THESIS

BACTERIAL CULTURE COMPONENTS ACTIVATING COLORIMETRIC TRANSITION IN POLYDIACETYLENE NANOFIBER COMPOSITES

Submitted by

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ABSTRACT

BACTERIAL CULTURE COMPONENTS ACTIVATING COLORIMETRIC TRANSITION IN POLYDIACETYLENE NANOFIBER COMPOSITES

Polydiacetylene (PDA) demonstrates colorimetric transition behaviors due to conformational changes in π conjugated backbone of PDA macromolecules at external stimuli of bacteria, suggesting potential applications in biosensors. However, the bacterial culture components activating colorimetric transition in PDAs are still undetermined due to the complexity of the bacterial system. In this study, PU-PDA nanofiber composite was prepared via electrospinning and tested with components from *Escherichia coli (E. coli)* culture including supernatant fluid, cell pellet, and extracellular polymeric substances (EPS). When PU-PDA nanofiber was tested with supernatant fluid, it changed color from blue to red. In contrast, bacterial cell pellets could not induce a color change, suggesting the color-changing substances (CCS) are not cell-associated, rather can be found in the spent media (supernatant fluid) generated by *E. coli* during its growth phase. Intense color change in the nanofiber by the autoclaved supernatant fluid indicated that the CCS may not be a protein, DNA, or RNA since they denature in high heat and pressure from the autoclaving process. With an increase in storage time of the supernatant fluid, the color-changing rate was reduced significantly, suggesting a degradation in CCS with time. Free EPS from the supernatant fluid could induce a color change in the nanofibers, which confirmed that EPS contains the CCS. No significant changes were found in the morphology of PU-PDA nanofibers before and after the exposure of *E. coli* culture components. Critical bacterial

concentration (CBC) was found approximately 9×10^8 CFU/ml, suggesting the efficiency of the PU-PDA nanofiber composite to be used as a biosensor. Additionally, solvatochromism of the nanofiber composite was investigated using organic solvents commonly used in extracting bacterial culture components. The results from this study provided a guideline for using PU-PDA nanofiber composite as a biosensor in point-of-care applications.

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DEDICATION

I would like to dedicate this thesis to my father Adinath Bhattacharjee.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 INTRODUCTION

π- Conjugated polymers (CP) have been investigated for many applications including solar cells (Günes, Neugebaur, & Sariciftci, 2007), light-emitting diodes (Pei, Yu, Zhang, Yang, & Heeger, 1995), photovoltaic cells (Coakley & McGehee, 2004), biosensors (Feng, Liu, Wang, & Zhu, 2010), chemical sensors (McQuade, Pullen, & Swager, 2000), artificial muscles (Smela, 2003), gas separation techniques (Anderson, Mattes, Reiss, & Kaner, 1991) and in laser technology (Kallinger, et al., 1999), since they were discovered in 1977 (Birgerson, Fahlman, Bröms, & Salaneck, 1996). Due to the delocalized π electrons along their backbone, CPs can transfer nonspecific interactions in the molecular structures into transducible responses (McQuade, Pullen, & Swager, 2000), resulting in interesting optical and electrical properties (Charych, Nagy, Spevak, & Bednarski, 1993), (Yunfeng, et al., 2001). They have great potential in developing biosensing devices due to easy fabrication, improved binding efficiency with ligands from living and non-living organisms along with better stability (Qian & Städler, 2019). CPs can be electrically controllable, which is advantageous in artificial muscle preparation (Smela, 2003). Polydiacetylene (PDA) is an excellent CP that has recently attracted significant attention due to its brilliant chromatic responses at various external stimuli such as mechanical stress, temperature, and chemicals (Chen, et al., 2018). PDAs have been used in vast applications, ranging from microorganism sensing (Charych, Nagy, Spevak, & Bednarski, 1993), (Yapor, et al., 2017) to drug delivery (Li, An, & Yan, 2015), from supercapacitors (Ulaganathan, et al., 2016) to an oil-water separator (Chen, et al., 2018). Alam et al. (2016) and Yapor et al. (2017) reported that PDA nanofiber composites show rapid blue-to-red color transition responding to bacteria, heat, and pH, suggesting great potential in biosensors for bacteria detection.

10, 12- pentacosadiynoic acid (PCDA) has been commonly used as a monomer to prepare PDAs via 1, 4- addition reaction of PCDAs followed by photo-polymerization with UV or γ irradiation. Substrate-based, solution-based, and nanofiber-based PDA sensors have been reported for sensor applications due to easy fabrication, cell-mimicry, stability, and enhanced sensitivity (Qian & Städler, 2019). PDAs can be drop or spin casted or coated onto a substrate such as a paper to prepare a substrate-based PDA sensor. PDAs can also be dissolved in a solvent such as dimethyl sulfoxide (DMSO) and buffer solution HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) to prepare solution-based PDA sensor (Wang, Wang, Wang, Zhang, & Chen, 2015). The solution-based PDA sensors provide advantages in biomedical applications especially due to a high degree of cell mimicry. On the other hand, the substrate-based PDA sensor is relatively stable and sensitive in many sensing applications (Qian & Städler, 2019). Recently, it was reported that PDA nanofibers made via an electrospinning method were able to increase the sensitivity of color changes due to the high aspect ratio inherent in nanofibers (Yapor, et al., 2017). The majority of the detection devices reported in the literature that uses PDAs, added one or more complex receptors to initiate a receptor-ligand interaction that induces a colorimetric transition which signals the stimuli detection. However, the PU-PDA nanofiber composite reported by Yapor et al. (2017) is the first PDA based sensor that can directly detect bacteria without the incorporation of complex receptors or lipids.

When Charych et al. (1993) first reported using PDAs for detecting influenza virus, they suggested that the PDA films undergo colorimetric changes via receptor-ligand interactions. Later, conformational changes in the π conjugated backbone structure in PDAs have been confirmed to be responsible for the color changes in the PDAs due to bacterial stimuli (Yapor, et al., 2017). However, little is known about the bacterial culture components capable of activating such

conformational changes in the PDAs. The activation mechanism of the colorimetric transition in PDAs is still undetermined due to the complexity of a bacterial culture. A laboratory-grown bacterial culture contains growth media, the bacterium, and biomaterials produced by the bacteria during metabolism. These biopolymers are collectively known as extracellular polymeric substances (EPS) and consist of proteins, nucleic acids, polysaccharides, and other polymers (Eboigdin & Biggs, 2008). The EPS that attach to bacterial cells are bound EPS and EPS released in the media during bacterial growth is known as free EPS (Eboigdin & Biggs, 2008). The EPS composition is determined by several factors such as bacterial growth phase, metabolism, and the interaction of a bacterial cell with the environment.

In this study, PDA nanofiber composites will be used to investigate the bacterial culture components that enable the colorimetric transition behaviors in the PDAs. *Escherichia coli* (*E. coli*) will be used in this study because it is commonly found in humans, animals, and the natural environment and is a representative of the *Enterobacteriaceae*, a family of bacteria that can cause serious infections in humans and animals (Jenkins, Rentenaar, Landraud, & Brisse, 2017). For preparing the PDA nanofiber composite, polyurethane (PU) will be mixed with the monomer PCDA as a matrix polymer because pristine PCDA is difficult to convert to nanofibers by electrospinning owing to its low viscosity and spinnability (Yapor, et al., 2017). Colorless PU-PCDA nanofibers will be prepared via electrospinning and then will be photo-polymerized, resulting in blue PU-PDA nanofibers. *E. coli* will be grown in liquid media and then centrifuged and separated, resulting in bacterial culture components such as supernatant fluid and bacterial cell pellets. Free EPS will be extracted, and PU-PDA nanofiber will be tested with a free EPS solution. Critical bacterial concentration (CBC) will be investigated to identify *E. coli* concentration that

can initiate a colorimetric transition in PU-PDA nanofiber. Additionally, PU-PDA nanofiber will be tested with common organic solvents to study the solvatochromism of PDA.

Chapter 2 LITERATURE REVIEW

2.1 Conjugated Polymers

Conjugated polymers (CPs) are popular in many applications such as light-emitting diodes and biosensors due to their superior electrical and optical properties (Yunfeng, et al., 2001). CPs offer opportunities to transfer nonspecific interactions into observable responses (McQuade, Pullen, & Swager, 2000) due to their color transition and fluorescent properties responding to external stimuli such as heat, pH and pathogens ((Chen, Zhou, Peng, & Yoon, 2012); (Yapor, et al., 2017)). CPs have overlapping π-orbitals in molecular structure, resulting in conductivity that can facilitate radiative energy transfer upon light absorption. The changes in electrochemical properties of CPs are demonstrated in visible and fluorescent (colorimetric) properties, which makes CPs an attractive option in sensing applications (Lee, Polvich, & Kim, 2010). A variety of CPs has been reported in the literature for sensing applications, including graphene (Li, Zhang, & Cui, 2015), poly (p-phenylene ethynylene) (Hill, Goswami, Evans, & Schanze, 2012), poly (phenylene vinylene) (Koenen, et al., 2014) and polydiacetylene (Jelinek & Ritenberg, 2013).

2.2 Biosensors

A biosensor is an analytical device that analyzes biological samples and detects their bio-composition, structure, and function by converting biological signals into electrical signals (Guo X. , 2012). The main characteristics of a biosensor include selectivity, precision, sensitivity, and response time (Lee, Polvich, & Kim, 2010). A common example is blood glucose meter, invented by Clarke and Lyons in 1962. A blood glucose meter contains a strip carrying glucose oxidase that can react with glucose from human blood. When a drop of blood is placed on the strip, the glucose in the blood reacts with the glucose oxidase, producing ferrocyanide- a cyanide salt. The

ferrocyanide enables an electrical signal that is converted into a readable digital signal by the glucose meter, indicating the amount of sugar content in the blood sample. Sugar measurements are widely used for self-monitoring of blood sugar levels in diabetic patients (Clarke & Foster, 2012). Recently, biosensors have attracted great interest in detecting pathogens in medical and food preparation (Yapor, et al., 2017). Pathogenic organisms can be identified using a pathogen-detecting biosensor in hospitals, food production, and environment protection. The detection can be fast and effective by using biosensors made from CP. Intensive research works have been conducted in developing biosensors detecting pathogens with conjugated polymers (Alocilja & Radke, 2003).

2.3 Polydiacetylene (PDA)

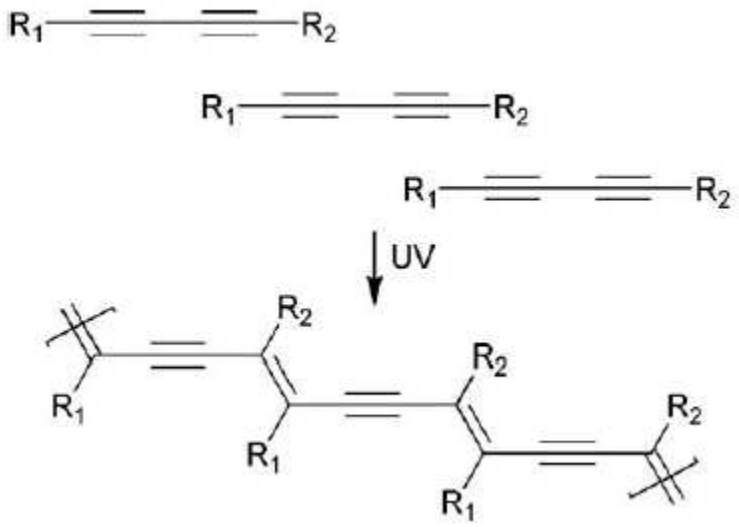


Figure 2-1 Photo-polymerization of diacetylene monomers into PDA *(Reppy & Pindzola, 2007)*

One CP of great interest in biosensor applications is polydiacetylene (PDA). PDA has alternating ene-yne backbone structures (Lee, Yarimaga, Lee, Choi, & Kim, 2011) and can be prepared from diacetylene monomers with a treatment of UV or γ irradiation. Figure 2-1 shows a schematic of the polymerization of the diacetylene monomer via UV-irradiation. Bright blue-to-

red color transitions in PDAs have been reported at environmental perturbations such as high temperature, mechanical stress, organic solvents and ligand-receptor interactions (Alam, Yapor, Reynolds, & Li, 2016); (Charych, Nagy, Spevak, & Bednarski, 1993); (Lee, Yarimaga, Lee, Choi, & Kim, 2011)). The color-changing characteristic of PDAs makes it useful in detecting influenza virus (Charych, Nagy, Spevak, & Bednarski, 1993), bacteria (Oliveiraa, Soaresa, Silva, Andrade, & Medeiros, 2013), proteins (Kolusheva, Kafri, Katz, & Jelinek, 2001) and many other micro-organisms (Pindzola, Nguyen, & Reppy, 2006).

PDAs can be synthesized from diacetylene (DA) monomers (Nava, Thakur, & Tonelli, 1990). Different solvents have been used in the preparation of PDAs such as chloroform (Tobias, Rooke, & Hanks, 2019), acetone (Park, et al., 2019), ethanol (Su, 2006), dimethylformamide (DMF) (Yapor, et al., 2017), tetrahydrofuran (THF) (Kang, et al., 2014), diethyl ether (Li, An, & Yan, 2015) and dichloromethane (Wang, Wang, Wang, Zhang, & Chen, 2015). PDAs have been used in the paper (Yoon, et al., 2011), hydrogel (Guo, et al., 2014), membrane (Li, et al., 2015) , and microbeads (Kang, et al., 2014) for detection applications. Many fabrication methods including casting (Pumtang, Siripornnoppakhun, Sukwattanasinitt, & Ajavakom, 2011), screen-printing (Shin, Yoon, Park, & Kim, 2014), inkjet printing (Yoon, et al., 2011) electrospinning (Yoon, Jung, & Kim, 2009), and microfluidic techniques (Hong, et al., 2015) have been used to fabricate PDA-based sensors. Recently, 3D printing technology was also used to prepare a 3D matrix of hydrogel containing PDAs (Guo, et al., 2014). Although the solution-based PDA sensors are advantageous due to cell mimicry of the nanoparticles in aqueous solutions, the substrate based sensors can overcome the shortcomings of solution-based sensors due to their high sensitivity and stability (Qian & Städler, 2019).

Nanofiber based PDA sensors has unique advantages in their colorimetric responses to external stimuli due to their high aspect ratio (length to diameter ratio) (Yapor, et al., 2017), high sensitivity and stability (Qian & Städler, 2019). The nanofibers can be prepared by a mixture of diacetylene (DA) monomer and a supporting matrix polymer via electrospinning, followed by photo-polymerization. Because the DA monomers have low spinnability owing to their impaired solubility and low viscosity (Yapor, et al., 2017), the use of a matrix polymer is an imperative method to prepare nanofibers. Polyurethane (PU) has been reported to be used in preparing PDA nanofiber composites for wound dressing applications (Khil, Cha, Kim, Kim, & Bhattarai, 2003). PU has superior mechanical properties and biocompatibility (Zdrahala & Zdrahala, 1999). The PU-PDA nanofiber composite is a superhydrophobic fibrous membrane that is mechanically-stable and air-permeable, as well as stable in strong acid or alkaline environment (Chen, et al., 2018). The PU-PDA nanofiber composites have been reported to detect bacterial presence rapidly and effectively (Yapor, et al., 2017).

2.4 Electrospinning

Electrospinning produces nanofibers and microfibers by pushing a charged polymer liquid through a nozzle (spinneret). The polymer forms a Taylor cone when it is released from the nozzle. When a high voltage is applied to the polymer and the electrical force becomes greater than the surface tension of the polymer, a jet is formed from the Taylor cone, and the liquid polymer stretches and turns into micro or nanofiber that is collected on a collector plate or rotating collector drum. A schematic of the electrospinning process is presented in Figure 2-2. Electrospinning produces fibers with diameters ranges in micro and nanometer scale. That is why fibers from electrospinning possess a high aspect ratio providing a large surface area for rapid sensing. In this study, PU-PDA nanofibers were produced using an electrospinning method.

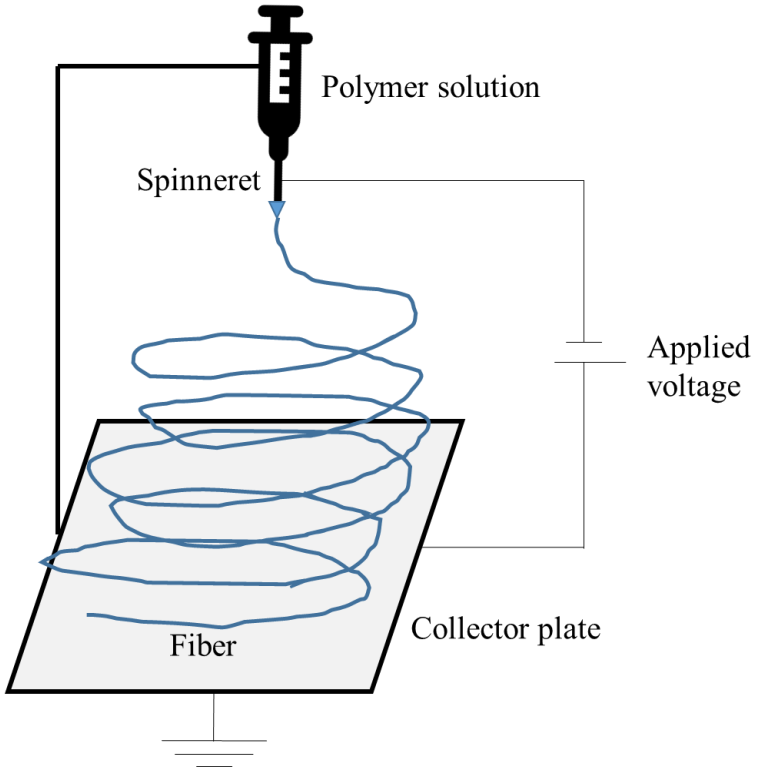


Figure 2-2 Schematic diagram of the setup of a basic electrospinning apparatus.

2.5 Bacteria and problems with a bacterial infection

Bacteria are single-celled organisms that populate the earth for almost 3.5 billion years (Srivastava & Srivastava, 2003, p. 1). They are primarily classified in two large groups, Gram-positive and Gram-negative based on the Gram Stain method developed by Christian Gram (Bartholomew & Mittwer, 1952). Gram-positive bacteria retain the crystal violet stain and appear blue in the Gram stain test (Figure 2-3). On the other hand, gram-negative bacteria lose the crystal violet stain and contain only the counter-stain safranin and thus appear red (Sandle, 2016) (Figure 2-4).

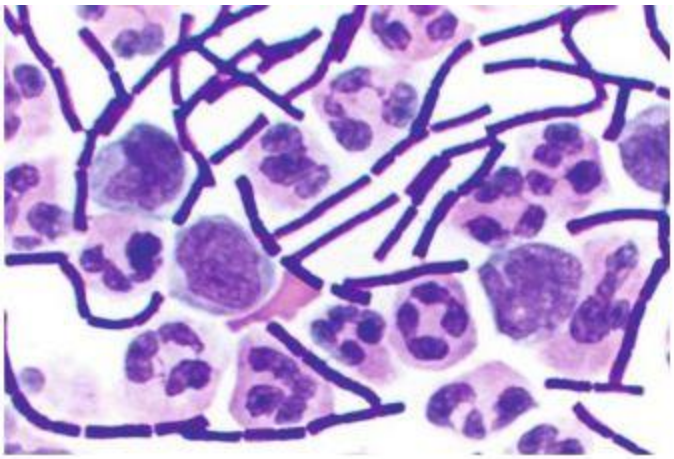


Figure 2-3 Gram-positive bacteria after staining technique *(Sandle, 2016)*.

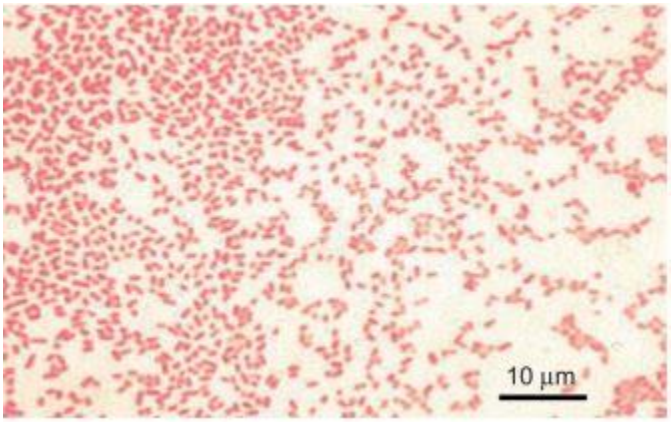


Figure 2-4 Gram-negative bacteria after staining technique *(Sandle, 2016)*.

The bacterial infection causes a significant medical and financial burden due to the rapid emergence of antibiotic resistance strains (Zhou, et al., 2018). Infections of the dermis, including burns, surgical-site infections, and non-healing diabetic foot ulcers, affect approximately 2 million people, cause more than 200,000 deaths, and account for more than $18 billion in direct medical costs in the United States annually (DeLeon, et al., 2014). Worldwide, infectious diseases account for nearly 40% of the total of 50 million annual estimated deaths (Ivnitski, Abdel-Hamid, Atanasov, & Wilkins, 1999). It was reported that PDAs can demonstrate a colorimetric response to *E. coli* (Yapor, et al., 2017), a Gram-negative bacterium commonly found in humans, animals,

and in the natural environment. *E. coli* is a representative of the *Enterobacteriaceae*, a family of bacteria that can cause serious infections in humans and animals (Jenkins, Rentenaar, Landraud, & Brisse, 2017).

2.6 Components in bacterial culture

Bacteria produce several materials in the growth and reproduction phase which can be found in the surrounding environment where the bacteria grow. Generally, a bacterial culture contains the bacterial cells, components of growth media, and the metabolites bacteria produce during the growth phase which includes various proteins, nucleic acids, polysaccharides, and other polymers. The biopolymers bacteria produce during culture are collectively known as extracellular polymeric substances (EPS) (Eboigdin & Biggs, 2008). The EPS that adheres to the bacterial cells are known as bound EPS, and the EPS that are found in the surrounding environment and generally forms the bacterial biopolymer are known as free EPS (Eboigdin & Biggs, 2008). Figure 2-5 shows a schematic representation of bacterial cell pellets and EPS. The EPS composition is determined by several factors such as bacterial growth phase, metabolism, and the interaction of a bacterial cell with the environment. For Gram-negative bacteria such as *E. coli*, lipopolysaccharides (LPS) can also be a component in the culture. LPS constitutes the outer layer of a Gram-negative bacteria and produces endotoxins that can trigger an endotoxic shock in humans resulting in medical complications and death (Qiao, Dong, Wang, Liu, & Ma, 2019).

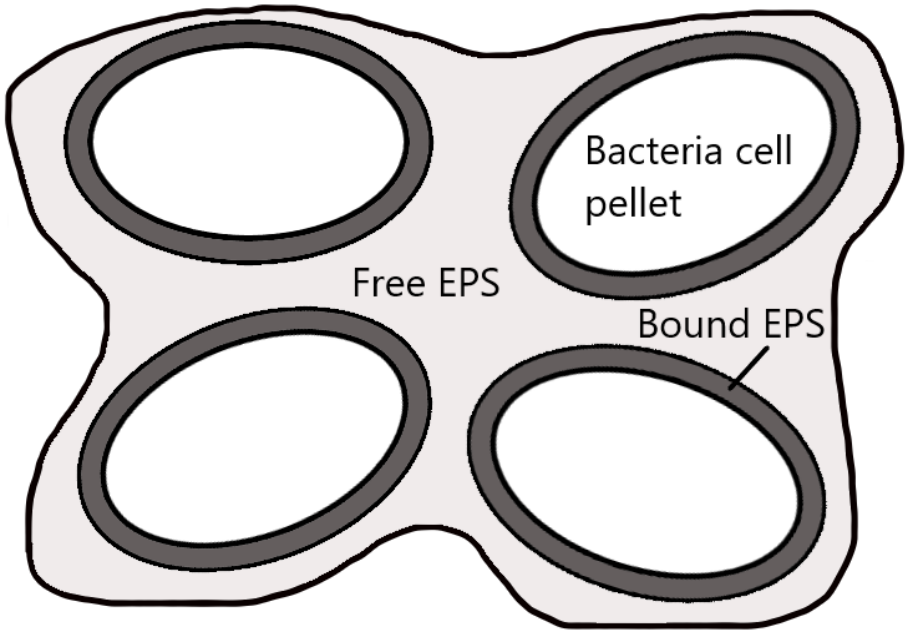


Figure 2-5 Schematic of bacterial cell pellets and EPS

Bacteria produce biofilms by secreting autoinducing agents (homoserine lactones or peptides) that act as a chemical communication system between the environment and bacteria (Barnett & Weir, 2012, p. 251). Bacteria require specific ingredients for growth. Success in bacterial growth relies on their ability to obtain essential nutrients such as a carbon source, an energy source, and other required nutrients. Several culture mediums are used for bacterial growth in vitro. These mediums vary in components and nutrition for bacterial culture and bacterial secretion pathways depend on the type of culture media. A common culture media is Lysogeny Broth (LB) which is nutritionally rich for fast bacterial growth. Sometimes defined culture media is used to specifically understand how bacteria use a component from the media during the growth phase. M9 minimal media is a common defined media that contains only salts and a nitrogen source and is often used to study nutritional aspects of *E. coli* growth when supplemented with different carbon sources and amino acids.

Chapter 3 EXPERIMENTAL SECTION

3.1 Materials

10, 12- pentacosadiynoic acid (PCDA, 98%, GFS Organics, Columbus, OH) will be used as a monomer for preparing PDA. Thermoplastic polyurethane (PU) will be purchased from The Lubrizol Corporation (Wickliffe, OH) to be used as a matrix polymer. Tetrahydrofuran (THF, 99%) and N, N-dimethylformamide (DMF, 99.8%, extra dry, AcroSeal) will be purchased from Fisher Scientific (Waltham, MA) for preparing the solvent system to synthesize PDA nanofibers.

E. coli ATCC25922 (1.0×10^4 CFU/pellet) will be purchased from ATCC (Manassas, VA). Lysogeny Broth (LB) and M9 minimal media will be used for the growth of *E. coli*. The ingredients of LB media will include tryptone (Fisher, Waltham, MA), NaCl (Fisher, Waltham, MA), yeast extract (Fischer, Waltham, MA), and distilled water. The ingredients of M9 minimal media will include M9 minimal salt (DIFCO, Sparks, MA), distilled water, 20% arabinose solution (Sigma-Aldrich, St. Louis, MO), 1.0 M $MgCl_2$ (Anhydrous, minimum 98%, Sigma Chemical Co., St. Louis, MO) solution, 1 mg/ml Thiamine (Sigma-Aldrich, St. Louis, MO) and 1.0 M $CaCl_2$ (Anhydrous, Granular, 12 Mesh, J. T. Baker Chemical Co., Philadelphia, PA) solution. For size fractionation test, Amicon® Ultra-4 Centrifugal Filter Units having different molecular weight cutoffs will be purchased from Sigma Aldrich (St. Louis, MO). Organic solvents that will be used to study solvatochromism include hexanes, xylenes, isopropyl alcohol (99.5%), 1-butanol, tetrahydrofuran (THF), methanol, ethanol (200 proof, molecular biology grade), glacial acetic acid, N, N- dimethylformamide (DMF), which will be purchased from Fisher Scientific (Waltham, MA); and Chloroform ($CHCl_3$, >99.8%), acetone (≥99.5%) and dimethyl sulfoxide (DMSO)

(≥99.9%) will be purchased from Sigma-Aldrich (St. Louis, MO). Distilled water will be used as a control.

3.2 Methods

3.2.1 Electrospinning of PU-PDA nanofiber composite

Electrospinning solutions will be prepared using a method adopted from Yapor et al. (2017). Briefly, a fixed amount of PU and PCDA will be mixed in THF and will stir overnight. Then, the mixture will be added to DMF and will stir overnight again until a fine pink homogeneous solution of PU-PCDA will be yielded. A customized electrospinning apparatus will be used to produce PU-PDA nanofiber composite mats. The apparatus primarily consists of a Gamma High Voltage Research ES50P power supply and a Harvard PHD 2000 syringe pump. For electrospinning, a PU-PCDA solution loaded in a syringe will be injected by the syringe pump at a constant speed and an electrical force will be generated by the power supply. The spinning time will be 1 h for each mat and nanofibers will be collected on an aluminum collector plate that will be placed at a collection distance of 21 cm. A non-woven mat consisting of colorless PU-PCDA nanofibers will be obtained. The PU-PCDA nanofiber mat will then be exposed to a UV light (Spectroline, Westbury, NY) at 254 nm for 3 min. The PCDAs in the nanofibers will be photo-polymerized by the UV irradiation, resulting in PDAs mixed with PU in the nanofibers. The color of the PU-PDA nanofiber mats will turn blue after the photo-polymerization. All the PU-PDA nanofiber mats after photo-polymerization will be stored in the dark to avoid unwanted continuous photo-polymerization for future tests.

3.2.2 Fiber morphology characterization

The morphology of PU-PDA nanofibers will be analyzed before and after the tests with the components of *E. coli* culture using a Scanning Electron Microscope (SEM) (JEOL JSM-6500F

Field Emission Scanning Electron Microscope). PU-PDA nanofiber mats will be kept in room temperature in dark for 24 h to evaporate any residual solvent or moisture. Before imaging, they will be sputter-coated with gold (Au) for 33 s using Denton Vacuum- DESK 2 Sputter Coater to improve conductivity. The average diameters of the fibers will be calculated from SEM images using ImageJ software.

3.2.3 Culture media preparation

LB culture medium will be prepared by combining tryptone, NaCl, and yeast extract in 500 ml of distilled water and will sterilize by autoclaving at 250° F at 23 psi pressure for 20 min. For preparing M9 minimal media, M9 minimal salt will be dissolved in 1 L distilled water and will sterilize at 250° F at 23 psi pressure for 20 min. Then, 200 ml of the minimal salt solution will be diluted by adding 800 ml of sterilized water to prepare a 1 L solution. After that, filter-sterilized 20% arabinose solution, 1.0 M $MgSO_4$, 1 mg/ml thiamine solution, and 1.0 M $CaCl_2$ solution will be added to the diluted minimal salt solution, resulting in M9 minimal media. All the filter-sterilization will be conducted using 0.2 µm syringe polyethersulfone (PES) filters. Both LB media and M9 minimal media will be used to grow *E. coli* for testing the colorimetric properties of PU-PDA nanofibers.

3.2.4 Preparation of components in *E. coli* culture

A premade *E. coli* ATCC25922 pellet will be hydrated in the hydration liquid following the manufacturer's procedures. After hydration, the mixture will be vortexed and added to a 250 ml LB medium following incubation at 37°C for 24 h. After that, an inoculation loop will be used to transfer the bacteria to an LB streak plate and will be placed in an incubator to grow overnight at 37°C. Next, a single colony will be taken from the streak plate and will be placed in 500 ml LB medium following incubation at 37°C by a shaker incubator for 24 h. The *E. coli* culture will be

centrifuged at 11,984 × g for 10 min at 27°C. The supernatant fluid will be collected, filtered (0.2 µm syringe PES filters), and divided evenly into two flasks. One half of the supernatant fluid will be autoclaved at 250°F at 23 psi for 20 min and the other half will be kept in the incubator at room temperature. On the other hand, the bacterial cell pellets after centrifuging will be washed twice using a 0.2% sterile saline solution and will be centrifuged again at 11,984 × g for 10 min at 27°C. The cell pellets will then be dispersed in a 0.9% saline solution and will be separated into two flasks. One half of the cell pellet solution will be autoclaved at 250°F at 23 psi for 20 min and the other half will be kept in room temperature for testing. Besides, two supplementary batches of supernatant fluids will be prepared following the same procedures described above. One batch of supernatant fluids (including both autoclaved and non-autoclaved) will be used to treat PU-PDA nanofibers at 24 h (t_s = 24 h) storage time in room temperature. The other batch will be kept for 36 h (t_s = 36 h) of storage time. *E. coli* culture in M9 minimal media will be prepared and divided into four component solutions following the procedure described for LB. To summarize, the components of bacteria culture will include (1) autoclaved supernatant fluids, (2) non-autoclaved supernatant fluids, (3) autoclaved bacterial cell pellets, and (4) non-autoclaved bacterial cell pellets from both LB and M9 minimal media. LB and M9 minimal media solutions will be used as controls.

3.2.5 Size fractionation of supernatant fluid

Supernatant and autoclaved supernatant fluids will be prepared using the methods described above. 4 ml of supernatant fluid will be taken into each filter and will be centrifuged at 4,000 × g for 15 min using a bucket centrifuge device. The filtrate fluid will be collected and will be used for testing with PU-PDA nanofibers. The same procedures will be followed for autoclaved supernatant fluid.

3.2.6 Free EPS extraction

Free EPS will be extracted from the supernatant fluid using a method adopted from Eboigbodin and Biggs (2008). The supernatant fluid will be centrifuged for 30 min at 11,984 × g at 4°C will be decanted and ethanol will be mixed with the decanted solution. The container will be inverted 24 times following storage at -20º C for 18 h. Then, the precipitation will be pelleted by centrifugation at 11,984 × g for 15 min at 4º C, resulting in free EPS. The free EPS pellet will be suspended in 70% ethanol and the suspension will be washed with distilled water. Residual ethanol will be evaporated from free EPS suspension by keeping it at room temperature for 48 h. 5 ml distilled water will be added to the suspension and then will be vortexed to make a solution. The resultant solution of free EPS will be used to test PU-PDA nanofibers.

3.2.7 Colorimetric transition behavior of PU-PDA nanofibers at bacterial culture components

The nanofiber samples will be immersed in bacterial component solutions including (1) autoclaved supernatant fluids, (2) non-autoclaved supernatant fluids, (3) bacterial cell pellets, and (4) non-autoclaved bacterial cell pellets. The immersion time will vary in a range of 1, 2, 3, 30, 60, 90, 120, 150, 180 min, and 24 h. Colorimetric properties of the PU-PDA nanofibers upon testing with bacterial components will be evaluated first visually and then using a spectrophotometer (ColorQuest, Hunter Lab). A PU-PDA nanofiber mat (2 inch X 2 inch) will be placed inside a sterile petri dish following photograph and spectrophotometer measurements before testing with a bacterial component. Then a bacterial component solution (approximately 25 ml) will be poured slowly on the nanofiber mat until the mat will completely be immersed. After 1 min, the nanofiber mat will be taken out and will be kept in another sterile petri dish to take photograph and spectrophotometer measurement (t = 1 min). After that, the nanofiber mat will be

immersed back into the bacterial component solution and will be taken out again after 1 min to take photograph and spectrophotometer measurement (t = 2 min). The process will be repeated to take photographs and spectrophotometer measurements for each of the measurement times (t = 1, 2, 3, 30, 60, 90, 120, 150, 180 min, and 24 h) and each of the treatment with bacterial components (autoclaved supernatant fluids, non-autoclaved supernatant fluids, autoclaved bacterial cell pellets, and non-autoclaved bacterial cell pellets). Fiber mats will also be immersed in LB and M9 minimal media (having no bacteria and bacterial secretions) for control testing. All the tests will be repeated 3 times and the average values of spectrophotometer measurements will be taken for further calculations. From the values of spectrophotometer measurements, colorimetric response percentage (CR%) will be calculated using the method described by Yapor et al. (2017). All the tests will be conducted at room temperature. Extra precautions will be taken to cool down the autoclaved fluids to room temperature before testing.

3.2.8 Lipopolysaccharide (LPS) solution preparation

Lipopolysaccharide (LPS) powder will be suspended in 1 ml of distilled water and 1 ml of LB broth at a 1:10 ratio for each solution. Then, the solutions will be used to test the PU-PDA nanofibers in a tissue culture well. The nanofiber mats will be immersed in the LPS solutions for 72 hours.

3.2.9 Critical bacterial concentration (CBC)

To calculate the concentration of *E. coli* required for initiating a colorimetric transition in PU-PDA nanofibers, the LB medium will be used to culture *E. coli*. From the beginning of the culture (0 h), optical density (OD) reading using Thermo Scientific™ GENESYS™ 20 Visible Spectrophotometer will be collected at 600nm with a one-hour interval. When a significant change in OD600 reading will be observed, the PU-PDA nanofiber will be tested with the *E. coli* culture solution instantly and reflectance data will be collected by the method described earlier. *E. coli*

CFU/ml and PU-PDA nanofibers' CR% will be calculated from OD600 readings and reflectance measurements respectively.

3.2.10 Colorimetric transition behavior of PU-PDA nanofibers in common organic solvents

A PU-PDA nanofiber mat (2 inch X 2 inch) will be placed inside a petri dish; photograph and spectrophotometer measurement will be taken before testing with an organic solvent. Then one organic solvent (approximately 25 ml) will be poured slowly on the nanofiber mat until complete immersion will be observed. The nanofiber mat will be immersed in the solvent for at least 30 min. Then the nanofiber mat will be taken out of the petri dish and will be placed in a new sterile petri dish for photography and spectrophotometer measurement. The experiment will be repeated three times for each of the solvents. Then CR% will be calculated from the spectrophotometer data.

Chapter 4 RESULTS AND DISCUSSION[1]

Synopsis

Polydiacetylene (PDA) demonstrates colorimetric transition behavior due to conformational changes in π conjugated backbone of PDA macromolecules at external stimuli of bacteria, suggesting potential applications in biosensors. To characterize the nature of bacterial culture components and their interaction with PDA, PU-PDA nanofiber composites were tested with different bacterial components produced by the growth of *E. coli* in both a defined and complex culture media following autoclave treatment. The results indicated that the color-changing substances (CCS) were not the bacterial cells but were present in the spent media (supernatant fluid) and were probably due to the extracellular polymeric substances (EPS) *E. coli* produced during culture. While treated with autoclaved supernatant fluid, the colorimetric transition of the nanofibers intensified significantly, suggesting a hypothesis that the CCS is not a protein, DNA, or RNA because they typically denature while autoclaving. Storage of the supernatant fluid for 24 and 36 hours resulted in a significant reduction of the CCS activities. The detection sensitivity of the PU-PDA nanofiber composite was determined to be approximately 9 x 10^8 CFU/ml *E. coli* concentration. Color changes in the PDA nanofiber composite occurred with exposure to several organic solvents with exceptions of hexane, methanol, and water. The study provides a guideline for developing point-of-care devices such as smart wound dressing using PDA nanofiber composites capable of in-situ bacterial-detection.

[1] The major content of chapter 4 is a manuscript that has been submitted to ACS Sensors for publication.

Keywords: Polydiacetylene (PDA), colorimetric transition, supernatant fluid, bacterial cell pellet, extra-cellular polymeric substances (EPS), color-changing substances (CCS), point-of-care, biosensor, solvatochromism.

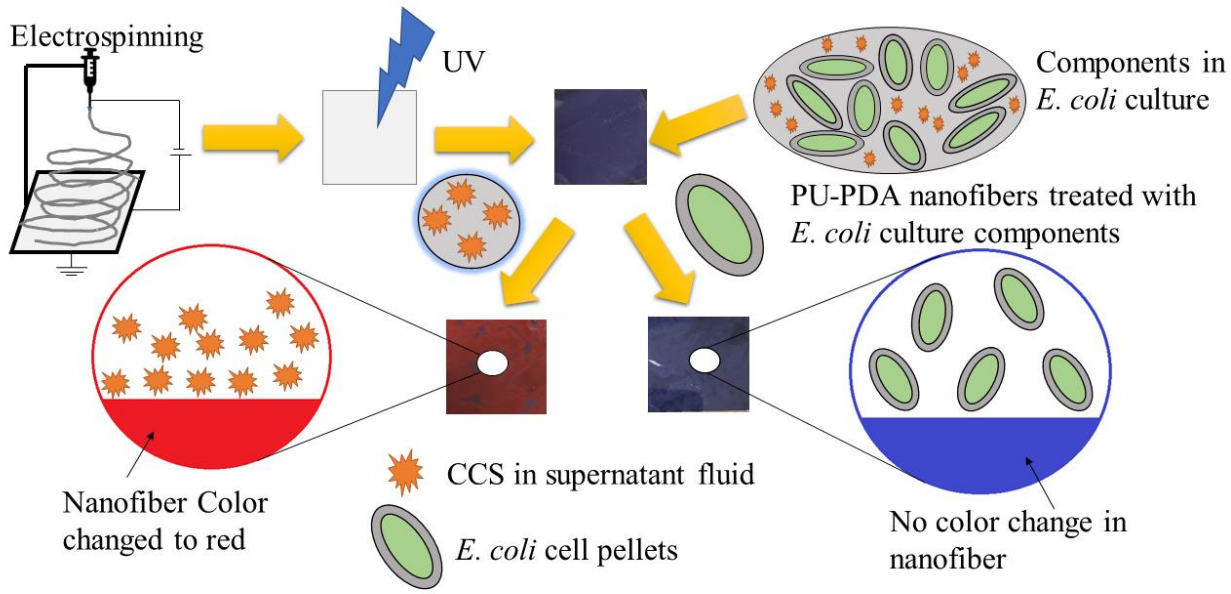


Figure 4-1 Graphical abstract

4.1 Introduction

π - conjugated polymers (CP) with unique optical and electrochemical properties have been used in several applications including solar cells [1], light-emitting diodes [2], photovoltaic cells [3], sensors [4], artificial muscles [5], and laser technology [7]. Due to the delocalized π electrons along the macromolecular backbone, CPs can transfer nonspecific interactions in the molecular structures into transducible responses [7], resulting in characteristic optical and electrical properties. Polydiacetylene (PDA) is an attractive CP due to its quick chromatic responses at various external stimuli such as solvents, temperature, electricity, light, mechanical stress, chemical stress, and bacteria [8]. PDAs have been used in microorganism sensing [9], drug

delivery [10], supercapacitor [11], and oil-water separations [12]. Alam et al. [13] and Yapor et al. [14] reported that PDA nanofiber composites show rapid blue-to-red color transition responding to bacteria, heat, and pH, suggesting their potential for use in biosensor applications, especially in developing point-of-care devices such as smart wound dressings that can be used to detect bacterial infection in wound care. Point-of-care devices are recently of great interest in hospitals, battlefields, and nursing homes owing to many advantages over traditional testing technologies, such as simplicity, affordability, and effective and efficient patient care [15].

10, 12- pentacosadiynoic acid (PCDA) has been commonly used as a monomer to prepare PDAs via 1, 4- addition reaction of PCDAs followed by photo-polymerization by UV or γ irradiation. Substrate-based, solution-based, and nanofiber-based PDA sensors have been reported for sensor applications due to easy fabrication, cell-mimicry, stability, and enhanced sensitivity [8]. PDAs can be drop/spin casted or coated onto a substrate such as a paper to prepare a substrate-based PDA sensor. PDAs can also be dissolved in a solvent such as dimethyl sulfoxide (DMSO) and buffer solution HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) to prepare a solution-based PDA sensor [16]. The solution-based PDA sensors provide advantages in biomedical applications especially due to a high degree of cell mimicry. On the other hand, the substrate-based PDA sensor is relatively stable and sensitive in many sensing applications [8]. Recently, it was reported that PDA nanofibers made via an electrospinning method were able to increase the sensitivity of color changes due to the high aspect ratio inherent in nanofibers [14].

Conformational changes in the π conjugated backbone structure in PDAs have been confirmed to be responsible for the color changes in the PDAs due to bacterial stimuli [14]. However, little is known about the bacterial culture components capable of activating such conformational changes in the PDAs. The activation mechanism of the colorimetric transition in

PDAs is still undetermined due to the complexity of a bacterial culture. A laboratory-grown bacterial culture contains growth media, the bacterium, and biomaterials produced by the bacteria during metabolism. These biopolymers are collectively known as extracellular polymeric substances (EPS) and consist of proteins, nucleic acids, polysaccharides, and other polymers [17]. The EPS that attach to bacterial cells are bound EPS and EPS released in the media during bacterial growth is known as free EPS [17]. The EPS composition is determined by several factors such as bacterial growth phase, metabolism, and the interaction of a bacterial cell with the environment.

In this study, PDA nanofiber composites were used to investigate the bacterial culture components that enabled the colorimetric transition behaviors in the PDAs. *Escherichia coli* (*E. coli*) was chosen in this study because it is commonly found in humans, animals, and the natural environment and is a representative of the *Enterobacteriaceae*, a family of bacteria that can cause serious infections in humans and animals [18]. For preparing the PDA nanofiber composite, polyurethane (PU) was mixed with the monomer PCDA as a matrix polymer because pristine PCDA is difficult to convert to nanofibers by electrospinning owing to its low viscosity and spinnability [14]. Colorless PU-PCDA nanofibers were prepared via electrospinning and then were photo-polymerized, resulting in blue PU-PDA nanofibers. *E. coli* was grown in liquid media and then centrifuged and separated, resulting in supernatant fluid and bacterial cell pellets. It was found that the colorimetric response (CR%) in the nanofibers tested with supernatant fluid was significant (CR = 10%). The colorimetric response of the nanofibers was further increased to 28% by the autoclaved supernatant fluid. In comparison, no color change was found in testing with bacterial cell pellets. The results indicated that the supernatant fluid contained the color-changing substances (CCS), which were in free EPS and most likely not the proteins, DNA, or RNA in bacterial culture because they typically (except for eukaryotic prion proteins) denature in extreme

heat and pressure from autoclaving. Further experiments also indicated that lipopolysaccharide (LPS) from the outer layer of gram-negative *E. coli* was not the CCS. The colorimetric response of the nanofibers significantly reduced with an increase in supernatant fluid's storage time, suggesting the CCS had a time-dependent decay. The extracted supernatant fluid was also filtered based on molecular weights. Results indicated that the CCS in the supernatant fluid can be a combination of multiple substances having different molecular weights. Interestingly, no color change was observed when a minimal media supplemented with arabinose (due to the auxotrophy of *E. coli*) was used, suggesting that the CCS were not released by *E. coli* in the spent media when grown in low nutrient culture. Critical bacterial concentration (CBC) that was defined as a concentration initiating a colorimetric transition in the nanofibers was found to be approximately 9×10^8 CFU/ml, suggesting the viability of using the PU-PDA nanofiber composite as a biosensor. Besides, color changes were observed when the nanofibers were tested with common organic solvents, demonstrating solvatochromism in PDAs.

4.2 Experimental Section

4.2.1 Materials

10, 12- pentacosadiynoic acid (PCDA, 98%, GFS Organics, Columbus, OH) was used as a monomer for preparing PDA. Thermoplastic polyurethane (PU) was purchased from The Lubrizol Corporation (Wickliffe, OH) to be used as a matrix polymer. Tetrahydrofuran (THF, 99%) and N, N-dimethylformamide (DMF, 99.8%, extra dry, AcroSeal) were purchased from Fisher Scientific (Waltham, MA) for preparing the solvent system to synthesize PDA nanofibers.

E. coli ATCC25922 ($1.0 \text{ X } 10^4$ CFU/pellet) was purchased from ATCC (Manassas, VA). Lysogeny Broth (LB) and M9 minimal media were used for the growth of *E. coli*. For size fractionation test, Amicon® Ultra-4 Centrifugal Filter Units having molecular weight cutoffs of 3k, 30k and 100k Da were purchased from Millipore Sigma (Burlington, MA). Prepared

lipopolysaccharide (LPS) from *E. coli* was purchased from Millipore Sigma (Burlington, MA). Organic solvents that were used to study solvatochromism included hexanes, xylenes, isopropyl alcohol, 1-butanol, tetrahydrofuran (THF), methanol, ethanol (200 proof), glacial acetic acid, N, N- dimethylformamide (DMF), chloroform ($CHCl_3$), acetone and dimethyl sulfoxide (DMSO). Distilled water was used as control. All the chemicals and solvents were used without further purification.

4.2.2 Methods

PU-PDA nanofibers were prepared via electrospinning and then tested with *E. coli* culture components as well as with organic solvents. Figure 4-2 shows a schematic of the experimental design.

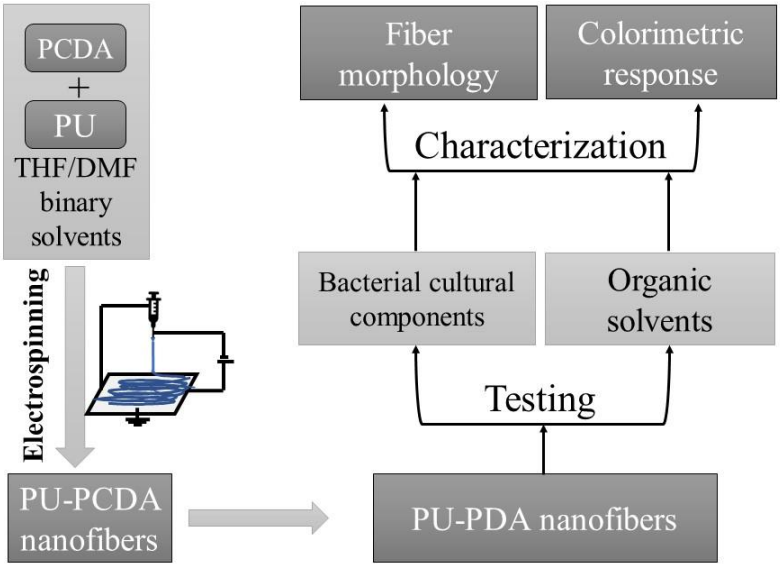


Figure 4-2 Schematic of experimental design

4.2.2.1 Electrospinning of PU-PDA nanofibers

Electrospinning solutions were prepared using a method adopted from Yapor et al. [14]. Briefly, 2 g PU and 1 g PCDA were mixed in 8.34 ml THF and stirred overnight at 1200 rpm and

55º C. Then, the mixture was added to 8.34 ml DMF and was stirred overnight until a fine pink homogeneous solution of PU-PCDA was yielded. A customized electrospinning apparatus was used to produce PU-PDA nanofiber mats. The apparatus primarily consists of a Gamma High Voltage Research ES50P power supply and a Harvard PHD 2000 syringe pump. For electrospinning, a PU-PCDA solution loaded in a syringe was injected by the syringe pump at a constant speed of 0.12 ml/h and a 12-kV electrical force was generated by the power supply. The spinning time was 1 h for each mat and nanofibers were collected on an aluminum collector plate placed at a collection distance of 21 cm. A non-woven mat consisting of colorless PU-PCDA nanofibers was obtained. The PU-PCDA nanofiber mat was then exposed to a UV light (Spectroline, Westbury, NY) at 254 nm for 3 min. The PCDAs in the nanofibers were photo-polymerized by the UV irradiation, resulting in PDAs mixed with PU in the nanofibers. The color of the PU-PDA nanofiber mats became blue after the photo-polymerization. All the PU-PDA nanofiber mats after photo-polymerization were stored in the dark to avoid any unwanted continuous photo-polymerization for future tests.

4.2.2.2 Fiber morphology characterization

The morphology of PU-PDA nanofibers was analyzed before and after the tests with E. coli culture components using a Scanning Electron Microscope (SEM) (JEOL JSM-6500F Field Emission Scanning Electron Microscope). PU-PDA nanofiber mats were kept in room temperature in dark for 24 h to evaporate any residual solvent or moisture. Before imaging, they were sputter-coated with gold (Au) for 33 s using Denton Vacuum- DESK 2 Sputter Coater to improve conductivity. The average diameters of the fibers were calculated from SEM images using ImageJ software.

4.2.2.3 Culture media preparation

LB culture medium was prepared by combining 5 g of tryptone, 2.5 g of NaCl, and 2.5 g of yeast extract in 500 ml of distilled water and sterilized by autoclaving at 250° F at 23 psi pressure for 20 min. For preparing M9 minimal media, 56.4 g M9 minimal salt was dissolved in 1 L distilled water and sterilized at 250° F at 23 psi pressure for 20 min. Then, 200 ml of minimal salt solution was diluted by adding 800 ml of sterilized water to prepare a 1 L solution. After that, 10 ml filter-sterilized 20% arabinose solution, 2 ml filter-sterilized 1.0 M $MgSO_4$, 10 ml filter-sterilized 1 mg/ml thiamine solution and 0.1 ml filter-sterilized 1.0 M $CaCl_2$ solution were added to the diluted minimal salt solution, resulting in M9 minimal media. All the filter-sterilization was conducted by using 0.2 µm syringe polyethersulfone (PES) filters. Both LB media and M9 minimal media were used to grow *E. coli* for testing the colorimetric properties of PU-PDA nanofibers.

4.2.2.4 Preparation of E. coli culture components

A premade *E. coli* ATCC25922 pellet was hydrated in the hydration liquid following the manufacturer's procedures. After hydration, the mixture was vortexed and added to 250 ml LB medium and incubated at 37°C for 24 h. After that, an inoculation loop was used to transfer the bacteria to an LB streak plate and placed in an incubator to grow overnight at 37°C. Next, a single colony was taken from the streak plate and placed in 500 ml LB medium and incubated at 37°C by a shaker incubator for 24 h. The *E. coli* culture then was centrifuged at 11,984 × g for 10 min at 27°C. The supernatant fluid was collected, filtered (0.2 µm syringe polyethersulfone filters), and divided evenly into two flasks. One half of the supernatant fluid was autoclaved at 121.11°C at 23 psi for 20 min and the other half was kept in the incubator at room temperature. On the other hand, the obtained bacterial cell pellet after centrifugation was washed twice using a 0.2% sterile saline solution and centrifuged again at 11,984 × g for 10 min at 27°C. The cell pellets were then dispersed in a 0.9% saline solution and separated into two flasks. One half of the cell pellet solution

was autoclaved at 121.11°C at 23 psi for 20 min and the other half was kept at room temperature. Besides, two supplementary batches of supernatant fluid were prepared following the same procedures described above. One batch of supernatant fluid (including both autoclaved and non-autoclaved) was used to treat PU-PDA nanofibers at 24 h (t_s = 24 h) storage time at room temperature. The other batch was kept for 36 h (t_s = 36 h) of storage time. *E. coli* culture in M9 minimal media was prepared and divided into four component solutions following the procedure described for LB. To summarize, the *E. coli* culture components included (1) autoclaved supernatant fluid, (2) non-autoclaved supernatant fluid, (3) autoclaved bacterial cell pellets, and (4) non-autoclaved bacterial cell pellets from both LB and M9 minimal media. LB and M9 minimal media solutions were used as controls.

4.2.2.5 Size fractionation of supernatant fluid

Supernatant and autoclaved supernatant fluid were prepared using the methods described above from LB culture media. 4 ml of non-autoclaved supernatant fluid added to each Amicon® Ultra-4 Centrifugal Filter Unit having molecular weight cutoffs of 3k, 30k, and 100k Da and centrifuged at 4,000 × g for 15 min according to the manufacturer's procedures. The filtrate fluid was collected and used for testing PU-PDA nanofibers. The same procedures were followed for autoclaved supernatant fluid. Six fluid samples containing molecules with a molecular weight less than 3k, 30k, and 100k Da were collected to test on PU-PDA nanofibers. The fluid that did not pass through the 100k filter device having a molecular weight greater than 100k Da was also collected from the supernatant and autoclaved supernatant fluid to test on PU-PDA nanofibers.

4.2.2.6 Free EPS extraction

Free EPS was extracted from the supernatant fluid using a method adopted from Eboigbodin and Biggs [17]. Supernatant fluid from LB culture media was centrifuged for 30 min at 11,984 × g and 4°C, decanted, and ethanol was mixed with the decanted solution. After the

container was inverted 24 times and stored at -20ºC for 18 h, the precipitate was pelleted by centrifugation at 11,984 × g for 15 min at 4ºC, resulting in free EPS. The free EPS pellet was suspended in 70% ethanol and the suspension was washed with distilled water. Residual ethanol was evaporated from free EPS suspension by keeping it at room temperature for 48 h. 5 ml distilled water was added to the suspension and vortexed. The resultant solution was used to test on PU-PDA nanofibers.

4.2.2.7 Colorimetric transition behaviors of PU-PDA nanofibers

The nanofiber samples were immersed in *E. coli* culture component solutions including (1) autoclaved supernatant fluid, (2) non-autoclaved supernatant fluid, (3) bacterial cell pellets, and (4) non-autoclaved bacterial cell pellets. The immersion time was varied in a range of 1, 2, 3, 30, 60, 90, 120, 150, 180 min, and 24 h. Colorimetric properties of the PU-PDA nanofibers upon testing with bacterial culture components were evaluated first visually and then using a spectrophotometer (ColorQuest, Hunter Lab). A PU-PDA nanofiber mat (2 inch X 2 inch) was placed inside a sterile petri dish, photographed and a spectrophotometer measurement was taken before testing. Then each culture component solution (25 ml) was poured slowly on the nanofiber mat until the mat was completely immersed. After 1 min, the nanofiber mat was taken out and placed in another sterile petri dish for photograph recording and spectrophotometry measurement (t = 1 min). After that, the nanofiber mat was immersed back into the same culture component solution for an additional 1 min, and then photographed and measured with a spectrophotometer (t = 2 min). This process was repeated, and photographs and spectrophotometer measurements were taken for each of the measurement times (t = 1, 2, 3, 30, 60, 90, 120, 150, 180 min, and 24 h) and treatment with each culture component. Fiber mats were also immersed in LB and M9 minimal media (having no *E. coli* cells or materials produced by *E. coli*) for control testing. Besides, the free EPS solution was used to test with PU-PDA nanofibers as well. All the tests were

repeated three times and the average values of spectrophotometer measurements were taken for further calculations. Colorimetric response percentage (CR %) was calculated using the method described by Yapor et al. [14]. Reflectance data obtained in the spectrophotometer measurement was first converted to absorbance using equation 1:

$$Absorbance = \log\left[\frac{1}{\frac{Reflectance}{100}}\right] \quad \text{Equation 1}$$

Then, the percentage blue (PB) values were calculated using equation 2:

$$PB = \frac{A_{blue}}{A_{red}+A_{blue}} \quad \text{Equation 2}$$

Where A_{red} is the absorbance value at the color of red (540 nm) and A_{blue} is the absorbance value at the color of blue (640 nm). The colorimetric response percentage (CR %) was calculated using equation 3:

$$CR\% = \left[\frac{PB_{\circ}-PB}{PB_{\circ}}\right] \times 100\% \quad \text{Equation 3}$$

Where PB_o is the percentage blue value for the nanofiber mats before testing with bacterial stimuli and PB is the percentage blue value after the testing. All the tests were conducted at room temperature. Extra precautions were taken to cool down the autoclaved fluids to room temperature before they were used in tests.

4.2.2.8 Lipopolysaccharide (LPS) solution preparation

Lipopolysaccharide (LPS) powder was suspended in 1 ml of distilled water and 1 ml of LB broth at a 1:10 ratio for each solution. Then, the solutions were used to test the PU-PDA nanofibers in a tissue culture well. The nanofiber mats were immersed in the LPS solutions for 72 hours.

4.2.2.9 Critical bacterial concentration (CBC)

To calculate the concentration of *E. coli* required for initiating a colorimetric transition in PU-PDA nanofibers, the LB medium was used to culture *E. coli*. From the beginning of the culture (0 h), optical density (OD) reading using Thermo Scientific™ GENESYS™ 20 Visible Spectrophotometer was collected at 600nm with a one-hour interval. When a significant change in OD600 reading was observed, the PU-PDA nanofiber was tested with the *E. coli* culture solution instantly and reflectance data was collected by the method described earlier. *E. coli* CFU/ml and PU-PDA nanofibers' CR% were calculated from OD600 readings and reflectance measurements respectively.

4.2.2.10 Colorimetric transition behaviors of PU-PDA nanofibers in common organic solvents

A PU-PDA nanofiber mat (2 inch X 2 inch) was placed inside a petri dish, photographed and spectrophotometer measurement was taken before testing with an organic solvent. Then one organic solvent (25 ml) was poured slowly on the nanofiber mat until it was completely immersed. The nanofiber mat was immersed in the solvent for at least 30 min. Then the nanofiber mat was taken out of the petri dish and placed in a new sterile petri dish for photography and spectrophotometer measurement. The experiment was repeated three times for each of the 12 solvents studied. Then CR% was calculated from the spectrophotometer data.

4.3 Results and Discussion

4.3.1 Fiber morphology

Figure 4-3 shows the SEM images of PU nanofiber, PU-PDA nanofiber, and the nanofibers interacting with different bacterial components. The PU nanofibers had a diameter ranging from 200-300nm. The addition of PDA in the PU nanofibers increased the resultant fiber diameter ranging from 200-2000nm. A flake-like structure on the PU-PDA fiber surface (Figure 4-3 (b-f)) was found compared to the PU nanofibers [19]. The interaction of the nanofibers with different

bacterial components did not significantly change the fiber morphology as shown in Figure 4-3 (c-f).

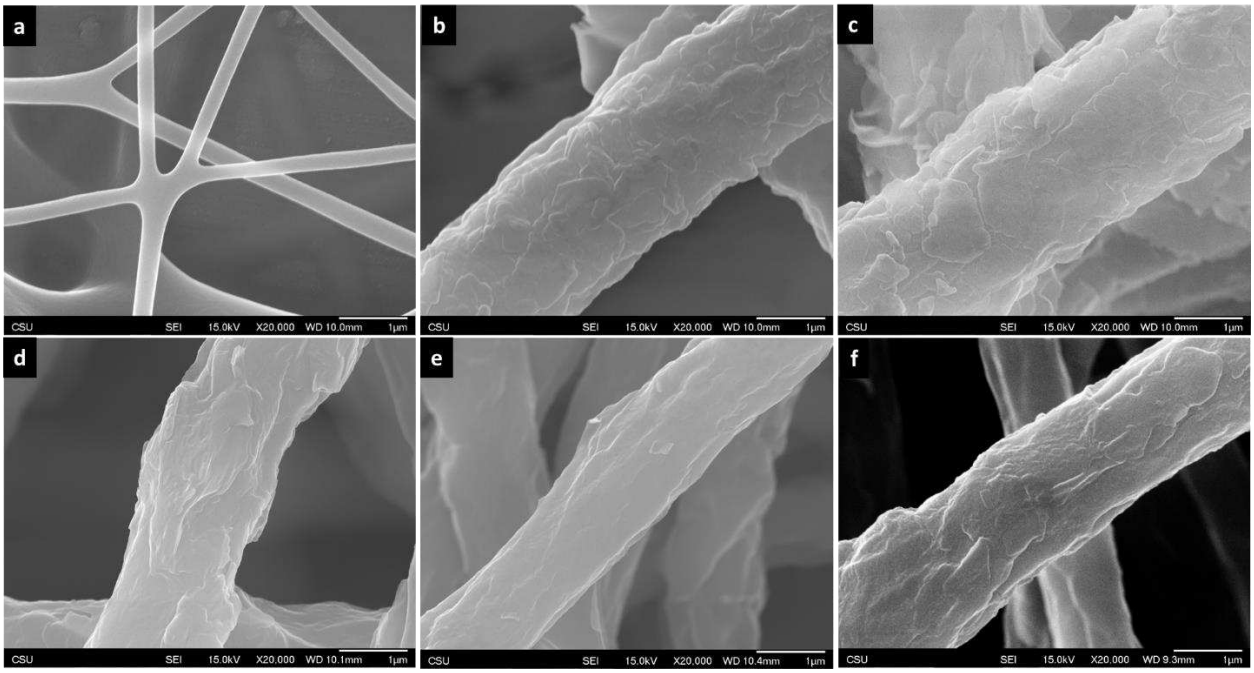


Figure 4-3 SEM image of (a) pristine PU nanofiber and (b) PU-PDA nanofibers before testing with bacterial components, PU- PDA nanofibers after testing with (c) autoclaved supernatant fluid, (d) non-autoclaved supernatant fluid, (e) autoclaved cell pellet and (f) n non-autoclaved cell pellet

4.3.2 Colorimetric responses of PU-PDA nanofibers to *E. coli* culture components

4.3.2.1 Color changing substance (CCS) in LB media

To determine whether the CCS was associated with the *E. coli* cells or with the supernatant fluid, PU-PDA nanofiber composites were tested with LB media only and four components of *E. coli* culture including (1) autoclaved bacterial cell pellets, (2) non-autoclaved bacterial cell pellets, (3) autoclaved supernatant fluid, and (4) non-autoclaved supernatant fluid. Table 4-1 shows the photographs of the nanofiber mats before and after testing. No color change was found when the nanofibers were tested with LB medium or with either non-autoclaved or autoclaved bacterial cell pellets while there was a significant colorimetric transition from blue to red when the nanofibers were tested in both autoclaved and non-autoclaved supernatant fluid. The color change started

initially at the edges of the nanofiber mats and progressed towards the center of the mats. After 30 min, the red covered most of the nanofiber mat decorated with some blue patches. The blue patches were due to the inability of the supernatant fluid to penetrate the nanofiber mat. PU-PDA is a superhydrophobic material [12] and hence the wettability of the nanofiber mat was poor. The blue patches eventually became red after 24 hours.

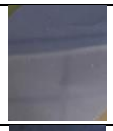
Autoclaving is a sterilization process where high-temperature steam is used along with high pressure to denature essential structural and enzymatic proteins, DNA, and RNA, resulting in the death of the bacteria. Proteins and nucleic acids in the bacterial supernatant fluid are denatured by the extreme heat and pressure during the autoclaving process [20]. It was found that the nanofiber tested with autoclaved supernatant fluid changed its color significantly faster than that tested with non-autoclaved supernatant fluid, suggesting that structural changes of components in the bacterial spent media occurred during autoclaving and accelerated the colorimetric response in the PDA molecules. These results indicate that the CCS was present in spent media (supernatant fluid) and was not cell-associated. Also, the lack of sensitivity to autoclaving strongly suggests that the CCS was not protein, DNA, or RNA.

The supernatant fluid is essentially made of materials produced by bacteria that are collectively known as extracellular polymeric substances (EPS). Bacterial cell pellets and LB media did not induce any color changes in PU-PDA nanofibers, indicating that the CCS was not inbound EPS. Therefore, free EPS were extracted from both autoclaved and non-autoclaved supernatant fluid to test with nanofibers. Table 4-1 shows the color change behaviors of the nanofibers immersed in free EPS solutions. The free EPS extracted from both autoclaved and non-autoclaved supernatant fluid changed the color of nanofibers. Due to the hydrophobic nature of the PU-PDA nanofiber mat, the free EPS solution could not quickly penetrate the nanofiber fiber mat

completely. Also, the concentration of CCS in free EPS might not be as high as in the supernatant fluid. For these reasons, some blue portion of the nanofiber remained after free EPS exposure as shown in the photographs. The difference in the red color between the nanofibers treated with free EPS collected from autoclaved and non-autoclaved supernatant fluid can be a result of structural differences in CCS due to the autoclaving process. The response time of color changes was 30 min for autoclaved supernatant fluid and 3 hours for non-autoclaved fluid, respectively.

Lipopolysaccharide (LPS) is a surface molecule found in gram-negative bacteria such as *E. coli* and maintains the structural integrity of the bacterial outer membrane [21]. When PU-PDA nanofibers were tested with LPS solutions prepared with distilled water and LB broth, the color change was not observed for 72 hours, confirming LPS is not the CCS that causes a colorimetric transition in PU-PDA nanofibers. Therefore, the CCS existed in the free EPS found in the supernatant fluid. Eboigbodin and Biggs [17] identified over 500 different types of protein in free EPS along with different functional groups such as amines, carboxyl, and phosphoryl groups. Further studies will be required to identify the CCS in free EPS.

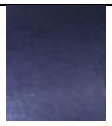
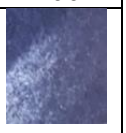
Table 4-1 Photographs of PU-PDA nanofibers before and after testing with E. coli culture components and free EPS. S= non-autoclaved supernatant fluid, AS= autoclaved supernatant fluid, P= non-autoclaved bacterial cell pellet, AP= autoclaved bacterial cell pellet, LB= LB media (control)

	Test with *E. coli* culture components (LB media)					Test with free EPS	
E. coli component	Control	AS	S	AP	P	AS	S
Before exposure							
After exposure							

4.3.2.2 Colorimetric responses to size-fractionated supernatant fluid

The supernatant fluid is complex mixtures of monomers and biopolymers. To determine a specific molecular weight range, the *E. coli* supernatant fluid was size-fractionated and tested with PU-PDA nanofiber composites. Table 4-2 shows the photographs of PU-PDA nanofibers tested with size-fractionated supernatant fluid. The color change was found for all fluids that passed through a filter with a molecular weight cutoff of 3k, 30k, and 100k Da. Color change also observed for the fluid that did not pass through the filter having a molecular weight cutoff of 100k Da. This result suggests that the CCS was composed of multimetric substances that were not single components in the fluid, but multiple components with different molecular weights.

Table 4-2 Photographs of PU-PDA nanofibers before and after testing with- supernatant and autoclaved supernatant fluids not retained by filters with molecular weight cutoffs of 3k, 30k, 100k Da, and fluid that retained by filter with molecular weight cutoff of 100k Da

	Molecular weight cutoffs (Da)							
	Supernatant fluid				Autoclaved supernatant fluid			
	<3k	<30k	<100k	>100k	<3k	<30k	<100k	>100k
Before								
After								

4.3.2.3 Stability of color-changing substance (CCS)

Supernatant fluids with different storage times (t_s = 0, 24, and 36 h) were evaluated for the ability to activate a colorimetric transition of PU-PDA nanofibers. Proteins and carbohydrates along with several other components in the supernatant fluid can undergo structural changes such as degradation over time as no new metabolites are produced in the absence of bacterial cells. Table 4-3 shows the photographs of PU-PDA nanofibers before and after testing with supernatant fluid with different storage time. Supernatant fluid with increased storage time changed the color of nanofibers significantly slower than the supernatant fluid with no storage time, suggesting a

degradation in CCS with increased storage. Besides, the degradation of CCS in autoclaved supernatant fluid with t_s = 36 h was significant because the vibrant red color was absent in the nanofibers, which was not observed in non-autoclaved supernatant fluid with t_s = 36 h (Table 4-3).

Table 4-3 Photographs of PU-PDA nanofibers before and after testing with supernatant fluid with different storage time.

Storage time (t_s)	Autoclaved supernatant fluid			Non-autoclaved supernatant fluid		
	0 h	24 h	36 h	0 h	24 h	36 h
Before exposure						
After exposure						

4.3.2.4 Time-dependence of colorimetric responses

Two representative reflectance spectra of the PU-PDA nanofibers are shown in Figures 4-4 (a-b). The spectra of the nanofibers tested in LB media showed no significant changes from t = 0 to t = 180 min during the test as shown in Figure 4-4 (a), which agrees with the lack of color change in the photographs shown in Table 4-1. In the case of autoclaved supernatant fluid, a peak at 540 nm (red) developed progressively and significantly on the spectra from t = 0 to t = 180 min during the test as shown in Figure 4-4 (b), suggesting a colorimetric transition from blue to red in the nanofiber mat as shown in the photographs. The reflectance intensity at blue (640 nm) and red (540 nm) on the spectra was used in calculating colorimetric response percentages (CR%) for the nanofibers tested with different *E. coli* culture components. Figure 4-4 (c) shows the CR% as a function of testing time for the nanofibers tested. No significant color transition occurred for any of the culture components during the first 3 minutes. The colorimetric transition started at t = 3

min, continued increasing, and plateaued, resulting in a peak colorimetric transition of 28% for the autoclaved supernatant fluid and 10% for the non-autoclaved supernatant fluid, respectively. The CR% was negligible for the PU-PDA nanofiber mats tested with *E. coli* cell pellets from t = 0 to t = 24 h. The results of both photographs and CR% suggest that the CCS were in the spent media (supernatant fluid) and not cell associated.

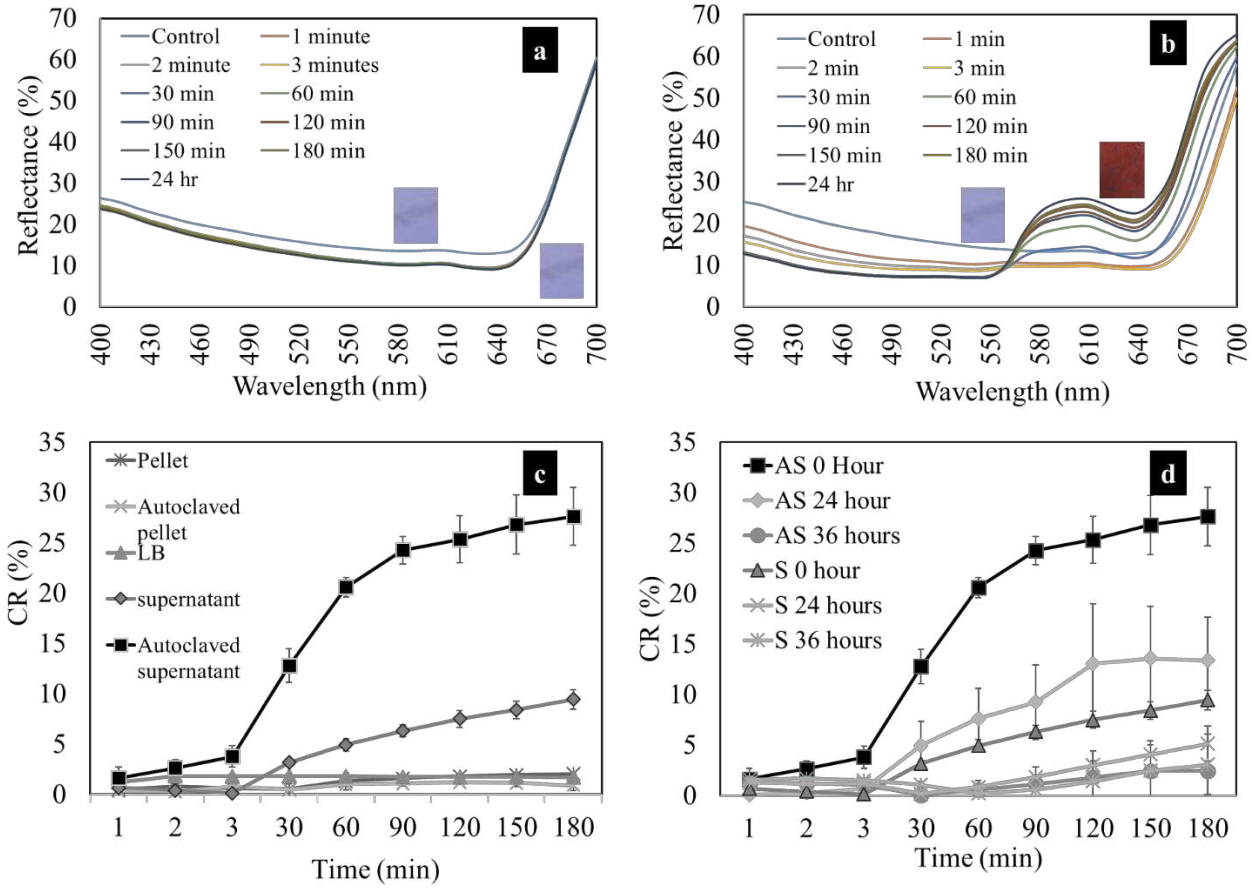


Figure 4-4 Representative reflectance spectra of PU-PDA nanofibers treated with (a) LB medium and (b) autoclaved supernatant fluid; (c) CR% of PU-PDA nanofibers treated with bacterial components and (d) CR% of PU-PDA nanofibers treated with supernatant fluid following storage for 0 h, 24 h and 36 h (AS = autoclaved supernatant fluid; S = non-autoclaved supernatant fluid).

Figure 4-4 (d) shows the CR% of the nanofibers tested with supernatant fluid with different storage times. Generally, color change started at t = 3 min and then increased in CR% followed by a plateau at t = 180 minutes. The response was proven rapid in comparison with solution-based or

paper-based PDA sensors previously reported, suggesting that the detection sensitivity was significantly improved due to high surface areas and aspect ratios in PDA nanofibers [22-23]. A reduction in CR% was found with an increase in the storage time of supernatant fluid, which agrees with the photographs presented in Table 4-3. The CR% at the plateau in the case of autoclaved supernatant fluid was 28% at t_s = 0 h, 14% at t_s = 24 h, and 2.5% at t_s = 36 h, respectively. CR% at the plateau decreased by almost 25.5% from t_s = 0 h to t_s = 36 h. On the other hand, the non-autoclaved supernatant fluid demonstrated CR% at the plateau of 10% at t_s = 0 h, 5 % at t_s = 24 h, and 3% at t_s = 36 h, respectively. CR% at the plateau decreased by almost 7%.

4.3.2.5 Critical bacterial concentration (CBC)

Critical bacterial concentration (CBC) is defined as the concentration of bacteria at which colorimetric transition in PDA occurs. The CBC determines the sensitivity of using PU-PDA nanofiber composite for the identification of bacterial presence in point-of-care devices such as smart wound dressing. The colony-forming unit per volume (CFU/ml) of *E. coli* is a common indicator of bacterial concentration. Figure 4-5 (a) shows a plot of colorimetric response (CR%) of the PU-PDA nanofiber as a function of *E. coli* CFU/ml. The color change in PU-PDA nanofibers began to occur when exposed to the *E. coli* concentration that was approximately 9×10^8 CFU/ml. The number of bacteria required to cause infection in wounds varies between $>10^5$ to 10^9 or more per gram of wound tissue [24-25]. Therefore, the PU-PDA nanofiber composite appears to be sufficiently sensitive for practical detection purposes.

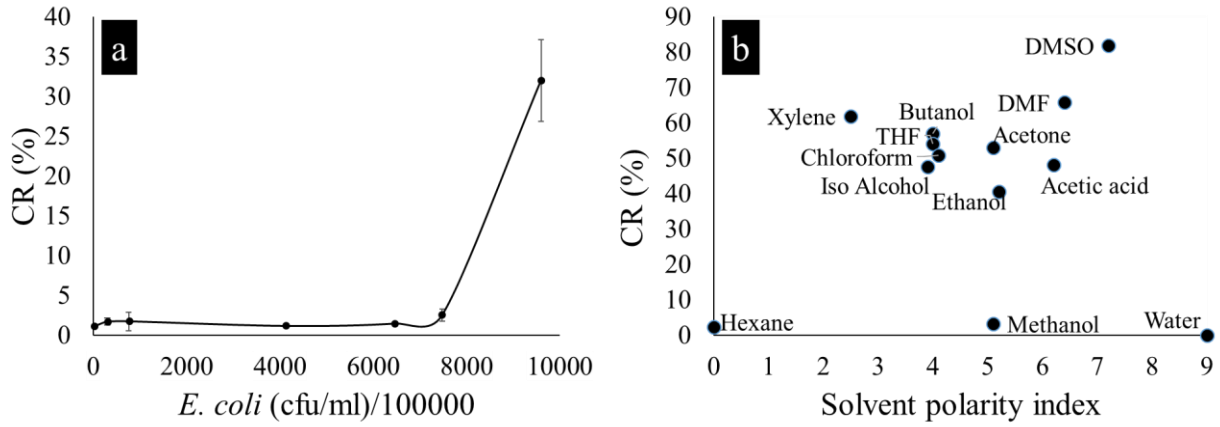


Figure 4-5 CR% of PU-PDA nanofibers with (a) E. coli concentration measured in (CFU/ml)/100000, and (b) solvent polarity index

4.3.2.6 CCS in minimal media

There are different secretion pathways for a gram-negative bacterium such as *E. coli* and these secretion pathways can change with variations in the surrounding environment such as culture media [26]. M9 minimal media contains only salts and a nitrogen source and is often used to study nutritional aspects of *E. coli* growth when supplemented with different carbon sources and amino acids. M9 minimal media is a defined growth media that lacks the peptides, amino acids, vitamins, and trace elements found in LB media. M9 minimal media supplemented with arabinose and different *E. coli* culture components were tested to compare the colorimetric properties of PU-PDA nanofibers with LB media. The photographs from the result are shown in Table 4-4. No color changes were promoted by any of the cultural components from M9 minimal media. Most likely, the minimal nutritional components that *E. coli* received from M9 minimal media were not enough to produce the CCS responsible for a color change of the PU-PDA nanofibers.

Table 4-4 Photographs of PU-PDA nanofibers before and after testing with E. coli culture components from M9 minimal media. S= non-autoclaved supernatant fluid, AS= autoclaved

supernatant fluid, P= non-autoclaved bacterial cell pellet, AP= autoclaved bacterial cell pellet, M9 = M9 minimal media solution (control)

Bacterial components	Control: M9	AS	S	AP	P
Before exposure					
After exposure					

4.3.3 Solvatochromism

PU-PDA nanofibers immersed in common organic solvents as well as distilled water were tested and the colorimetric response (CR%) as a function of the solvent polarity index is shown in Figure 4-5 (b). The majority of the solvents except for hexane and methanol were able to cause a color change in the nanofibers instantly, which is known as solvatochromism of PDAs. With an increase of polarity index from 0 (Hexane) to 7.2 (DMSO), the CR% of the nanofibers was increased exponentially by almost 80%, resulting in the highest CR% (82%) in DMSO. It was also found in the tests that the fiber mats began to disrupt with an increase in solvent polarity index. The solvents with a polarity index higher than 4, disassembled the fibers, resulting in serious damages at the edges of the fiber mats. The fiber mats were nearly completely dissolved in THF, acetic acid, and DMF after 30 min of exposure. The exception of methanol to change the color while having polarity index the same as acetone indicates that the solvent polarity may not be the sole responsible factor for a color change in PU-PDA nanofibers. The solvatochromism of PDAs suggests that extra precautions are needed when PDA is used to detect external stimuli including bacteria.

4.4 Conclusion

Extensive studies have been found in the literature for the colorimetric behavior of PDA when bacteria were detected, but with a limited focus on investigating the CCS in a bacterial

complex. In this study, components of *E. coli* culture were investigated and tested with a PU-PDA nanofiber composite. The results showed that *E. coli* cell pellets and culture media did not change the color of PU-PDA nanofibers. The CCS was found in the *E. coli* supernatant fluid, more specifically the free EPS being produced during the growth phase. The CCS that was produced in rich culture media such as LB media was unlikely a protein, DNA, RNA, or LPS and was likely the composition of multimetric components with different molecular weights. On the other hand, the color-changing rate of the nanofibers decreased significantly with increasing storage time, suggesting that the CCS degraded or lost the ability to interact with the PU-PDA nanofibers with an increase of storage time. The critical bacterial concentration (CBC) that triggered color changes in the nanofibers suggested a sensitivity appropriately adopted by point-of-care devices such as smart wound dressings that detect bacterial infection via instant colorimetric indication. Finally, the solvatochromism of PDA nanofibers with common organic solvents suggested that extra precautions are required when PDA is used in the development of point-of-care devices.

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Chapter 5 CONCLUSION

Polydiacetylene has been extensively studied in the literature for its rapid blue-to-red color transition at external stimuli. Extensive research works have been conducted to incorporate PDAs into several mediums for identifying environmental stimuli such as bacteria, viruses, electricity, heavy metal, hazardous materials, and solvents. For the rapid identification of bacteria via PDAs, several substrate-based and solution-based PDA sensors have been reported. However, little is known about the interaction of PDAs with a bacterium system due to the complexity of the materials found in a typical bacteria culture. In this report, the complex bacteria system was studied to understand the color-changing substances (CCS) responsible for a colorimetric transition in PDA nanofiber composite. The colorimetric transition initiation within 3 min of introducing the *E. coli* culture components strongly suggests the rapid identification of bacteria by the PU-PDA nanofiber composite system. Previous reports of PDA based systems claimed to detect bacteria within seconds (Ma, et al., 1998) and minutes (Scindia, Silbert, Volinsky, Kolusheva, & Jelinek, 2007) along with a recent report that used a portable paper-based band-aid to detect bacteria after 4 h (Sun, Zhao, Niu, Ren, & Qu, 2020). However, the PU-PDA nanofiber system described in this report can detect *E. coli* within 3 min and without incorporating any complex receptor system such as lipid, which made this system easily synthesizable. Additionally, as the PU-PDA nanofiber composite is a substrate-based PDA sensor, it is more stable than solution-based sensors, making it an effective and superior biosensor that can be easily incorporated into wound dressing for bacterial detection.

Results from the experiments showed that CCS is not associated with *E. coli* cells, rather the CCS was found in the spent media (supernatant fluid) produced by *E. coli* during the growth phase. The CCS was heat-stable after autoclaving treatment, indicating that proteins, DNA, or RNA cannot be the responsible CCS, however, with an increase in storage for the supernatant fluid, the degradation of CCS was found which reduced the colorimetric transition in the PU-PDA nanofiber composite. The size-fractionation of the supernatant fluid indicated that the CCS is a possibly multimetric component having a combination of molecules with various molecular weights. As *E. coli* is a Gram-negative bacterium, lipopolysaccharide (LPS) from the outer layer can be found in the supernatant fluid of *E. coli* culture. When PU-PDA nanofiber composite was treated with LPS solution, no color change was observed confirming LPS is not the CCS. More extensive experiments confirmed that CCS can probably be found in the extracellular polymeric substances (EPS) bacteria produce during the growth phase which is a complex biopolymer consists proteins, nucleic acids, polysaccharides, carbohydrates, and other materials. The critical bacterial concentration (CBC) to initiate a colorimetric transition in the nanofibers confirmed the usability of the PU-PDA nanofiber composite as an efficient biosensor to detect bacterial presence in the wound. Additionally, solvatochromism property of the nanofiber composite was studied and it was found that most of the organic solvents can change the color of PU-PDA nanofiber and therefore, extra-precautions should be taken while using PU-PDA nanofibers in smart wound dressings and point-of-care devices.

Chapter 6 FUTURE WORK

Future investigations can be included to further study the CCS and to understand the mechanism of the CCS inducing a colorimetric transition in the PDA macromolecules. Suggestions for future studies are listed below.

1. The major finding of the current study is that the CCS can be found in free EPS. Therefore, the free EPS composition can be studied further to extract various components and test with PU-PDA nanofiber composite. The result will help to identify the CCS that plays key roles in the colorimetric transition of PDAs.

2. The storage of supernatant fluid reduced the colorimetric transition rate. Further studies can be conducted to understand the molecular changes that happen in the components of supernatant fluid with time. It will future imply the mechanism of the CCS interact with PDA nanofibers in the presentation of different colorimetric responses.

3. Because the components in M9 minimal media was not able to induce a colorimetric transition in PU-PDA nanofibers, additional components such as amino acids and vitamins can be introduced to the minimal media to continue track color change in the nanofibers. Therefore, the secretion pathways of the bacteria for additional amino acid or vitamin can be studied and a better understanding of the mechanism of color change can be obtained.

4. In other previous reports of PDA sensors, the majority of the systems used complex receptors to catch bacteria and thus induced a colorimetric transition. However, the PU-PDA nanofiber composite reported in this study detected bacteria without any use of the receptor. Therefore, a comparison study between a substrate-based PDA sensor and PU-PDA nanofiber composite can be conducted in terms of colorimetric response percentage (CR%) and response

time. The results will provide a better understanding of PU-PDA nanofibers and the mechanism of colorimetric detection to bacteria.

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