrication of DNA biosensors (Taylor, Smith, Windle, & Guiseppi-Elie, 2003). Stability plays a crucial role in

withstanding long shelf lives since food storage can provide various ranges of humidity and pH for the food packaging biosensors. Although the chemistries that we selected for our work have been widely studied, there is limited research on post immobilization stability, which is a crucial requirement for food packaging. In order to study these properties, DNA-printed slides were incubated under different pH conditions for 24 hours. Fluorescence intensities of the slides were measured before and after the incubation in order to identify any changes in DNA concentration. **Figure 2.3** summarizes the stability test results. Although DNA is covalently attached to every substrate, the DNA-coated epoxy surfaces showed the highest stability under harsh pH conditions. As shown in the previous section, epoxy-coated COP foils are highly hydrophobic and that covalent DNA immobilization is denser on them compared to other chemistries. Therefore, these findings can justify the high coupling efficiency of epoxy foils.

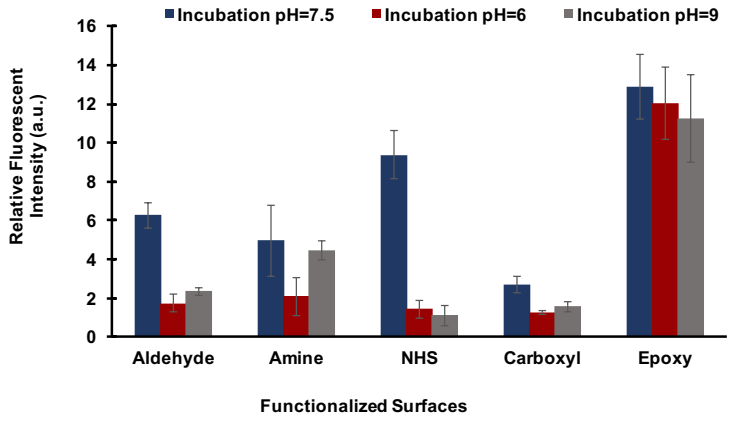

Figure 2.3 Covalent attachment reaction efficiency after immobilization. DNA immobilized surfaces were incubated in different pH conditions (pH= 6,7.5,9) to simulate the food condition for 24 hours. Epoxy-coated COP films were the only group of chemistries that showed a high stability under different pH conditions.

Evaluation of reusability through fluorescent probe hybridization.

Reproducibility is one of the most important characteristics of biosensors used for monitoring food quality as most of the current biosensors need to be replaced with new biomolecules after each detection. To study the reproducibility of our biosensors, amine-terminated DNA probes were immobilized onto epoxy slides, followed by hybridization of fluorescent complementary DNA probes as explained in methods. We performed this hybridization and de-hybridization reaction on the same epoxy surfaces for up to 8 times using complementary DNA strand containing a fluorescent tag. Hybridization results were assessed using a fluorescence scanner to provide information regarding the relative density and homogeneity of the immobilized and the complementary fluorescent probes. As shown

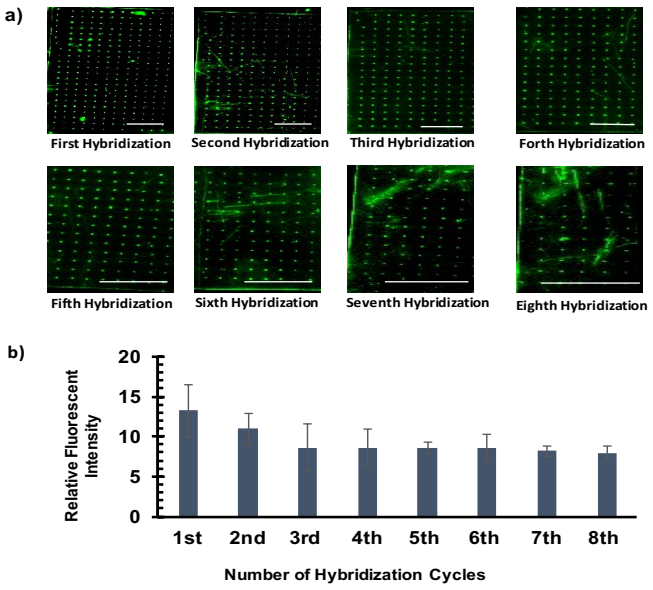

Figure 2.4 Sequential DNA hybridization steps on epoxy surfaces. **a)** Fluorescence imaging of the slides after each hybridization step shows the consistent DNA density and successful re-hybridization. Scale bar: 200µm. **b)** Fluorescence intensity measurements of the areas printed with DNA after hybridization with fluorescently labelled complementary probe. Although there was a decrease in fluorescence intensity in first few steps, it showed a constant value afterward.

in **Figure 2.4a**, the presence of fluorescent DNA after 8 hybridization cycles showed that

the DNA probes remain functional and that the washing and heating procedures in the de-hybridization process did not affect covalent attachment of DNA on the epoxy substrate. **Figure 2.4b** shows the fluorescence intensity measurements of the complimentary DNA probe after each hybridization reaction. It is seen that there is a slight drop in fluorescence intensity over the first three measurements, followed by a stable and consistent reading for all of the remaining cycles. This initial drop in fluorescence intensity can be attributed to the removal of nonspecifically attached DNA from the surface. The stability of the immobilized DNA allows for the creation of a reloadable biosensor for detecting food-borne pathogens. This easy to use, reusable and stable platform would enable both consumables and store owners to reload and create their personalized biosensors based on the need (*e.g.* when there is an outbreak of a specific pathogen).

Conclusions

We investigated several substrate and surface chemistry options to be used as food packaging biosensors. Although other substrates contain useful properties such as shorter reaction time for covalent attachment, we demonstrated that overall, epoxy coated slides are the best candidates for the producing DNA-based biosensors. These epoxy surfaces showed promising performances for covalent immobilization, binding strength, stability, durability, and low non-specific immobilization. We also showed that these slides are suitable substrates for reloadable biosensors in food packaging because of their consistent efficiency after several hybridization processes. Finally, COP slides can be transformed from thick slides to thin films to be used inside food packaging wraps.

Materials and methods:

Chemicals

Epoxy-coated plastic and carboxyl plastic slides were purchased from AutoMate Scientific Inc. Aldehyde and amine glass slides were purchased from Arrayit. N-Hydroxysuccinimide (NHS) glass slides were purchased from MicroSurfaces Inc. Phosphate-buffered saline was purchased from BioShop. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), 2-(N-Morpholino) ethanesulfonic acid (MES), Sodium dodecyl sulfate (SDS) and N-Hydroxysuccinimide (NHS) was purchased from Sigma-Aldrich. Sodium Phosphate Monobasic was purchased from EMD. Sodium Phosphate Dibasic and 50% Glutaraldehyde Solution were purchased from Fisher Scientific. All synthetic oligonucleotides were obtained from Integrated DNA Technologies and were purified using denaturing polyacrylamide gel electrophoresis (dPAGE). 5'-aminated DNA probe bearing a 3'-FAM label [5'-/5AmMC12/TTT TTC ACG GAT CCT GAC AAG GAT/36-FAM/-3'], 5'-aminated DNA probe [5'-/5AmC12/ TTT TTT TTT TAG GAA GAA GTT TCA AGG AAA GGA-3'], and a FAM labeled probe without terminal amine and was complement to the aminated probe [5'-/56-FAM/TCC TTT CCT TGA AAC TTC TTC CT-3'] were used in this work.

DNA immobilization on selected surfaces

Five immobilization chemistries that are commonly used in biosensors, namely epoxide, carboxyl, amine, aldehyde, and N-hydroxysuccinimide (NHS) reactive ester, were selected for this work (Ramakrishnan et al., 2002). Epoxy and carboxyl functionalized surfaces

utilized a cyclo olefin copolymer (COP) substrate, while NHS, amine, and aldehyde slides were made of glass.

In this study, Scienion SciFlexArrayer, a pico liter sized droplet-dispensing non-contact printer, was used to print solutions containing DNA probes onto the different surfaces. Using the Scienion printer, we were able to print droplets as small as 500 picoliter, which produced DNA microarrays. Following printing, the DNA was rehydrated through incubation in 75% relative humidity at room temperature overnight. The humidity chamber used in this work was prepared by placing a 100% sodium chloride solution in a sealed box. Humidity inside the box was monitored by a humidity meter, which was also installed inside the box.

Aldehyde Slides: a 5μM single stranded DNA in 0.3M sodium phosphate buffer at pH 9.0 were added onto the functionalized surface. Following the overnight reaction in the humid chamber, the samples were washed once with 0.1% SDS, twice with Milli-Q water, then incubated in sodium borohydride solution containing 2.5mg of $NaBH_4$, 750μL of PBS, and 250μL of 100% ethanol for 2 hours under agitation for reduction of Schiff base. *Amine Slides:* the functionalized surfaces were activated through incubation in solution containing 2.5% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.0 for 2 hours. The slides were then rinsed in sodium phosphate buffer. Following the activation, 5μM of single-stranded DNA in 0.1M sodium phosphate buffer at pH 7.0 was added onto functionalized surface. *Carboxyl Slides:* the substrates were treated in a CO_2 plasma cleaner for 2 minutes prior to immobilization in order to induce carboxyl functional groups on the surface. A 5μM single-stranded DNA in 0.1M MES buffer, with 25mM of EDC, and a 25mM NHS at pH 4.3 were

then added onto the functionalized surface. *Epoxy Slides:* a 5μM single stranded DNA in 0.1M sodium phosphate buffer at pH 7.5 was added onto the functionalized surface. *NHS Slides:* a 5μM single-stranded DNA in 0.1M phosphate buffer solution at pH 8.3 was added onto the functionalized surface.

After the immobilization of DNA probes, the substrates were rinsed for 30 seconds with Milli-Q water and imaged at pH 7.5 using the ChemiDoc and fluorescence microscope. Oligonucleotides lacking amine functional groups can also attach to surfaces via physisorption (*e.g.*, combinations of hydrogen bonding, acid-base, hydrophobic, electrostatic interactions).

Surface characterization

Contact angle measurement. Contact angles of water droplets on the substrates were measured by Future Digital Scientific Corp contact angle measurement system (Biointerface Institute, McMaster University). A micro-needle was used to dispense 2 μl droplets of deionized (dI) H_2O on all substrates before and after the DNA immobilization.

X-ray Photoelectron Spectroscopy (XPS). XPS measurements were performed using a Physical Electronics (PHI) Quantera II spectrometer equipped with an Al anode source for X-ray generation and a quartz crystal monochromator was used to focus the generated X-rays (Biointerface Institute, McMaster University). For XPS measurements, DNA was hand printed to cover a large surface area allowing proper analysis. A minimum of 3 areas containing DNA were analyzed on each substrate.

Stability test

Incubation buffers at different acidity were prepared, including PBS buffer at pH 6, sodium phosphate buffer at pH 7.5, and carbonate buffer at pH 9. After DNA immobilization, slides were incubated in each buffer for 24 hours and imaged using a fluorescence microscope and ChemiDoc.

Hybridization and de-hybridization cycle using complementary probes

In order to determine the stability and reproducibility of the immobilized DNA strand on the surface, we conducted 8 rounds of hybridization and de-hybridization using complimentary fluorescent DNA strand and compared the fluorescence intensity after each cycle. After initial immobilization of amine-terminated DNA probes on the epoxy slides, fluorescent complimentary strand was incubated on the surface in 1x SDS buffer for 2 hours. Following the reaction, the substrates were rinsed with water and imaged at pH 7.5 using fluorescence scanner. De-hybridization of the complimentary fluorescent DNA strand involved incubating the substrates in 4M Urea solution at 70°C for 1 hour.

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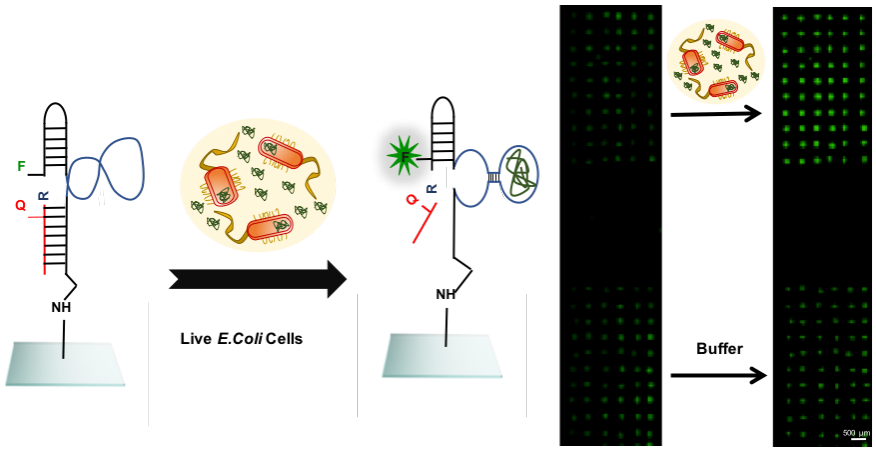
Chapter 3

Sentinel wraps; smart biosensors in food packaging for real-time on-the-shelf pathogen detection



In chapter 3, all experiments were done by myself. Dr. Sana Jahanshahi Anbuhi, a postdoc fellow of prof. Filipe, helped me with data analysis. Dr. Ali Monsur helped me with DNAzyme designing and preparations as well as experiments planning. Prof. Filipe and Prof. Didar gave many helpful suggestions with experimental deigns and data analysis. I started writing the paper draft. Dr. Monsur, Prof. Filipe and Prof Didar helped me revise the draft and prepare the final version.

3. Sentinel wraps; smart biosensors in food packaging for real-time on-the-shelf pathogen detection

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Abstract

Microbial pathogens can grow in food at any point in food processing chain, causing foodborne illnesses. Biosensors, developed based on liquid phase sensors or lab-on-a-chip devices cannot easily be used for real-time food examinations after packaging without taking the sample out of the stock. Packaged food such as meat, apple and juice are directly in touch with the surface of their containers or covers. Therefore, real-time on surface sensing mechanisms, installed inside the food packaging, tracing the presence of pathogens inside the packaged food are much needed to examine food safety. Here we report on developing thin, transparent, flexible and durable sensing surfaces based on DNAzyme biosensors, that generate fluorescent signal in the presence of a target non-pathogenic bacteria in food or water samples. The covalently attached DNAzyme probe glowed upon

contact with the packaged food (meat, sliced apple and apple juice) contaminated with target bacterium (*Escherichia coli*). We were able to detect *Escherichia coli* in food packaging with concentrations as low as 10^3 CFUs/mL. The developed sensing surfaces remained stable up to 10 days under varying pH conditions (pH 5 to 9). In addition to detecting pathogens on packaged food or drinking bottles, the developed sensing surfaces has the potential to be applied for a variety of other applications such as health care settings, environmental monitoring, food production chain, and biomaterials like wound dressing.

Introduction

Microbial growth in food products, derived from packaging deficiencies or incorrect manipulation by consumers during distribution and storage period, results in an increased prospect of consuming contaminated food and large-scale outbreaks. This threat assigns shelf storage period a paramount importance throughout the whole food supply chain. On-the-shelf, real-time, precise and simple tracing of pathogens can provide a powerful tool in addressing this issue. Traditional microbiological identification methods for pathogens in food, are well known to be prolonged and challenging (Nicholson et al., 1998; R.-F. Wang, Cao, & Cerniglia, 1996). These methods are progressively being recognized as insufficient to meet the requirements of real-time response and remote analysis in food storing conditions.

In addition, an important aspect of reliable sensors that makes them suitable for real-time measurements, is their stability, which is normally expected to be longer than a few hours, preferably days or weeks (Wilson & Gifford, 2005). Therefore, designing innovative

sensing devices which can be used in food packaging and kept in storage conditions to evaluate real-time freshness of food products are highly desired. During the past several years, researchers have designed and constructed a number of sensors for on-the-shelf detection of pathogens using magnetic nanobeads (L. Xu et al., 2017), specific food targeted sensors based on polyaniline (Kuswandi, Restyana, Abdullah, Heng, & Ahmad, 2012) and humidity based wireless sensors (Tan, Ng, Shao, Pereles, & Ong, 2007) in order to indicate food rotting or infection in a real-time manner. However, these sensors do not meet the main requirements for packaged food monitoring, which are mainly stability and self-reliance (L. Xu et al., 2017). Therefore, it demands appropriate solutions for smart packaging in food controlling. Thus, there is a greater need for developing faster, more sensitive, and more stable food monitoring instruments that can be located inside the food packaging during the storage time (Yoo & Lee, 2016).

Synthetic catalytic DNA molecules (DNAzymes) as functional nucleic acids, are artificial single-stranded DNA molecules that have a catalytic ability (Breaker & Joyce, 1994; Gong et al., 2015; Lu & Liu, 2006). Among the DNAzymes, RNA-cleaving ones have become attractive particularly in developing detection methods for a wide variety of targets (Pun et al., 2004; Schubert et al., 2003). Recently RNA-cleaving fluorescent DNAzymes (RFD) were generated by *in vitro* selection for specific bacteria and optimized for real-time bacterial detection purposes (Sergio D Aguirre et al., 2012; Li, 2011; W. Zhang et al., 2016). These DNAzymes cleave a fluorogenic DNA substrate at a single ribonucleotide embedded in the substrate. The cleavage junction is surrounded by a fluorophore and a quencher so that the intact substrate prior to cleavage reaction possesses minimal

fluorescence signal. When the substrate is cleaved by the DNAzyme in the presence of the target bacterium, the fluorophore and the quencher separates away from each other and enhances the fluorescence signal. High sensitivity and selectivity of these DNAzyme probes combined by their facile real-time behavior in bacterial detection (S. D. Aguirre et al., 2013), and, higher stability, make them a great candidate for food packaging sensors. DNAzymes were previously optimized in liquid phase as pathogen sensing agents, on magnetic beads (H. Zhang et al., 2016), on metal organic frameworks (MOF) (Chen et al., 2017) and with liquid crystals (Liao et al., 2016). However, there is no report on attaching DNAzymes to flexible polymer based surfaces so far. In addition, these DNAzyme sensors were shown to respond to crude extracellular mixtures (CEM) (Ali et al., 2011) and crude intracellular mixtures (CIM) (S. D. Aguirre et al., 2013) of specific bacteria but their application for detecting live cells of pathogens has not been demonstrated so far.

Here we demonstrate, for the first time, employing DNAzyme biosensors on flexible surfaces, for detecting bacteria inside packaged food. Thin, transparent and flexible COP (cyclo olefin copolymer) films functionalized with epoxy, were used as the substrate to immobilize DNAzyme probes. This work is the first microarray production of DNAzyme probes on customary surfaces which can provide the food industry with on the package sensing and tracking opportunities. In addition, our novel DNAzyme based sensors showed a high range of stability which proved them as a reliable candidate for on-the-shelf bacterial detection. In this work, DNAzyme based surfaces showed a sensitive feature to detect live bacterial cells in both liquid (juice) and solid (meat and apple) food supplies without a need for high-level monitoring system. Furthermore, the developed biosensors are able to detect

live bacterial cells eliminating the need to lyse the bacterial cells in order to detect them. Moreover, their high stability, in different environments with varying pH, makes them a perfect candidate to be used in different food packaging.

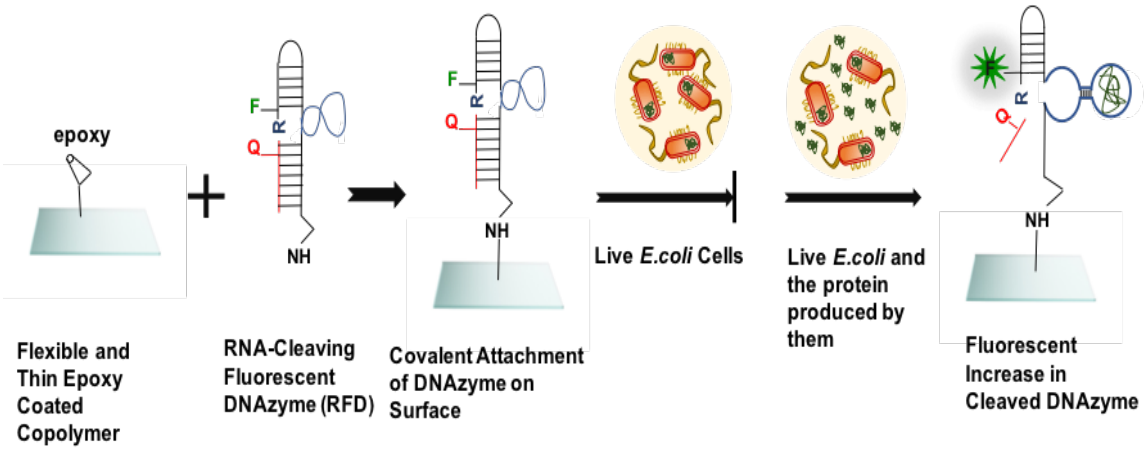

Figure 3.1 Illustration of highly sensitive DNAzyme sensors cleaving in presence of live E. coli cells. Amine terminated DNAzyme probes were covalently attached to flexible, transparent epoxy films. In presence of bacteria, RNA cleaving section is detached, consequently, the fluorescence intensity is increased.

Results and discussion

Sensors fabrication, characterization and stability assays

As mentioned above, long-term stability is an important issue in sensing devices that are designed to be used in consumer packages (Scott, 1998). We used a previously reported DNAzyme that cleaves a fluorogenic DNA substrate in the presence of CEM or CIM of *E. coli*. The fluorogenic substrate consists of the three parts: fluorophore, the quencher molecules and the cleavage junction. The substrate is located at the 3'-end of the DNAzyme. Therefore, the DNAzyme was synthesized with an amine group at the 5'-end, so that, after cleavage reaction, the fluorophore remains bounded on the surface losing the quencher to increase the fluorescence. To minimize the waste of DNAzyme reagent and

achieve mass production with ease, we used a picoliter inkjet printer which allows us to rapidly produce DNA microarrays. The amine group of the printed DNAzyme reacts with the epoxy group on the surface of COP foil forming covalent bond (**Figure 3.1**). The covalent attachment after printing was investigated by alkaline treatment (see methods). It is assumed that the alkali labile ribonucleotide in the DNAzyme substrate is hydrolyzed and removes the quencher fragment resulting in enhancement of fluorescence signal on the printed spot (**Figure 3.2a, left side)**, shows the cleaved DNAzyme surface. In addition, a control experiment without amine modified DNAzyme was carried out. After washing with alkaline solution, no fluorescence signal was observed (**Figure 3.2a, right side**) indicating the complete wash off of the DNAzyme with no amine groups. DNAzyme probes were also printed on non-functionalized COP foils to further confirm the covalent attachment. Results confirmed successful attachment of DNAzyme probes when conjugated with amine groups. Since packaged food reside under different pH conditions, we tested the surface attached DNAzyme reaction efficiency and stability at different pH conditions. Our experimental results indicated that the DNAzyme is stable under a broad range of pH conditions (**Figure 3.2b**). The relative fluorescence is the ratio of the immobilized amine terminated DNA signal to the control. The fluorescence imaging results are reported in relative fluorescence format to emphasize on detectability of the signals.

To evaluate the activity of DNAzyme probes after the attachment to the plastic surfaces in the presence of the target bacteria, DNAzyme-COP surfaces were incubated with live *E. coli* cells in reaction buffer for 4 h. A negative control was also conducted wherein the DNAzyme-COP surfaces were incubated in the reaction buffer without *E. coli*. After

removing the substrates from the reaction tube the surfaces were washed and imaged using a fluorescence imager (ChemiDoc^{TM}, Bio-Rad). **Figure 3.2c** shows the fluorescence intensity difference between *E. coli* and buffer incubated COP surfaces. These results indicate that the coupling process (printing, incubation and washing) do not affect DNAzyme functionality producing a reliable sensing surface similar to the solution phase.

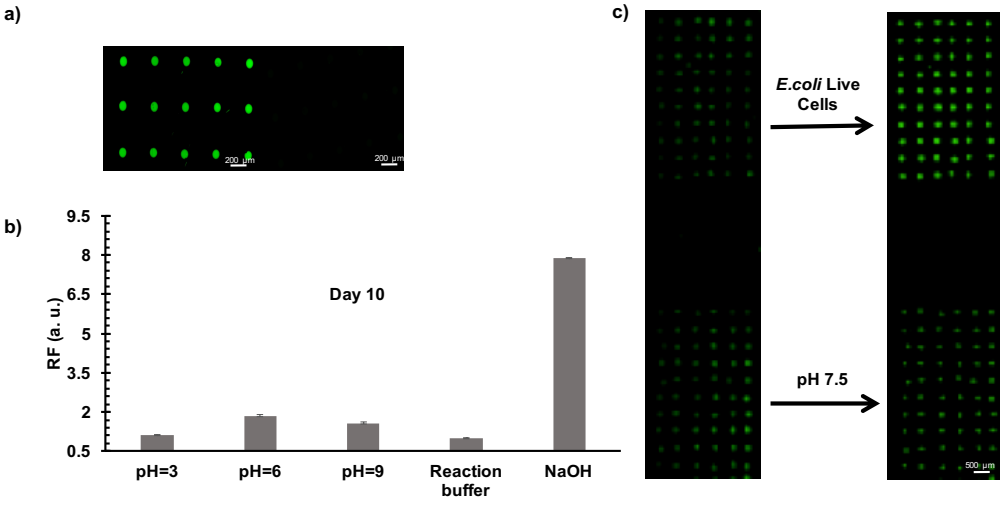

Figure 3.2 DNAzyme based surfaces characterization and stability assay: **a)** Amine terminated DNAzyme and amine free DNA probes were mixed with reaction buffer and printed with picoliter sized droplets, on transparent and epoxy functionalized flexible copolymers. Amine terminated DNAzyme probes were covalently attached to the epoxy slides and were then cleaved by NaOH solution. Slides were washed thoroughly with water and PBS buffer. DNA probes without amine at the end had no non-specific attachment to the epoxy surface. **b)** DNAzyme sensors' stability under different pH conditions. DNAzyme slides were incubated under different ranges of pH for 10 days to monitor their stability. Both covalent attachment and DNAzyme function were stable after the incubation period. DNAzymes did not lose their activity after the incubation period. **c)** Upper section of sensors was incubated with live *E. coli* cells and the bottom section were incubated in reaction buffer. After incubation, the upper side showed a significantly higher fluorescence intensity.

Real-time fluorescence assay

Another key consideration for the real-time on-site detection, is the rate of interaction between the developed sensor and bacteria. The real-time activity of DNAzyme sensors was investigated and measured by introducing the sensing surfaces to *E. coli* cells and

collecting the fluorescence signal at different time points see experimental section for detail procedure). NaOH and the reaction buffer without adding cells were used as a positive and negative controls respectively. The results indicated that the fluorescence intensity increased by 7 folds in less than 2 hours with the bacterial sample (**Figure 3.3a**).

Specificity

Although the DNAzyme, RFD-EC1, has been reported to be specific against *E. coli* in the previously published reports, we were further interested to investigate specificity with its surface immobilized form. In order to examine specificity gram-positive bacteria (*Pediococcus acidilactici, Bacillus subtilis)* and gram-negative bacteria (*Yersinia ruckeri* and *Achromobacter xylosoxidans)* were used. All bacteria samples were incubated

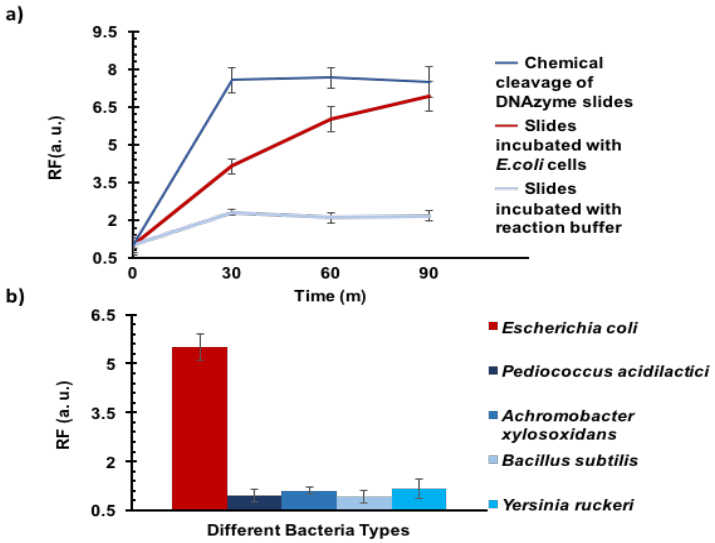

Figure 3.3 Response of DNAzyme biosensors to bacteria incubation. **a)** Results of experiments show that bacteria presence can lead to a high fluorescence increase in DNAzyme sensors which was measured as 7 times higher fluorescence after only two hours. **b)** Specificity test. E. coli cells and two gram negative bacteria and two gram positive bacteria were tested with DNAzyme slides to show the specific attachment of DNAzyme probes to E. coli cells.

overnight in TSB growth media and prepared as described in methods. DNAzyme slides were immerged into the cell suspension and incubated for 2 hours. The fluorescence

intensity of the slides was measured. Results demonstrated high specificity of DNAzyme probes to *E. coli* (**Figure 3.3 b**).

Sensitivity

DNAzyme probes previously showed a limit of detection of 10^3 CFU/mL when CIM and CEM were extracted from live bacterial cells. In order to measure the Limit of detection (LOD) of the developed biosensors, they were incubated with live *E. coli* in reaction buffer for 4 hours and overnight (**Figure 3.4**). Overnight incubation of DNAzyme with cells yielded a LOD of 10^3 CFU/mL. This indicates that the developed surfaces are capable of detecting bacterial concentrations as low as 10^3 CFU/mL during the initial storage days of the packaged food (1-3 days).

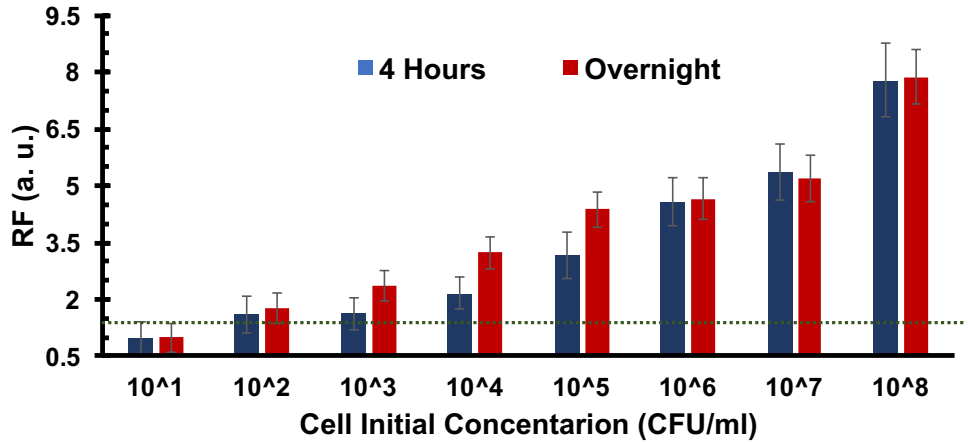

Figure 3.4 Limit of Detection of DNAzyme biosensors. E. coli cells with different initial concentrations were incubated with DNAzyme slides in reaction buffer for four hours (navy columns) and fourteen hours (red columns). Results show that with the overnight incubation the sensor can detect concentrations as low as 10^3CFU/ml. Four-hour incubation of live cells and DNAzyme led to a detection limit of 10^4 CFU/ml. The dotted line indicates no fluorescence difference between buffer incubate and *E. coli* incubated slides (RF=1).

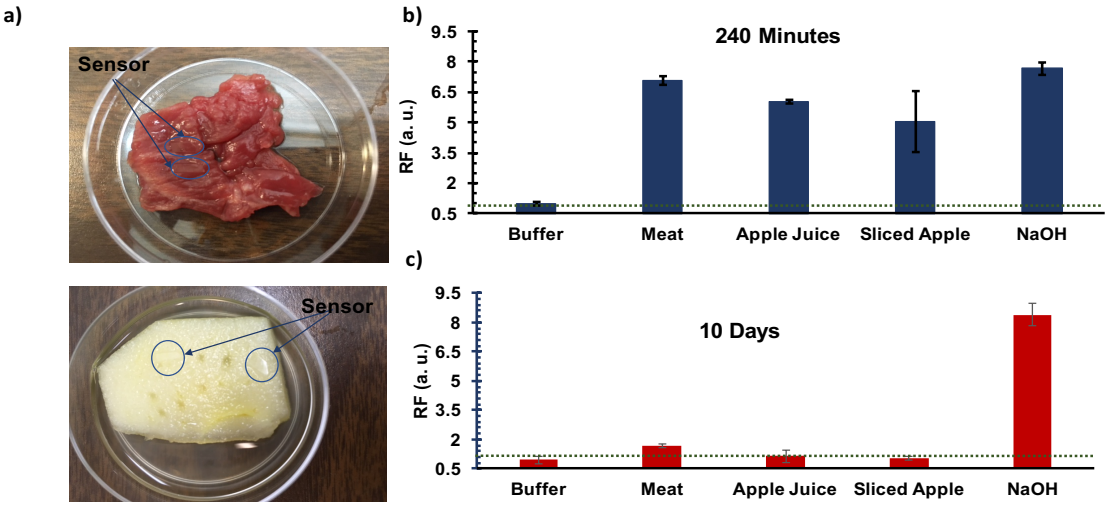

Figure 3.5 Biosensors' application with food material and bacteria. **a)** DNAzyme slides were introduced to *E. coli* infected meat and apple. **b)** Biosensors were incubated with *E. coli* infected apple juice, meat and sliced apple for four hours. Food supply without *E. coli*, NaOH buffer and reaction buffer were used as controls. Results show the response of sensors which proves the functionality of them under different environmental conditions. **c)** DNAzyme biosensors were incubated in raw meat, sliced apple, apple juice, 1M NaOH and reaction buffer for ten days. DNAzyme sensors showed high stability under different environmental pH and the covalent attachment and quencher attachment were not affected after ten days. The dotted line indicates no fluorescence difference between buffer incubate and *E. coli* incubated slides (RF=1).

Food supply spoilage trial

Finally, we tested the performance of the immobilized sensors with contaminated foods and drinks. The DNAzyme-COP films were placed in touch with the samples of solid food supply (raw beef and sliced apple) as well as on the wall of liquid food container (apple juice pH 3) (**Figure 3.5a**). First, surfaces were incubated with food samples in room temperature for 10 days to test the stability of the sensors to simulate on-the-shelf storage conditions. Slides were taken out, washed and imaged for their fluorescence intensity. There was no significant increase in their fluorescence intensity compared to the control (reaction buffer) which demonstrates high stability of the developed sensors under different pH conditions (**Figure 3.5c**). Following the stability test, 100 µL of live *E. coli* cells (10^6

CFU/mL) were spiked with the food samples, incubated for 4 h and tested for fluorescence signals. Each COP slide had and area of 10^{-5} m^2. Although the total number of cells added to each food sample were 10^5 colonies, the sensors were in touch with a small part of the food sample (approximately 3%) which means not all the bacteria were needed to reach the slides and activate the DNAzyme on them. Fluorescence intensity of the sensors was compared to the controls and results showed significant increase (up to 7 time) in fluorescence intensity of the sensors indicating successful detection of pathogens in the food samples **(Figure 3.5b**). These properties make the sensors a great candidate for smart packaging applications.

Conclusion

The DNAzyme-based sensing surfaces described here appear to have several promising features for on-the-shelf health monitoring in food supply such as: (1) no need to lyse the cells (2) no need for liquid handling, pipetting, flow or external accessories, (3) real-time response to bacterial growth (4) sensitivity (LOD of 10^3 CFU/mL) and (5) high stability in food storage conditions. On the other hand, fluorescence sensing software are being developed to enable the cellphones to detect fluorescence signals. This gives us the hope for real usage of our sensors in food packaging as smart packaging. The cleavage-based RNA detection presented here is suitable for use with diverse bacterial targets, because the modified DNAzyme probes can be designed to target different pathogens due to their high specificity to each RNA of bacteria.

Materials and methods

Chemicals

The amine modified DNAzyme, ligation template (NH-EC1 and LT in Table 3.1) oligonucleotides were purchased from Integrated DNA Technologies (IDT). The fluorogenic substrate (FS1) was purchased from Yale University (Sequence is provided in Table 3.1). Epoxy coated COP foils were purchased from PolyAn molecular surface engineering. NaCl, $MgCl_2$, Tween 20, Na_2PO_4, $NaHPO_4$, Tryptic Soy Broth (TSB), KCl, Na_2CO_3 (99.99%), $NaHO_3$, NaOH and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were purchased from Sigma Aldrich. ATP, PEG4000, T4 DNA ligase and polynucleotide kinase (PNK) and their respective buffers were purchased from Thermo Scientific, Canada. *E. coli* K12 strains are regularly maintained in our laboratory. Other bacteria types (*Pediococcus acidilactici, Bacillus subtilis, Yersinia ruckeri* and *Achromobacter xylosoxidans)* were donated by Dr. Yingfu Li laboratory at McMaster University. ChemiDoc imaging system, Zeiss and Olympus inverted microscopes were used to image DNAzyme slides. Scienion FLEXARRAYER was used to print DNAzyme on epoxy slides.

Preparation of RFD-EC1

NH-EC1 was enzymatically ligated to FS1 as follows: 500 pmol of FS1 was phosphorylated in 100 µL volume containing 1x PNK buffer A for 35 min at 37 C. The enzyme was inactivated by heating at 90 C for 5 min and cooled down to room temperature

(RT) for 20 min. Next, equivalent amount of NH-EC1 and LT sequences were added to the FS mixture, vortexed and spun down. The mixture was heated at 90 C for 1 min and cooled down to RT for 20 min. To this mixture, 20 µL each of PEG4000 and T4 DNA ligase buffer were added. After adding 4 µL of T4 DNA ligase (20 units) the volume was adjusted to 200 µL and mixed by pipetting. The tube was incubated at RT for 2 h and the DNA molecules were isolated by ethanol precipitation. The ligated DNA molecules (RFD-EC1) was purified by 10% denaturing gel electrophoresis (dPAGE), dissolved in ddH2O and quantified by nano-quant (TECAN) and stored at -20 C until used. Final concentration of storage for the DNAzyme solution was 3 µM. DNAzyme probes functionality was tested before further processes by adding *E. coli* CIM to the mixture and measuring the fluorescence intensity increase over incubation the time.

Table 3.1 Synthesized oligonucleotides (5'-3') used to prepare the biosensing agent (DNAzyme)

Probe	Sequence
NH-EC1	5'-NH_2TTTTTCACGGATCCTGACAAGGATGTGGTTGTCGAGAC CTGCGACCGGA ACACTACACTGTGTGGGATGGATTTCTTTACAGTTGTGTGCAGCTCCGTCC G -3'
LT	5'- CTAGGAAGAGTCGGACGGAGCTG -3'
FS1	ACTCTTCCTAGCFrAQGGTTCGATCAAGA (F: fluorescein-dT, rA: riboadenosine, Q: dabcyl-dT)
RFD-EC1	5'-NH_2TTTTTCACGGATCCTGACAAGGATGTGGTTGTCGAG ACCTGCGACCGGAACACTACACTGTGTGGGATGGATTTCTTTACAGTTGT GTGCAGCTCCGTCCG -3' 5'/5AmMC12/TTTTTCACGGATCCTGACAAGGATGTGGTTGTCGAGACCTGC GAC CGGAACACTACACTGTGTGGGATGGATTTCTTTACAGTTGTGTGCAGCTCC GTC CG ACTCTTCCTAGCFrAQGGTTCGATCAAGA-3'

Bacteria preparation

E. coli K12, *bacillus subtilis* (BS), *yersinia ruckeri* (YR), *pediococcus acidilactici* (PA) and *achromobacter xyloxsoxidans* (AX), were cultured overnight (14 hours at 37 °C with shaking at 250 rpm) in TSB culture media. In order to measure the colony formation unit (CFU/ml) of *E. coli* cells, the cells were grown in TSB media overnight and a fresh culture was conducted until the OD600 reached to ~1. Next, a serial dilution (10 fold) was conducted with 1 mL volume. 100 µL from dilution tube 8 was spread onto a TSA (tryptic soy agar) plate and incubated at 37 C overnight. This was done in triplicate samples. The CFUs were counted and averaged to obtain the number of CFUs. *E. coli* cells concentration was calculated to be 7.7 $*10^8$ CFU/mL in the culture. Other bacteria colonies were plated onto a TSA plate and grown for 14 h at 37 °C. A single colony was taken and inoculated into 2 mL of TSB and grown for 14 h at 37 °C with shaking at 250 rpm. The final concentration of all the bacteria were adjusted on OD_{600} of ≈ 1. Live cells were collected by centrifuge at 5000 rpm for 5 minutes and added to reaction buffer (1× RB; 100 mM HEPES, 300 mM NaCl, 30 mM MgCl2, 0.1% Tween 20, pH 7.5) to obtain the same concentration and ready to use.

Covalent immobilization of RFD-EC1 onto the surfaces

A 5 µL of DNAzyme probes were mixed with 5µL of 2x printing buffer (autoclaved sodium phosphate buffer at pH 7.5). Scienion printer was used to print the reaction solution onto epoxy coated slides following by overnight incubation (14 hours) at room temperature and 75% relative humidity. Then, slides were washed thoroughly to make sure unreacted

DNAzymes are washed out. Washing process was two minutes rinsing by autoclaved Milli-Q water and one minute rinsing by PBS buffer at pH 7.5.

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4. Conclusions and future works

Conclusions

The major contributions of this work are given as follows:

In chapter 2, we demonstrated that epoxy coated polymer substrates are the best candidates for producing DNA-based biosensors amongst the current available functionalized surfaces. The following is to support this claim based on the experimental results:

- Epoxy coated surfaces have easy DNA immobilization protocol.
- DNA immobilized epoxy surfaces showed the highest stability under different environmental conditions.
- Epoxy coated surfaces showed the highest binding strength without influencing DNA probes functionality, which led to consistent efficiency in several hybridizations and dehybridizations cycles on the same substrates (up to 8).
- Reloadable sensing surfaces makes them suitable for monitoring outbreaks by being able to change their functionality.
- COP substrates can easily be made of thin films which makes them ideal for food packaging.

In chapter 3, we developed DNAzyme-based sensing devices as potential candidates for on-the-shelf monitoring of contamination in food packaging. The following is to support this claim based on the experimental results:

- Surface based DNAzyme biosensors are established by covalently immobilizing DNAzyme probes on the surfaces.

- Surfaces are made of polymer-based, thin, flexible and transparent functionalized substrates. These physical properties make the substrates ideal for food packaging purposes.
- Prepared biosensors perform an easy and fast *E. coli* detection that can be expanded to other bacteria and pathogens.
- Prepared biosensors are applied for real-time detection of bacteria.
- DNAzyme biosensors are stable both in ambient condition and in contact with food supply which makes them suitable for on-the-shelf storage.

Future Works

The results and findings in this thesis present a great potential to bring the thin sensing films to the real applications in biosensors. In order to do so, the following research suggestions can play a vital role:

- Developing pathogen specific DNAzyme probes and implementing them in the developed platform to target desired pathogens in food packaging.
- Developing reloadable DNAzyme biosensors that can be switched depending on the need in order to monitor different pathogens. This can be a step forward towards both prevention and monitoring of outbreaks in food industry.
- Developing multiplex microarrays of various sensing agents on the presented thin films in order to provide a multiplex high-throughput pathogen monitoring in packaged food.