Evaluation of Surface Acetylated Bacterial Cellulose for Antibacterial Wound Dressing Applications

Timothy Joseph Bertucio

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Maren Roman, Committee Chair
Kevin Edgar
Yong Lee

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ACADEMIC ABSTRACT

Complications during the healing process of skin wounds often arise due to infection by pathogenic bacteria. Bacterial hydrolytic enzymes degrade the host tissue while biofilms can shield the bacterial cells from the host's immune response. Wound dressings with bacteriostatic or bactericidal properties are a promising solution. This study investigated the potential of surface acetylated bacterial cellulose as a novel antibacterial wound dressing. Hydroxyl groups on the surface of bacterial cellulose were substituted with acetyl groups using acetic anhydride in a citric acid-catalyzed reaction. The resulting ester linkages between the acetyl groups and bacterial cellulose surface were hypothesized to be cleaved by bacterial esterases or other hydrolytic enzymes such that acetic acid, a well-known antibacterial compound, will be produced leading to the death of the bacterial cells. Surface acetylation was confirmed via FTIR and its effect on the morphology of bacterial cellulose was analyzed with FESEM and XRD while the degree of substitution was determined by HPLC-UV. Indirect contact human cell cytotoxicity assays using extracts from surface acetylated bacterial cellulose showed no cytotoxic effect on human umbilical vein endothelial cells. Two types of antibacterial assays were performed in which surface acetylated bacterial cellulose was exposed to *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa* which were selected as model bacteria for Gram-positive, Gram-negative, and pathogenic bacterial species, respectively. Neither assay showed a reduction of bacterial cell viability. Further research is needed to determine if the acetyl ester linkages on the surface of bacterial cellulose are susceptible to cleavage by bacterial esterase enzymes.

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GENERAL AUDIENCE ABSTRACT

The healing of skin wounds is frequently complicated by infection of the wound with harmful bacteria. A potential remedy could be wound dressings that kill such bacteria. Bacterial cellulose is a naturally occurring biomaterial with multiple properties that make it an ideal material for wound dressings. Pure bacterial cellulose has no inherent antibacterial properties but can be chemically modified with a separate substance that is antibacterial such as acetic acid. This study investigates the potential of chemically modified bacterial cellulose in antibacterial wound dressing applications. The material may release acetic acid in the presence of bacteria and cause cell death. A series of human cell and antibacterial assays were carried out to test the ability of the modified bacterial cellulose to inhibit bacterial growth as well as any potential harmful effect on human cells. While it showed no adverse effects on human cells, the modified bacterial cellulose did not reduce bacterial cell viability.

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Chapter 1: Introduction

1.1 Motivation

Bacterial infection of skin wounds remains a common medical affliction. Secretion of hydrolytic enzymes and toxins into a skin wound by pathogenic bacteria causes the degradation of the human cells surrounding the wound. This in turn can lead to longer healing times or even more serious medical complications. Wound dressings can aid in the healing process but many of those currently in use do not have antibacterial properties and therefore have little to no effect on infected wounds. For this reason, the development of novel antimicrobial wound dressings remains of continual and considerable interest to the medical field.

Due to certain properties of the biomaterial bacterial cellulose, including its greater tensile strength and water holding capacity in comparison to plant cellulose, its potential suitability as a wound dressing material has been the focus of numerous publications. However, bacterial cellulose itself has no inherent antibacterial properties and thus provides limited benefits when used for infected wounds.

Many researchers have attempted to chemically modify bacterial cellulose in order to create a new bacterial cellulose derivative with antibacterial properties. While many have been successful in doing so it must be noted that some of the antibacterial substances used in these cases, such as silver nanoparticles, may themselves have adverse effects on human cells.

One common antibacterial substance is acetic acid. The antibacterial properties of acetic acid at concentrations well under 1% is well documented. Prior to this work, the antibacterial properties of acetic acid had yet to be used in conjunction with bacterial cellulose for wound dressing applications.

1.2 Goals and Objectives

The overall goal of this project was to evaluate surface acetylated bacterial cellulose as a novel wound dressing material that exhibits antibacterial properties through the release of acetic acid in the presence of bacteria. If successful, this project would potentially lead to the development of a new antibacterial wound dressing that could serve as a more effective alternative to those already commonly used by medical professionals. The specific objectives of this project are:

1. To synthesize surface acetylated bacterial cellulose and qualitatively and quantitatively compare its morphological and chemical properties to those of unmodified bacterial cellulose.
2. To determine if surface acetylated bacterial cellulose has cytotoxic effects on human umbilical vein endothelial cells.
3. To determine if surface acetylated bacterial cellulose has bacteriostatic or bactericidal effects upon several species of bacteria.

1.3 Thesis Outline

This thesis contains five chapters. Chapter 2 reviews background information relevant to this project. More specifically, the review introduces the two materials that are the primary focus of this project, bacterial cellulose and acetic acid, and presents the state-of-the-art of antibacterial wound dressings. Lastly, the chapter discusses the potential benefits of combining the wound healing properties of bacterial cellulose with the antibacterial properties of acetic acid to obtain a novel antibacterial wound dressing.

Chapter 3 describes the materials and methods used in this project, specifically the synthesis of bacterial cellulose by fermentation, the topochemical acetylation of the bacterial cellulose, the characterization of the unmodified and surface acetylated bacterial cellulose, the cytotoxicity assays, and the antibacterial assays.

Chapter 4 discusses the results of FTIR spectroscopy, high-performance liquid chromatography, scanning electron microscopy and X-ray diffraction analyses of the unmodified and surface acetylated bacterial cellulose as well as the effects of these materials on the viability of human umbilical vein endothelial cells and *Escherichia coli*, *Staphylococcus epidermis*, and *Pseudomonas aeruginosa* bacterial cells.

Finally, chapter 5 summarizes the conclusions of this research project.

Chapter 2: Literature Review

2.1 Introduction

The adverse health effects of skin wounds are not limited to physical trauma as the bacterial infection of wounds often leads to further medical complications (Ki and Rothstein, 2008). The limitations of conventional wound dressings in healing infected wounds have led to the development of a new class of wound dressings with antibacterial properties (Zheng, 2020). While the base material of most modern wound dressings is a bandage or some variety of gauze, commonly one that is cotton-based, the biomaterial bacterial cellulose shows great promise as a superior substitute (Moradpoor et al., 2022). In this study, the potential of surface acetylated bacterial cellulose in antibacterial wound dressing applications will be investigated.

2.2. Bacterial Cellulose

Due to its function as a key component in plant cell walls, cellulose is one of the most common organic polymers on the planet. Almost fifty years after cellulose was first extracted from plant matter and its chemical structure determined by Anselme Payen in 1838 (Payen, 1838), a substance derived from bacteria with the same chemical structure as cellulose was observed. This substance would come to be known simply as bacterial cellulose, or microbial cellulose. First reported by A.J. Brown in 1886 but not identified as cellulose until the 1930's [(Brown, 1886) (Mühlethaler, 1949)], bacterial cellulose is a versatile biomaterial produced by several species of bacteria with the most prominent species being *Komagataeibacter xylinus*, formerly known as *Gluconacetobacter xylinus* and *Acetobacter xylinum* (Romling and Galperin, 2015). While the exact biological function of this biomaterial is currently unknown, the leading theories are that it provides protection against ultraviolet light or serves as a flotation device that gives the bacteria access to oxygen (Da Silva et al., 2022). Like plant cellulose, bacterial cellulose consists of anhydroglucose units (AGU) connected by β-1,4 glycosidic linkages as seen in **Figure 2.1** (Delmer and Amor, 1995). On a microscopic level, bacterial cellulose exists as a complex network of nano-sized fibrils as shown in **Figure 2.2** (Stanislawska, 2016).

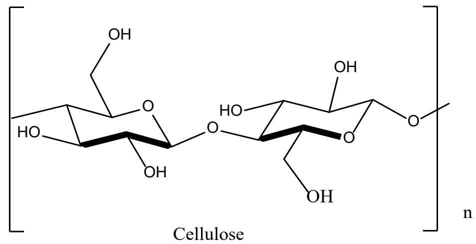

Figure 2.1: Molecular structure of cellulose

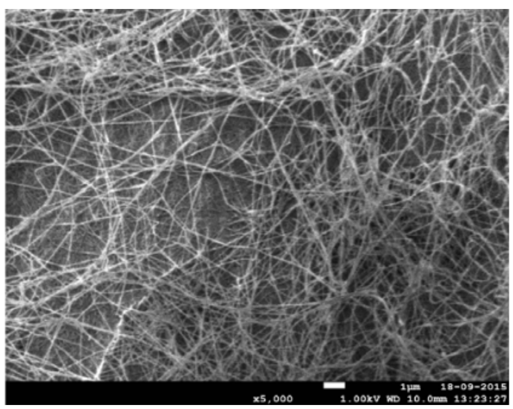

Figure 2.2: Bacterial cellulose fibril network

Reprinted from Stanisławska, A. (2016). Bacterial nanocellulose as a microbiological derived nanomaterial. *Advances in Materials Science*, *16*(4), 45–57. Used under fair use.

2.2.1. Properties

Unlike plant cellulose, bacterial cellulose contains no lignin or hemicellulose though living bacterial cells and endotoxins may be present (Zhong, 2020). Its degree of polymerization and crystallinity index are both higher than those of plant cellulose (Ullah et al, 2016). Bacterial cellulose microfibrils are larger than those of plant cellulose and it also has greater tensile strength and water holding capacity such that as much as 98% of a never dried bacterial cellulose pellicle may be water (Rebelo et al., 2018). **Figure 2.3** lists the different properties of bacterial cellulose in comparison to plant cellulose.

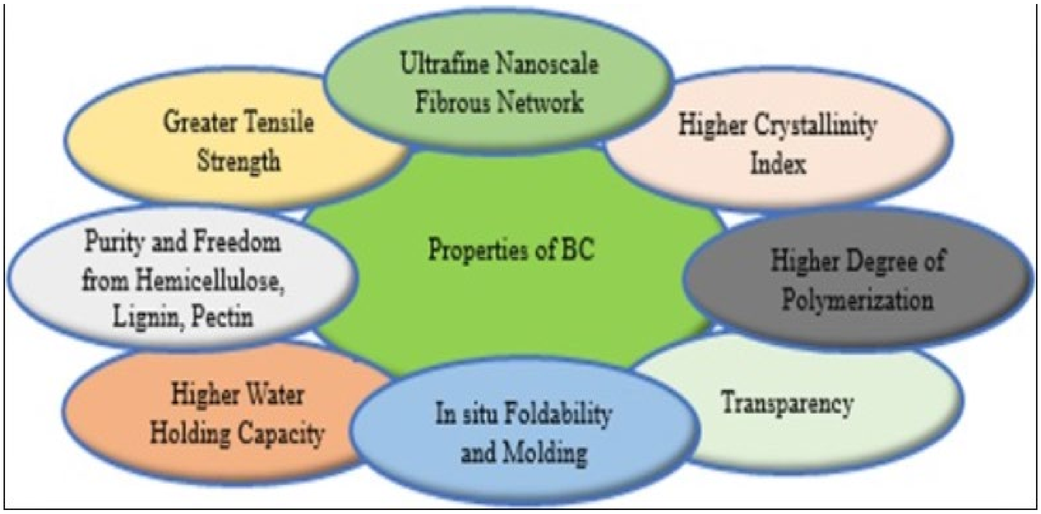

Figure 2.3: Properties of Bacterial Cellulose

Reprinted from Ullah, H., Wahid, F., Santos, H. A., & Khan, T. (2016). Advances in biomedical and pharmaceutical applications of functional bacterial cellulose-based nanocomposites. *Carbohydrate Polymers*, *150*, 330–352. Used under fair use.

2.2.2. Bacterial Cellulose Production

While several species of bacteria produce cellulose only *K. xylinus* does so in quantities large enough for practical applications (Liu et al., 2018). Bacterial cellulose growth media must contain some type of sugar substrate such as glucose or mannitol that can be converted by the bacteria into cellulose (Ramana et al., 2000). More specifically, the sugar molecules are joined together to form glycan chains that are extruded from the bacteria into the growth media where the chains then aggregate into microfibrils (McNamara et al., 2015). A pH in the 4.0-6.0 range is also desirable as *K. xylinus* cellulose production is most optimal in mildly acidic environments though it should be noted that organic acids may be produced as a byproduct causing a further drop in pH to a level that inhibits cellulose production (Masaoka, 1993). A common growth media for bacterial cellulose cultures in use since the 1950's is Hestrin-Schramm media (Hestrin and Schramm, 1954). These cultures can be either static or non-static in terms of agitation. The former leads to the formation of a single piece of bacterial cellulose on the surface of the growth medium that is known as a pellicle as shown in **Figure 2.4** while the latter produces individual pieces of bacterial cellulose (Kim et al., 2007).

Figure 2.4: Bacterial cellulose pellicle

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Current yields of bacterial cellulose are low, resulting in high production costs. Many recent studies have focused on finding suitable alternative feedstocks to lower the growth media cost, particularly feedstocks that are a waste product of current industrial processes (Thompson and Hamilton, 2001). The time intensive nature of bacterial cellulose production and the limited number of production facilities that currently exist are also impediments to large-scale production. Despite the general biochemistry of bacterial cellulose fermentation being well understood it has proven difficult to utilize this knowledge to increase bacterial cellulose production (Liu et al., 2018). Due to these factors bacterial cellulose remains significantly more expensive than plant cellulose.

2.2.3. Chemical Modification

Like cellulose derived from plants, bacterial cellulose can be chemically modified in a variety of ways. Such modifications can be made either *in situ* during cell culture or *ex situ* after the bacterial cellulose has been formed (Stumpf et al., 2018).

The large amount of hydroxyl groups on the surface become easy targets for functionalization with a variety of chemical groups. Naturally, the properties of the bacterial cellulose are altered by this change in chemical structure. For instance, surface modification with organic acids leads to the initially hydrophilic surface of the bacterial cellulose becoming more hydrophobic (Lee et al., 2009).

2.2.4. Cellulose Acetate

One of the most common chemical modifications of cellulose is its esterification with acetic anhydride to form cellulose acetate (Cheng et al., 2009). As there are three hydroxyl groups in an AGU that can be substituted, the average number of substituted hydroxyl groups per AGU, known as the degree of substitution, ranges from 0-3 (Fei et al., 2017).

Cellulose acetate is used in the production of a variety of widely used films, fibers, and plastics. It should be noted that the cellulose acetate used in industry is produced from plant cellulose. At present, cellulose acetate obtained from the acetylation of bacterial cellulose appears to be restricted to academic research projects (Ramirez et al., 2016).

2.2.5. Applications

Despite its high cost, bacterial cellulose is still studied for a variety of applications including electronics, food, and even clothing. The most extensive and promising use of bacterial cellulose has been in the medical field. Coupled with its biocompatibility, the ability of bacterial cellulose to be grown into essentially any useful shape or thickness has led to its usage in multiple medical applications (Ullah et al., 2016). Tissue engineering scaffolds made from bacterial cellulose have been the focus of numerous research publications. More ambitious researchers have even begun to fashion artificial blood vessels out of bacterial cellulose as an alternative to polytetrafluoroethylene and other synthetic polymers that are currently used to produce artificial blood vessels (Weber et al., 2017). Like those from synthetic polymers, blood vessels and other artificial organs made from bacterial cellulose are biocompatible and stable when implanted in a human patient (Da Silva et al., 2022). So far, the most common medical application of bacterial cellulose is as a material for wound dressings.

2.3. Wound Dressings

The suitability of bacterial cellulose for wound dressings is due in part to its high biocompatibility. The high water holding capacity and water permeability of bacterial cellulose are also relevant as many skin wounds have been shown to heal faster and more successfully when kept moist (Zheng et al., 2020). In addition, never dried bacterial cellulose wound dressings can be somewhat transparent allowing medical professionals to visually monitor the healing of a wound without having to remove the wound dressing leading to lower patient discomfort (Tabaii and Emtiazi, 2018). **Figure 2.5** shows a burn victim covered in a bacterial cellulose wound dressing that was molded to fit the shape of the patient's body. These properties may make bacterial cellulose a possibly more suitable choice than traditional wound dressing materials such as cotton gauze.

Figure 2.5: Bacterial cellulose wound dressing on a burn victim

Reprinted with permission from Czaja, W. K., Young, D. J., Kawecki, M., & Brown, R. M. (2007). The future prospects of microbial cellulose in biomedical applications. *Biomacromolecules*, *8*(1), 1–12. Copyright 2007 American Chemical Society."

2.3.1 Bacterial Wound Infections

As effective as bacterial cellulose wound dressings have proven to be for the healing process, bacterial infection of skin wounds remain a significant issue. Normally, an acute skin wound will heal after passing through four major stages: hemostasis, inflammation, proliferation, and remodeling as illustrated in **Figure 2.6** (Wallace et al., 2021). When a wound fails to heal within a timely manner it is because the healing process becomes arrested in one of these phases, typically the inflammation phase, and such a wound is then classified as a chronic wound (Frykberg and Banks, 2015).

Figure 2.6: The four stages of wound healing

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The reason for the disrupted healing process of a chronic wound can vary, but it is often due to the presence of bacteria within the wound. Open wounds lack the defensive protection of intact skin and are thus easy targets for bacterial colonization (Negut et al., 2018). Gram-positive bacteria are the most common type of bacteria found within an infected skin wound but Gram-negative bacteria are often present as well (Stulberg et al., 2002). Therefore, the bacterial population of a chronic wound will not be limited to a single type but will instead exhibit a diversity of species from both the Gram-positive and Gram-negative classifications. Pathogenic bacteria within a wound frequently form biofilms that are impenetrable to host immune cells and certain antibiotics (Sharma et al., 2019). These biofilms therefore allow for the survival of the bacteria and continual secretion of bacterial toxins into the wound to the detriment of the afflicted person.

2.3.2. Bacterial Hydrolytic Enzymes

Further medical complications of infected wounds arise from the presence of bacterial hydrolytic enzymes. Protease, lipase, and esterase enzymes all contribute to degradation of host proteins and tissues (McCarty et al., 2012). The mere presence of such hydrolytic enzymes within an infected wound is not cause for concern as the controlled expression of hydrolytic enzymes by the afflicted person’s own body is key to normal wound healing. In an acute wound a balance between the degradation of the extracellular matrix by the host hydrolytic enzymes and its regeneration by other host enzymes is soon established leading to a timely healing of the wound (McCarty and Percival, 2013). This balance is disrupted in infected wounds as the bacteria contribute to elevated levels of hydrolytic enzymes. As a result, tissue degradation consistently occurs at a faster rate than tissue regeneration leading to a chronic wound that heals only after a prolonged period and often recurs (Lindsay et al., 2017).

2.4. Antimicrobial Compounds

While bacterial cellulose has no antibacterial properties of its own it has previously been modified with antimicrobial compounds (Zheng et al., 2020). **Figure 2.7** shows the variety of different antimicrobial compounds have been incorporated into the structure of bacterial cellulose in order to develop antibacterial wound dressings.

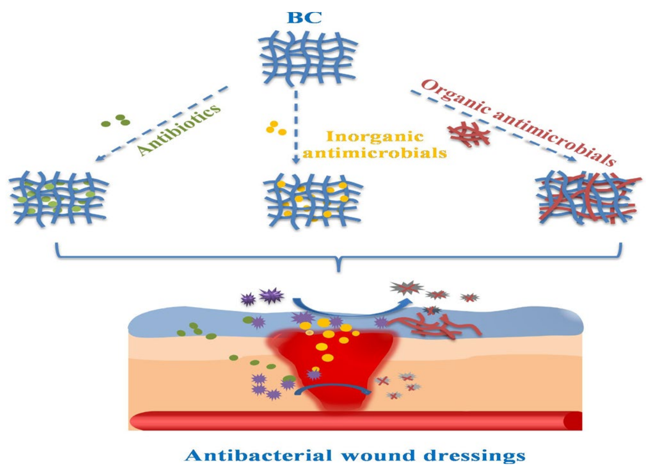

Figure 2.7: Different methods of adding antibacterial activity to bacterial cellulose

Reprinted from Zheng, L., Li, S., Luo, J., & Wang, X. *Frontiers in Bioengineering and Biotechnology*. 2020, *8*, 1334. Used under fair use.

Antimicrobial compounds that are not antibiotics are of particular interest due to the threat posed to public health by the current antibiotic resistance crisis resulting from antibiotic overuse (Ventola, 2015). For some inorganic antimicrobial compounds there are concerns relating to their potential cytotoxicity. For instance, the antibacterial properties of silver nanoparticles against both Gram-negative and Gram-positive bacteria are well documented but current literature includes conflicting reports on whether silver nanoparticles are toxic to human cells as well (Liao et al., 2019). These factors are of great concern for the development of antibacterial bacterial cellulose wound dressings as much of the research on this topic appears to have focused on using either antibiotics or inorganic compounds that may adversely affect human cells as the antibacterial component of bacterial cellulose wound dressings (Zheng et al., 2020). For this reason, new fusions of bacterial cellulose with other types of antibacterial compounds are of interest. Certain organic acids with antibacterial properties and little to no cytotoxic effect against human cells have shown great potential for this function, particularly acetic acid.

2.4.1 Acetic Acid

Acetic acid (**Figure 2.8**) is a carboxylic acid that is widely used for chemical and food applications. The use of acetic acid as an antibacterial agent is not a novel idea as acetic acid is the main component of vinegar which has long been applied to skin wounds as a household remedy. When in its protonated form with a neutral charge, acetic acid has been proven to have the ability to penetrate bacterial biofilms and cell membranes and eradicate bacterial colonies for a wide range of bacterial species [(Ryssel et al., 2009), (Nagoba et al., 2013)]. The biochemical explanation for the toxicity of acetic acid to bacteria is as follows: upon entry into the bacterial cell interior the acetic acid molecule will deprotonate with the resulting influx of protons contributing to a decrease in the intracellular pH and an inhibitory effect on the cell's metabolism leading to cell death (Halstead et al., 2015). This deprotonation is ensured by the fact that the internal pH of most bacteria is higher than the pKa of acetic acid. (Kundukad et al., 2017).

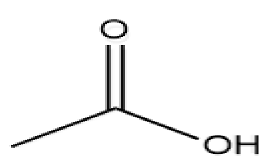

Figure 2.8: Molecular structure of acetic acid

Bacteria are susceptible to the antibacterial activity of acetic acid even when in environments in which the concentration of acetic acid is well under 1% [(Halstead et al., 2015), (Fraise et al., 2013)]. This antibacterial activity can be quantified with the concept of the minimum inhibition concentration (MIC) of a given bacterial species which is defined as the lowest concentration of a chemical that will inhibit visible growth after overnight incubation (Andrews, 2001). Common variations include MIC50 and MIC90 which represent the concentration at which at least 50% or 90% of bacterial isolates in a test population of given species experience inhibited growth (Schwarz et al., 2010). **Figure 2.9** illustrates the antibacterial activity of acetic acid by showing the effect of different concentrations of acetic acid on the production of *P. aeruginosa* biofilm biomass. The magnitude of this antimicrobial activity can be influenced by multiple factors including pH, acid concentration, type of bacterial strain, and environmental conditions such as the growth phase and temperature. The current practice for applying acetic acid to infected wounds typically involves soaking cotton gauze with vinegar, of which acetic acid is a small percentage v/v, and placing it on the wound, repeated several times a day, for a period of several days or weeks (Elhage et al., 2021). It should be noted that certain bacterial strains have developed a level of resistance to acetic acid ([Diez-Gonzalez et al., 1997], [Trcek et al., 2015]).

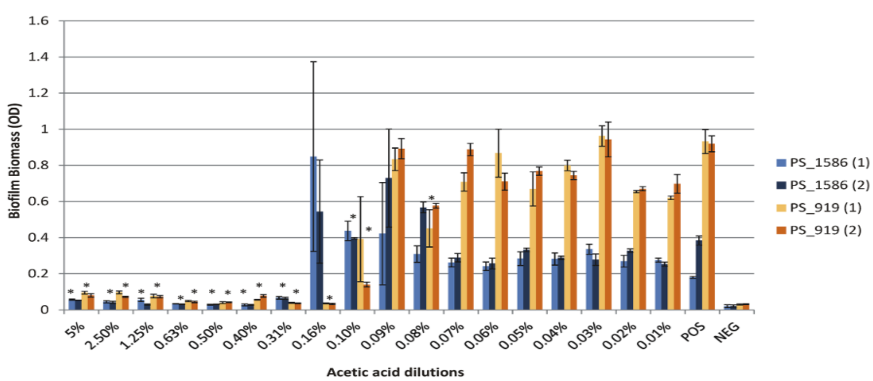

Fig 2. Graph showing the MBIC results for isolates PS_919 and PS_1586. Optical density on the y axis refers to the average biofilm biomass for isolates PS_919 and PS_1586 at the range of AA dilutions tested. POS: positive control, NEG: negative (broth only) control. The error bars represent the standard error, and asterisks denote dilutions with statistically significant reductions in biofilm production according to the t-test.

Figure 2.9 Antibacterial Activity of Acetic Acid on *P. Aeruginosa* Biofilm Biomass

Reprinted from Halstead, F. D., Rauf, M., Moiemen, N. S., Bamford, A., Wearn, C. M., Fraise, A. P., Lund, P. A., Oppenheim, B. A., & Webber, M. A. (2015). The Antibacterial Activity of Acetic Acid against Biofilm-Producing Pathogens of Relevance to Burns Patients. *PloS One*, *10*(9), e0136190. Used under fair use.

2.5. Significance

Many previous publications have studied the potential of bacterial cellulose to be used as a material for antibacterial wound dressings [(Kucinska-Lipka et al., 2015), (Liyaskina et al., 2017)]. While bacterial cellulose has previously been modified with a wide variety of antibacterial substances, notably silver nanoparticles [(Maneerung et al., 2008), (Pal et al., 2017)], this project will be the first attempt to use bacterial cellulose in conjunction with acetic acid to develop such a wound dressing. The acetylation of bacterial cellulose with acetic anhydride has previously been achieved (Ramirez et al., 2016) but the biocompatibility and antibacterial properties of the resulting material have yet to be evaluated. It is noteworthy that even if the surface acetylated bacterial cellulose proves to be slightly cytotoxic to human cells it still may be of interest for antibacterial wound dressing applications so long as it kills bacterial cells to a much greater extent than mammalian cells.

The potential of bacterial enzymes commonly found in skin wounds to cleave acetyl groups from surface acetylated bacterial cellulose is also yet to be determined. There is evidence to indicate that this may be possible as many microorganisms have been found to produce enzymes that deacetylate cellulose acetate and produce acetic acid such as acetyl xylan esterases and cutinases [(Puls et al., 2011), (Haske-Cornelius et al., 2017)]. However, these acetyl esterase enzymes are produced by species of bacteria and fungi that are found in soil [(Altaner et al., 2003), (Moriyoshi et al., 2005)]. It therefore remains to be determined if any enzymes derived from bacteria found on the skin are also capable of producing acetic acid by the deacetylation of acetylated cellulose. It should also be noted that in these instances the cellulose acetate studied was plant-based. For this reason, this project will be the first investigation of potential bacteria-mediated release of acetic acid from bacterial cellulose.

In broader terms, the success of this project has the potential to benefit society by catalyzing the development of a new class of antibacterial wound dressings that improve patient care, lower health care costs, and moderate the antibiotic resistance crisis by serving as an alternative to antibiotics and lessening the current overuse of antibiotics.

Chapter 3: Materials and Methods

3.1. Materials

The bacterial cellulose (BC) producing strain *K. xylinus* (ATCC 10245), *E. coli* (ATCC 1129) and *S. Epidermidis* (ATCC 12228), were purchased from the American Type Cell Collection (ATCC). Peptone, yeast extract, and acetic anhydride were purchased from Fisher Chemical. Citric acid was purchased from Research Products International. Sodium hydrogen phosphate was purchased from Alfa Aesar. D-Mannitol was purchased from Sigma-Aldrich. Phosphate buffered saline (PBS) was purchased from Gibco.

3.2. Synthesis of Bacterial Cellulose

BC was produced by cultivation of *K. xylinus* in pH 5 Hestrin-Schramm (HS) media (Hestrin and Schramm, 1954). Mannitol was used as the sugar source. The bacterial preculture was prepared by inoculating 2 mL of bacterial glycerol stock to 50 mL of HS medium in a 250 mL Erlenmeyer flask and incubated stationary at 28 º C for 5 days. BC mats were produced in 100 x 15 mm Petri dishes filled with 25 mL HS medium. The production culture was inoculated with 10% preculture (v/v) and incubated stationary at 28 º C for 7 days with BC mats grown in 100 x 15 mm Petri dishes filled with 25 mL HS media. After the incubation period the BC mats were removed from the petri dishes and washed in 0.5 M NaOH to remove the *K. xylinus*, HS media, and any toxins or other impurities. The BC was then washed in water to remove the NaOH.

3.3 Surface Acetylation of Bacterial Cellulose

The BC mats were solvent exchanged from water through acetic acid to acetic anhydride prior to reaction. The solvent exchange involved washing the BC in acetic acid for 30 minutes (2x) followed by washing in acetic anhydride for 30 minutes (2x) with mixing. The surface acetylation of the BC mats was done with acetic anhydride by adapting a method previously reported in the literature. Acetic anhydride (500 ml) was transferred to a 1000 ml round bottom flask attached to a condenser containing a magnetic stirrer. 15 never dried BC mats (dry weight 350 mg) and 1.30 g citric acid (a 3 mol citric acid / 1 mol AGU ratio) were then added to the flask and heated 120 º C for 4 hours with mixing. The reaction scheme is depicted in **Figure 3.1**. Once the reaction was complete the surface acetylated BC mats were removed, and a solvent exchange from acetic anhydride to water by washing in acetic acid and then water with mixing. (twice each).

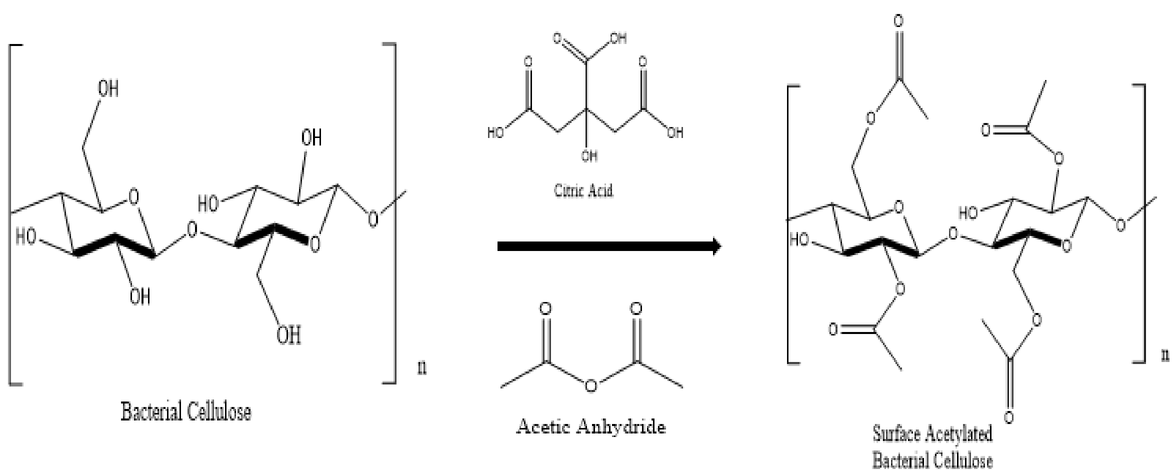

Figure 3.1: Surface acetylation of bacterial cellulose reaction scheme (Ramirez et al., 2016)

3.4 Structural Characterization of Surface Acetylated Bacterial Cellulose

3.4.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of unmodified and surface acetylated BC were acquired using transmission FTIR. Dried and ground up samples (5 mg) were mixed with dried KBr (95 mg) and pressed into a disk. Samples were scanned 256 times in the 4000-500 cm^{-1} range. Measurements were taken on a Nicolet 8700 instrument and data analyzed using the software Omnic.

3.4.2 High Performance Liquid Chromatography- Ultraviolet (HPLC-UV)

HPLC-UV was used to determine the degree of acetylation of the surface acetylated bacterial cellulose following a saponification reaction that releases acetate from the BC surface. This technique was chosen as the HPLC component allows for the separation of the acetate from the other molecules in the saponification solution and the UV component allows for the concentration of acetate to be quantified. Samples of the BC were transferred to vials containing 70% ethanol and a single BC mat (63 mg dry weight). The flasks were covered and heated at 60 º C for 30 minutes at which point an equal volume of 0.5 N NaOH was added. The flasks were kept at the same temperature for an additional 15 minutes after which the flasks were left to sit out at room temperature for 48 hours with mixing. Samples with only the ethanol/water/NaOH saponification solution with no BC were prepared in the same process for a comparison.

HPLC-UV measurements were taken on a Nexera X2 system using a Rezex Organic Acid column (150 x 4.6 mm) with a flow rate of 1 mL/minute and sample injection volume of 10 microliters. Data analysis was done with Shimadzu LabSolutions 5.96. Surface acetylated BC went through the same saponification process as described in the previous section. A 1 ml aliquot from the saponification solution was removed, 300 microliters of 1.8 M sulfuric acid were added, and the sample was then cooled in ice. After cooling the samples were put through centrifugation for 10 minutes at 4 degrees Celsius and 13X. 200 microliters were removed and added to a chromatography vial and then put through HPLC-UV with 2.5 mM sulfuric acid as the mobile phase. By using a calibration curve derived from acetic acid standards of known concentrations run through HPLC-UV, the concentration of acetate released by the saponification was determined. The acetate concentration was in turn used to calculate the degree of acetylation.

3.4.3 X-ray Diffraction (XRD)

X-ray diffraction patterns were collected using a D8 Discovery XRD operating at 40 kV and 40 mA with a 1 mm slit. Data collection was done using Diffrac Plus XRD Commander version 2.6.1. The data was collected using a scan speed of 0.2 sec/step with a step increment of 0.02. Samples were placed on a zero diffraction SiO2 plate for analysis. For analysis, Diffrac Plus Eva version 13.0.0.3 by Bruker was used and Profex software was used for Rietveld refinement.

3.4.4 Field Emission Scanning Electron Microscopy (FESEM)

Samples of unmodified and surface acetylated BC were observed under a LEO (Zeiss) 1550 scanning electron microscope. Images were taken at magnifications of 10X, 30X, and 100X.

3.5 Human Cell Cytotoxicity Assays (Indirect Contact Method)

Unmodified and surface acetylated BC mats were cut into 9 cm^2 pieces and sterilized in 70% ethanol for 12 hours, washed in a sodium phosphate buffer, and then put under ultraviolet light. Samples were then incubated in the human endothelial cell growth media V2 (HECGM V2) in 3 cm^2 / mL for 24 hours at 37 °C to produce BC extracts. Human umbilical vein endothelial cells (HUVECs) were used as a model for human primary cells. HUVECs were plated in a 96-well plate at a density of 5000 cells per well in 200 µL HECGM V2 per well. After culturing for 24 hours, cells were washed with 1X PBS three times. The culture medium was then replaced with 200 µL BC extracts per well. Normal HECGM V2 with no BC was the negative control and 0.5% DMSO was used for the positive control. The incubation periods were 1, 3, and 7 days. For the 7-day trial, 200 µL fresh bacterial cellulose extracts, positive and negative control media were replaced on the 4th day. After each incubation time, cells were washed three times with 1X PBS and then treated with 100 µL serum free HEC media and 10 µL Cell counting kit 8 solution (CCK-8, Dojindo, Rockville, MD). After incubation for 3 h to allow the development of the CCK-8 dye, absorbance was recorded at 450 and 750 nm using a BioTek Synergy Mx plate reader (BioTek, Winooski, VT).

3.6 Antibacterial Assays

Two types of antibacterial assays were performed:

1. The first was a variation of a disk diffusion assay. Lysogeny broth (LB) agar plates were coated with *E. coli* (ATCC 1129) or *S. epidermidis* (ATCC 12228) and divided into four sections. Both species are commonly found on human skin with the former represented Gram-negative bacteria and the latter representing Gram-positive bacteria. Section A contained unmodified BC, Section B contained surface acetylated BC, Section C was a negative control with no BC, and Section D was a positive control with filter paper soaked in 5% acetic acid. The agar plates were incubated overnight at 37 º C after which the bacterial growth was observed.

2. Samples of unmodified and surface acetylated BC were cut into small pieces and washed in PBS. These BC pieces were transferred to M63 media inoculated with *P. aeruginosa* of the pathogenic strain P14 at a dilution factor of 25. Inoculated media with neither type of BC was used as a control for a total of three sample sets. Each sample set was incubated at 37 º C for 24 hours. The bacterial growth was measured with OD measurements at 600 nm after 24 hours.

3.7 Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine the statistical significance of the data pertaining to the human cell cytotoxicity assays and antibacterial assays. ANOVA was considered statistically significant when P-value < 0.01.

Chapter 4: Results

4.1 Structural Characterization

Figure 4.1 gives a comparison of unmodified (left) and surface acetylated BC (right). Evidently, pigments were produced during the heating of acetic anhydride and citric acid in the surface acetylation reaction. The reaction solution was initially colorless before turning purple for a time and then eventually black giving the surface acetylated BC a brownish tint. This has previously been reported in the literature [Odland, 1971]. Aside from the color change, surface acetylation caused no immediately observable change in physical properties.

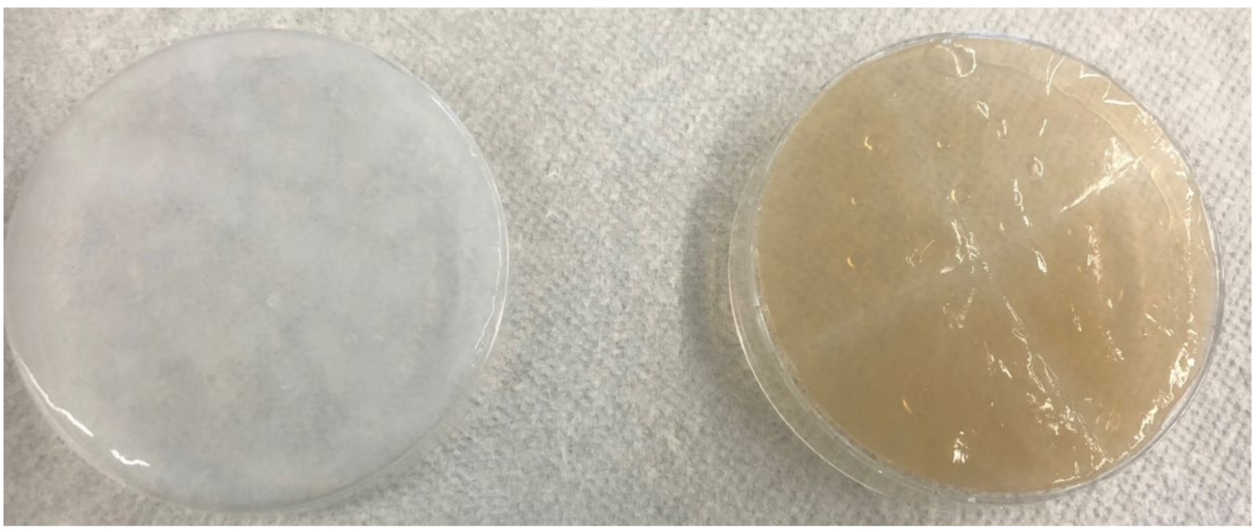

Figure 4.1: Unmodified (left) and surface acetylated bacterial cellulose (right)

4.1.1 FTIR Spectroscopy

The grafting of acetyl groups onto the surface of BC was qualitatively confirmed by FTIR spectroscopy. **Figure 4.2** shows the FTIR spectra of unmodified (**a**) and surface acetylated (**b**) BC. Spectra 4.2(a) is typical of unmodified BC. The broad peak at 3347 cm^{-1} is the result of OH stretching, 2896 cm^{-1} is due to C-H stretching, the peaks in the 1000-1200 cm^{-1} range indicate C-O-C and C-O stretching, and 898 cm^{-1} results from the β-1,4 bond vibration (Atykyan et al., 2020). Surface acetylation is confirmed by the presence of three new peaks in Spectra 4.2(b) corresponding to the addition of the acetyl groups:1729 cm^{-1} (the most prominent peak) corresponds to the acetyl C=O stretching, 1372 cm^{-1} corresponds to C-H bending, and 1249 cm^{-1} corresponds to C-O stretching of the acetyl groups (Fei et al., 2017).

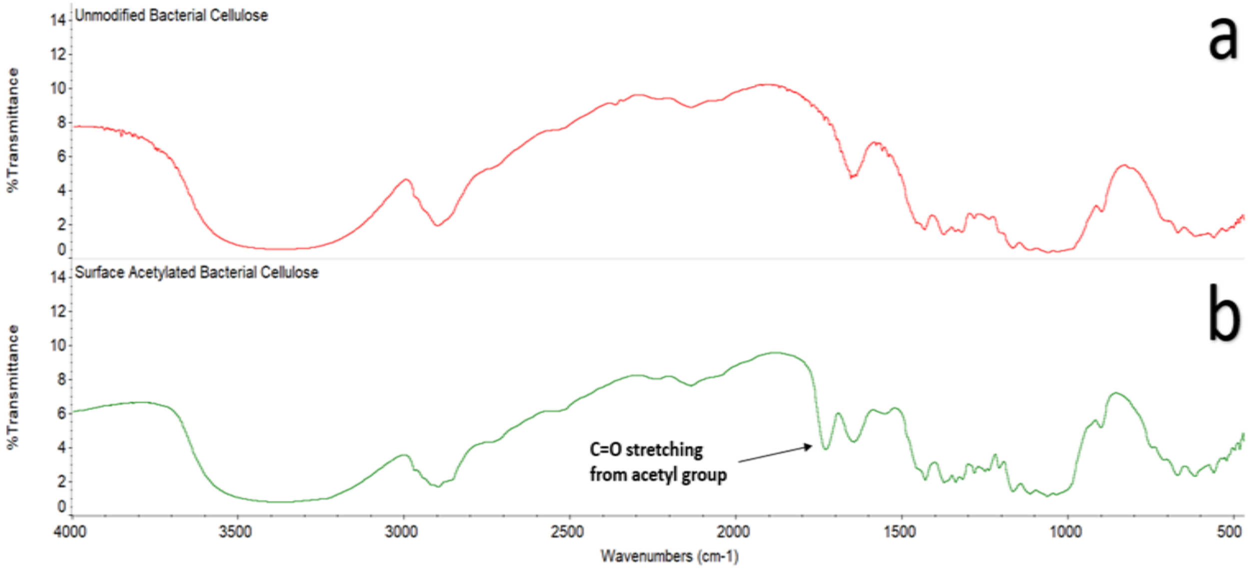

Figure 4.2: Comparison of the FTIR spectra of unmodified (a) and surface acetylated (b) bacterial cellulose.

4.1.2 HPLC-UV

Confirmation of surface acetylation by FTIR was followed by the quantification of the degree of acetylation by HPLC-UV. More specifically, HPLC-UV was used to determine the concentration of acetate released from the surface of acetylated BC after a saponification reaction and from this concentration the degree of substitution (degree of acetylation) was calculated. Individual BC mats (63 mg dry weight) were put through a saponification reaction. This process is quantitative as it completely removes the acetyl groups from the BC surface (Joshi et. al, 2016).

A 1 mL sample of the saponification solution was then run through HPLC-UV. **Figure 4.3** shows the chromatograms of the saponification solution from surface acetylated BC (top), a 0.1% acetic acid standard (middle), and the plain saponification solution of ethanol/water/NaOH with no BC (bottom).

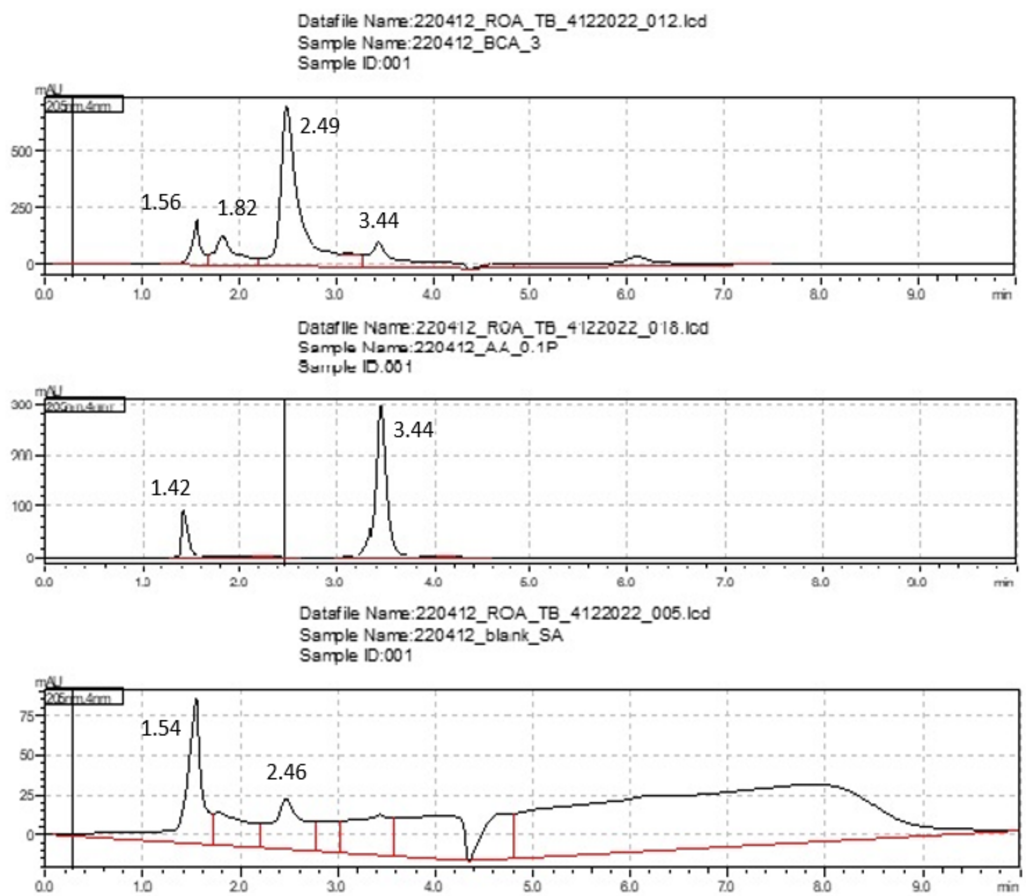

Figure 4.3:

HPLC-UV chromatograms of saponification solution with surface acetylated bacterial cellulose (top), 0.1% acetic acid standard (middle), and saponification solution (bottom).

The peaks at the beginning of each chromatogram with a retention time of approximately 1.50 min are the solvent fronts of the chromatograms. The solvent front is a consistent feature of chromatograms and should be neglected when analyzing the data. The surface acetylated BC solution (top) shows a large peak at 2.49 min and a small peak at 3.44 min. The 3.44 min peak is assumed to correspond to an acetate peak as the 0.1% acetic acid (middle) also produces a peak at 3.44 min.

The 2.49 min peak is assumed to correspond to the saponification solution of ethanol/water/NaOH. This is due to the presence of a peak at 2.46 min in the saponification solution with no BC chromatogram (bottom). The corresponding UV spectra for each peak in in this figure are included in **Appendix A**.

Figure 4.4 shows a calibration curve derived from a series of acetic acid standards of known concentrations. Based on this curve the average concentration (v/v) of acetate released by the surface acetylated BC upon saponification was determined to be 0.0625% (see intersection of red lines).

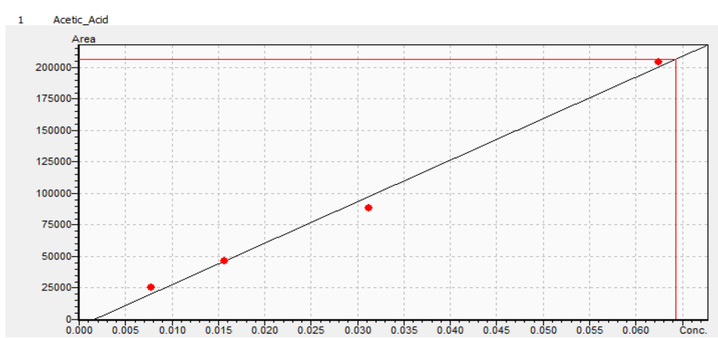

Figure 4.4: Calibration curve of acetic acid standards. The intersection of the red lines corresponds to the concentration of acetate released from the surface acetylated BC.

From this value the degree of acetylation was determined to be 0.29. The relevant calculations are shown below:

[Acetate]= 0.0625% (v/v) Total Saponification Volume= 8 mL

HPLC-UV Sample Volume: 1.3 mL (1 mL from saponification solution + 0.30 mL H_2SO_4)

$$0.000625 \times 1.3 \text{ mL} = 0.0008125 \text{ mL Acetate}$$

$$0.0008125 \text{ mL Acetate} \times 1.05 \text{ g/ mL (Density of Acetic Acid)} = 0.000853 \text{ g Acetate}$$

$$0.000853 \text{ g Acetate} \div 59.04 \text{ g / mol (Acetate Molecular Weight)} = 0.0000144 \text{ mol Acetate}$$

$$(0.0000144 \text{ mol Acetate / 1 mL}) \times 8 \text{ mL} = 0.000115 \text{ mol Acetate total}$$

$$0.063 \text{ g BC} \div 162.14 \text{ g/mol (AGU molecular weight)} = 0.00039 \text{ mol AGU}$$

$$\text{Degree of Acetylation} = [\text{Acetate mol}] / [\text{AGU mol}] = [0.000115 \text{ mol}] / [0.00039 \text{ mol}] = \mathbf{0.29}$$

Comparing the calculated degree of acetylation value (0.29) to the theoretical limit that can be achieved by surface acetylation is also of interest. The theoretical limit was calculated as follows:

Step 1: Average dimensions of BC fibrils [Width: 40 nm, Height: 8 nm] (Tokoh et al., 1998)

Step 2: Number of cellulose chains per fibril cross section

Spacing between cellulose molecules:

0.53 nm [Along Width], 0.62 nm [Along Height] (Sugiyama et al., 1991)

40 nm / 0.53 nm = 75 cellulose chains along fibril width

8 nm / 0.62 nm = 13 cellulose chains along fibril height

75 x 13 = 975 cellulose chains per cross section

Step 3: Number of cellulose chains on surface of fibril

(13 x 2) + (75 x 2) = 160 cellulose chains on surface

176 / 975 = 18% of cellulose chains on surface

Step 4: Number of OH groups on surface

Assuming only half are exposed to surface. Therefore, upper limit of surface degree of acetylation is 1.5 as this is half of the maximum degree of substitution of cellulose (3.0).

1.5 x 0.18= 0.27

The upper limit of degree of acetylation on BC surface is **0.27**

The theoretical degree of acetylation of 0.27 is very close to the determined value of 0.29. This indicates that the method of surface acetylation used in this project was essentially able to achieve the maximum degree of acetylation.

4.1.3. X-ray Diffraction

The effect of the acetylation procedure on the crystallinity of the BC fibrils and crystallite size was determined by diffraction. The diffraction patterns of unmodified and surface acetylated BC are shown in **Figure 4.5**. As expected, BC displays a typical Cellulose I structure with three peaks centered at $2\theta = 14.4°$, 16.7°, 22.6°. These peaks arise from the 100, 010, and 110 crystallographic planes of cellulose I_α, respectively [(Sugiyama et al., 1991), (Bootten et al., 2007)]. After surface acetylation, the XRD pattern of the acetylated BC showed the same peaks as unmodified BC but with the intensity of the peaks at 14.4° having decreased relative to the other two peaks. This agrees with previously reported trends in which the peak at 14.4° decreased in intensity as the degree of acetylation increased (Ramirez et al., 2016).

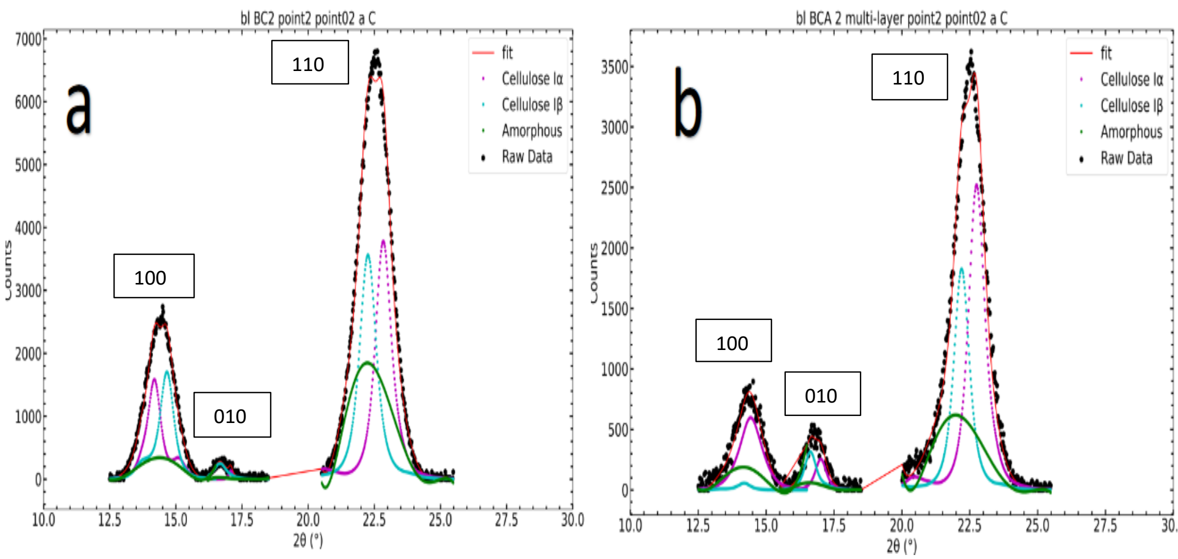

Figure 4.5: X-ray diffraction patterns of unmodified (a) and surface acetylated (b) bacterial cellulose

Further analysis of the patterns was performed using the Rietveld Refinement and reported in **Table 4.1**. The dominant allomorph in BC is Cellulose I_α. The crystallinity index for the unmodified BC was 64.8% (standard deviation 8.2) while that of surface acetylated BC was 67.4% (standard deviation 11.9). The difference in crystallinity is 2.6% but as this is lower than the standard deviation values it cannot be concluded that there is truly a difference in crystallinity between unmodified and surface acetylated BC. Using the Scherrer Equation (See **Appendix B**) with the full width at half maximum (FWHM) of the peak at 22.6°, the average crystallite size of the unmodified and surface acetylated bacterial cellulose calculated to be 6.58 nm (standard deviation 0.30) and 6.74 nm (standard deviation 0.44), respectively. The crystallite size difference of 0.16 is within the standard deviation values and thus it cannot be concluded that there is truly a difference in crystallite size between unmodified and surface acetylated BC. In summary, the acetylation procedure did not cause any significant changes in crystallinity and crystallite size.

Table 4.1: Crystallinity index and crystallite size values of unmodified and surface acetylated bacterial cellulose

Bacterial Cellulose Sample	Unmodified	Surface Acetylated
Crystallinity Index (%)	64.8 (± 8.2)	67.4 (± 11.9)
Crystallite Size (nm)	6.58 (±0.30)	6.74 (± 0.44)

4.1.4 FESEM

The effect of the acetylation procedure on the morphology of the BC fibril network was qualitatively determined by field emission scanning electron microscopy (FESEM). **Figure 4.6** displays the FESEM images of unmodified (**A-C**) and surface acetylated BC (**D-F**) at magnifications of 10x, 30x, and 100x. As demonstrated by these images, BC exists as a matrix of nanosized, thin fibrils. Visual comparison of the two image sets shows that the morphology remains essentially unchanged post-surface acetylation with no visible damage to the fibrils themselves and the overall fibril matrix is being maintained.

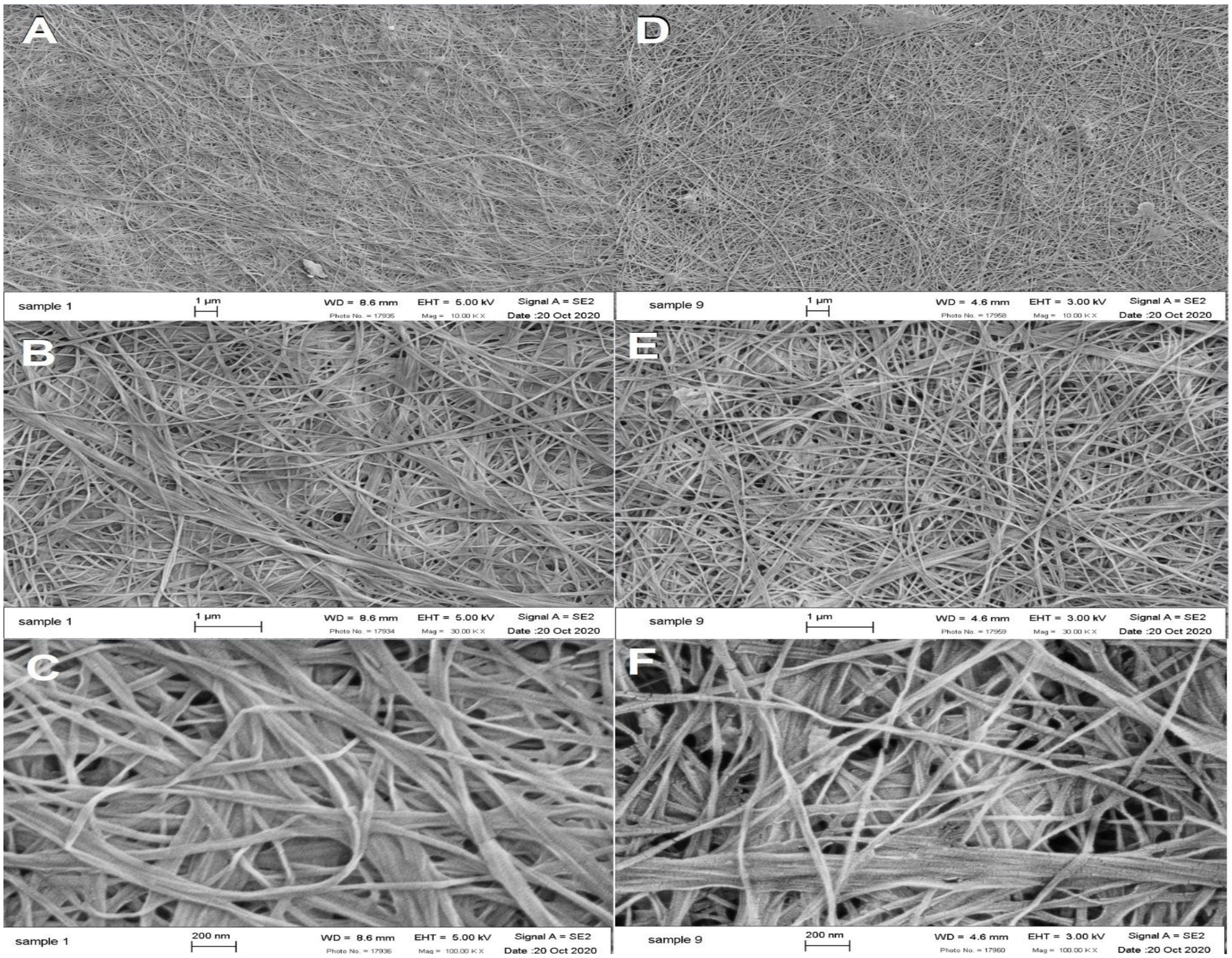

Figure 4.6: FESEM image of unmodified (A-C) and surface acetylated (D-F) at magnifications of 10X (A, D), 30X (B, E), 100X (C, F).

4.2 Cytotoxicity Assays

The potential cytotoxic effect of surface acetylated BC on human cells was assessed with cytotoxicity assays. **Figure 4.7** shows the percent viability of human umbilical vein endothelial cells (HUVECs) after exposure to unmodified and surface acetylated BC extracts after 24 hours, 72 hours, and 1 week. HUVECs were used because this cell line is a model for human primary cells. The percent viability is relative to the negative control sample set with no bacterial cellulose. The negative control therefore has a percent viability of 100%. In comparison to the negative control the percent viability of both unmodified and surface acetylated bacterial cellulose was above 100% after 24 and 72 hours indicating no cytotoxic effect. The percent viability of the surface acetylated sample set after 1 week exposure was lower than 100% but is not statistically significant as confirmed by ANOVA testing (**Table 4.2**). Only the positive control of DMSO showed a statistically significantly lower percent viability relative to the negative control.

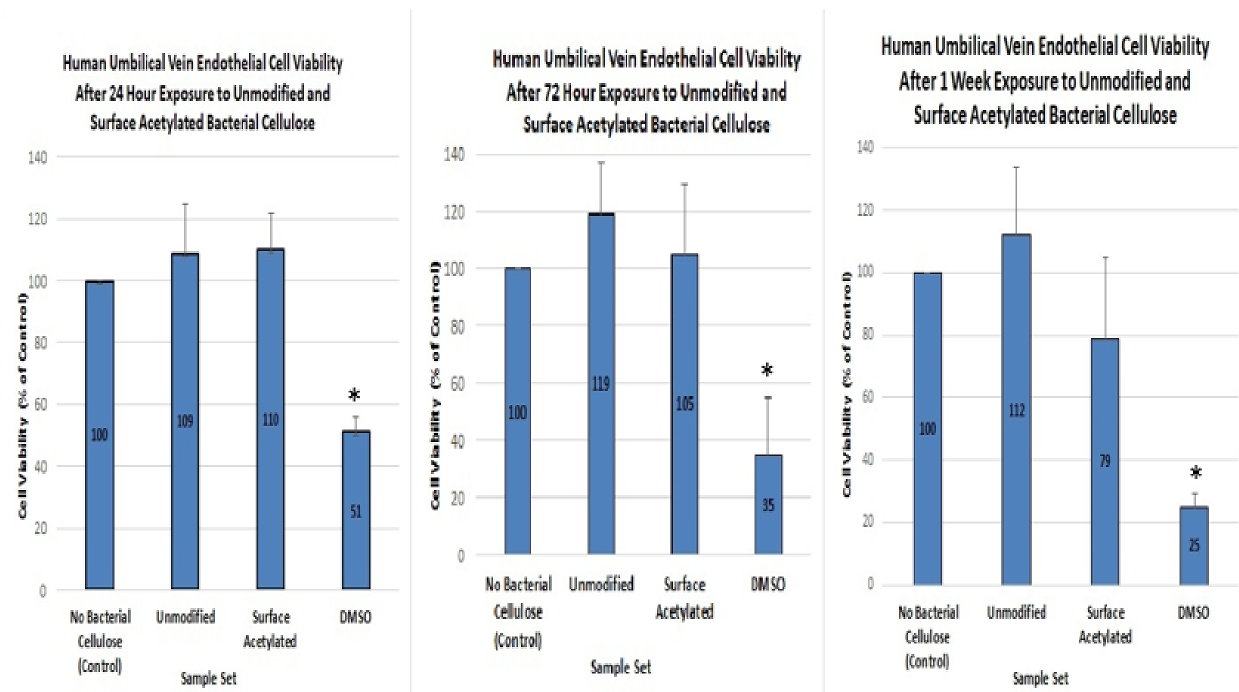

Figure 4.7: The effect of unmodified and surface acetylated bacterial cellulose on human umbilical vein endothelial cells after 24 hours (left), 72 hours (center), and 1 week. Data shown are mean ± SD for each group (n=10 for positive and negative controls and n=20 for the cellulose samples) (*p < 0.01)

Table 4.2: ANOVA for HUVEC cytotoxicity assay (1 week exposure to surface acetylated bacterial cellulose)

	Degrees of Freedom	Sum of Squares	Mean Square	F-Statistic	P-Value
Between Groups	1	0.287	0.287	6.4748	0.0167
Within Groups	28	1.2413	0.0443		
Total	29	1.5283			

4.3 Antibacterial Assays

The antibacterial effect of surface acetylated BC was assessed with antibacterial assays. **Figure 4.8** displays the effect of BC and surface acetylated BC when in direct contact with a layer of *E. coli* and *S. epidermidis* on agar plates. The agar plate coated with *E. coli* in shows no inhibition zones whatsoever surrounding either type of BC as bacterial growth encompasses the entire agar plate except for the section exposed to the positive control of 5% acetic acid. This observation holds true for the agar plates coated with *S. epidermidis* as well indicating that this surface acetylated BC has no antibacterial effect on either species of bacteria. In fact, in both cases there appears to be bacterial growth on both the unmodified and surface acetylated samples themselves. This may indicate that the BC fibril matrix itself may serve as a suitable template for bacterial growth.

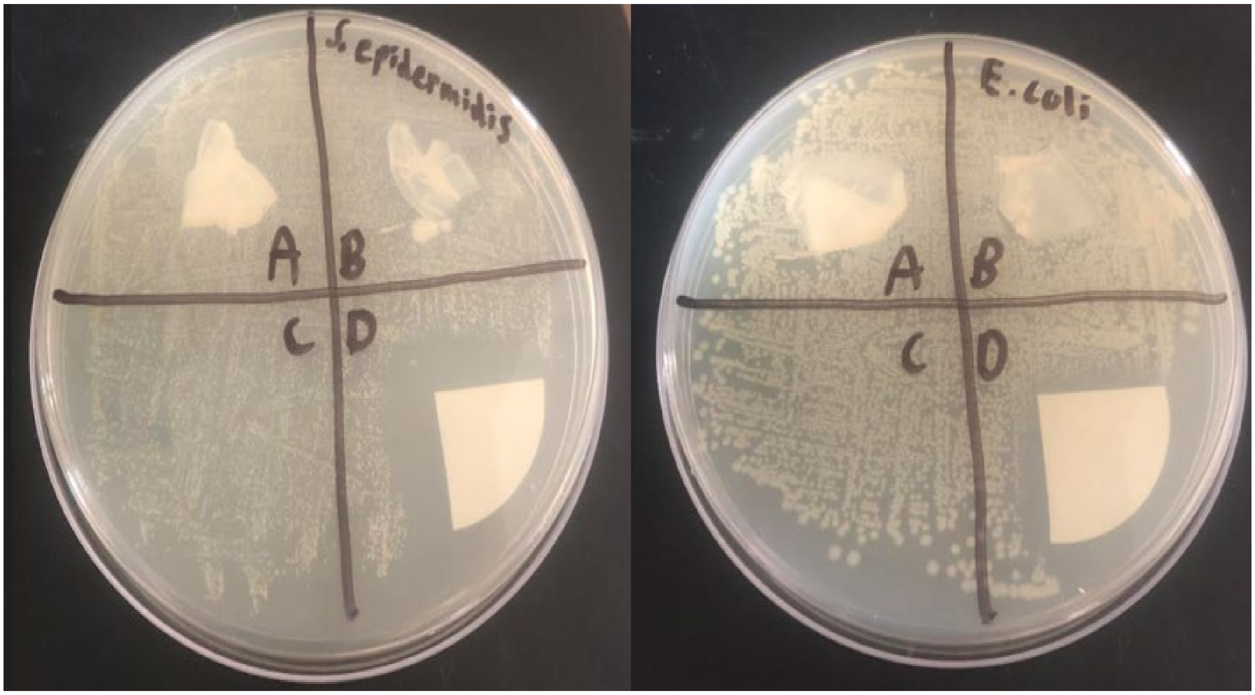

Figure 4.8: Antibacterial assays showing the effect of unmodified (A) and surface acetylated (B) bacterial cellulose on *E. coli* and *S. epidermidis*. No bacterial cellulose (C) is used as the negative control and 5% acetic acid (D) serves as the positive control. Except for the acetic acid quadrant, no zone of inhibition was observed.

Figure 4.9 presents the absorbance value of *P. Aeruginosa* (Strain P14) in the presence of unmodified, surface acetylated, and no BC after a period of 24 hours. The greater the absorbance value, the greater the bacterial growth. Relative to the control of no BC, neither the unmodified nor surface acetylated BC showed a statistically significant reduction in bacterial growth as determined by one-way ANOVA testing (**Table 4.3**). This implies that the surface acetylated BC causes no reduction in bacterial cell viability and thus exhibits no antibacterial effects.

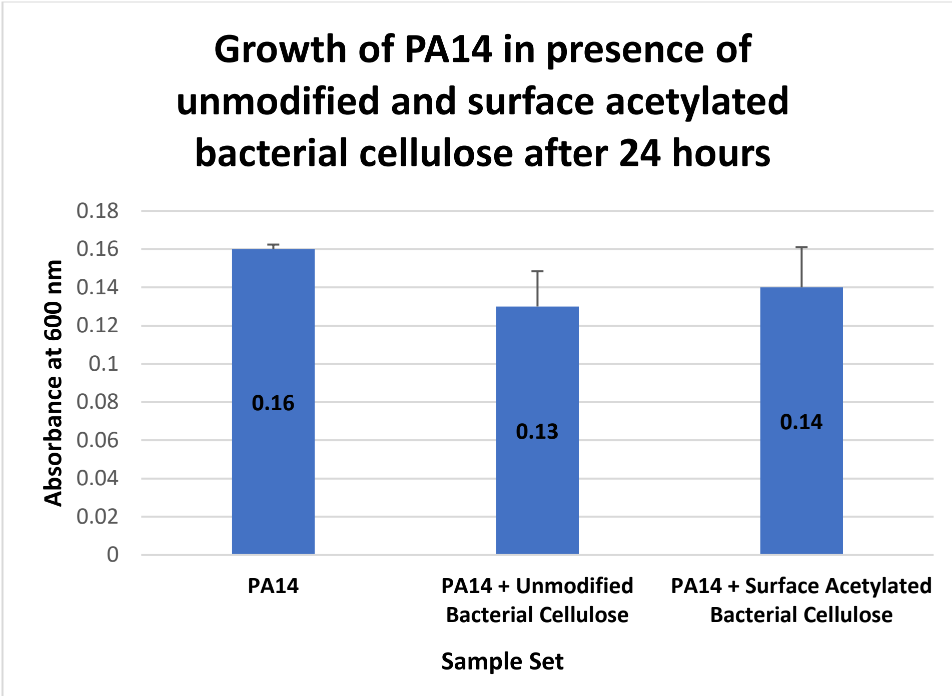

Figure 4.9: Growth of *P. aeruginosa* in the presence of unmodified and surface acetylated bacterial cellulose after 24 hours. Data shown are mean ± SD for each group (n=9 for control and n=18 for the cellulose samples) (*p <0.01)

Table 4.3: ANOVA for Antibacterial Assay #2

	Degrees of Freedom	Sum of Squares	Mean Square	F-Statistic	P-Value
Between Groups	2	0.0016	0.0008	2.0163	0.1758
Within Groups	12	0.0047	0.0004		
Total	14	0.0063			

Chapter 5: Conclusions and Discussion

5.1 Conclusions

This project studied the potential of surface acetylated bacterial cellulose as a novel antibacterial wound dressing. More specifically, this project was an investigation into the ability of surface acetylated bacterial cellulose to reduce bacterial cell viability through the production of acetic acid via the enzymatic cleavage of the ester linkage between the acetyl groups and the surface of the bacterial cellulose

Structural characterization experiments indicate the chosen acetylation method (Ramirez et al., 2016) can acetylate the surface of never dried bacterial cellulose mats to a low degree of acetylation of 0.29. These experiments also give evidence that achieving this DS value has no significant effect on the preservation of the bacterial cellulose fibril network and crystalline structure. The preservation of the physical structure of bacterial cellulose is likely critical as the morphology gives bacterial cellulose its unique properties relative to plant cellulose, including its greater tensile strength and water holding capacity, that make it such an ideal wound dressing material.

Evidently, the acetylation method used in this project is not intensive enough to penetrate and acetylate the interior of the bacterial cellulose leading to an upper limit on the degree of acetylation. Still, limiting acetylation to the surface is intended. Any interactions between bacterial hydrolytic enzymes and this material would likely be limited to its surface meaning the presence of acetyl groups in the interior of the bacterial cellulose would be irrelevant. It is also possible that higher levels of acetylation may lead to greater disruption of the physical structure the occurrence of which has been already explained as something to possibly be avoided. If true, then finding an appropriate balance between increasing the degree of acetylation and maintaining the physical structure would be of importance.

Cytotoxicity assays showed no evidence of any cytotoxic effect of surface acetylated bacterial cellulose on HUVECs. This cell line is a suitable model for human primary cells. This was expected as plant-derived cellulose acetate has no adverse effect on human cells and is in fact commonly used in medical applications. Still, the surface acetylated bacterial cellulose was synthesized in a novel manner and therefore any potential cytotoxic effects needed to be evaluated. The potential for human enzymes to release acetic acid from the surface of the bacterial cellulose was also a potential concern. This could have a cytotoxic effect on human cells, in which case this material would not be suitable for wound dressing applications, no matter how effective it may prove at killing bacteria. Since no cytotoxic effect was detected, this is no longer a concern.

Finally, multiple antibacterial assays showed no evidence that the surface acetylated bacterial cellulose could reduce the cell viability of *S. epidermidis*, *E. coli*, or *P. aeruginosa*. *S. epidermidis* and *E. coli* served as non-pathogenic model bacteria to represent Gram-negative and Gram-positive bacteria, respectively, while *P. aeruginosa* functioned as a model for pathogenic bacteria. There are two potential reasons for the lack of antibacterial activity. First, the ester linkage between the acetyl groups and bacterial cellulose chain may simply not be susceptible to cleavage by bacterial hydrolytic enzymes, in which case no acetic acid will be produced and a major assumption of this project has been proven false. Second, conversion of the acetyl groups to acetic acid via enzymatic activity may occur but the concentration of the released acetic acid is too low to lead to significant bacterial cell death. This would be a natural consequence of the degree of acetylation being quite low due to acetylation only occurring on the surface of the bacterial cellulose. In conclusion, the first explanation reveals a flaw in the underlying hypothesis of this project while the second explanation indicates that the hypothesis of this project may have value in theory but not in practice.

5.2 Project Limitations

This project may have some limitations. As mentioned, the antibacterial assays for *E. coli* and *S. epidermidis* showed no antibacterial activity. It is possible that any secreted bacterial hydrolytic enzymes may not have been able to encounter the ester linkages on the surface acetylated bacterial cellulose due an inability to diffuse through the solid LB agar. As opposed to the antibacterial assay with *P. aeruginosa* in which liquid media was used and thus enzyme movement was far less restricted. If true, then the lack of antibacterial activity may be a false negative. Though this possibility is rendered moot if the enzymatic cleavage of the ester linkage does not occur even if the ester linkages and bacterial enzymes are in full contact. Repeating these agar plate assays with loose agar such as Mueller-Hinton agar may allow for greater diffusion of any hydrolytic enzymes secreted by the bacteria and in turn may increase the likelihood of observing antibacterial activity, if any.

5.3 Future Research

There are several potential extensions of this project. Perhaps another method of acetylation would have achieved a greater degree of surface acetylation and in turn a greater concentration of acetic acid released by enzymatic cleavage that may have been great enough to lead to reduced bacterial cell viability. This is assuming such enzyme-mediated production of acetic acid does occur. Still, these other acetylation methods typically involve potentially harmful solvents as was the case in Kim et al., 2002. The usage of such solvents would ideally be avoided in the synthesis of a material meant for a biomedical application. Though an extensive washing method may be able to remove traces of such solvents left on the bacterial cellulose. Also, as previously discussed, a higher degree of acetylation may not necessarily be beneficial.

This project did not fully establish whether bacterial hydrolytic enzymes, or any enzyme for that matter, can cleave the ester linkage between the acetyl groups and bacterial cellulose and convert the acetyl to acetic acid. An extensive in vivo study of the ability of a variety of enzymes to do so may be a beneficial extension of this project. The specific enzymes produced by the pathogenic bacteria commonly found in infected wounds may be difficult to obtain, and for this reason, non-bacterial enzymes may need to be used as model enzymes. The potential differences in enzymatic activity between in vivo and in vitro environments would need to be considered, as would the limitations in using non-bacterial enzymes as substitutes for bacterial enzymes.

Other methods of attaching acetyl groups to the surface of bacterial cellulose are also of interest. The specific acetyl linkages produced in this project may not be acted upon by bacterial enzymes, but this may not be the case for other variations of acetyl linkages. Different methods of surface modification may be explored in order to synthesize a bacterial cellulose derivative that can be confirmed to release acetic acid in the presence of bacterial enzymes.

5.4 Significance

Based on the results of this project, it cannot yet be concluded that surface acetylation is a suitable way to modify bacterial cellulose with antibacterial properties. This project appears to be the first instance in which the acetylation method first reported in Ramirez et al., 2016 was used to acetylate the surface of never-dried bacterial cellulose. The lack of observed antibacterial activity does not necessarily mean it is not possible to combine the suitability of bacterial cellulose as a wound dressing material with the antibacterial properties of acetic acid. Other methods of incorporating acetic acid into the bacterial cellulose fibril matrix may prove more successful. One possible implication of this project may be the inability of bacterial enzymes to act upon synthetic acetyl linkages. The practical significance of this study is its contribution to the overall body of knowledge on the utilization of bacterial cellulose as a wound dressing material.

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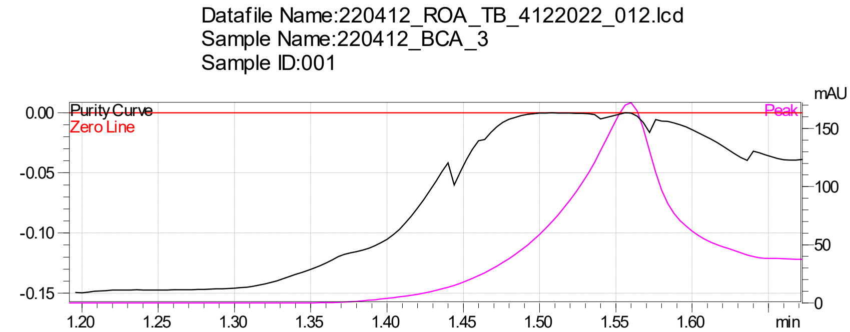
Appendix A

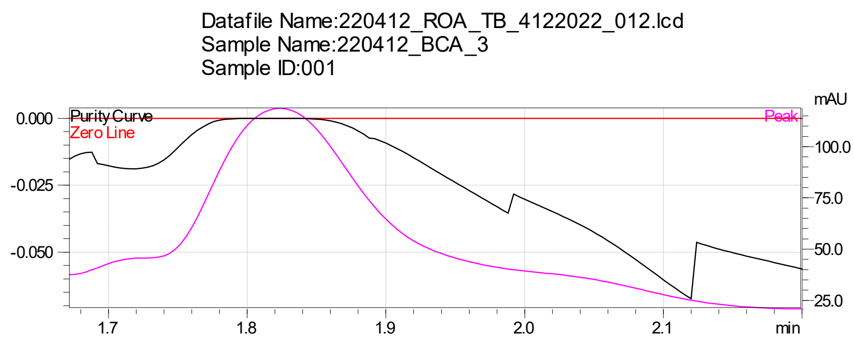
HPLC-UV Spectra for Figure 4.4

Figure 4.4 (Top)

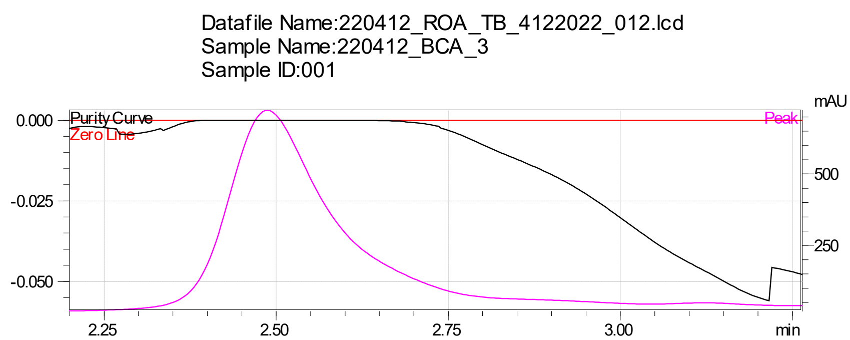
Peak at 1.56 min



Peak at 1.82 min



Peak at 2.49 min



Peak at 3.44 min

Datafile Name:220412_ROA_TB_4122022_012.lcd
Sample Name:220412_BCA_3
Sample ID:001

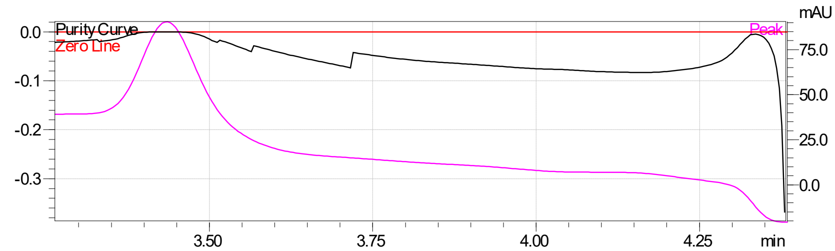
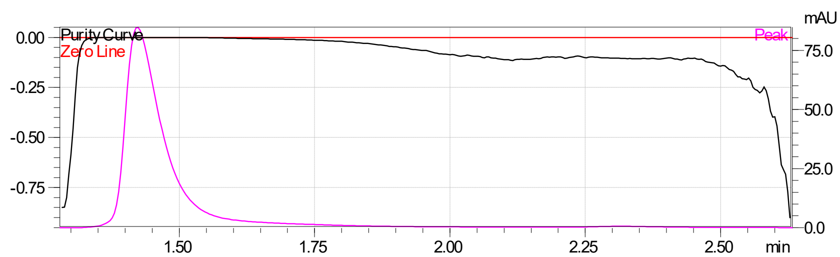


Figure 4.4 (Middle)

Peak at 1.42 min

Datafile Name:220412_ROA_TB_4122022_018.lcd
Sample Name:220412_AA_0.1P
Sample ID:001



Peak at 3.44

Datafile Name:220412_ROA_TB_4122022_018.lcd
Sample Name:220412_AA_0.1P
Sample ID:001

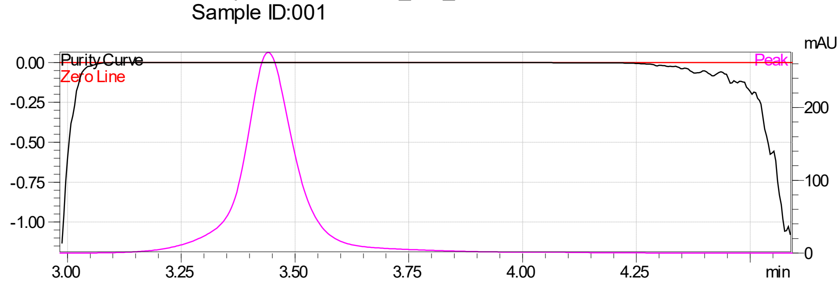
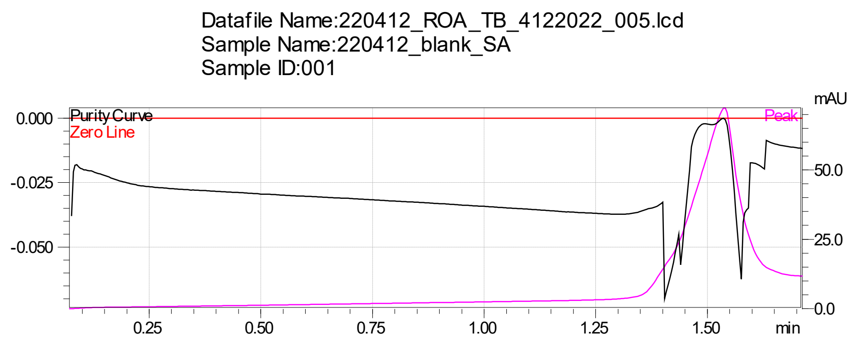
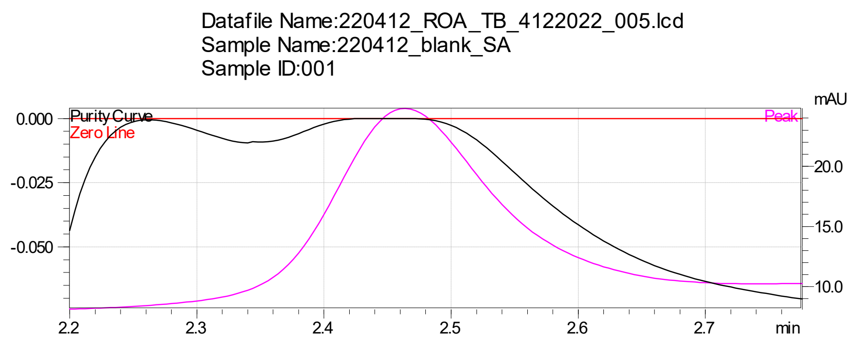


Figure 4.4 (Bottom)

Peak at 1.54 min



Peak at 2.46 min



Appendix B

Scherrer Equation

$$D = \frac{K\lambda}{\beta \cos\theta}$$

D is the mean size of the ordered (crystalline) domains

λ is the X-ray wavelength

K is the shape factor. It is dimensionless with a value of 0.94.

β is the full width at half the maximum intensity (FWHM) in radians

θ is the Bragg angle