Applications of Nanotechnology for Microbial Diagnostics to Combat Infectious Diseases

A DISERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Vinni Novi Thekkudan

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: Dr. Abdennour Abbas

March 2024
Acknowledgements

There are several people to thank for their guidance and support throughout this program. First, my deepest gratitude to my academic advisor, Dr. Abdennour Abbas, for being a supportive mentor throughout my doctoral journey. His constant guidance in my academic and professional development is invaluable. My sincere gratitude to my committee members Dr. Brett Barney, Dr. William Tze, and Dr. Brett Arenz for their time and commitment in providing feedback to bring the best out of this work. I thank Dr. Benham Lockhart, Dr. Ping Wang and Dr. Roger Ruan for their valuable insights during the formative years of my program. I also thank my research collaborators Dr. Hamada Aboubakr for his valuable contribution to the initial LAMP design as well as Dr. Jennifer Juzwik and Melanie Moore for their support in sample preparation in Chapter 4. I also thank my colleagues Dr. Anil Meher and Akli Zarouri for extending their support. I would like to thank Dr. Snober Ahmed, Dr. John Brockgreitens and Andrew Gonzalez for their significant contributions to sample preparation and characterization in Chapter 5. I thank Juer Liu for her support in NMR experiments in chapter 6.

I am extremely grateful to Schwan’s Company for providing me with the Schwan’s Graduate Fellowship that covered my PhD education. Funding for various projects was provided by the Minnesota Environment and Natural Resource Trust Fund as recommended by the LCCMR, through the MITPPC, and the USDA National Institute of Food and Agriculture, the CDC, the USDA Small Business Innovation Research program, and the US Army Department of Defense Small Business Technology Transfer program (Claros Technologies Inc.). Portions of this work were conducted in the Bio-Nano Labs at the Minnesota Nano Center, which is supported by the NSF through the National Nanotechnology Coordinated Infrastructure (NNCI). Parts of this work were carried out in the Characterization Facility, UMN, which receives partial support from the NSF through the MRSEC and the NNCI programs. Some of the work presented in chapter 5 is the subject of an International Patent Application No. PCT/US2016/056850 and US Patent Application No. 63/123,814 “Antimicrobial and Antiviral Nanocomposite Sheets”.

The protocol development and testing would not have been possible or complete without the support of the United States Forest Service Northern Research Station, NRS-16, the Plant Disease Clinic at UMN and the Biophysical Technology Center.

I would also like to thank my other lab members Dr. Quichen Dong and Dr. Kaige Zhang, all my friends in both BBE North and South, and especially my best friend Koushik Sampath for making this journey of learning so much more fun. Further thanks to the group of undergraduate students who diligently assisted me in this work.

Finally, I would like to express my love and gratitude to my family and friends in India and the United States, particularly my parents and my sister Evina for their constant encouragement and strength. This would not be possible without all your care and attention.
Dedication

I dedicate this dissertation to my parents and grandparents, their relentless prayers and faith in my success has been a constant source of strength.
Abstract

Various pathogens cause disease outbreaks in plants, animals and humans that have led to fatalities and economic losses. To effectively prevent these outbreaks, disease surveillance and early diagnosis is crucial. While there are some standard detection methods used currently, they are expensive and suffer from long turnaround times that delay treatment and disease control actions. Since nucleic acid detection techniques are preferred as they offer target gene specific diagnosis, significant research has been directed to simplifying them for faster and more accurate identification. Several isothermal amplification methods have been explored for this purpose, among which loop mediated isothermal amplification (LAMP) offers a simple, cost-effective, and reliable approach for rapid onsite pathogen detection. This study discusses the design and optimization of a LAMP assay for the detection of the oak wilt fungus, Bretziella fagacearum, as the model organism. Oak wilt disease is a significant threat to oak (Quercus spp.) tree health in the United States and eastern Canada. Without management the disease may cause dramatic changes to natural and urban ecosystems. Early and accurate diagnosis is necessary for timely treatment. The LAMP assay developed for oak wilt takes 30 min to complete and shows 100% sensitivity and specificity. Recent studies have investigated colorimetric visualization of LAMP products for their adaptability to onsite microbial detection, but they suffer from reproducibility and varied perceptions of color change. Therefore, this study also discusses the development of a novel LAMP visualization method by exploiting the optical properties of gold nanoparticles (AuNPs) to overcome those challenges. Oligonucleotide-coated AuNPs (AuNP-oligos)
hierarchically assemble on DNA networks in positive samples to form globular nanostructures, that settle into a visible red pellet upon inducing precipitation whereas, the negative samples do not show this. This LAMP assay coupled with AuNP-oligos visualization is a promising method for accurate and rapid molecular-based diagnosis in field settings.

Additionally, other applications of nanomaterials in combating infectious diseases are also discussed. This includes the study of zinc oxide nanoparticle coated textiles for their antimicrobial activity against human bacterial and fungal pathogens, *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Candida albicans*. 
Table of Contents

Chapter 1. Introduction ...........................................................................................................1

Chapter 2. Literature Review ...............................................................................................6
  2.1 Turbidity-based LAMP detection .................................................................................6
  2.2 Fluorescence-based LAMP detection .........................................................................8
    2.2.1 Fluorescent dyes ..................................................................................................8
    2.2.2 Fluorescent nanoparticles ....................................................................................11
  2.3 Bioluminescence-based LAMP detection .................................................................13
  2.4 Colorimetric LAMP detection ....................................................................................16
    2.4.1 Dye-based colorimetry .......................................................................................16
      2.4.1.1 Intercalating dyes ........................................................................................16
      2.4.1.2 pH-sensitive dyes ........................................................................................21
      2.4.1.3 Metal-indicating dyes ....................................................................................25
    2.4.2 Nanoparticle-based colorimetry .........................................................................27
  2.5 Conclusions ..................................................................................................................32

Chapter 3. Research Objectives ..........................................................................................33

Chapter 4. Naked-eye visualization of nucleic acid amplicons using hierarchical nanoassembly ..................................................................................................................35
  4.1 Introduction ................................................................................................................35
  4.2 Experimental Section .................................................................................................38
    4.2.1 Materials ............................................................................................................38
    4.2.2 Fungal culture and DNA extraction .....................................................................38
    4.2.3 Obtaining LAMP amplicons ...............................................................................39
    4.2.4 LAMP visualization using agarose gel electrophoresis ......................................39
    4.2.5 Synthesis of gold nanoparticle (AuNP)-oligos .....................................................39
    4.2.6 Optical detection of LAMP products with AuNP-oligos .....................................41
    4.2.7 Evaluation of the AuNP-oligos-amplicon assembly ............................................42
  4.3 Results and Discussion ...............................................................................................42
  4.4 Conclusions ................................................................................................................51

Chapter 5. A rapid LAMP assay for the diagnosis of oak wilt with the naked eye ...53
  5.1 Introduction ................................................................................................................53
  5.2 Experimental section .................................................................................................57
    5.2.1 Materials ............................................................................................................57
    5.2.2 Fungal culture and DNA extraction .....................................................................58
    5.2.3 DNA extraction from red oak samples ...............................................................58
    5.2.4 LAMP assay ......................................................................................................60
    5.2.5 LAMP visualization using agarose gel electrophoresis .....................................63
Chapter 6. Magnetic relaxation switching and chemiluminescence assays for rapid diagnostics ............................................. 79

6.1 Magnetic relaxation switching (MRSw) assay using magnetic nanoparticles .... 79
6.1.1 Experimental section .............................................................................. 82
   6.1.1.1 Materials ......................................................................................... 82
   6.1.1.2 Synthesis of magnetic nanoparticles (MNPs) .................................. 83
   6.1.1.3 Characterization of magnetic nanoparticles .................................... 84
   6.1.1.4 Conjugation of NH$_2$-Fe$_3$O$_4$ nanoparticles with anti *E. coli*
       antibodies .................................................................................................. 84
   6.1.1.5 Bradford assay ................................................................................ 84
   6.1.1.6 Preliminary experiments with T$_2$ NMR: MRSw assay ................. 85
6.1.2 Summary of preliminary results .............................................................. 86
   6.1.2.1 Characterization results of NH$_2$-MnFe$_2$O$_4$ nanoparticles .......... 86
   6.1.2.2 Characterization results of NH$_2$-Fe$_3$O$_4$ nanoparticles ............... 89
   6.1.2.3 Bradford assay results ................................................................... 91
   6.1.2.4 MRSw assay results ..................................................................... 92

6.2 Nanoaggregation-enhanced chemiluminescence (NEC) assay using AuNPs ..... 93

Chapter 7. Highly efficient and durable antimicrobial nanocomposite textiles ...... 97

7.1 Introduction ............................................................................................... 97
7.2 Experimental section ............................................................................... 100
   7.2.1 Nanocomposite synthesis ................................................................. 100
   7.2.2 Bacterial culture ............................................................................... 101
   7.2.3 Quantitative antimicrobial tests ......................................................... 101
   7.2.4 Textile laundering ............................................................................. 102
   7.2.5 Statistical analysis ........................................................................... 103
7.3 Results and discussion ............................................................................. 103
   7.3.1 Textile zinc nanocomposite synthesis ............................................. 103
7.3.1.1 Characterization ................................................................. 107
  7.3.1.1.1 Structure and characterization .................................... 107
  7.3.1.1.2 Synthetic precipitate leachate procedure .................... 108
7.3.2 Antimicrobial application test ............................................. 109
  7.3.2.1 Before-wash antimicrobial test ..................................... 110
  7.3.2.2 After-wash antibacterial test ....................................... 113
    7.3.2.2.1 Third-party wash durability analysis ....................... 113
    7.3.2.2.2 Laboratory wash durability analysis ....................... 114
7.3.3 Safety and skin irritation test ........................................... 115
7.4 Conclusions ........................................................................... 115

Chapter 8. Conclusions ................................................................ 116

Bibliography .................................................................................. 118

Appendix I: PhD outcomes and achievements ............................... 133
List of Tables

Table 1. List of colorimetric LAMP assays using pH-sensitive dyes .........................23
Table 2. Some LAMP visualization assays using metal nanoparticles as colorimetric indicators ......................................................................................................................................31
Table 3. Nucleic acid sequences of LAMP primers sets used for specificity tests including the chosen set ......................................................................................................................................................61
Table 4. The T_2 relaxation measurements using NMR ..........................................................................................93
Table 5. Synthetic Precipitate Leachate Procedure results from Pace Analytical for three types of cotton samples. The samples were washed 1, 50 and 100 times after functionalization ................................................................................................................................................109
Table 6. Microbial reduction (%) of Pseudomonas aeruginosa (PA), methicillin resistant Staphylococcus aureus (MRSA) and Candida albicans (CA) after 0 hours of incubation (immediate elution) and after 24 hours with zinc nanocomposite textiles (The zinc nanocomposite textiles were washed once as part of the manufacturing process to remove loose particles; microbial reduction in terms of log reductions is provided for some of the samples in brackets) ........................................................................................................................................112
Table 7. Microbial reduction (%) of Klebsiella pneumoniae (KP) and Staphylococcus aureus (SA) after 0 hours of incubation (immediate elution) and after 24 hours with zinc nanocomposite textiles subjected to 50 wash cycles. Results obtained from third party independent testing (Independent evaluation conducted by Vartest Laboratories LLC).114
List of Figures

Figure 1. Schematic of the dual-color fluorescence LAMP assay [32] ................. 10

Figure 2. Schematic of the one-step LAMP assay with cysteamine-modified CdSeS/ZnS quantum dots (amine-QDs) [34] ........................................... 13

Figure 3. Schematic of the LUMID sensor [37] .................................................. 15

Figure 4. Mechanism of LAMP detection using the triphenylmethane dye, crystal violet [45] ................................................................................................. 18

Figure 5. The microdevice design. (a) Sample chamber, (b) reaction chamber, and (c) detection chamber with fuchsin stained strips [50] ........................................ 20

Figure 6. (a) Mechanism of calcein, (b) Colorimetric results after LAMP reaction [78] ... 26

Figure 7. Schematic of the LAMP-AuNPs detection [84] ........................................... 29

Figure 8. Design of the LAMP-AgNPs colorimetric assay using the foldable microdevice [85] ........................................................................................................... 30

Figure 9. Oligonucleotide-coated gold nanoparticles hierarchically assemble on DNA networks to form globular nanostructures, which precipitate into a distinct visible red pellet. This aims to overcome challenges associated with nanoparticle aggregation and dye-based colorimetric detection in LAMP assays ........................................... 37

Figure 10. Naked-eye visualization of DNA amplicons using nanoassembly. From top to bottom: (a) DNA amplicons form globular networks under the effect of salts and ethanol, (b) uncoated gold nanoparticles (AuNPs) aggregate under the same conditions, (c) AuNPs coated with oligonucleotides (AuNP-oligos) bind non-specifically to the amplified DNA causing assembly of single nanoparticles with the DNA amplicons to form globular nanostructures, which precipitate into visible red pellet, (d) In the absence of the DNA amplicons, the AuNP-oligos remain suspended in solution as single nanoparticles. ...... 37

Figure 11. The process of using ImageJ software to analyse the amount of AuNP-oligos remaining in the supernatant by measuring the gray value. The yellow arrow on the sample image shows the direction in which the measurement was done. The graph on the left indicates the gray value between the supernatant (highest value) and the pellet (lowest value). A higher difference between the two indicates that more AuNP-oligos assembled with the DNA amplicons to form the red pellet. The measurements were done in triplicates for every sample ................................................................. 42
Figure 12. Agarose gel image of LAMP products. Well M serves as a marker. Well 1 contains *B. fagacearum*. LAMP amplicons and wells 2-8 are 2x, 4x, 6x, 8x, 10x, 20x and 40x dilutions of the amplicons in well 1.

Figure 13. Transmission electron microscopy (TEM) images of AuNPs-oligos DNA conjugates (a) before and (b) after nanoparticle assembly following the addition of DNA amplicons. In the presence of the amplicons, the AuNP-oligos conjugates (10 nm) assemble into globular nanostructures of 300-700 nm.

Figure 14. (a) Agarose gel image of LAMP products obtained from amplifying *Listeria ivanovii* gene. Well M serves as a marker. Wells L1 to L3 contains *L. ivanovii* maintained as triplicate. Well W indicates the negative control, which is nuclease free water. (b) Visualization of *Listeria ivanovii* DNA amplicons (2.44 mg/ml) by nanoassembly with nuclease free water as negative control.

Figure 15. Visualization of *Bretziella fagacearum* (*B. fag*) DNA amplicons by nanoassembly. (a) AuNPs were coated with oligonucleotide probes that are specific to *B. fag*, then mixed with the *B. fag* DNA amplicon to cause nanoassembly and precipitation into a visible red pellet. The control sample (b) was obtained by replacing the *B. fag* DNA amplicon with nuclease free water.

Figure 16. Different control experiments to confirm the specificity of the nanoassembly and red pellet formation for the presence of DNA amplicons. Samples that did not contain all the necessary ingredients (AuNP-oligos, salts, ethanol, DNA amplicons) showed no visible red precipitation. P depicts the presence of oligonucleotides on the AuNPs. The “Sample” represents DNA amplicons obtained for LAMP reactions with primers specific to *B. fagacearum*, the oak wilt fungus.

Figure 17. Serial dilutions of the DNA amplicons and corresponding changes caused by AuNP-oligo assembly. The y axis represents the amount of AuNP-oligos that assemble with the DNA amplicons. Measurements were done in triplicates.

Figure 18. LAMP reaction steps. The reaction uses 4-6 primers targeting 6-8 regions on the target DNA strand. The strand displacing DNA polymerase starts the amplification process followed by two of the primers forming a loop structure to facilitate further rounds of amplification.

Figure 19. Agarose gel electrophoresis of nested PCR products obtained from testing red oak samples using crude NaOH-extracted DNA. (a) Infected samples; (b) healthy samples, water control, positive control. Samples were from the same branches as those used for the LAMP assay.
Figure 20. Characterization of the developed LAMP assay using spectrofluorometric analysis. (a) Selectivity of the assay for *Bretziella fagacearum* (BF). No detection is observed for other fungi including *Dicarrella* sp. (DS), *Fusarium sporotrichoides* (FS), *Graphostroma* sp. (GS), *Querciphoma carteri* (QC), and *Epicoccum nigrum* (EN). (b) Limit of detection of the LAMP assay (LOD: 30 fg/µl).................................67

Figure 21. Primer specificity tests using different fungal species: *Dicarrella* sp. (DS), *Fusarium sporotrichoides* (FS), *Graphostroma* sp. (GS), *Querciphoma carteri* (QC), *Epicoccum nigrum* (EN), *Bretziella fagacearum* (BF). (a) LAMP reaction results for tests with primer set 42, (b) LAMP reaction results for tests with primer set 55 ......................67

Figure 22. Detection of the fungus *Bretziella fagacearum* in wood chip samples from healthy and infected oak trees. The wood chip samples were treated to extract and purify the fungal DNA which was then amplified using LAMP. The product of the LAMP assay was characterized using fluorospectroscopy. The results show 100% specificity and 100% sensitivity. (a) Results from LAMP assay conducted using StepOnePlus™ Real-Time PCR System for heating and fluorescence detection. (b) Results from LAMP assay conducted using Thermo Scientific™ Compact Digital Dry Bath/Block Heater for heating and Agilent-Cary Eclipse Fluorescence Spectrophotometer for fluorescence detector. The difference in the fluorescence intensity ranges in each set of experiments reflects the varied sensitivities of the fluorescence devices used.................................70

Figure 23. Agarose gel electrophoresis of LAMP products obtained from testing red oak samples. Infected samples tested on (a) real-time PCR instrument, and (b) portable dry heat block. Healthy samples along with nuclease-free water as control tested on (c) the same PCR device as a), and (d) the same heat block as b). .........................................................71

Figure 24. (a) Concept of amplicon visualization using hierarchical nanoparticle assembly. (b) The visible pellet at the bottom of the tube is formed following induced precipitation of assembled AuNP-oligo with the DNA amplicons, and (c) transmission microscope image of globular nanostructures, and (d) conceptual diagram of one assembled globule. The assembly of hundreds of these structures yields a red pellet visible to the naked eye.........................................................73

Figure 25. Detection of the fungus *Bretziella fagacearum* in wood chip samples from healthy and infected oak trees. After DNA extraction and amplification using LAMP, visualization of the amplicon is performed using oligos-conjugated gold nanoparticles. In the presence of the target DNA, the nanoparticles bind to the DNA amplicons, and assemble into globular nanostructures that can be easily precipitated to form a visible red pellet at the bottom of the microtube. (a) Results from LAMP assay conducted using a
real-time PCR instrument, (b) Results from LAMP assay conducted using a portable dry heat block .........................................................................................................................74

Figure 26. LAMP detection of the fungus *Bretziella fagacearum* in crude samples, without nucleic acid purification. (a) Amplicon characterization using spectrofluorometry, and (b) Amplicon visualization using the hierarchical assembly of oligos-conjugated gold nanoparticles ........................................................................76

Figure 27. (a) Gel electrophoresis results showing the typical LAMP amplicon bands for positive controls with *B. fagacearum* DNA, and faint bands at the bottom of the gel, indicative of no amplification in negative control and the samples containing the Cladosporium herbarum DNA. (b) The results from the AuNP-oligos visualization test, where only the positive control shows the red precipitate. ............................................................77

Figure 28. Energy levels of a nucleus with $\frac{1}{2}$ spin [149] ........................................80

Figure 29. Schematic of the magnetic relaxation switching bioassay ..........................81

Figure 30. (a) The synthesized magnetic nanoparticles attracted to a magnet; (b) TEM image of NH$_2$-MnFe$_2$O$_4$ nanoparticles ..............................................................................................................................86

Figure 31. Dynamic light scattering results for NH$_2$-MnFe$_2$O$_4$ nanoparticles ........87

Figure 32. FTIR spectrum comparison between non-functionalized MnFe$_2$O$_4$ and functionalized NH$_2$-MnFe$_2$O$_4$ nanoparticles ........................................................................................................87

Figure 33. Results from zeta potential analyzer for NH$_2$-MnFe$_2$O$_4$ ......................87

Figure 34. TEM image of the synthesized NH$_2$-Fe$_3$O$_4$ nanoparticles ..................88

Figure 35. FTIR spectrum comparing non-functionalized and amine functionalized Fe$_3$O$_4$ nanoparticles ................................................................................................................90

Figure 36. Results from zeta potential analyzer for NH$_2$-Fe$_3$O$_4$ ..........................91

Figure 37. Standard curve for Bradford assay ..........................................................92

Figure 38. Carboxdiimide chemistry steps [159]. ..................................................92

Figure 39. Chemiluminescence reaction catalyzed by metal particles in utilizing luminol to produce light [160] .......................................................................................................94

Figure 40. NEC assay working principle: The DNA probes 1 and 2 are in grey and the target DNA is in red. The orange spheres represent AuNPs [113] .................................................95

Figure 41. Comparison of (a) conventional dip-coating process with (B) thermal Crescoating technology. A) Wet synthesis of nanoparticles by chemical reduction (1),
dip-coating of the textile in the nanoparticle (2), followed by washing and drying (3). (b) Impregnation of the textile in precursor solution (1), Thermal reduction by heating the textile at 100°C (2), followed by washing and drying (3).

Figure 42. SEM images of plastic nanocomposites produced with “in situ growth” process. (a) Zinc-polyurethane nanocomposite film. The blue arrows show two pieces of nanocomposite thin film. Image amplification at the film cross-section shows the presence of zinc nanoparticles inside the film. (b) Zinc-nylon nanocomposite showing zinc nanoparticles embedded with the nylon fibers. (c) Silver-polyester/cotton nanocomposite

Figure 43. Versatility of the in situ nanoparticle growth process using different nanoparticles on different textiles. nSe: nanoselenium, nb: nanoboron, nCe: nanocerium, nFe: nanoiron, nAg, nanosilver

Figure 44. SEM images of nanoparticles harvested from zinc treated polyester fabric.

Figure 45. SEM images of untreated polyester control fabric

Figure 46. Selected photos of cell culture plates used in antimicrobial testing via cell counting for samples eluted after 24 hours with $10^5$X dilution
Chapter 1. Introduction

A pathogen can be defined as an organism that infects and causes diseases in a host body. Most pathogenic organisms are usually microscopic and come under the four categories – fungi, bacteria, viruses and microscopic parasites. Depending on their characteristics, these pathogens can affect a wide range of living beings such as plants, animals, humans and even other microorganisms.

While viruses require a living cell to thrive, many bacteria and fungi can propagate through contamination of environmental samples such as water, soil, air and food. An infectious disease is usually caused by such pathogens through their transmission from a contaminated source or from infected organisms to a susceptible host [1] (This study specifically focusses on plant pathogens and the following discussion will be streamlined towards that).

Several environmental and human pathogens have caused outbreaks that have led to loss of life and economic disruption. As of 2019, the Food and Agriculture Organization of the United Nations (FAO) has estimated a global loss of around $220 billion due to plant diseases annually [2]. Apart from crop and food loss, there are also economic losses due to infections among commercial plant species such as oaks [3]. The value of just the red oak timber in Michigan alone is estimated to be $1.6 billion [4]. Similar losses have also been reported in livestock production due to animal diseases. One of the most significant losses occurs due to the spread of infectious diseases among humans, which has led to hospitalizations and death among people. In most cases of
human disease outbreaks, the source of infection is the consumption of food or water contaminated by pathogens or their toxins. Common foodborne pathogens include *Salmonella* spp., *Listeria* spp., etc. [5]. The Centers for Disease Control and Prevention (CDC) has estimated that roughly 48 million people fall sick annually, out of which there are around 128,000 hospitalizations and 3,000 deaths due to foodborne diseases [6].

Such losses due to infectious diseases can be avoided by preventing their spread, which requires successful disease surveillance and detection. Disease surveillance includes collection, analysis, interpretation, and distribution of data pertaining to newly emerging or reemerging infectious diseases, which aids in taking preventive actions against the spread of the disease. The most effective way to achieve this goal is through early detection and diagnosis of the disease onset [7]. The Institute of Medicine defines diagnosis as “the cornerstone of effective disease control and prevention efforts, including surveillance” [8]. Though there are standard tests to diagnose plant, animal and human diseases, their applications are limited when it comes to fast and accurate identification of the causal agent. This in turn leads to improper and delayed diagnosis and treatment, which makes it harder to contain the disease [9].

Plant disease surveillance occurs in three steps, starting with detection of the pathogen, followed by estimation and targeting. Estimation involves gathering information on the route of disease spread to understand the extent of the problem. This is followed by targeting, which involves identifying as many sites of infection as possible to implement control measures. Proper surveillance and preventive measures, therefore, relies on highly accurate and sensitive detection of the causal agent and the
disease. While this is currently achieved by sampling and laboratory-based testing, faster decision making and implementation of preventive measures demand field tests that allow rapid, sensitive, and cost-effective disease diagnosis [7, 10, 11]. Similarly, foodborne outbreak investigations in the United States (U.S.) rely on DNA fingerprinting to match pathogens from infected individuals to those in the suspected food product. However, the sample processing step takes time and can often lead to delayed decision-making on recalls of contaminated food products. The process also suffers from lack of confirmatory evidence in most cases and so is less effective in preventing the spread of diseases [12].

Plant diseases continue to be detected using standard laboratory culture-based techniques in addition to molecular and serological approaches. While these techniques provide accurate information, they are also extremely slow, require expensive equipment and trained personnel for operation [13]. Given that plant diseases occur much faster and may result in the complete loss of economically important foliage and other plant tissues, faster detection and prevention is essential. Further, this is also significant to maintain food security [7]. The World Health Organization’s (WHO) definition of an effective diagnostic tool for human diseases is that they should be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable (ASSURED). For the reasons mentioned above this is, therefore, also significant in achieving better surveillance and preventive measures for the spread of plant diseases [13]. Similarly, current standard methods for the detection of foodborne contamination also depend on plate culture, which takes days to confirm pathogen
identity [14], and molecular techniques such as polymerase chain reaction (PCR), which, although accurate, has longer turnaround times. Therefore, the identification of pathogens requires faster methods of detection to improve food quality and safety.

Several nucleic acid (NA) detection techniques have been developed for the diagnosis of diseases caused by a wide range of pathogens including viruses, bacteria, and fungi. The NA-based detection method has been considered a more accurate form of diagnosis since it targets specific gene sequences on the microorganism for identification. This makes them highly specific for detection. Although PCR is one among these, it can only be done in a laboratory setting due to the need to use equipment that requires multiple cycles of temperature variations. Not only does this make them expensive but also less accessible in resource-limited countries along with requiring trained personnel. This may not fit all types of microbial detection requirements and so, alternative methods have been studied that can cut down the costs while still maintaining the same or higher sensitivity [15].

Isothermal nucleic acid amplification methods have been studied as an alternative to PCR. Isothermal methods eliminate the need to use expensive thermocycling equipment for nucleic acid amplification since they do not require the maintenance of varying temperatures for each amplification cycle. Further, the simplicity of the method gives flexibility to conduct the tests anywhere and by anyone, which is significant in reducing the cost and time taken. Some such methods include nucleic acid sequence-based amplification (NASBA), primer-generation rolling circle amplification (PG-RCA), strand displacement amplification (SDA), helicase-
dependent amplification (HDA), and recombinase polymerase amplification (RPA). Among these NASBA, SDA, HDA and RPA require multiple enzymes to function. This greatly increases the complexity in optimizing the assay for various pathogen detection along with increasing the cost of reagents. RCA is specifically applied to copying target DNA of circular nature, which does not cover most of the pathogens generally tested.

Loop mediated isothermal amplification (LAMP) as the name suggests, is one other method that comes under this category. It is most popularly used for the development of rapid onsite tests due to its simplicity, reliability, and flexibility in optimizing reaction conditions and detection [18]. Similar to PCR this can be divided into two parts – a nucleic acid amplification step and a detection step. The reaction mixture uses four to six primers for target sequence amplification that makes it highly sensitive and Bst polymerase enzyme that enables strand displacement in constant temperature [15]. This allows the reaction to be carried out using a simple heating block that can maintain a constant temperature between 60-65° C, which cuts down the cost and time taken to conduct the test. Therefore, this research is focused on designing a rapid onsite pathogen detection assay based on LAMP by overcoming the limitations of its current reporting techniques.

The next chapters give a comprehensive review of literature on using LAMP coupled with rapid reporting methods for microbial testing and their shortcomings, followed by the objectives of this work and the subsequent outcomes.
Chapter 2. Literature Review

While the amplification process in LAMP can be conducted using a simple heating block, the detection of the amplicons has been done using several techniques. The most common/standard methods of LAMP detection include fluorescence, turbidity, and gel electrophoresis. However, other detection methods have also been developed based on colorimetric techniques including the use of dyes and nanoparticles. Tools such as smartphone applications, multiplex assays, microfluidic devices, etc. [18] were also developed to aid in the visualization process using the above mechanisms. The following sections will discuss these techniques.

2.1 Turbidity-based LAMP detection

Turbidity after a LAMP reaction occurs due to the precipitation of magnesium pyrophosphate which is a byproduct of nucleic acid replication. New DNA/RNA strand synthesis is accompanied by the formation of negatively charged phosphates which binds to the divalent magnesium cations from the salts in the buffer. Therefore, measuring this turbidity directly correlates to the number of amplicons formed during the reaction [19].

\[(\text{DNA})_{n-1} + \text{dNTP} \rightarrow (\text{DNA})_{n} + \text{P}_2\text{O}_7^{4-}\]
\[\text{P}_2\text{O}_7^{4-} + 2\text{Mg}^{2+} \rightarrow \text{Mg}_2\text{P}_2\text{O}_7\]

Usually, turbidity could be seen using the naked eye. However, people with poor eyesight may have difficulty with distinguishing positive and negative samples. It must also be noted that the sample size for LAMP reactions is usually around 25 µl [20, 21], which is too small for such accurate distinctions. For this purpose, conventionally,
turbidity from a LAMP reaction product is measured using a turbidimeter and compared for confirmation.

Some studies have included advancements to the turbidity-based LAMP detection for fast and reliable results. One study uses real time monitoring of LAMP induced turbidity using an LA-230 Loopamp real-time turbidimeter (Eiken Chemical Co., Ltd.) for the detection of blaKPC producing *Serratia* spp [22]. This instrument measures the optical density of the sample at a wavelength of 650 nm every 6 seconds as the reaction progresses. A turbidimeter software then records the turbidity based on this measurement. Another study also used a real-time turbidimeter to monitor turbidity in LAMP reactions containing single, duplex and triple templates for chicken parvovirus, chicken infectious anaemia virus, and fowl aviadenovirus serotype 4. The turbidity signal came out the fastest when multiple reactions were conducted. This shows that the real time turbidity measurement system can be used for monitoring multiple reactions simultaneously. However, it could not identify the pathogen template that caused the positive result, which may be necessary to diagnose diseases [23]. In yet another study, *Streptococcus pneumoniae*, the causal agent of pneumonia, was detected using real time turbidity monitoring of LAMP assay. A turbidity measurement of >0.1 was considered positive [24].

The advantage of using turbidity as an indicator is that it does not require opening the reaction tubes for post processing to enable detection of DNA. This reduces the possibility of cross contaminations while processing multiple samples over time. However, the turbidity does not last too long [25], making it necessary to analyze the
sample immediately after the reaction is complete [26]. While this would save time in getting results, it could also cause variability in the sample readings if not detected within a certain period. Real time turbidity measurement overcomes this problem and is simpler than conventional real time fluorescence detection of LAMP [27]. However, measuring the turbidity this way uses an additional instrument which increases the cost and time of the test and may not be user-friendly when testing on site. Turbidity measurement may also not be suitable for samples containing protein in the background such as blood due to their ability to precipitate and cause turbidity, resulting in false positives [28].

2.2 Fluorescence-based LAMP detection

Fluorescent resonant energy transfer (FRET)-based detection of LAMP products generally relies on the use of fluorescent dyes, probes, or nanoparticles as signal transducers to detect amplicons. As nucleic acid amplification occurs, the intensity of fluorescence from LAMP products increases, which is measured at the end of the reaction using an appropriate fluorescence device. This type of detection also allows real time monitoring of the fluorescence similar to the turbidity measurement [15]. In laboratory settings, the fluorescence can be measured using a spectrofluorometer, which has a broad range of excitation and emission wavelengths that make it flexible for applications with most fluorescent agents used in LAMP. It is also proven to be highly specific and sensitive.

2.2.1 Fluorescent dyes
Ethidium bromide, SYBR Green I and EvaGreen are some common dyes used to generate fluorescence signals in LAMP reactions. Their fluorescence mechanism is based on their ability to intercalate within double-stranded DNA (dsDNA) structures that usually get generated in the form of amplicons in LAMP [29, 30]. Since ethidium bromide is more carcinogenic in nature, most LAMP-based fluorescence detection methods have used SYBR Green I dye. However, one of its major drawbacks is its non-specific interaction with any dsDNA. This means that this method not only measures fluorescence from dye bound to specific amplicons generated in the LAMP but also to background and non-specifically formed dsDNA. This creates problems in getting accurate results. It must also be noted that the real time fluorescence curves obtained in LAMP using such dyes look like a baseball hat as opposed to the curve obtained from the more conventional real time PCR, which is sigmoidal. This could be due to the simultaneous increase in turbidity of the LAMP mixture that could potentially mask the fluorescence generated [31]. This can be more profound towards the end of the reaction and may be significant when considering accurate data collection.

One study explored the possibility of combining two different dyes – hydroxynaphthol blue (HNB) and SYTO 9 in certain proportions to act as indicators of the amplification process. The SYTO 9 was chosen for its ability to intercalate between dsDNA similar to SYBR Green and HNB for its ability to chelate Mg\(^{2+}\) ions required for DNA replication. In this case, the dyes were added before the reaction began, at which point the samples emitted light green fluorescence. This is due to the intercalation of SYTO 9 dye with background dsDNA before the reaction began. Once the reaction was
complete, the positive samples emitted brighter green fluorescence at 610 nm due to the increasing accumulation of SYTO 9 on the generated dsDNA target amplicons. During this time the Mg\(^{2+}\) ions were used up in the replication process and were absent for HNB binding. However, the negative samples emitted red fluorescence at 505 nm due to the formation of the HNB-Mg\(^{2+}\) complex. This is because of the absence of a target DNA strand that is required for DNA replication to occur, which leaves the Mg\(^{2+}\) ions free to bind to the HNB dye. Such distinct changes in fluorescence emission between the positive and negative samples solve issues associated with inaccurate reporting arising from wrong color perception. However, it still does not rule out the possibility of detecting a false positive sample as discussed earlier since the SYTO 9 dye does not differentiate between specific and non-specific dsDNA [32]. The mechanism of detection is shown in Figure 1.

Figure 1. Schematic of the dual-color fluorescence LAMP assay [32]
SYTO 9 has also been used for the detection of SARS-CoV-2 RNA in saliva samples. Since this involves the detection of RNA, reverse transcription LAMP was employed for amplification to make cDNA copies. The fluorescence dye then intercalates between the cDNA copies and fluoresces, which is later quantified [33].

Microfluidic platforms have garnered attention in the development of diagnostic systems and have not gone unnoticed for applications involving LAMP assays. One study demonstrated the possibility of detecting multiple foodborne bacterial pathogens in milk using a microfluidic chip containing ten chambers. Each chamber is preloaded with specific primer sets corresponding to a different pathogen along with positive and negative controls. The microfluidic chip was designed to allow a single injection of the reaction mixture, including the sample, into a distribution channel, after which the reaction was started. Although the reaction takes 45 min, which is longer than most LAMP assays that take 30-35 min, the microfluidic platform with the preloaded primers saves a lot more time in sample preparation. Both fluorescence and visual detection options were explored for quantitative and qualitative analysis, respectively. EvaGreen dye (another nucleic acid intercalating dye) served as an indicator for fluorescence-based detection, which was measured at the end of the reaction using a LAMP instrument. While this may not be ideal for field testing, the development of an appropriate qualitative visualization for the microfluidic platform can prove to be effective for point-of-care tests [16].

2.2.2 Fluorescent nanoparticles
Semiconductor fluorescent nanoparticles such as quantum dots (QDs) have been studied for their photostable property. Several LAMP studies have used QDs modified with proteins or oligonucleotide/primer sequences to act as fluorescent labels for the generated amplicons. However, most of these have multiple steps that increase the time to result or require post-amplification open-tube procedures which increases the risk of carryover contamination. Although some studies have focused on eliminating these problems, the preparation of the modified QD probes is expensive. To overcome these issues, Lee et al. synthesized amine functionalized QDs that can be added to the reaction mixture before amplification and are less expensive due to the simplicity of the synthesis. Specifically, cysteamine-modified CdSeS/ZnS QDs were synthesized. Here, the negatively charged amine group acts as a link between the QDs and magnesium pyrophosphate crystals (Mg$_2$P$_2$O$_7$) that are generated during nucleic acid amplification in positive samples. This interaction causes the QDs to coprecipitate with the crystals and settle to the bottom of the tube, which look like green, fluorescent precipitates under fluorescence photography. In a negative sample, the Mg$_2$P$_2$O$_7$ crystals are absent and so, the negatively charged amine-QDs remain dispersed due to the interparticle electrostatic repulsion and show uniform green fluorescence (Figure 2). This study shows the possibility of using both qualitative and quantitative readout of the results [34].
Figure 2. Schematic of the one-step LAMP assay with cysteamine-modified CdSeS/ZnS quantum dots (amine-QDs) [34]

While fluorescence photography allows final qualitative confirmation, real time fluorescence monitoring is highly efficient, sensitive, and specific and allows quantitative analysis. However, given the need for a detection instrument for real time fluorescence monitoring, this may not be suitable for field tests [35].

A spectrofluorometer is hard to use in field-based tests and instead would require portable fluorescence devices without real time monitoring. These have a limited range of fluorescence measurements, forcing the user to restrict their choice of dyes for assay development.

2.3 Bioluminescence-based LAMP detection

Like fluorescence, bioluminescence resonance transfer (BRET) has been applied to detect LAMP amplicons in some studies. Although not as commonly used as fluorescence-based detection, its mechanism remains similar, where, instead of an external light source that is used to excite a fluorescent marker, light is produced when
the enzyme luciferase oxidizes its substrate luciferin. This excites the protein marker used to indicate the presence of dsDNA generated using the LAMP assay. Since the light is produced inherently in BRET, this may overcome some of the problems associated with FRET such as autofluorescence, light scattering, or photobleaching [36].

One study that explored the application of BRET for LAMP amplicon detection uses a luminescent multivalent intercalating dye (LUMID). Here, the intercalating dye used for dsDNA detection is conjugated to a NanoLuc luciferase enzyme, which is blue light-emitting. When the dye binds to dsDNA generated during a LAMP reaction, energy transfer from the luciferase to the dye occurs, causing them to emit green luminescence (Figure 3). This change is captured using a smartphone camera to record the results of the test. To improve the signal and make the dsDNA binding stronger, the researchers combined multiple dyes with positively charged lysine linkers and conjugated them closer to the active site of the luciferase enzyme. This seems to be a requisite for better signal emissions and stronger binding of the dyes to the dsDNA. However, apart from the advantage of not needing an external light source for the detection of the LAMP amplicons, [37] the working of this visualization technique is the same as using any intercalating fluorescent dye described in literature. The need to use multiple dye components, the additional reagents, including the enzyme, and the varying conjugation routes, seem to make the adoption of this potentially off-the-shelf visualization kit expensive.
Other studies have combined real time LAMP (RT-LAMP) monitoring with bioluminescent assay in real time (BART) for the detection of various viral pathogens. A group of researchers studied the application of this RT-LAMP-BART system to detect different strains of SARS-CoV-2. The assay relies on the use of Lyophilized BART Master™ Reagent (Erba Mannheim, Ely, UK) which is added to the LAMP reaction mixture prior to amplification [27, 38]. In another study, hepatitis A virus was detected in inoculated food samples [38]. Given that the viral template is RNA, reverse transcription LAMP was performed, during which the generation of dsDNA caused precipitation of magnesium pyrophosphate in the reaction mixture. It is the formation of these inorganic pyrophosphates that is monitored using the bioluminescence signal [27, 38, 39].

The mechanism of BART starts when the inorganic pyrophosphate is converted to adenosine triphosphate (ATP) by the enzyme ATP sulfurylase. Then the thermostable luciferase enzyme uses this ATP to oxidize the substrate luciferin and produce bioluminescence. A peak in the light signal is observed in positive samples when this
phenomenon occurs, whereas the negative samples do not show any such peak. However, BART system still requires detection devices to continuously keep track of these light signals from the LAMP reaction to identify that peak [40, 41]. The need for such an instrument could add to the cost of operation and the dependency on a laboratory setup [35].

Alternatively, colorimetric detection methods have been explored for LAMP visualization in field-based applications.

2.4 Colorimetric LAMP detection

2.4.1 Dye-based colorimetry

2.4.1.1 Intercalating dyes

Generally, nucleic acid intercalating dyes used in LAMP assays tend to be fluorescent in nature and would require a fluorometer to detect the results. However, certain fluorescent dyes such SYBR Green I, if used at a higher concentration, can indicate the presence or absence of a positive reaction through visual color change from orange to green. Some studies have explored this option to develop naked eye reporting of LAMP results. But the amplification process gets inhibited when the dye is used at such high concentrations. This makes it necessary to add the dye after the LAMP reaction unlike when it is used for fluorescence detection at low concentrations. They sometimes would also require a UV lamp for better visualization. The malarial parasite, *Plasmodium knowlesi*, and SARS-CoV-2 are examples of some pathogens detected using this SYBR Green I LAMP assay [30, 42]. Typically, SYBR Green I function as a DNA indicator due to the presence of positive charges that help with binding to the negatively charged
dsDNA. This DNA-dye complex then absorbs blue light and emits green light, which is used as a signal for positive reactions. [43]. Other fluorescent dyes that could be used as colorimetric indicators based on similar mechanism include Quant-iT PicoGreen, which could be expensive, and EvaGreen dye [26].

Certain triphenylmethane dyes such as crystal violet, methyl green, fuchsin, and malachite green also operate based on their affinity to specifically bind with the major grooves of dsDNA [44]. When crystal violet interacts with sulfite ions, they turn colorless due to the formation of leuco crystal violet (LCV). In the presence of dsDNA, the colorless LCV turns back to its colored form crystal violet upon its strong binding to the DNA amplicon (Figure 4) [45]. Based on this observation, an RT-LAMP colorimetric assay was developed for the detection of SARS-CoV-2. The study explains the development of a single and two-stage LAMP assay. While the single-stage follows a conventional reverse transcription LAMP process, the two-stage assay termed Penn-RAMP starts with recombinase polymerase amplification (RPA) in the first step and LAMP in the second. Although the LAMP reaction was conducted with real time monitoring of fluorescence signals, LCV was included in the reaction mixture for a final colorimetric detection [46].
Other studies described the use of malachite green for the detection of LAMP reaction products. In the presence of the specific *Plasmodium* sp. DNA, the dye shows a green/blue color upon binding, whereas it remained colorless in negative samples [47]. Reverse transcription LAMP was performed along with the addition of malachite green in...
the initial mixture for the detection of SARS-CoV-2 with a similar visualization procedure at the end of the process [48].

Fuchsin is another intercalating dye, which works similarly. It is magenta in color and upon interaction with sulfite ions turns to its colorless leucofuchsin form due to the loss of its chromophoric structure. However, when the acid-hydrolyzed DNA binds to this dye, the sulfite gets removed and the dye returns to its chromophoric structure. During this interaction, the dye looks purple [49]. This mechanism was exploited in one study to develop a foldable microdevice for the colorimetric LAMP detection of Magnaporthe oryzae and Sarocladium oryzae rice seeds. Figure 5 shows the design of this biosensor. The microdevice was made of a PCR sealing film and consisted of three chambers – sample, reaction, and detection. The sample chamber was where the template DNA was loaded. The LAMP reaction mixture was loaded into the reaction chamber consisting of wells for different samples. To start the LAMP reaction, the sample chamber was pressed onto the reaction chamber by folding the microdevice for a minute and then removed. The reaction chamber was then covered with a sealing film for the reaction to be closed. After the LAMP process, the wells in the reaction chamber were opened to add HCl and sodium sulfite. Following this, the reaction chamber was folded onto the detection chamber that already contained the fuchsin dye. Upon interaction with the LAMP products, the dye turned purple in the presence of positive samples and magenta to colorless in the presence of negative samples [50]. While this process uses a single portable microdevice to carry out the LAMP process, the possibility of cross reaction or contamination seems high, with the reagents being added in proximity. Among the above
discussed intercalating dyes, the advantage of triphenylmethane dyes over fluorescent dyes is that they do not inhibit the amplification process. This makes them flexible enough to be added to the LAMP mixture prior to amplification, reducing additional post amplification steps. Therefore, simpler LAMP assay designs could be developed for various pathogen detection.

Schiff’s reagent is a dye formulation obtained by the combination of fuchsin and sodium bisulfite. One study directly used this reagent for the detection of hair loss related single nucleotide polymorphism (SNP) to eliminate the additional step of adding sodium sulfite. The study also describes the use of a foldable hand-sized chamber to conduct the colorimetric LAMP assay, which was then analyzed using the ImageJ software [51].

**Figure 5.** The microdevice design. (a) Sample chamber, (b) reaction chamber, and (c) detection chamber with fuchsin-stained strips [50]
Although the intercalating dyes described above directly detect the dsDNA through visible color change in theory, they do suffer from showing a clear distinction between positive and negative samples in practice. Given that the sample size for a LAMP assay could be as small as 20 µL, not being able to discern the color change can lead to false positives or negatives [52]. To overcome these issues, some studies have considered using image analysis software to record the changes in those hues using Red-Green-Blue (RGB) and Commission Internationale de l’Eclairage (CIE) Lab color spaces models [44]. However, having this additional step requires a visualizing instrument, which goes against the concept of a rapid cost-effective visualization method. Another potential problem with the use of intercalating dyes is that they could be toxic/mutagenic in nature. This is because they operate through their affinity for nucleic acids [53]. Care must be taken in handling and disposing of these reagents. While not all intercalating dyes are considered dangerous, and there are indeed safer dyes commercially available, the user must be cautious before choosing the right one.

2.4.1.2 pH-sensitive dyes

To overcome the issues posed by intercalating dyes, many studies have explored the use of pH-sensitive dyes for detecting positive samples in a LAMP assay [52, 54-57]. The mechanism of detection is based on pH changes induced by the amplification process, where the generation of dsDNA strands leads to a significant release of protons (H⁺ ions), causing the solution to turn acidic. When the pH drops, the dye in the solution changes color [26]. This is usually a sharp contrast that is easily discernible. Additionally, most pH-sensitive dyes do not inhibit LAMP and can be added to the reaction mixture
prior to amplification, making them more convenient to employ. The following reaction sequences show the mechanism of pH-based dyes for the detection of nucleic acids [58].

With Bst DNA polymerase: \( fDNA + dNTPs \rightarrow DNA^{+1} + P_2O_4^{-7} + H^+ \)

Hydrolysis reaction: \( P_2O_7^{4-} + H_2O \rightarrow 2PO_4^{3-} + 2H^+ \)

The most used pH sensitive dye is phenol red, which changes from dark pink to orangish yellow color in a positive sample. Due to its distinct color change and ease of use, it has been included in the commercial LAMP kits developed by New England Biolabs, a company that specifically provides reagents and master mixes for LAMP assays. The WarmStart\textsuperscript{®} Colorimetric LAMP 2X Master Mix (DNA & RNA) consists of phenol red as the indicator dye and Bst polymerase enzyme that only activates at temperatures above 60°C [59]. Several studies that used phenol red as an indicator, have used this colorimetric master mix to conduct their LAMP assays. At the end of the reaction, the results are recorded based on the color change observed in the LAMP mixture [54, 60-64].

Alternatives to phenol red were explored by a group of researchers to overcome the issue of confusion in color perception by different individuals. This is because of the slow change from red to yellow with phenol red could be shallow and ambiguous. For this purpose, LAMPshade Magenta and LAMPshade Violet were used to develop a JaneliaLAMP (jLAMP) for the detection of SARS-CoV-2 that showed steep and highly contrasting color changes. Apart from naked eye detection, the color change can also be
detected under UV lamp due to their fluorescent nature. However, the availability of these dyes may not be widespread, which could limit their application [65].

Several studies have also explored the use of other pH-sensitive dyes to develop rapid LAMP visualization assays for various pathogens. Table 1 below summarizes recent research in this area.

**Table 1. List of colorimetric LAMP assays using pH-sensitive dyes**

<table>
<thead>
<tr>
<th>pH-sensitive dye</th>
<th>pH range for color change</th>
<th>Colorimetric indication for positive</th>
<th>Target detected</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromothymol blue</td>
<td>8.8 to 6.8</td>
<td>Blue to yellow</td>
<td>Toxoplasma gondii</td>
<td>[58]</td>
</tr>
<tr>
<td>Cresol red</td>
<td>8.8 to 7.2</td>
<td>Purple/pink to yellow</td>
<td>Food allergens; Human Papilloma viruses (HPV)</td>
<td>[56, 66]</td>
</tr>
<tr>
<td>Neutral red</td>
<td>8 to 6.8</td>
<td>Light orange/yellow to pink</td>
<td>African swine fever virus (ASFV); Singapore grouper iridovirus (SGIV); Chicken</td>
<td>[52, 67, 68]</td>
</tr>
<tr>
<td>Xylenol orange</td>
<td>&lt;6.7</td>
<td>Purple to yellow</td>
<td>Phytophthora Species</td>
<td>[55, 69]</td>
</tr>
<tr>
<td>Phenolphthalein</td>
<td>&lt;8.5</td>
<td>Pink to colorless</td>
<td>Vancomycin-resistant <em>Enterococcus</em> (VRE)</td>
<td>[57]</td>
</tr>
</tbody>
</table>

Apart from the straightforward LAMP reaction for the visual indication of amplification using dyes, other studies have explored some innovative platforms to conduct the colorimetric assay. One study developed an electrowetting-on-dielectric (EWOD) microfluidic platform for lab-on-a-chip colorimetric LAMP assay using xylenol orange as an indicator. The study focused on developing a diagnostic test for the detection of early mortality syndrome in shrimp [70]. In yet another study, a novel dye was formulated using xylenol orange and malachite green named lavender green. This was used along with xylenol orange to enhance the accuracy of the colorimetric test. Further, the study also incorporated the use of an artificial intelligence (AI) operated tool (RT-LAMP-
DETR) for precise analysis of the color change [69]. Phenolphthalein-based test swabs were developed in another study as a post-reaction kit. An osteoarthritis marker MTF1 gene was tested using LAMP, which was then evaluated using the phenolphthalein swab. A change from pink to colorless on the swab indicated a positive reaction [71].

Most pH-sensitive dyes show a color change involving a short range of colors such as purple, blue, pink, and yellow. To expand the color spectrum for people affected by color weakness, one study explored the possibility of combining two or more dyes for LAMP visualization [72]. This study combined pH-sensitive dyes with some pH insensitive dyes such as phenol red-azure II, and phenol red-methylene blue that change from blue purple to green, and bromothymol blue-cresol red, and bromothymol blue-phenol red that changes from green to yellow. Though this study aimed at expanding the color spectrum, the tested combinations were not enough to add to that range. Further, the combinations of dye would just increase the complexity and the price of the indicators used [72].

Another group of researchers tested the combination of different pH-sensitive dyes for LAMP assay. This was done with the aim of reducing errors in sample addition by tracking it through the inclusion of dyes. Both the reaction mixture (16 µL) and the sample solution (4µL) had different dyes added to them. The study tested eight different pH sensitive dyes, of which bromothymol blue for the reaction mixture and phenol red for the sample was found to show the best contrast at the end of the LAMP assay. However, it seems that varying volumes of the reagents may produce variations in the
result and must be optimized each time before use. This complexity undermines the need to track the appropriate addition of LAMP reagents and sample solution [73].

While pH sensitive dyes have some advantages, there are some major limitations to their application for accurate colorimetric analysis. The first problem is the indirect nature of detection, where changes in the pH could occur due to several factors. This increases the chances of false positives. Due to this, LAMP reaction with crude DNA extracts may not give desirable results since there could be interfering factors that alter the pH of the reaction [74]. Another significant problem is the slow color change of the dyes under high buffer conditions. This is because the change in pH is lowered under high buffered conditions. This necessitates the application of low buffer concentration for LAMP reaction [75]. Furthermore, the perception of color change using the pH-sensitive dyes can vary between users causing variations in color reporting.

2.4.1.3 Metal-indicating dyes

The amplification of nucleic acids produces pyrophosphate ions that bind to magnesium ions (Mg$^{2+}$) already present in the LAMP reaction buffer forming Mg$_2$P$_2$O$_7$. Certain dyes are also capable of binding to these Mg$^{2+}$ ions and depending on their availability, change color. This behavior is used as an indication of amplification in each tested sample.

Calcein, which is also a fluorescent dye, has been used for the naked eye colorimetric detection of LAMP due to their ability to bind to Mg$^{2+}$ ions. The colorimetric mechanism of calcein is usually combined with the addition of MnCl$_2$, at
which point the dye appears orange due to fluorescence quenching. When pyrophosphate ions are produced, the Mn\(^{2+}\) ions from MnCl\(_2\) replace the Mg\(^{2+}\) ions, letting the calcein dye turn yellow/green. Based on this process, the negative samples remain orange whereas the positive samples turn yellow/green [76, 77]. Figure 6 illustrates the mechanism of metal-indicating dyes for detecting LAMP assays [78]. Although some research has used calcein as an indicator, there have also been reports of the difficulty in distinguishing color change [79]. Another major problem is the need to use MnCl\(_2\), which at certain concentrations inhibits polymerase activity.

Figure 6. (a) Mechanism of calcein, (b) Colorimetric results after LAMP reaction [78]

Hydroxynaphthol blue (HNB) is another metal binding dye that is commonly used for LAMP detection. HNB binds to Mg\(^{2+}\) ions and turns violet in a negative sample. During amplification in a positive sample, the generation of pyrophosphates takes away the Mg\(^{2+}\) ions leading to the dye turning sky blue [76]. An RT LAMP assay was
developed for the detection of porcine epidemic diarrhea virus. This study also explored the use of HNB for the final visualization of the LAMP results through color change [80]. Although similar studies have used HNB as an indicator dye, [81, 82] some studies have tried to combine its application with other dyes for better contrast. This is because the change from violet to sky blue is sometimes subtle and not enough for conclusive reporting of LAMP outcome [66].

Other less commonly used metal-sensitive dye includes Eriochrome Black T (EBT), which also changes color based on the presence or absence of Mg$^{2+}$ [76]. To overcome the problems associated with the use of phenol red and other similar pH-sensitive dyes, one study employed the use of murexide (MX), a complexometric indicator. This compound interacts with the divalent Zn$^{2+}$ which is provided as ZnCl$_2$. This Zn-MX initially appears pink. During the generation of pyrophosphates, they form a complex with the Zn$^{2+}$ causing the free murexide to turn yellow. This way a positive reaction is identified. However, just like MnCl$_2$, ZnCl$_2$ can also inhibit polymerase activity and must be added post-amplification [74].

Similar to the mechanism of pH sensitive dyes, the metal indicating dyes change color due to an indirect phenomenon as a result of DNA amplification. These dyes do not directly detect the generated nucleic acids, rather indicate the presence or absence of free divalent cations in the buffer that interacts with the byproducts of amplification. Due to this the same problems occurring with pH sensitive dyes could potentially affect the results of the LAMP assay with these dyes.

2.4.2 Nanoparticle-based colorimetry
Most colorimetric LAMP assays are based on metal nanoparticles, especially noble metals such as gold (AuNPs) and silver (AgNPs). Among these the most popular choice is AuNPs due to their simple and straightforward approach to color change. These nanoparticles exhibit certain optical properties that are different from their bulk counterparts. Surface plasmon resonance-based colorimetry is a phenomenon in which absorption of light in the visible range gives the nanoparticles a certain color that changes with the size of the nanoparticles or with their assembly. This property has been exploited in several diagnostic assays for the detection of microorganisms or their genetic material [83]. Visual detection of LAMP assay is no exception to this. A basic mechanism of visualization involves the use of the metal nanoparticles conjugated with target-specific oligonucleotide sequences that bind to the amplified DNA after a LAMP reaction. Depending on the conditions induced in this mixture, the nanoparticles tend to remain dispersed or aggregate, causing them to change color from red to purple in case of AuNPs and colorless to deep brown in AgNPs. This way both positive and negative samples are distinguished from each other.

One study explained the use of AuNPs conjugated with target-specific oligonucleotides for the detection of the shrimp pathogen *Vibrio parahaemolyticus*. After the LAMP assay was conducted, the amplification products were incubated with the AuNP-oligonucleotides and a certain concentration of NaCl. In the case of positive samples, the generated dsDNA hybridized with the oligonucleotides on the AuNPs causing them to remain dispersed and red even with NaCl in solution. However, in negative samples, due to the absence of dsDNA, the free AuNP-oligonucleotides
aggregated in the presence of the NaCl solution and turned purple. Based on this activity, the LAMP samples were distinguished as positive or negative which is illustrated in Figure 7 [84].

Another study explained the use of silver nitrate and quercetin to produce AgNPs in the presence of LAMP amplicons. The mechanism of detection was based on the formation of a complex between the nitrogenous bases of LAMP amplicons and silver ions. When quercetin is introduced to this complex under basic conditions, it acts as a reducing agent leading to the formation of AgNPs. At this point the solution turns deep brown indicating a positive LAMP reaction. In negative samples, because of the absence
of LAMP amplicons, the silver nitrate remains in solution and no color change is observed when quercetin is added. Based on this mechanism, a foldable microdevice was fabricated that contained a reaction and detection chamber. The LAMP assay was carried out in the reaction chamber followed by addition of silver nitrate. Then the device was folded so that the detection chamber containing quercetin would interact with the components in the reaction chamber causing a color change, if positive (Figure 8). The final color change was analyzed using the ImageJ software [85].

Figure 8. Design of the LAMP-AgNPs colorimetric assay using the foldable microdevice [85]

Similar studies have been conducted by other research groups for rapidly visualizing LAMP amplicons at the end of the reaction. These studies use the same mechanism of nanoparticle aggregation and color changes but with some modifications on how they are induced and the method of fabricating the test kit. Table 2 provides a
selective list of LAMP visualization assays developed over the last three years based on color changes exhibited by metal nanoparticles.

**Table 2.** Some LAMP visualization assays using metal nanoparticles as colorimetric indicators

<table>
<thead>
<tr>
<th>Nanomaterial used</th>
<th>Color change</th>
<th>Testing method</th>
<th>Target detected</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver nanoplates</td>
<td>Red for positive and yellow for negative</td>
<td>Paper-based analytical device</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em> (MRSA)</td>
<td>[86]</td>
</tr>
<tr>
<td>Gold nanoparticles</td>
<td>Red for positive and purple for negative</td>
<td>CRISPR-Cas12a empowered LAMP</td>
<td><em>Fusarium temperatum</em></td>
<td>[87]</td>
</tr>
<tr>
<td>Gold nanoparticles</td>
<td>Red for positive and grayish purple for negative</td>
<td>Heating block based conventional LAMP assay</td>
<td>Microsporidian <em>Enterocytozoon hepatopenaei</em></td>
<td>[88]</td>
</tr>
<tr>
<td>Gold nanoparticles</td>
<td>Red for positive and grayish blue for negative</td>
<td>Reverse transcription LAMP</td>
<td><em>Tilapia lake virus</em> (TiLV)</td>
<td>[89]</td>
</tr>
<tr>
<td>Gold nanoparticles</td>
<td>Red for positive and pale purple with dark precipitate for negative</td>
<td>Conventional LAMP</td>
<td><em>Leishmania</em></td>
<td>[90]</td>
</tr>
<tr>
<td>Gold nanoparticles</td>
<td>Red for positive and purple for negative</td>
<td>CRISPR/Cas12-assisted RT-LAMP</td>
<td>SARS-CoV-2</td>
<td>[91]</td>
</tr>
</tbody>
</table>

In all these studies, the colorimetric detection of LAMP amplicons depended on the state of the nanoparticles – dispersed or aggregated. There are several factors that could induce aggregation of nanoparticles, leading to a color change. This may include varying buffer concentrations in the sample and other biological interferences. In some
studies, changes in pH and/or ionic strength following amplicon generation lead to the aggregation of the AuNPs, resulting in a color change from red (single AuNPs) to purple (aggregated AuNPs) for detection [90, 92-95]. However, this type of aggregation can occur due to other changes in the reaction medium that alters its pH or ionic strength irrespective of the presence or absence of amplified DNA. This could give rise to higher false negatives and decrease the sensitivity of the assay [96]. Additionally, given that the sample size is small for most of these post-amplification visualization tests, the changes observed in color most times are not as distinct, or may require longer than the stipulated time to be visible. This causes the interpretation of results to be varied among users.

2.5 Conclusions

LAMP products are conventionally detected using a turbidimeter or a fluorescence spectrophotometer, which may not be adaptable for field applications. More recently, several studies have explored the possibility of using colorimetric dyes and nanoparticles for rapid detection of LAMP reaction products to save time and make them field deployable. However, these methods suffer from problems with varying color perception and inconsistencies. Most of them do not directly detect the amplified product, with the color changes caused largely by pH variations or additional byproducts of a LAMP reaction. Hence, these methods are prone to false results. Thus, it is important to develop alternative visual identification methods for LAMP that are fast and easier to interpret to make them cost-effective and adaptable for onsite microbial testing.
Chapter 3. Research Objectives

This dissertation focusses on the design and implementation of a rapid, accurate and sensitive nucleic acid-based assay for the onsite detection of microbial pathogens. Specifically, the application of LAMP was explored because of their simple and cost-effective operation and the flexibility to incorporate a rapid visualization test at the end of the process. This work also addresses the challenges associated with the current visualization tools used for LAMP and describes the development of a naked eye amplicon detection assay using gold nanoparticles (AuNPs) assembly. The combination of the optimized LAMP assay and the novel optical visualization test is expected to (1) be an efficient tool for the early diagnosis of infectious diseases onsite, (2) eliminate reliance on a laboratory setting to conduct tests and be easily accessible in resource poor settings, and (3) reduce costs associated with diagnosing diseases.

This dissertation focusses on the design of a LAMP assay for the targeted detection of Bretziella fagacearum as a model organism since they are the causal agent of oak wilt disease. Oak trees (Quercus spp.) play a significant role in the ecosystem and are considered economically important for several reasons [97]. However, these trees are prone to oak wilt disease, which is of significant concern. The following objectives were identified to achieve the goal of this work:

1. Develop a rapid optical visualization test for LAMP reaction products.
2. Design and optimize a LAMP assay for the detection of Bretziella fagacearum in red oak tree samples.
3. Apply the naked eye visualization test for the detection of *Bretziella fagacearum* ITS amplicons generated from the designed LAMP assay.
Chapter 4. Naked-eye visualization of nucleic acid amplicons using hierarchical nanoassembly

This study reports on the development of a rapid visualization method for DNA amplicons.

4.1 Introduction

Loop-mediated isothermal amplification (LAMP) assays have gained growing interest over the last few years due to their ability to achieve high sensitivity and specificity while offering rapid detection and portability [98-101]. Requiring only a heating block, LAMP reactions can be visualized with the naked eye using a variety of methods. These include the use of pH sensitive metal indicating dyes such as hydroxynaphthol blue or fluorescent intercalating dyes such as [102], SYBR green I [103] that react with the LAMP amplification products to produce a change in color, which is detected visually. However, there have been reports of the inability to reproduce color changes at certain concentrations of reagents used in LAMP reactions [104]. Other dyes used for colorimetric detection include PicoGreen, propidium iodide, and ethidium bromide [31]. However, these methods depend on the use of certain concentrations of the dye to produce a desirable color change, which oftentimes can cause inhibition of the LAMP amplification reaction without extensive optimization [105]. Another significant disadvantage of these methods is the difficulty in distinguishing subtle color changes, especially in the field with varying natural light [106, 107]. Color perception by different individuals is also different and has to account for users with color blindness, which most times makes it necessary to use standard color quantification methods for secondary validation [108].
Nanomaterials are different from their bulk counterparts with better physical properties. Some of them include improved optoelectronic, magnetic, antireflection properties and super-hydrophobicity. The most significant difference is attributed to their large surface-to-volume ratio due to the small size, which enhances their surface properties [109]. This makes them desirable for several applications including the development of rapid detection assays. A limited number of studies have explored the use of gold nanoparticles (AuNPs) due to their optical properties [110]. Most of these studies exploit the color change of AuNPs from red to purple that occurs when they aggregate [90, 92-95]. However, as discussed earlier several factors could induce this aggregation that may not directly depend on the differences between positive and negative samples [96]. In addition, the color change from red to purple is often hard to perceive in small volumes such as 25 µL, which is usually the sample size of LAMP assays, and takes a long time to be detected. Such problems question the applicability of most colorimetric tests for onsite detection and creates a need to develop alternative visual identification methods that are fast and easier to interpret.

In this study, AuNPs functionalized with non-complementary oligonucleotides (AuNP-oligos) are used to detect nucleic acid amplicons through their rapid and non-specific assembly. A subsequent precipitation results in a distinctive red pellet visible to the naked eye (Figures 9 and 10). AuNPs were used for their desirable optical property (distinct red color) and flexible synthesis mechanism. However, unlike the previous studies that use colorimetric change for target detection, this study uses AuNPs as a stable colorant for the DNA precipitate in a positive sample.
Figure 9. Oligonucleotide-coated gold nanoparticles hierarchically assemble on DNA networks to form globular nanostructures, which precipitate into a distinct visible red pellet. This aims to overcome challenges associated with nanoparticle aggregation and dye-based colorimetric detection in LAMP assays.
**Figure 10.** Naked-eye visualization of DNA amplicons using nanoassembly. From top to bottom: **(a)** DNA amplicons form globular networks under the effect of salts and ethanol, **(b)** uncoated gold nanoparticles (AuNPs) aggregate under the same conditions, **(c)** AuNPs coated with oligonucleotides (AuNP-oligos) bind non-specifically to the amplified DNA causing assembly of single nanoparticles with the DNA amplicons to form globular nanostructures, which precipitate into visible red pellet, **(d)** In the absence of the DNA amplicons, the AuNP-oligos remain suspended in solution as single nanoparticles.

The fungus, *Bretziella fagacearum*, that has a significant ecological importance [97], was used as a model organism to conduct the LAMP assay and generate amplicons.

4.2 Experimental Section

4.2.1 Materials

WarmStart® LAMP Kit (DNA & RNA) was purchased from New England Biolabs (Ipswich, MA). The oligonucleotide sequences for AuNP synthesis and primers for LAMP experiments were fabricated by Integrated DNA Technologies (Coralville, IA). All solutions were prepared using nuclease-free water purchased from Stemcell Technologies (Vancouver, Canada). Gold (III) chloride trihydrate, trisodium citrate dehydrate, Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), and sodium chloride (NaCl) were obtained from Sigma-Aldrich (St. Louis, MO). GelRed® Nucleic Acid Stain 10000X Water and phosphate saline buffer (PBS) were purchased from Millipore Sigma (Burlington, MA). GeneRuler Low Range DNA Ladder, 10X UltraPure TAE Buffer and UltraPure DNase/RNase-Free Distilled Water were purchased from Thermo Fisher Scientific (USA). Ethylenediaminetetraacetic acid (EDTA) was obtained from Boston Bioproducts (Milford, MA) and ethanol was procured from Fisher Scientific (USA).

4.2.2 Fungal culture and DNA extraction
Bretziella fagacearum (B. fagacearum) isolates were provided by the United States Forest Service Northern Research Station, NRS-16, Saint Paul, USA. B. fagacearum DNA was extracted using a commercial kit (QIAamp DNA Mini Kit) with modifications in the buffer volumes provided in the manufacturer’s instructions. This was used as the sample for LAMP reactions.

4.2.3 Obtaining LAMP amplicons

The LAMP reaction mixture was prepared according to the manufacturer’s instructions for the WarmStart® LAMP Kit (DNA & RNA). The total reaction volume was 25 µl and the amplification was carried out at 65°C for 30 min followed by enzyme denaturation at 85°C for 5 min. The final LAMP products were used for testing the developed assay.

4.2.4 LAMP visualization using agarose gel electrophoresis

Agarose gel electrophoresis of the obtained LAMP products was done to confirm the generation of DNA amplicons. Serial dilutions of the amplicons (2x, 4x, 6x, 8x, 10x, 20x and 40x) were also prepared and run alongside the original amplicons.

4.2.5 Synthesis of gold nanoparticle (AuNP)-oligos

All the glassware used for nanoparticle synthesis was cleaned using Nochromix solution followed by Aqua Regia based on standard laboratory protocol. The AuNPs were then synthesized using a modified version of the Turkevich’s method [111]. Initially, 0.25mM HAuCl₄ was prepared in 90 mL of deionized water and boiled at 100°C with vigorous stirring. Then, 10 mL of preheated trisodium citrate (7.352 g/L) was added. The
mixture was maintained at the same temperature and vigorously stirred until the solution turned deep red in color. The nanoparticles were later stored at 4°C and characterized using UV – visible spectrophotometer (Shimadzu 1800) and transmission electron microscope (TEM, FEI Tecnai T12).

Two different oligonucleotide sequences with disulfide modifications were designed for conjugation with the AuNPs. The oligonucleotides were determined based on the primer and probe sequences used for RT-PCR in a previous study to test for *B. fagacearum* [112]. These were:

Oligos 1: Thiol 5'-TGGCAGGGACTTCTTTCTTCA- 3'

Oligos 2: 5'-ATGTTTCTGCCAGTAGTATT-3' Thiol

It must be noted that although the chosen sequences were part of the target fungal DNA, they were non-complementary to the target amplicons obtained from the chosen primer set. This helped with understanding the nature of the oligonucleotide stabilized AuNPs’ attachment to the LAMP amplicons to enable naked eye detection of infection. The conjugation of the oligonucleotides with AuNPs was done using a modified version of a previously developed protocol [113]. Briefly, stock solutions of the oligonucleotides were prepared at 100 µM concentration in PBS-EDTA buffer. This was followed by incubation with TCEP for 1 h at room temperature with gentle shaking at 400 rpm. This solution was then added to 5 mL of AuNPs solution and incubated at room temperature for 5 h. After this, 600 µL of 1M NaCl was added dropwise to stabilize the conjugates and incubated for another 16 h. Finally, the solution was centrifuged at 14,000 rpm for 30
and resuspended in PBS-EDTA buffer. The AuNP-oligos conjugates (AuNP-O1 and AuNP-O2) were characterized by UV – visible spectrophotometer and TEM.

The same process was carried out with two oligonucleotide sequences that were complementary to the target amplicon sequence to see if the visualization technique worked differently. The following are the sequences used for this test:

Probe 1: 5’-ATGCCTAGCAGAATACTGC-3’ Thiol

Probe 2: Thiol 5’-ACCTGATCCGAGGTAAC-3’

4.2.6 Optical detection of LAMP products with AuNP-oligos

Initially, a small volume of each LAMP reaction product, including the diluted amplicons, was incubated separately for 5 min with AuNP-O1 and AuNP-O2 to enable attachment to amplicons. Then, 2 M NaCl and 100% ethanol were added in equal amounts (same as AuNP-O1 and AuNP-O2 volumes) and incubated for 2 min to precipitate the amplified DNA-AuNP-oligos conjugates in target samples. The concentration of NaCl and ethanol were chosen to allow maximum precipitation of DNA molecules in the small volume of sample. This was based on conditions usually applied during a DNA extraction process with some modifications [114]. Finally, the samples were centrifuged for 1 minute at 6000 rpm (MyFuge™ Mini Centrifuge with 2 rotors from Benchmark Scientific) and distinguished as positive or negative based on their final appearance. Given the objective was to develop a rapid and simple method to visualize the amplicons, the tested incubation times were chosen to be lower to start with, and slowly increased until a difference between positive and negative samples could be seen.
4.2.7 Evaluation of the AuNP-oligos-amplicon assembly

To quantify the amount of AuNP-oligos that formed an assembly with the varying concentrations of the LAMP amplicons, the difference in the gray value of the supernatant and the red pellet was evaluated using the ImageJ software. Measuring the variation in the color intensity of the supernatant with varying amplicon concentration helped measure the amount of unassembled AuNP-oligos left in the supernatant. **Figure 11** illustrates this process.

**Figure 11.** The process of using ImageJ software to analyse the amount of AuNP-oligos remaining in the supernatant by measuring the gray value. The yellow arrow on the sample image shows the direction in which the measurement was done. The graph on the left indicates the gray value between the supernatant (highest value) and the pellet (lowest value). A higher difference between the two indicates that more AuNP-oligos assembled with the DNA amplicons to form the red pellet. The measurements were done in triplicates for every sample.

4.3 Results and Discussion

LAMP reactions for pure *B. fagacearum* DNA extracts were conducted to yield DNA amplicons that served as samples for the development of the new visualization method. Serial dilutions of the amplicons were used to evaluate the limit of detection
(LOD). **Figure 12** shows a typical bright ladder-like pattern of DNA copies obtained from a LAMP reaction in well 1, which confirms the presence of the amplicons. It should be noted that with each subsequent well the bands grow fainter as they represent serial dilutions of the amplicons. Fluorescence detection of the amplicons and their dilutions were also done using a Qubit fluorometer to confirm amplification and to quantify the DNA concentrations.

![Agarose gel image of LAMP products](image)

**Figure 12.** Agarose gel image of LAMP products. Well M serves as a marker. Well 1 contains *B. fagacearum*. LAMP amplicons and wells 2-8 are 2x, 4x, 6x, 8x, 10x, 20x and 40x dilutions of the amplicons in well 1.

The visualization test begins with the addition of the non-complementary AuNP-oligos to the LAMP reaction products, which gives it a uniform pink color. This was followed by the addition of salt and ethanol to induce precipitation of the DNA copies which eventually settled down as red pellets upon centrifugation. The high salt concentration causes DNA molecules to become insoluble in alcohol, causing them to
precipitate [114, 115]. The AuNP-oligos, in this case, are used as a coloring agent for the amplicons, owing to their natural red color, where they assemble closely to form a red pellet. This makes the amplicons distinctly visible to the naked eye while the rest of the solution remains clear. The solutions of samples that did not contain the DNA amplicons, remained uniformly pink with no detectable change. The time taken to conduct this test was less than 10 min.

It is important to note that when AuNPs aggregate due to Van der Waals interactions, they usually form dark blue or gray precipitates in solution [116]. The formation of a red pellet in the presence of the amplicons indicates that AuNP-oligos did not aggregate but assembled in an organized fashion through DNA clustering. Transmission electron microscopy (TEM) imaging was conducted to confirm the binding of the AuNP-oligos to the DNA amplicons. **Figure 13** shows single AuNP-oligos (around 10 nm) inside and around the DNA amplicon network, forming globular nanostructures of hydrodynamic diameter around 300-500 nm. Here, the oligonucleotide sequences serve two purposes. One is facilitating the non-specific assembly of the AuNPs with the DNA amplicons and two is stabilizing the AuNPs to prevent aggregation.
Figure 13. Transmission electron microscopy (TEM) images of AuNPs-oligos DNA conjugates (a) before and (b) after nanoparticle assembly following the addition of DNA amplicons. In the presence of the amplicons, the AuNP-oligos conjugates (10 nm) assemble into globular nanostructures of 300-700 nm.

The precipitation and agglomeration of these nanostructures result in the red pellet observed at the bottom of the centrifuge tube. The red color suggests that most nanoparticles still maintain an interparticle distance higher than 20 nm. An interparticle distance below 20 nm would have resulted in a change of color from red to purple due to localized surface plasmon resonance effect as we have previously reported [116, 117]. Maintaining the red color and generating a visible red pellet is important to distinguish between AuNP-oligos-amplicon assembly and other non-specific aggregation of AuNPs.
To verify the nature of this interaction, a series of control tests were conducted. First, LAMP products were incubated with the prepared AuNP-oligos for 5 min at 65°C (LAMP reaction temperature), followed by amplicon precipitation using salt and ethanol. Tests at room temperature and at 95°C (DNA melting temperature) were also conducted. The results showed no difference in AuNP-oligos-amplicon interactions at different temperatures. This confirms that the binding of the DNA amplicons with the oligonucleotides on the surface of the AuNPs is non-specific. This is because higher temperatures are required to melt the double-stranded LAMP products before specific hybridization, which was not the case at RT or 65°C. Such non-specific interaction is likely due to a combination of factors including low stringency conditions created by high concentration of salts for non-complementary hybridization [118]. Furthermore, the positive sodium ions from the salts interact with the negative charges of the DNA amplicons’ phosphate backbones, creating a salt bridge [119]. At this point, the DNA molecules likely form an expanded network with the bound AuNP-oligos. When ethanol was added, the DNA with bound AuNPs started clustering into globular nanostructures due to low solubility in ethanol, which then precipitated as a distinctive and visible red pellet at the bottom of the centrifuge tube. Tests with a different target amplicon obtained from conducting LAMP assay on *Listeria ivanovii* gene [120], a foodborne bacterial pathogen, yielded similar results using the same AuNP-oligos (Figure 14). Further, tests with AuNPs functionalized with complementary oligonucleotide sequences that are specific to the target fungal sequence (*B. fagacearum*) also yielded a similar result (Figure 15). This means that under these conditions synthesized AuNP-oligos can act as
labels for any amplicons obtained from a DNA amplification reaction irrespective of complementarity and is not selective for the source of amplicons. Therefore, as long as the designed LAMP assay primers are highly specific to the target template sequence, the final reaction with AuNP-oligos would serve as a rapid non-specific visual confirmation. Furthermore, the non-specific binding of AuNP-oligos with the amplicons for naked-eye detection eliminates the need to maintain high temperatures for hybridization, making it more feasible for field testing. Triplicates were maintained throughout.

Figure 14. (a) Agarose gel image of LAMP products obtained from amplifying Listeria ivanovii gene. Well M serves as a marker. Wells L1 to L3 contains L. ivanovii maintained as triplicate. Well W indicates the negative control, which is nuclease free water. (b) Visualization of Listeria ivanovii DNA amplicons (2.44 mg/ml) by nanoassembly with nuclease free water as negative control.
Figure 15. Visualization of *Bretziella fagacearum* (*B. fag*) DNA amplicons by nanoassembly. (a) AuNPs were coated with oligonucleotide probes that are specific to *B. fag*, then mixed with the *B. fag* DNA amplicon to cause nanoassembly and precipitation into a visible red pellet. The control sample (b) was obtained by replacing the *B. fag* DNA amplicon with nuclease-free water.

Tests were also conducted with samples containing AuNPs synthesized without any oligonucleotides. Samples without the addition of NaCl and ethanol were also used as controls (Figures 10 and 16). The addition of AuNPs without the oligonucleotides caused the nanoparticles to aggregate in the presence of the salt and settle down as a dark gray aggregate irrespective of whether the sample was positive or negative. This showed that the oligonucleotide sequences on the AuNPs were necessary for their stability and attachment to the DNA amplicons for a successful distinction between positive and negative samples. Moreover, without the addition of the salt and ethanol, both positive and negative samples remained pink in color for samples with AuNP-oligos and amplicons. This showed that the addition of NaCl and ethanol was necessary to cause the nanoassembly of the DNA amplicons and AuNP-oligos, which led to the appearance of
the red pellet in positive samples. Therefore, the AuNP-oligos, and adequate concentrations of salt and ethanol were essential ingredients for a visual detection of LAMP amplicons at the end of the amplification reaction. Results from this test are shown in **Figure 16**.

![Figure 16](image)

**Figure 16.** Different control experiments to confirm the specificity of the nanoassembly and red pellet formation for the presence of DNA amplicons. Samples that did not contain all the necessary ingredients (AuNP-oligos, salts, ethanol, DNA amplicons) showed no visible red precipitation. P depicts the presence of oligonucleotides on the AuNPs. The “Sample” represents DNA amplicons obtained for LAMP reactions with primers specific to *B. fagacearum*, the oak wilt fungus.

To determine the minimum concentration of amplicons that is required to induce the formation of the red pellet, serial dilutions of the LAMP products were also tested. The concentrations of the amplicons were determined using a Qubit fluorometer. Given that the sample volume and concentration of the AuNP-oligos in solution was too low for UV-visible-based quantification using the nanodrop, AuNP-oligo assembly with the DNA
amplicons was evaluated using ImageJ software. The change in the gray value between
the free nanoparticles in solution (supernatant) and the nanoparticles assembled into a red
pellet was quantified. The process is illustrated in Figure 11. This approximates the ratio
of nanoparticle assembly caused by the DNA amplicons. The results depicted in Figure
17 show that the lowest concentration of the amplicons that yielded a visible red pellet in
the assay was found to be around 45 µg/mL, which was calculated to be $1.87 \times 10^{11}$
copies/µl using an online tool
(https://www.technologynetworks.com/tn/tools/copynumbercalculator). It is important to
note that this concentration is up to 20 times lower than the DNA amplicon
concentrations usually produced by nucleic acid amplification during LAMP assays,
confirming that the proposed process can be a powerful visualization method for LAMP
assays.
Figure 17. Serial dilutions of the DNA amplicons and corresponding changes caused by AuNP-oligo assembly. The y axis represents the amount of AuNP-oligos that assemble with the DNA amplicons. Measurements were done in triplicates.

4.4 Conclusions

The nanoassembly of AuNP-oligos and DNA amplicons into a distinct structure visible to the naked eye is demonstrated to be a simple and efficient method for rapid visualization of LAMP reaction products. The new approach overcomes the limitation faced by current visual colorimetric detection methods, including nuanced color change, and concentration-dependent reaction of dyes. Unlike other studies that explored the use of nanoparticles for LAMP assay detection, the approach reported here relies on a novel
method of applying AuNP-oligos, where visual detection is defined by the behaviour of the AuNP-oligos-amplicon complex rather than color changes. While the binding of the oligonucleotides to the DNA amplicon is non-specific, the nanoassembly and subsequent formation of a red pellet is highly dependent on the presence of DNA amplicons in the sample. Furthermore, the nanoassembly approach allows visualization of amplicons at concentrations down to $1.87 \times 10^{11}$ copies/µl (45 µg/mL), which is up 20 times lower than the average amplicon concentration obtained from LAMP-based nucleic acid amplification.
Chapter 5. A rapid LAMP assay for the diagnosis of oak wilt with the naked eye.

5.1 Introduction

Oak trees (*Quercus*, Fagaceare) are widely distributed across the United States being found in both natural, planted, and urban forest ecosystems [121]. They are estimated to account for 11 percent of all US trees. In the Eastern US, oaks are most abundant in the oak-hickory forest type group [3]. Besides their economic importance as a source of high-quality lumber [122], oaks are ecologically important for wildlife (e.g., habitat, food) and provide many ecosystem services (e.g., reducing soil erosion and air pollution) [97]. They are also valued for their aesthetics and for cultural purposes [121].

Oak wilt, caused by the fungus *Bretziella fagacearum*, is a destructive vascular wilt disease of *Quercus* in the Eastern USA and was recently found in southern Ontario, Canada. The red oak species (Section Lobatae) are highly susceptible to oak wilt compared to the moderate to low susceptibility of the white oak species (Section Quercus) [3]. The pathogen spreads from diseased trees to healthy ones below-ground through connected roots and above-ground via insect vectors [123]. Although *B. fagacearum* cannot be eradicated from a tree once it is infected, treatments are available to prevent or reduce the probability of spreading to healthy trees. Early detection and timely treatment of single or small clusters of infected trees offer the best chance of controlling oak wilt [124].

Unfortunately, damage to oaks caused by other biotic agents and abiotic factors may be confused with foliar symptoms of oak wilt. For example, drought stress coupled with twolined chestnut borer (*Agrilus bilineatus*) infestations causes symptoms in red
oaks that can be hard to distinguish from those caused by oak wilt. Laboratory testing of woody samples from suspected diseased trees is advisable, particularly if treatment is warranted. Traditional fungus culture and polymerase chain reaction (nested PCR, real-time PCR, qPCR) techniques are currently used by US plant diagnostic laboratories for testing both red oak and white oak species for *B. fagacearum*. Small wood chips or drill shavings from sapwood of suspect oaks are generally assayed. Culture assay requires 14 to 21 days to provide results, while results from PCR-based methods may be possible within less than one day [112]. In Canada, two real-time PCR / qPCR TaqMan® assays were developed for laboratory settings; high sensitivity and reliable results in detecting *B. fagacearum* in environmental samples were reported [125, 126]. In addition, a DNA endonuclease-targeted CRISPR trans reporter (DETECTR) assay using DNA amplified by recombinase polymerase amplification (RPA) was developed and validated using environmental samples, specifically, oak wilt fungal mats and *B. fagacearum*-infested insects [126]. The method was then optimized for cost-effective and “point-of-care” (i.e., field) use based on the results of beta testing by multiple potential users. Research by others has yielded preliminary or potential techniques for rapid DNA assay of sapwood samples in the field [113, 127-129].

Nucleic acid amplification tests (NAATs), such as PCR-based assays, have been considered as one of the most sensitive methods for pathogen detection due to their ability to amplify very small amounts of the pathogen’s genetic material [130, 131]. However, PCR-based assays require expensive equipment to maintain multiple cycles of varying temperatures for the amplification process. They also require trained laboratory
personnel for operation, which makes their adaptability to field tests nearly impossible [132]. To overcome this challenge, several isothermal nucleic acid amplification methods have been developed to eliminate the necessity for sophisticated equipment and to save time and costs. Recombinase polymerase amplification (RPA) [133], helicase-dependent amplification (HDA) [134], and loop-mediated isothermal amplification (LAMP) [135] are examples of such methods that have been utilized for the diagnosis of plant diseases [107]. Among these, LAMP-based assays were found to be highly sensitive and more flexible for optimizing reactions based on the target pathogen [136]. Since this method requires at least four primers with the inclusion of loop primers, the nucleic acid amplification is highly specific to the target and occurs much faster under the right conditions [137]. For these reasons, several plant pathogen detection studies based on LAMP have been reported for rapid onsite testing [138]. A schematic of the LAMP reaction is shown in Figure 18.
Figure 18. LAMP reaction steps. The reaction uses 4-6 primers targeting 6-8 regions on the target DNA strand. The strand displacing DNA polymerase starts the amplification process followed by two of the primers forming a loop structure to facilitate further rounds of amplification.

In this study, we report the development, characterization, and testing of the first LAMP assay for rapid and sensitive detection of *Bretziella fagacearum*, the causal agent of oak wilt, in *Quercus rubra* (northern red oak) samples. Furthermore, the coupling of our LAMP assay with a recently developed amplicon visualization technique employing nanoparticle assembly [129] enables naked-eye detection of the oak wilt fungus.

5.2 Experimental section

5.2.1 Materials

WarmStart® LAMP Kit (DNA & RNA) was purchased from New England Biolabs (Ipswich, MA), which contains Warmstart LAMP 2X Master Mix and LAMP fluorescent dye (for fluorescence detection). Primers for LAMP experiments and oligonucleotide sequences for AuNP conjugation were fabricated by Integrated DNA Technologies (Coralville, IA). All solutions were prepared using nuclease-free water purchased from Stemcell Technologies (Vancouver, Canada). Sodium chloride (NaCl), gold (III) chloride trihydrate, trisodium citrate dehydrate and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Sigma-Aldrich (St. Louis, MO). GelRed® Nucleic Acid Stain 10000X Water was purchased from Millipore Sigma (Burlington, MA). GeneRuler Low Range DNA Ladder, 10X UltraPure TAE Buffer and UltraPure DNase/RNase-Free Distilled Water were purchased from Thermo Fisher Scientific (USA) for gel electrophoresis visualization of amplicons. Ethanol was procured
from Fisher Scientific (USA). Ethylenediaminetetraacetic acid (EDTA) was obtained from Boston Bioproducts (Milford, MA).

5.2.2 Fungal culture and DNA extraction

Fungal cultures were isolated from oak wilt-infested Q. ellipsoidalis wood samples. Wood tissues exhibiting the distinctive streaking of oak wilt infection were removed using sterile procedures and placed on Barnett’s media [139] for approximately 14 days at 25°C. B. fagacearum cultures and other fungi that were frequently obtained during the procedure were then grown on half-strength PDA to get pure cultures. The DNA from the other fungal colonies was then extracted following manufacturer’s instructions from a commercial kit (Qiagen DNeasy Plant Mini Kit). Through PCR using universal ITS1F/ITS4 fungal primers, Sanger sequencing, and BLAST searches, the other fungi were determined to be Dicarpella sp., Fusarium sporotrichoides, Graphostroma sp., Querciphoma carteri, and Epicoccum nigrum. These were used in the specificity studies described below. B. fagacearum was propagated on potato dextrose agar (PDA) for 7 to 14 days at 25°C. The DNA from the pure fungal colonies was then extracted following manufacturer’s instructions from a commercial kit (QIAamp DNA Mini Kit) with slight modifications in the buffer volumes.

5.2.3 DNA extraction from red oak samples

Drill shavings of Northern pin oak (Q. ellipsoidalis) samples were obtained from various locations of known oak wilt infection centers in central Minnesota in the summer of 2022. Specifically, they were found in Anoka, Chisago and Sherburne counties. The DNA from the wood samples were extracted using a previously developed extraction
protocol [127]. This method uses NaOH to extract crude DNA then dilutes it in Tris buffer. Optionally, it is then purified using a spin filter process. Each extraction yielded about 340 µL of DNA in elution buffer with the DNA concentrations ranging from 100-300 ng/ml. The samples were predetermined to be healthy or infected by *B. fagacearum* using nested PCR (Figure 19) [112]. This is the benchmark procedure to which this new method is compared. Ten samples each of healthy and infected drill shavings were used for the study using the purified protocol. Additionally, five samples of healthy wood tissue and five of diseased wood tissue from the Plant Disease Clinic of the University of Minnesota were sent to Frontline Biotechnologies Inc., Saint Paul, MN, USA. They were extracted and purified using the same technique [127] and used in this study for confirmatory tests. A subset of crude extracts of two samples, each of healthy and diseased trees were used to test the feasibility of using the simpler and less time-consuming method for obtaining DNA for the LAMP assay. It must be noted that the quality of wood collected may vary depending on the sampling period, location, and method and could affect the amount of DNA extracted. However, maintaining a standard protocol for collection and obtaining samples from multiple locations in the suspected tree could reduce those discrepancies.
Figure 19. Agarose gel electrophoresis of nested PCR products obtained from testing red oak samples using crude NaOH-extracted DNA. (a) Infected samples; (b) healthy samples, water control, positive control. Samples were from the same branches as those used for the LAMP assay.

5.2.4 LAMP assay

Three sets of LAMP primers, targeting the ribosomal DNA (rDNA) internal transcribed spacer (ITS) of *B. fagacearum*, were designed using the NEB® LAMP primer Design Tool, version 1.3.1 (New England Biolabs Inc.). The ITS is a nonfunctional DNA region located between the small and large subunits of ribosomal DNA (rDNA). It is generally chosen for phylogenetic studies due to the low evolutionary pressure on such a non-coding sequence and high variability between closely related fungal species.

The nucleotide sequence of *B. fagacearum* strain CBS130770 (GenBank accession no. MH865866) was used as a reference.

AACAAGGTTTCCGTGTTGTAACCAGCGGAGGGATCATTACTGAGTTTTCAACTCTTTAAAACCATTTGTGAACATACCATTTTTTTCTCTAATACTGCTTTGGCAGGGACTTCTTTCTTCAGGGGATGTTTCTGCCAGTAGTATTTACAAACTCTTTT
Each set included 6 different primers – forward and backward inner primers (FIP & BIP), forward and backward outer primers (F3 & B3), and forward and backward loop primers (LF & LB). These primer sets were chosen for the study based on the following criteria:

1. The initial primer design has enough space between F2 and F1c to design the loop primers.

2. Design with the largest Gibbs free energy (ΔG) value for dimerization.

   Dimerization happens when the primers self-hybridize. A positive ΔG value would mean that the chance of dimerization is lowered.

3. Design with ΔG < -4 for the ends of the primers.

Based on the results of preliminary screening experiments, one set of primers was chosen to be used for the rest of the study (Table 3).

Table 3. Nucleic acid sequences of LAMP primers sets used for specificity tests including the chosen set

<table>
<thead>
<tr>
<th>Primer type</th>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward outer</td>
<td>P42L28-F3</td>
<td>GAGAATTTAATTCAATTGCTGCAGTTG</td>
</tr>
<tr>
<td></td>
<td>P55L1-F3</td>
<td>TGAAGAACGCAGCGAAAT</td>
</tr>
<tr>
<td></td>
<td>P87L1-F3</td>
<td>GCAGTTACCTGTGACCCCTCCCTGCGTAGTTTTTTGG</td>
</tr>
<tr>
<td>Backward outer</td>
<td>P42L28-B3</td>
<td>CAAGCAAGGCTTGAGTTG</td>
</tr>
<tr>
<td></td>
<td>P55L1-B3</td>
<td>AGTTTTACTACGCCAGCGGAAG</td>
</tr>
<tr>
<td></td>
<td>P87L1-B3</td>
<td>ACTTTGTGTTACCGCTGGAAACTC</td>
</tr>
<tr>
<td></td>
<td>P42L28-FIP</td>
<td>CGCATTTCGCTCAGTGGACCTGAGTTGAAACTTCAACACGGAAT</td>
</tr>
</tbody>
</table>
The LAMP reaction mixture was prepared according to the manufacturer’s instructions for the WarmStart® LAMP Kit (DNA & RNA). Briefly, 1µL of the sample DNA was added to a mixture of 12.5 µL of the 2X LAMP master mix, 2.5 µL of 10µM primer mix, 0.5 µL of 50X fluorescent dye with the final volume made up to 25 µL using nuclease-free water. The reaction mixture was incubated at 65°C for 30 min to allow amplification, followed by termination at 85°C for 5 min. The fluorescence intensity of the final amplicons was measured at the end of this period. StepOnePlus™ Real-Time PCR System was used both for incubation and fluorescence measurements. To show that LAMP can be done without a sophisticated PCR thermocycler, a set of experiments were also conducted using Thermo Scientific™ Compact Digital Dry Bath/Block Heater for incubation and Agilent-Cary Eclipse Fluorescence Spectrophotometer for fluorescence detection. All experiments were conducted in triplicates.

To assess the specificity of the designed primers for target DNA amplification, experiments were conducted using extracted DNA samples from other selected fungal species. The selected fungi were common species detected in sapwood of logs cut from oak wilt killed Q. ellipsoidalis trees. Specifically, the experiments included DNA

| Forward inner | P55L1-FIP | AGCAGAAATCTGCTAGGCCTAGT5TAGATTCAGT |
| Backward inner | P42L28-BIP | CAGTGAATCATCGAATCTTTTGAAACCGAATGACGCTCGGACAG |
| Forward loop | P42L28-LF | TCGATGCTAGAGCCAAAG |
| Backward loop | P42L28-LB | CCTAGCAGTTATTCTGTAGGCA |

The LAMP reaction mixture was prepared according to the manufacturer’s instructions for the WarmStart® LAMP Kit (DNA & RNA). Briefly, 1µL of the sample DNA was added to a mixture of 12.5 µL of the 2X LAMP master mix, 2.5 µL of 10µM primer mix, 0.5 µL of 50X fluorescent dye with the final volume made up to 25 µL using nuclease-free water. The reaction mixture was incubated at 65°C for 30 min to allow amplification, followed by termination at 85°C for 5 min. The fluorescence intensity of the final amplicons was measured at the end of this period. StepOnePlus™ Real-Time PCR System was used both for incubation and fluorescence measurements. To show that LAMP can be done without a sophisticated PCR thermocycler, a set of experiments were also conducted using Thermo Scientific™ Compact Digital Dry Bath/Block Heater for incubation and Agilent-Cary Eclipse Fluorescence Spectrophotometer for fluorescence detection. All experiments were conducted in triplicates.

To assess the specificity of the designed primers for target DNA amplification, experiments were conducted using extracted DNA samples from other selected fungal species. The selected fungi were common species detected in sapwood of logs cut from oak wilt killed Q. ellipsoidalis trees. Specifically, the experiments included DNA
extracted from *Dicarpella* sp., *Fusarium sporotrichoides*, *Graphostroma* sp., *Querciphoma carteri*, and *Epicoccum nigrum*.

To determine the limit of detection (LOD) of the designed LAMP assay, tests were conducted for different concentrations of *B. fagacearum* DNA using the chosen primer set. Pure culture of *B. fagacearum* was used for the DNA extraction and diluted to the required concentrations for this experiment using nuclease free water. Initial concentrations ranged from 0.15 pg/mL to 15 ng/mL in logarithmic increments to determine the range of LOD. Based on the results, further experiments were conducted with DNA concentrations of 0.015, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.15 ng/mL for accurate determination of the LOD. The DNA concentrations were confirmed using a Qubit Fluorometer.

The designed LAMP assay was conducted on healthy and infected red oak samples to test their applicability in detecting oak wilt in actual trees. The equations 1 and 2 were used to calculate the sensitivity and specificity of the assay based on the obtained fluorescence results,

\[
Sensitivity = \left( \frac{a}{a+c} \right) \times 100
\]

\[
Specificity = \left( \frac{d}{b+d} \right) \times 100
\]

where, \(a\) is the number of true positives, \(b\) is the number of false positives, \(c\) is the number of false negatives and \(d\) is the number of true negatives.

5.2.5 LAMP visualization using agarose gel electrophoresis
The agarose gel electrophoresis of the LAMP products obtained from red oak samples was done to confirm the fluorescence test results. GelRed® Nucleic Acid Stain 10000X Water agarose gel (1%) was prepared, and a low-range DNA ladder was included as a reference alongside the samples for comparison.

5.2.6 Optical detection of LAMP products with AuNP-oligos

Both healthy and *B. fagacearum* infected red oak samples were tested using the designed LAMP assay and their fluorescence intensities were measured. To enable a faster identification of target amplicons, a rapid naked eye detection method was employed with laboratory-synthesized nanoparticles [129]. The final appearance of the solution was used to distinguish between positive (red pellet) and negative samples (uniform pink supernatant). Non-complementary AuNP-oligonucleotides were added to the LAMP reaction products. Salt solution and ethanol were then added to induce precipitation of the gold-conjugated target amplicon. Both crude and purified DNA samples from red oak were used for this test. All experiments were conducted in duplicates.

5.2.7 Tests with a common contaminant *Cladosporium herbarum*

*Cladosporium herbarum* seems to be a common contaminant that causes false positive results in a PCR test for *Bretziella fagacearum*. This is especially the case when the CF01/CF02 primers were used to specifically test for *Bretziella fagacearum* [140]. The reason for this is that the primer sequences are complementary to similar sequences in the *Cladosporium herbarum* genome. The designed primer sequences for the LAMP assay presented here are not similar to this contaminant’s gene sequences when checked.
using the NCBI Blast tool [141]. However, possible contamination had to be ruled out. Therefore, LAMP was performed using DNA extracted from *Cladosporium herbarum* fungus obtained from the Plant Disease Clinic at UMN under the same reaction conditions described above. DNA from *Bretziella fagacearum* was used as the positive control, and nuclease-free water was the negative control. The LAMP reaction products were analyzed using both gel electrophoresis and the developed AuNP-oligos visualization test.

### 5.2.8 Statistical analysis

The LAMP assays using fluorescence measurements were conducted in triplicates. For the experiment with healthy and infected oak trees, the fluorescence intensities were compared for each of the samples and the significant difference was analyzed using t-test with significance level \( \alpha = 0.05 \).

### 5.3 Results and discussion

#### 5.3.1 Primer selection

The specificity of LAMP detection relies on the specificity of the primers targeting the pathogen of interest, here *B. fagacearum*. We designed and evaluated three different sets of six primers each, labelled as P42, P55 and P87. Here, DNA from different fungal species isolated from sapwood of oak wilt killed trees – *Dicarpella* sp., *Fusarium sporotrichoides*, *Graphostroma* sp., *Querciphoma carteri*, and *Epicoccum nigrum* were used as controls against *B. fagacearum* DNA to test the specificities of the primers. Nuclease-free water was used as negative control, where no amplification is expected due to the absence of a template DNA strand. The LAMP reaction and
fluorescence detection for this experiment was conducted using the StepOnePlus™ Real-Time PCR System. The indicator used in the LAMP assay is an intercalating dye detectable via the SYBR/FAM channel. With DNA amplification, the fluorescence intensity is expected to be high due to the dye binding to the double-stranded DNA. Based on the results, among the three primer sets, P87 exhibited the highest specificity for amplifying oak wilt DNA.

**Figure 20a** shows the fluorescence intensities measured for LAMP products obtained from P87 in the amplification reaction. The control DNA samples showed similar results to nuclease-free water, while samples containing *B. fagacearum* DNA displayed the highest intensity, reaching around 1900000 a.u. This indicates the absence of amplification in the controls despite the presence of fungal DNA, except for the target fungus. It is to be noted that the intensity of nuclease-free water and other fungal species is just above zero, likely due to the residual fluorescence contributed by the dye and the primers present in the reaction mixture. The other primer sets P42 and P55 either failed to show specific detection of the target DNA or had cross reactions and were therefore eliminated from further studies. The results from P42 and P55 are shown in **Figure 21**.
Figure 20. Characterization of the developed LAMP assay using spectrofluorometric analysis. (a) Selectivity of the assay for Bretziella fagacearum (BF). No detection is observed for other fungi including Dicarpella sp. (DS), Fusarium sporotrichoides (FS), Graphostroma sp. (GS), Querciphoma carteri (QC), and Epicoccum nigrum (EN). (b) Limit of detection of the LAMP assay (LOD: 30 fg/μl).
Figure 21. Primer specificity tests using different fungal species: *Dicarpella* sp. (DS), *Fusarium sporotrichoides* (FS), *Graphostroma* sp. (GS), *Querciphoma carteri* (QC), *Epicoccum nigrum* (EN), *Bretziella fagacearum* (BF). (a) LAMP reaction results for tests with primer set 42, (b) LAMP reaction results for tests with primer set 55.

5.3.2 Limit of detection

The limit of detection of the assay was studied using the P87 primer set by testing various concentrations of DNA extracted from a pure culture of *B. fagacearum*. Nuclease-free water was used to achieve the appropriate dilutions to be used for the test. StepOnePlus™ Real-Time PCR System was used for the LAMP reaction and fluorescence detection. The LOD was found to be 30 fg/µL with the fluorescence intensity in the detectable range of around 1,200,000 a.u (Figure 20b). As the DNA concentration increased, the fluorescence intensity also increased, and reached a plateau at around 1,700,000 a.u. Although the LOD is a hundred times higher than the one achieved using qPCR [112], it is much lower than other oak wilt detection assays developed to date [113, 126]. Moreover, this LOD is significantly below the concentrations of DNA usually extracted from an infected oakwood sample. Further improvements in primer design and target DNA preparation could potentially improve the LOD of the LAMP assay.

5.3.3 LAMP detection of oak wilt in red oak

The efficiency of the developed LAMP assay in detecting the target pathogen in real oak samples was investigated. Experiments were conducted using nucleic acids extracted from 10 healthy and 10 infected red oak drill shavings. Based on the results (Figure 22a), all infected samples showed high fluorescence intensity at the end of the LAMP reaction, indicating the occurrence of DNA amplification. To ensure that only the
target fungal DNA is being amplified irrespective of the presence of DNA of the plant and of any other fungal species present in the diseased samples, healthy wood DNA samples were also tested as controls. The DNA amplification did not occur in any of the healthy samples, which was indicated by the absence/very low fluorescence at the end of the LAMP assay. The statistical analysis also showed a significant difference between the intensities obtained from infected and healthy samples with the $p$ value being less than 0.05. Based on this, the sensitivity and specificity of the assay were calculated to be 100%. One of the healthy samples showed an average intensity of around 40000 a.u., which is slightly higher than the remaining healthy ones. This could be due to carryover contamination that may occur when the LAMP products are exposed in the same room as where the sample preparation is done. To avoid this problem, any post amplification steps were conducted in a different location. This set of experiments were conducted using the StepOnePlus™ Real-Time PCR System, which was used for both incubation and fluorescence measurement.

Tests were also conducted on a different set of DNA samples obtained from 5 healthy and 5 infected oak tree samples using the Thermo Scientific™ Compact Digital Dry Bath/Block Heater for incubation and Agilent-Cary Eclipse Fluorescence Spectrophotometer for the final measurements. This was done to exhibit the adaptability of the developed LAMP assay in any field testing, where transportation and use of a PCR instrument could be challenging. Both positive and negative controls were used to standardize the fluorescence measurements. The results obtained using this method correlated with the previously tested samples and are shown in Figure 22b. The
difference in the range between the fluorescence intensities from both experiments can be attributed to the different instruments used. The infected tree samples showed a higher fluorescence intensity compared to the healthy tree samples. In all cases, the residual fluorescence seen in the healthy samples comes from the primer interaction with the DNA binding fluorescence dye used with the LAMP master mix. All tests were conducted in triplicates.

Figure 22. Detection of the fungus *Bretziella fagacearum* in wood chip samples from healthy and infected oak trees. The wood chip samples were treated to extract and purify the fungal DNA which was then amplified using LAMP. The product of the LAMP assay was characterized using fluorospectroscopy. The results show 100% specificity and 100% sensitivity. (a) Results from LAMP assay conducted using StepOnePlus™ Real-Time PCR System for heating and fluorescence detection. (b) Results from LAMP assay conducted using Thermo Scientific™ Compact Digital Dry Bath/Block Heater for heating and Agilent-Cary Eclipse Fluorescence Spectrophotometer for fluorescence detector. The difference in the fluorescence intensity ranges in each set of experiments reflects the varied sensitivities of the fluorescence devices used.

Agarose gel electrophoresis was conducted to confirm the results obtained with fluorescence. Figures 23a and 23b show a typical ladder-like pattern for DNA amplicons obtained from a LAMP reaction in all the infected samples. Whereas the controls,
including all the healthy samples showed a single light band at the bottom of the gel, indicating the absence of amplicons (Figures 23c and 23d). The ladder-like pattern for positive samples is characteristic of LAMP products due to the production of the large concentration of DNA copies of varying sizes [142].

Figure 23. Agarose gel electrophoresis of LAMP products obtained from testing red oak samples. Infected samples tested on (a) real-time PCR instrument, and (b) portable dry heat block. Healthy samples along with nuclease-free water as control tested on (c) the same PCR device as a), and (d) the same heat block as b).

5.3.4 Optical detection of LAMP products using hierarchical nanoparticle assembly
While fluorescence detection is extremely sensitive, it does require expensive instrumentation and thus is not suitable for field detection. Apart from fluorescence, amplification after a LAMP reaction can also be indicated by turbidity in the sample. This is because nucleic acid amplification causes the release of magnesium pyrophosphate as a by-product proportionally to the number of amplicons, which causes turbidity [19]. However, without a turbidimeter, distinguishing positive and negative samples with the naked eye was difficult, especially when the sample size is as small as 25 µL. Therefore, to improve the detection of this turbidity with the naked eye, a newly developed method involving hierarchical gold nanoparticle assembly was employed here [129]. The addition of AuNP-oligos gave the amplicons solution a pink color, which upon precipitation using NaCl and alcohol resulted in a red pellet after centrifugation of the sample tube, while the remaining solution was clear.

The binding of AuNP-oligos with the DNA amplicons was confirmed using transmission electron microscopy. Figure 24 shows the DNA precipitates that are bound to the AuNP-oligos causing them to form closely spaced globular structures that are seen as the large pellet in a positive sample tube (Figure 24a). The attached AuNP-oligos can be seen as tiny dots in and around the larger DNA globules (Figures 24b-d). When there are not enough DNA copies due to lack of amplification, no precipitation occurred, and the solution remained uniformly pink. The AuNP-oligos act as amplicon labels by forming an assembly through the non-specific interaction of the short amplicon sequences with the oligonucleotides under low stringency conditions [118]. Here, the precipitation of DNA was induced by the presence of salt and alcohol, which is generally
used in the nucleic acid extraction process since DNA in a salty solution cannot
dissociate without a polar solvent [115]. Based on this test, all infected samples showed a
red pellet after centrifugation due to the presence of the *B. fagacearum* DNA copies
attached to the AuNP-oligos. On the other hand, the solution in all the healthy sample
tubes remained pink even after centrifugation (Figures 25a and 25b). The visualization
process required less than 10 min and did not require any instrumentation.

**Figure 24.** (a) Concept of amplicon visualization using hierarchical nanoparticle assembly. (b) The visible pellet at the bottom of the tube is formed following induced precipitation of assembled AuNP-oligo with the DNA amplicons, and (c) transmission microscope image of globular nanostructures, and (d) conceptual diagram of one assembled globule. The assembly of hundreds of these structures yields a red pellet visible to the naked eye.
Figure 25. Detection of the fungus *Bretziella fagacearum* in wood chip samples from healthy and infected oak trees. After DNA extraction and amplification using LAMP, visualization of the amplicon is performed using oligos-conjugated gold nanoparticles. In the presence of the target DNA, the nanoparticles bind to the DNA amplicons, and assemble into globular nanostructures that can be easily precipitated to form a visible red pellet at the bottom of the microtube. (a) Results from LAMP assay conducted using a real-time PCR instrument, (b) Results from LAMP assay conducted using a portable dry heat block.

Amplified DNA resulting from LAMP or RPA assays can be visualized using changes in turbidity, visible color, or fluorescence. Methods that require opening of sample tubes after assay reaction in order to add solutions for visualization step may allow for cross contamination due to aerosolized products [143]. This concern is relevant to colorimetric and fluorescent-based assays as well as the AuNP-oligos assay reported
here. However, some studies have attempted to overcome this problem by designing sample tubes to contain the post amplification reagents separately from the LAMP reagents. This way a centrifugation step post amplification can be done without opening the sample tube for the reagents to interact with the LAMP products and show results [144-147].

5.3.5 Rapid optical detection for crude DNA extracts

The LAMP assay and visualization can be completed in 40 min. However, the first step in any detection assay is nucleic acid extraction and purification, which can be time-consuming. We have previously confirmed that nested PCR detection of *B. fagacearum* in red oak sapwood can be successfully performed using a crude DNA extract, i.e., without any DNA purification step [127]. To evaluate the possibility of LAMP detection directly on a crude sample, crude DNA extractions from two infected and two healthy red oak wood samples were tested using the developed LAMP assay. Both the fluorescence measurements and the nanoparticle assembly tests were able to differentiate between the healthy and infected samples as depicted in Figure 26. Given that most nucleic acid-based tests require purified samples for detection, the ability to identify infection without the need for the purification step significantly reduces the preparation time. Therefore, with the availability of a portable heating block, this assay could be conducted in the field along with rapid visual detection of oak wilt.
Figure 26. LAMP detection of the fungus *Bretziella fagacearum* in crude samples, without nucleic acid purification. (a) Amplicon characterization using spectrofluorometry, and (b) Amplicon visualization using the hierarchical assembly of oligos-conjugated gold nanoparticles.

5.3.6 Results from the *Cladosporium herbarum* tests

To test whether the designed LAMP assay was able to avoid false positive results arising from the fungal contaminant *Cladosporium herbarum*, experiments were conducted with DNA extracted from the fungus in triplicates. Based on the gel electrophoresis and the AuNP-oligos visualization tests, no amplicons were generated for samples containing the *Cladosporium herbarum* DNA (Figure 27). This means that the developed LAMP assay was highly specific for *Bretziella fagacearum* and was better than the PCR test in avoiding false positive results.
Figure 27. (a) Gel electrophoresis results showing the typical LAMP amplicon bands for positive controls with *B. fagacearum* DNA, and faint bands at the bottom of the gel, indicative of no amplification in negative control and the samples containing the *Cladosporium herbarum* DNA. (b) The results from the AuNP-oligos visualization test, where only the positive control shows the red precipitate.

5.4 Conclusions

This work reports the first development and application of a LAMP assay for the detection of *Bretziella fagacearum*, the causal agent of oak wilt disease. The total time for the LAMP reaction was 30 min, achieving a limit of detection down to 30 fg/µL. Testing with real red oak samples showed 100% sensitivity and specificity, indicating the absence of false negatives and false positives. In addition, a ten-minute reaction with oligonucleotide-coated gold nanoparticles enables a naked-eye visualization of *B.*
B. fagacearum DNA amplicons. This rapid visual differentiation of positive samples obtained at the end of the LAMP assay eliminates the need for any instrumentation. Further, the LAMP assay coupled with the rapid visualization technique was able to specifically detect B. fagacearum DNA from crude DNA extracts of infected red oak wood samples, overcoming the need for DNA purification. Therefore, with just the use of a simple hand-held heating block, this assay holds potential for rapid field testing of oak wilt. A logical next step in method development would be to test the combined protocol on known diseased red oak in a field setting. The protocol would include NaOH extraction of DNA, LAMP assay using dry heat block, and visualization with oligonucleotide-conjugated gold nanoparticles. Evaluation of the same protocol for sapwood samples from known diseased white oak species (e.g., Q. macrocarpa, Q. alba) is also needed since NaOH extraction of DNA from these species may require a purification step [128].
Chapter 6. Magnetic relaxation switching and chemiluminescence assays for rapid microbial diagnostics

Before the development of the rapid LAMP assay for microbial diagnostics, other techniques were explored for the same purpose. They are briefly described in this section along with the associated challenges that did not make them ideal options for the goal of this dissertation.

6.1 Magnetic relaxation switching (MRSw) assay using magnetic nanoparticles

The MRSw assay relies on the use of benchtop NMR relaxometers that are specifically used for the measurement of the relaxation rates. They are low frequency NMR devices, typically in the range of 20 MHz [148]. NMR spectroscopy has been used for determining the chemical structure of molecules. The technique uses information from the spin states of the atomic nucleus in the molecule to determine its chemical environment in the presence of an external magnetic field. Some isotopes such as \(^1\)H and \(^{13}\)C have a net nuclear spin due to odd number of proton and neutrons, respectively. A nucleus with either an odd number of protons or neutrons possesses a half integer spin. \(^1\)H nucleus has \(\frac{1}{2}\) spin with two possible orientations. In the presence of an external magnetic field, the nucleus behaves like a magnet. Those in the higher energy level align in the direction opposite to the external magnetic field, whereas those in the lower energy level align along the direction of the external magnetic field (Figure 28). Usually, the nuclei in lower energy are comparatively more in number. To flip the nuclei to higher energy state, the NMR instrument applies radio frequency waves to the samples in the presence of the external magnetic field. This causes the nuclei in the samples to resonate at specific frequencies, which will be displayed as a spectrum. The resonance happens...
when the nuclei from a lower energy level move to a higher energy level until the population in both levels are equal, which is the saturation point. Beyond this, the excited nuclei return to their original state and the time taken would vary. This process is called relaxation, which occurs as longitudinal (z direction), $T_1$, and transverse (xy direction), $T_2$.

![Energy levels for a nucleus with spin quantum number 1/2](image)

**Figure 28.** Energy levels of a nucleus with $\frac{1}{2}$ spin [149]

Secondly, in the presence of an external magnetic field, magnetic nanoparticles (MNPs) can act as small magnets with spatial dependence. They can change the spin-spin relaxation of the water protons in their surroundings. This ability of MNPs is exploited in the MRSw application to detect the presence of a target molecule in solution [150].

In a typical MRSw assay, MNPs coated with the target specific molecules are introduced into the sample in solution. In the presence of the target biological molecules, the surface modified MNPs start aggregating due to their affinity for the target. Depending on whether the MNPs are dispersed or aggregated, the $T_2$ relaxation time of the surrounding water protons changes, which causes a change in the magnetic resonance signal. This change is used as an indication for the presence of the target [151]. It is also
hypothesized that the concentration of the target is linear with the change in the T$_2$ relaxation time of the water protons in the sample.

**Figure 29.** Schematic of the magnetic relaxation switching bioassay

The major advantage of the MRSw detection is the low signal to noise ratio while making measurements of target concentration in sample. The signal obtained is based on changes in the magnetic behavior of MNPs and the surrounding water protons, in the presence or absence of the target. Since biological samples do not have any inherent magnetic activity, the signal is purely based on the magnetic label’s interaction with the target. However, the following challenges prevented the adoption of this technique for further studies in this work:
1. The need to use a sophisticated instrument such as a relaxometer/low frequency NMR makes this method expensive and inaccessible for field applications. In addition to this, data analysis will also be required using an appropriate software before the results could be interpreted, which adds to the time.

2. The synthesis and stabilization of the MNPs was hard to achieve in practice due to their strong magnetic properties. The size of the nanoparticles plays a crucial role in the success of the MRSw assay. But the short stability period of the nanoparticles made it difficult to move forward with those tests. Even if the nanoparticles were successfully synthesized and functionalized with the biological markers, they did not remain stable for too long. This could pose problems for the development of a reliable diagnostic test.

The following sections discuss the experimental methods and preliminary results obtained during the period of this study.

6.1.1 Experimental section

6.1.1.1 Materials

Manganese chloride tetrahydrate (MnCl$_2$·4H$_2$O) was obtained from Chem Impex International Inc. (Wood Dale, IL). Ferric chloride hexahydrate (FeCl$_3$·6H$_2$O) and ethanolamine were purchased from Sigma Aldrich (St. Louis, MO). Anhydrous sodium acetate and ethylene glycol were obtained from Thermo Fisher Scientific (USA) and 1, 6-hexanediamine was purchased from Spectrum Chemicals Mfg. Corp (New Brunswick, NJ). Premixed PBS buffer 10X was obtained from Roche, deuterium oxide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS)
were purchased from TCI America (USA). *Escherichia coli* (BSL 1) was obtained from ATCC (USA), anti-*E. coli* goat polyclonal antibody was obtained from Fitzgerald Industries International Inc. (Acton, USA) and Pierce™ Coomassie (Bradford) protein assay kit was purchased from Avantor (Radnor Township, PA).

### 6.1.1.2 Synthesis of magnetic nanoparticles (MNPs)

All glassware was first cleaned using Nochromix solution followed by Aqua Regia based on standard laboratory practice before nanoparticle synthesis. Two types of magnetic nanoparticles were synthesized and functionalized with amine (NH$_2$) groups using a one pot solvothermal method for comparison. Manganese ferrite nanoparticles (MnFe$_2$O$_4$) were synthesized using a modified protocol published in literature [152]. Initially, MnCl$_2$$\cdot$4H$_2$O and FeCl$_3$$\cdot$6H$_2$O were added to 30 mL ethylene glycol in 1:2 ratio. To this 0.5 g of anhydrous sodium acetate was added and mixed thoroughly for 20 min at 80°C until the solution turned clear. This was followed by the addition of 15 mL ethanolamine to achieve one pot functionalization of the nanoparticles with amine groups. Following homogenization using a magnetic stirrer, the solution was transferred to a teflon-lined stainless steel autoclave and the mixture was heated at 200°C for 8 hours. The obtained NH$_2$-MnFe$_2$O$_4$ nanoparticles were then washed with water and ethanol three times each and left to dry overnight in oven at 100°C. Similarly, magnetite nanoparticles (Fe$_3$O$_4$) were synthesized using a protocol from literature [153, 154]. In this case, 1 g of FeCl$_3$$\cdot$6H$_2$O was added to 30 mL of ethylene glycol along with 2 g of anhydrous sodium acetate and 7 g of 1,6-hexanediame for amine functionalization. The solution was vigorously mixed at 50°C until the solution became clear. This was then
transferred to a teflon-lined stainless steel autoclave and heated at 200°C for 6 hours. The obtained NH$_2$-Fe$_3$O$_4$ nanoparticles were washed thrice with water and ethanol, respectively and dried overnight at 100°C.

6.1.1.3 Characterization of magnetic nanoparticles

The obtained nanoparticles were characterized using Thermo Scientific Nicolet iS50 FT-IR spectrometer to confirm functionalization of the nanoparticles with amine groups. Microtrac NanoFlex dynamic light scattering (DLS) particle analyzer and transmission electron microscope (TEM, FEI Tecnai T12) were used to check the size of the nanoparticles and Stabino zeta potential analyzer was used to determine the distribution of the nanoparticles.

6.1.1.4 Conjugation of NH$_2$-Fe$_3$O$_4$ nanoparticles with anti E. coli antibodies

Based on the results from the characterization studies, NH$_2$-Fe$_3$O$_4$ nanoparticles were chosen for further studies involving E. coli detection. For this purpose, the chosen nanoparticles were bio-conjugated based on a standard protocol involving EDC and NHS [153]. Briefly, 3 mg of the dried nanoparticles were sonicated in 6 ml of 10 mM PBS (pH 7.4). Then 250 µL of anti-E. coli goat polyclonal antibodies at a concentration of 4 mg/mL, 2.3 mg EDC and 5.2 mg NHS were added to the nanoparticle solution and incubated at 37°C for 24 h with gentle shaking. Following this the antibody-NH$_2$-Fe$_3$O$_4$ nanoparticles were washed twice with PBS buffer using an external magnet, resuspended in 3 mL PBS and stored at 4°C.

6.1.1.5 Bradford assay
To confirm conjugation of the antibodies to NH₂-Fe₃O₄ nanoparticles, Bradford assay was conducted, which is generally used to determine the concentration of proteins in a sample. The assay is based on the color change observed when Coomassie blue dye interacts with large proteins, leading to a shift in absorbance from 465 nm to 595 nm using UV-visible spectrophotometer. A standard curve was first generated based on the instructions provided with the assay kit at varying concentrations of a known protein, bovine serum albumin (BSA), [155] using Shimadzu 1800 UV-visible spectrophotometer. Applying this curve, the concentration of antibodies on the magnetic nanoparticles were determined before and after the conjugation step.

6.1.1.6 Preliminary experiments with T₂ NMR: MRSw assay

This experiment was done for the purpose of learning and did not include triplicate samples. However, measurements for every sample were done in triplicates. The proton relaxation measurements were done using a low field PCT-20/30 NMR Analyzer (20 MHz, Process Control Technology Corporation). E. coli was cultured in tryptic soy broth (TSB) at 37°C for 4 hours and diluted 10 times using PBS for this study. The sample was prepared by adding 1 mL of antibody conjugated NH₂-Fe₃O₄ to 9 mL of E. coli in PBS. The following controls were used for the study – 1) 1 mL antibody-NH₂-Fe₃O₄ and 9 mL PBS, 2) 10 mL PBS, and 3) 10 times diluted E. coli culture in PBS. The T₂ relaxation rates were measured using the NMR instrument following a standard protocol. First, the T₂* value was measured using the free induction decay (FID) process. Following this measurement the T₂ relaxation values were obtained using the Carr-
Purcell-Meiboom-Gill (CPMG) process. Both measurements were done one after the other for the sample and the controls, respectively.

6.1.2 Summary of preliminary results

6.1.2.1 Characterization results of NH$_2$-MnFe$_2$O$_4$ nanoparticles

![Image](image.png)

**Figure 30.** (a) The synthesized magnetic nanoparticles attracted to a magnet; (b) TEM image of NH$_2$-MnFe$_2$O$_4$ nanoparticles.

The synthesized NH$_2$-MnFe$_2$O$_4$ nanoparticles showed superparamagnetic properties, where they behave like a magnet in the presence of a magnetic field. The particles were attracted to a magnet when brought in contact, confirming their usability for NMR studies. The TEM image of the nanoparticles in **Figure 30** indicates their hydrodynamic diameter to be around 200 nm. As can be seen in the image, several smaller nanoparticles seem to come together to form a larger nanoparticle due to Van der Waal’s interaction.
The particle size distribution for the synthesized nanoparticles was determined using dynamic light scattering method. Based on the results (Figure 31), the average size of the particles was found to be around 245 nm.

To confirm the presence of the amine groups on the manganese ferrite nanoparticles, FTIR was done. MnFe$_2$O$_4$ nanoparticles were synthesized using the solvothermal method described above without the addition of ethanolamine to avoid functionalization. These particles were collected similar to the NH$_2$-MnFe$_2$O$_4$ nanoparticles and used for FTIR. The spectrum obtained from both the samples (Figure 32) showed characteristic peaks for amine functionalities.
showed a metal-O vibration at around 556 cm\(^{-1}\) coming from the formation of manganese and iron oxides. The N-H stretching vibration at 1680 cm\(^{-1}\) is prominently visible in the amine functionalized nanoparticles indicating the presence of NH\(_2\) groups on the nanoparticle surface [156]. The C-O and OH stretching vibrations at around 1071 cm\(^{-1}\) and 3293 cm\(^{-1}\), respectively, are visible for both particles but seems to be larger for the amine functionalized nanoparticles, indicating a difference between functionalized and non-functionalized nanoparticles. These stretching vibrations could be due to the presence of residual ethylene glycol used for the preparation of the nanoparticles [157].

![Zeta Potential for MnFe\(_2\)O\(_4\) (solvothermal; amine functionalized)](image)

**Figure 33.** Results from zeta potential analyzer for NH\(_2\)-MnFe\(_2\)O\(_4\).

The zeta potential indicates the potential stability of a colloidal solution. Given that the prepared magnetic nanoparticles dispersed in water are colloidal in nature, this tool was used to determine their surface properties and predict long-term stability. The zeta potential value was determined to be around -13 mV for NH\(_2\)-MnFe\(_2\)O\(_4\) nanoparticles. Generally, a zeta potential value below -30 mV (strongly anionic) or above +30 mV (strongly cationic) is ideal due to the presence of strong repulsive forces between
the particles. Any value between -30 mV and +30 mV could mean that the particles in solution cannot remain a colloidal suspension and will aggregate due to Van der Waal’s forces [158].

Based on the size distribution and zeta potential values, the NH$_2$-MnFe$_2$O$_4$ nanoparticles were not considered for studies involving the conjugation of antibodies and T$_2$ relaxation rate determination.

6.1.2.2 Characterization results of NH$_2$-Fe$_3$O$_4$ nanoparticles

Figure 34. TEM image of the synthesized NH$_2$-Fe$_3$O$_4$ nanoparticles.

Based on the TEM image (Figure 34), the size of the NH$_2$-Fe$_3$O$_4$ nanoparticles was determined to be around 50 nm. No visible aggregation of the particles was noticed indicating better stability compared to the NH$_2$-MnFe$_2$O$_4$ nanoparticles. The smaller size of the nanoparticles is also ideal for the T$_2$ relaxation experiments.
To confirm the presence of the amine groups on the synthesized magnetite nanoparticles, FTIR was done (Figure 35). Fe₃O₄ nanoparticles were synthesized using the solvothermal method described above without the addition of 1,6-hexanediamine to avoid functionalization. These particles were collected similar to the NH₂-Fe₃O₄ nanoparticles and used for FTIR. The spectrum obtained from both the samples showed Fe-O vibration at around 575 cm⁻¹ coming from the formation of manganese and iron oxides [154]. However, most of the distinguishing peaks in amine functionalized magnetite nanoparticles are missing and seem to be similar to the non-functionalized particles. There seem to be some undefined differences between the two spectra as indicated by the arrows in Figure 35. However, these results are inconclusive to determine the presence of amine groups and may require repetition. Nevertheless, further experiments were conducted with the synthesized nanoparticles due to their ideal size.
The zeta potential value for the NH$_2$-Fe$_3$O$_4$ nanoparticles was found to be around -21 mV which is lower than that of NH$_2$-MnFe$_2$O$_4$. However, this value also lies between the range of -30 mV to +30 mV and can aggregate over time due to Van der Waal’s interactions.

6.1.2.3 Bradford assay results

The Bradford assay was conducted to confirm the conjugation of antibodies to the NH$_2$-Fe$_3$O$_4$ nanoparticles. Figure 37 shows the standard curve generated to conduct the assay. Based on the absorbance values obtained for free antibodies present in the antibody-MNP conjugation solution before and after conjugation, the concentration of antibodies on NH$_2$-Fe$_3$O$_4$ was calculated. The free antibody concentration before conjugation was 41.18 µg/mL and after conjugation was 31.55 µg/mL indicating that some of the antibodies have been conjugated to the nanoparticle surface.
The conjugation occurs through carbodiimide chemistry as shown in Figure 38 where the COOH group on the antibody interacts with EDC and then NHS to form intermediates, that then crosslinks with the NH₂ groups on the nanoparticle surface [159].

Figure 37. Standard curve for Bradford assay.

Figure 38. Carbodiimide chemistry steps [159].

6.1.2.4 MRSw assay results

Table 4 shows the results from the T₂ relaxation measurements. The T₂* value is the observed value that can occur due to inhomogeneity in the magnetic field while applied initially. The T₂ value explains the natural T₂ relaxation behavior of the water.
molecules in the solution, which is of interest in the MRSw assay. The T2 values correspond to water molecules that are bound to the sample. As can be seen from Table 4, the sample and each control has varying T2 values. The T2 trend for PBS, and E. coli in PBS are similar, indicating that the presence of the bacteria itself shows little change in the T2 relaxation rates for PBS. This is potentially due to the low frequency of the NMR system which prevents it from picking up signals from bacteria. The T2 values of control with only antibody-NH2-Fe3O4 nanoparticles in the solvent, is higher than the other two controls, showing that their presence have changed the relaxation rate of the water molecules in PBS. This T2 value increased much higher when E. coli was included. This could potentially be due to the interaction of the antibodies on the nanoparticles with E. coli. Since the antibodies have affinity to the bacteria, they could have bound together to change the spatial positions of the magnetic nanoparticles. This in turn changed their behavior with respect to the water molecules around them and could have caused the big change in the T2 value as seen in Table 4. However, further tests with other bacterial species may be required to check the specificity of this assay.

Table 4. The T2 relaxation measurements using NMR

<table>
<thead>
<tr>
<th>Samples</th>
<th>T2* (ms)</th>
<th>T2 (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli + MNP + PBS</td>
<td>794.96</td>
<td>12906.7</td>
</tr>
<tr>
<td>PBS</td>
<td>507.75</td>
<td>2813.97</td>
</tr>
<tr>
<td>E. coli + PBS</td>
<td>324.29</td>
<td>2567.96</td>
</tr>
<tr>
<td>MNP + PBS</td>
<td>327</td>
<td>4732.27</td>
</tr>
</tbody>
</table>

In conclusion, though some of the preliminary results in this work showed promise, the challenges described earlier prevented further confirmatory experiments.

6.2 Nanoaggregation-enhanced chemiluminescence (NEC) assay using AuNPs
The NEC assay depends on the application of gold nanoparticles (AuNPs) conjugated with pathogen-specific biomolecules on its surface. In the presence of the target in the sample, the recognition molecules on the AuNPs bind to the target biomolecules and cause the nanoparticles to aggregate. This aggregated form of AuNPs can catalyze a chemiluminescence reaction involving luminol and hydrogen peroxide (Figure 39), which will be detected by a luminometer.

**Figure 39.** Chemiluminescence reaction catalyzed by metal particles in utilizing luminol to produce light [160].

The intensity of this signal is expected to be directly proportional to the concentration of the target in the sample. For samples without the target/pathogen-specific biomolecule, there will not be any signal.

This work was built on a previous study done in the lab [113]. The objective of this work was to optimize the parameters of the NEC assay to make it applicable for real samples such as infected oak wood.
Figure 40. NEC assay working principle: The DNA probes 1 and 2 are in grey and the target DNA is in red. The orange spheres represent AuNPs [113].

Although this assay takes less than 10 min to conduct, there were some significant limitations that rendered this technique not feasible for field testing. The chemiluminescence signal arising from the catalysis of luminol lasts only for a few seconds with the peak signal occurring rapidly. If not measured at the right time this intensity would be lost and can lead to false results. Additionally, different users may handle the samples differently and the reliance on a few seconds of luminescence intensity seems too short of a time for accurate readings. Although theoretically the clustering of the AuNPs due to the presence of the target is supposed to catalyze the luminescence process, the assay did not work as expected for most samples. Therefore, the mechanism of luminescence may have to be investigated further, especially with respect to potential interferences in the sample. A third limitation of the assay is its
reliance on luminol, which is very sensitive to light and if not contained well, can cause issues with the results. This may bring additional challenges for field applications.

The challenges observed while exploring the MRSw and chemiluminescence assays for rapid microbial diagnostics drove this research towards the application and development of the rapid LAMP assay in this dissertation.
Chapter 7. Highly efficient and durable antimicrobial nanocomposite textiles

This chapter discusses a side project that focused on the application of zinc oxide nanocomposite textiles for combating infectious diseases. Unlike previous studies that used nanomaterials for developing rapid onsite microbial detection assays, this study involves investigating antimicrobial applications against human bacterial and fungal pathogens.

7.1 Introduction

Healthcare-associated infections (HAIs) are of major public health concern with at least one in 31 patients getting infected during or after receiving treatment in hospitals in the United States [161]. Textiles in healthcare facilities (curtains, bedding, workers’ clothing, carpets, patient gowns, towels, furniture) are known to harbor microorganisms and facilitate the spread of HAIs including SARS-CoV-2 [162, 163]. Periodic cleaning and applying disinfectants to all textiles in the hospital environment is not sufficient to prevent transmission. Even with regular cleaning, healthcare workers’ clothing was found to have significant amount of microbial load after a typical work schedule of 8-12 hours, and about 92% of hospital curtains contained pathogens within a week after cleaning [164].

The magnitude of this problem has further increased since the COVID-19 pandemic that kept healthcare facilities at maximum capacity and prone to the spread of more infections. Among the common HAIs seen in hospitals in the United States, central line-associated bloodstream infection (CLABSI), catheter-associated urinary tract
infections (CAUTI) and ventilator-associated pneumonia increased by 47%, 19%, and 45%, respectively, in the year 2020. Apart from these, the Centers for Disease Control and Prevention (CDC) also reported that infections associated with methicillin-resistant *Staphylococcus aureus* (MRSA) increased in the year 2020, with the onset of the COVID-19 pandemic [165].

In the United States, transmission of SARS-CoV-2 has been predominant in hospitals, nursing homes and medical facilities resulting in over 1037,400 healthcare workers contracting COVID-19 in the US as of March 18, 2022 [166, 167]. While the focus of healthcare workers has shifted to combating this problem, some of the leading causes of nursing home deaths have taken a backseat, such as respiratory infections, urinary tract infections (UTIs), gastroenteritis, sepsis, and skin diseases, which involve multidrug-resistant bacterial and fungal pathogens [168, 169]. This makes these facilities “hotspots” for such infections resulting in an urgent need for self-disinfecting antiviral/antimicrobial textiles that curb transmission where ideally low capital investment is required.

The use of nanotechnology and nanomaterials is one of the most promising approaches for the development of the next generation of functional textiles [170, 171]. The importance of nanomaterials, particularly nanoparticles, lies in their ability to confer multiple functionalities with remarkable enhancement of these functionalities due to increased surface-to-volume ratio and high surface energy [172]. Silver nanoparticles in particular have seen growing interest from the textile industry due to their wide-spectrum antimicrobial properties. Between 2004 and 2011, the silver market share of
antimicrobial textiles increased from 9% to 25%, progressively replacing synthetic organic compounds [173]. Several methods have been developed to incorporate metal and metal-oxide nanoparticles into textiles [174]. The most common approaches include spraying of a nanoparticle solution [175], layer-by-layer deposition with polymers or polyelectrolytes [176], sonochemical coating [177], plasma deposition [178], and electrospinning [179].

Current methods of nanoparticle functionalization have significant limitations in terms of long-term durability [180]. Nanoparticle surface coatings are subject to leaching as nanoparticles are released from the fiber material. This is particularly pertinent in textiles and clothing that are subject to mechanical strain, abrasion as well as repeated wash and dry cycles. Silver nanoparticle emission from textiles varies among products and can be as high as 80% during the first wash [180, 181]. Furthermore, silver and other nanoparticles utilized in textiles, such as copper, have demonstrated toxicity in environmental systems [182-184]. These antimicrobial nanoparticles can also disrupt biological treatment processes used in municipal and industrial water treatment facilities [185].

The work presented here focuses on the use of a novel process called Crescoating, meaning coating by growth [-cresco], for the introduction of nanoparticles into textile to form stable nanocomposites and the demonstration of the antimicrobial effect of zinc nanocomposite textiles. Typically, nanocomposites are described as at least two-phased materials with one of the components being in the nanoscale. This could either be a metal or a non-metal nanoparticle phase embedded in a macroscale support such as the fabrics
in this case. In the Crescoating treatment process nanoparticles are grown directly on and within the bulk of polymeric materials. This bulk growth enables improved nanoparticle retention, preventing both textile performance losses and nanoparticle emissions into water systems.

Zinc has been selected due to its numerous advantages over nanoparticles currently used in textiles. Besides its antimicrobial properties it is Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA), does not exhibit environmental or human toxicity, and is commonly used in commercial products ranging from food to cosmetics [186]. The demonstration of the antimicrobial properties was performed by testing different textiles against different microorganisms including Gram-negative, Gram-positive bacteria, and fungi.

7.2 Experimental section

7.2.1 Nanocomposite synthesis

Zinc acetate dihydrate (Sigma Aldrich, USA) was used as a precursor. Textile fabric supports used were polyester cotton, nylon cotton (50/50 nylon cotton camouflage ripstop fabric) (Rockywoods Fabrics LLC), silk adjacent fabric, 60 g/m² (ISO 105-F06, Testfabrics Inc.), and synthetic polyester adjacent fabric, 130 g/m² (ISO 105-F04, Testfabrics Inc.). For synthesis on zinc nanocomposite textiles, solutions of 0.25M zinc acetate were prepared in filtered deionized water with ~18 megohm cm⁻¹ conductivity (SpectraPure, USA). In a typical synthesis process, fabrics were submerged in the solutions for 30 min at room temperature. Next, the fabrics were heated at 100°C in a convection oven (Model FDL 115, BINDER GmbH, Tuttingen, Germany) for 4 hours.
For wash cycle testing, the concentration of zinc acetate was increased to 0.5M for the ones tested in lab and decreased to 0.2M for the ones sent to Vartest Laboratories LLC.

7.2.2 Bacterial culture

The bacterial strains used for testing were the Gram-positive *Staphylococcus aureus subsp. aureus* (ATCC 6538) and methicillin resistant *Staphylococcus aureus*, the Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and the fungus *Candida albicans*. All organisms were cultured in tryptic soy broth (TSB) followed by serial dilution to reach a final concentration of $10^5$ CFU/ml for fabric inoculation. The concentration was confirmed by plate counting method prepared in triplicates. The Gram-positive *Staphylococcus aureus subsp. aureus* (ATCC 6538) and the Gram-negative *Klebsiella pneumoniae* (ATCC 4352) were used for third party tests.

7.2.3 Quantitative antimicrobial tests

Antimicrobial performance was quantified using a modified form of the plate counting method outlined in AATCC Test Method 100-2004. The materials tested for antimicrobial activity were zinc nanocomposite textiles, which include silk, nylon cotton, and polyester (PE) fabrics. The tests were conducted on swatches that have been machine washed once after fabrication, considered as “before washing” samples and those that were subjected to machine washing and drying cycles considered as “after washing” samples. The controls were untreated fabrics. One swatch of untreated fabric and one swatch of treated fabric were each placed in separate 60 mm x 15 mm petri dishes. The swatches were uniformly cut to the measurement of 4 X 2.5 cm. The untreated and treated fabrics were then inoculated with a concentration of $10^5$ CFU/ml of each
microbial strain as prepared previously. The purpose of this inoculation is to completely soak the swatch of fabric with the culture. The petri dishes were then sealed with parafilm and incubated for 24 hours at 37°C. For 0-hour immediate elution tests, samples were prepared similarly and immediately transferred to tubes containing 5 mL of Hank’s Balanced Salt Solution (HBSS buffer). The microbial culture was then completely eluted from the swatches via thorough vortexing. Following this, the swatches were removed from the HBSS buffer solution. The 24-hour incubated samples were similarly eluted. One tube of 5 mL of HBSS solution was directly inoculated with the microbial culture and served as a control. All the eluted samples were serially diluted using HBSS, thrice for immediate elution samples and five times for 24-hour elution samples and plated on tryptic soy agar (TSA) followed by incubation at 37°C for 18-24 hours.

The number of microbial colonies were counted after incubation, and the reduction percentage was calculated as described previously.

7.2.4 Textile laundering

For further laundering tests, nanocomposite polyester cotton textiles were subjected to washing cycles per AATCC LP1: Home Laundering method in a machine washer (Vortex M6, SDL Atlas) followed by drying cycles in a tumble dryer (Vortex M6D, SDL Atlas). This method involves a 16-minute wash cycle with warm water at high agitation followed by rinsing for 2 min 30 seconds, spinning for 5 min and finally high heat tumble drying cycles. The total load weight of the fabrics was 1.8 ± 0.1 kg and the water level was 72 ± 4 L. AATCC High Efficiency Liquid Standard Reference
detergent was used for all washing steps. The samples were subjected to 50 and 100 wash
cycles to test their durability and longevity.

7.2.5 Statistical analysis

The antimicrobial tests were conducted in triplicates for both 0-hour and 24-hour
contact periods. Geometric means and standard deviations were calculated and used for
statistical analysis. One-way ANOVA was used to do the analysis of variance and the
significant differences in the means were tested at significance = 0.05.

7.3 Results and discussion

7.3.1 Textile zinc nanocomposite synthesis

The nanocomposite textiles were fabricated by soaking the textiles in an aqueous
ionic precursor solution, which is thermally treated to form nanoparticles in the bulk and
throughout the surface of the fabrics. The technology used here is called Crescoating
(Figure 41) and begins with solid seed formation from the ionic solution under heating
followed by their nucleation to form defined nanoparticles. The thermal treatment was
done using a convection oven which leads to the evaporation of water followed by seed
formation and nucleation of the particles. The size of the particles could range from 5-
500 nm, depending on the process conditions and they get embedded in the textile
supports. This method improves the durability and longevity of the nanocomposite
textiles, unlike conventional surface-coated products, which lose their nanoparticles over
time due to multiple washing cycles.
**Figure 41.** Comparison of (a) conventional dip-coating process with (B) thermal Crescoating technology. A) Wet synthesis of nanoparticles by chemical reduction (1), dip-coating of the textile in the nanoparticle (2), followed by washing and drying (3). (b) Impregnation of the textile in precursor solution (1), Thermal reduction by heating the textile at 100°C (2), followed by washing and drying (3).

This method’s efficacy was previously demonstrated for environmental remediation using polyurethane foam, and polypropylene, polycotton and nylon cotton fabrics for their antiviral properties [187-190]. **Figure 42** shows SEM images of nanocomposite polyurethane, nylon and polyester materials.
Figure 42. SEM images of plastic nanocomposites produced with “in situ growth” process.
(a) Zinc-polyurethane nanocomposite film. The blue arrows show two pieces of nanocomposite thin film. Image amplification at the film cross-section shows the presence of zinc nanoparticles inside the film. (b) Zinc-nylon nanocomposite showing zinc nanoparticles embedded with the nylon fibers. (c) Silver-polyester/cotton nanocomposite.

To demonstrate the versatility of the Crescoating technology the process has been adapted to various fabrics using different metal nanoparticles as well (Figure 43).
Figure 43. Versatility of the in situ nanoparticle growth process using different nanoparticles on different textiles. nSe: nanoselenium, nb: nanoboron, nCe: nanocerium, nFe: nanoiron, nAg, nanosilver.

In this study the fabric supports used were silk, synthetic polyester, nylon cotton and polyester cotton. Natural, synthetic and blended textiles were selected to analyze how well the antimicrobial treatment would work on different types of fabrics. Specifically, the above chosen set of fabrics are commonly used in the consumer apparel and textile industry and incorporating antimicrobial activity in them would be beneficial. The synthesis of zinc nanocomposite textiles was based on a previously developed method [190]. Briefly, the fabrics were initially submerged in precursor ionic solutions of zinc salt for 30 min at room temperature. They were then heated in a convection oven at 100°C for 4 hours by maintaining a thin layer of the solution over them. White precipitates were formed on the zinc supports. Any unbound precipitate was washed
away by following the American Association of Textile Chemists and Colorists (AATCC) LP1: Home Laundering method and dried [191].

7.3.1.1 Characterization

7.3.1.1.1 Structure and characterization

The synthesized nanocomposites were characterized using scanning electron microscopy (6700 SEM, JEOL Inc.). Figure 44 depicts zinc nanocomposites grown in polyester fabrics. Very small zinc nanoparticles in a size range of ≤100 nm can be seen on the textile support. Nanoparticle coverage on the support is not uniform nor widespread. SEM images of untreated control fabrics is shown in Figure 45. Unbound nanoparticles collected through hand washing the nanocomposite fabrics immediately after synthesis were analyzed using x-ray crystallography (D8 Discover, Bruker Corp.) in our previous study [190]. Pattern fitting was conducted with JADE for XRD software (Materials Data Inc.). Zinc particles were primarily comprised of two crystalline phases of zinc hyroxide (Zn(OH)$_2$) and zinc carbonate hydroxide (Zn$_5$(CO$_3$)$_2$(OH)$_6$ hydrozincite).

![Figure 44](image.png)

**Figure 44.** SEM images of nanoparticles harvested from zinc treated polyester fabric.

The formation of these nanoparticles follows a three step process similar to the formation of iron oxide nanoparticles previously studied [188]. Thermal synthesis of
supported nanoparticles is achieved through the formation of solid phase seeds that nucleate to form nanoparticles. For zinc, the salt precursors undergo hydrolysis to form hydroxide intermediates in the form of Zn(OH)₂. In the presence of carbonate contributed from the atmosphere and/or from the degradation of the acetate counterion, zinc carbonate hydroxide particles form. The hydrozincite and zinc hydroxide phases can be seen in x-ray diffraction patterns [190, 192].

![SEM images of untreated polyester control fabric](image)

**Figure 45.** SEM images of untreated polyester control fabric

### 7.3.1.1.2 Synthetic precipitate leachate procedure

The retention of zinc nanoparticles in the textiles after several wash cycles was tested by a third party testing company, Pace Analytical LLC. Samples of several types of nanocomposite cotton fabrics were subjected to the synthetic precipitation leachate procedure (SPLP). These fabrics were previously washed once post manufacturing to remove loose nanoparticles from their surface. The test based on the EPA 3010A preparation and EPA 6010B analytical method was then applied to study the leaching of zinc nanoparticles from the fabrics [193]. The results from the study showed that the highest zinc leaching of 106,000 µg/L occurs after the first post-fabrication wash for one
of the cotton types. All other values are lower and are followed by significantly lower leaching after every subsequent wash cycle (Table 5). This shows that the nanocomposite fabrics prepared using the novel Crescoating method are highly durable. The highest initial leachate concentration is found to be much lower than 250,000 µg/L, which is the soluble threshold limit concentration (STLC) value for California state [194]. Based on this limit it can be concluded that the concentration of zinc leaching from the fabrics after each laundering cycle is not significant enough to pose a health or environmental hazard. The different values of leaching observed for the three different cotton types could be a result of differences in their fiber sizes and pretreatment processes such as mercerization (oxidization of fibers for metal easier metal binding), which was done for cotton types 1 and 2.

Table 5. Synthetic Precipitate Leachate Procedure results from Pace Analytical for three types of cotton samples. The samples were washed 1, 50 and 100 times after functionalization.

<table>
<thead>
<tr>
<th></th>
<th>Zinc Discharge (µg/L)</th>
<th># of Washes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Cot #1</td>
<td>5030</td>
<td>5610</td>
</tr>
<tr>
<td>Cot #1</td>
<td>543</td>
<td>575</td>
</tr>
<tr>
<td>Cot #1</td>
<td>1150</td>
<td>848</td>
</tr>
<tr>
<td>Cot #2</td>
<td>12800</td>
<td>11100</td>
</tr>
<tr>
<td>Cot #2</td>
<td>519</td>
<td>371</td>
</tr>
<tr>
<td>Cot #2</td>
<td>571</td>
<td>558</td>
</tr>
<tr>
<td>Cot #3</td>
<td>57100</td>
<td>106000</td>
</tr>
<tr>
<td>Cot #3</td>
<td>1500</td>
<td>1180</td>
</tr>
<tr>
<td>Cot #3</td>
<td>1180</td>
<td>1140</td>
</tr>
</tbody>
</table>

7.3.2 Antimicrobial application test

For antimicrobial testing, zinc nanoparticles were grown on polyester, silk and nylon/cotton (50:50) textile swatches obtained from Testfabrics Inc. and Rockywoods Fabrics LLC, respectively. AATCC Test Method 100-2004 was used for antibacterial and antifungal testing of functionalized fabrics. The experiment was done in triplicates. Two
bacterial species *Pseudomonas aeruginosa* (PA, ATCC 27853) (Gram-negative) and methicillin resistant *Staphylococcus aureus* (MRSA) (Gram-positive) and a fungal species *Candida albicans* (CA) were selected for antimicrobial testing. The results have been separated into two categories: a “before washing” test conducted on swatches that have been machine washed soon after treatment and an “after washing” test with swatches that were subjected to more machine washing and drying cycles. The “after washing” tests were conducted by a third-party testing company. The fabric used was polyester cotton blend and the tests were conducted on the bacterial species *Staphylococcus aureus* (SA, ATCC 6538) (Gram-positive) and *Klebsiella pneumoniae* (KP, ATCC 4352) (Gram-negative). This was done to check the antimicrobial efficacy of different zinc nanocomposite fabrics against various pathogens under different conditions.

### 7.3.2.1 Before-wash antimicrobial test

Briefly, the fabric samples were inoculated with suspensions of bacteria in nutrient broth and rinsed. These samples were divided into two sets with different elution times, a 0-hour immediate elution and an elution after a 24-hour incubation period. The eluted solution from the inoculated fabrics was then plated and incubated for 24 hours at 37°C. Bacterial growth was quantified through colony counting on plates as pictured in Figure 46. The results are reported in % reduction and calculated by the following formula (*Equation 3*),

\[
100 \times \frac{(B-A)}{B} = \% \text{ Reduction}
\]

where \(A\) is the number of bacterial colonies recovered from the inoculated treated sample
fabric and \( B \) is the number of bacterial colonies recovered from the inoculated untreated control fabric, both incubated over a set contact period. For all experiments, Equation 4 was used to determine test efficacy. This calculation is a qualitative check to confirm that the initial concentration of bacteria used was enough to do the antimicrobial test. This number must be higher than 1.5.

\[
\log(b) - \log(a) > 1.5
\]  

(4)

where \( a \) is the number of bacterial colonies recovered from untreated control fabric immediately after inoculation and \( b \) is the number of bacterial colonies recovered from untreated control fabric after 24-hour incubation post inoculation. For all reported experiments, these efficacy values ranged from 1.5-3.5, confirming the effective bacterial concentration.

The above procedure was followed similarly for tests against the fungal pathogen Candida albicans. Although AATCC recommended antifungal tests for textiles differ from that of bacteria, the growth cycle of Candida albicans resembles that of bacteria, which enables antifungal testing using this modified version of the AATCC Test Method 100-2004 [195].

The 0-hour data, shown in Table 6, for each textile showed varied antibacterial properties for Pseudomonas aeruginosa (PA), methicillin resistant Staphylococcus aureus (MRSA) and Candida albicans (CA). This could be due to the quick elution of microbial inoculations from the fabrics, leaving much less time for the nanoparticles to interact with the pathogenic cells and reduce their growth consistently. Despite this, the microbial reduction percentage for almost all samples ranged from 32-91% indicating that the
nanocomposite fabrics can still induce some antimicrobial properties. For nanocomposite polyester treated with MRSA, negative value was observed and indicate that there were more bacteria recovered than control. This could be possible because of variable absorption properties of each fabric or might be because of improper mixing of bacterial concentration. “NA” is used for either negative value or indicates contamination. The 24-hour elution results on the other hand showed significant microbial reduction in samples treated with zinc nanocomposite textiles compared to untreated controls (ANOVA, \( p < 0.05 \)). The microbial reduction percentages were far less variable and ranged from 98 to \( >99.999\% \) (\( >1 \) log10 to \( >5 \) log10) reduction. The percentages obtained are higher than or similar to those reported in the literature for antimicrobial cotton fabrics against Gram-positive \( S. \) aureus and Gram-negative bacteria [196, 197].

**Table 6.** Microbial reduction (%) of *Pseudomonas aeruginosa* (PA), methicillin resistant *Staphylococcus aureus* (MRSA) and *Candida albicans* (CA) after 0 hours of incubation (immediate elution) and after 24 hours with zinc nanocomposite textiles (The zinc nanocomposite textiles were washed once as part of the manufacturing process to remove loose particles; microbial reduction in terms of log reductions is provided for some of the samples in brackets).

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Reduction after 0 hours</th>
<th>% Reduction after 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PA</td>
<td>MRSA</td>
</tr>
<tr>
<td>Nylon cotton</td>
<td>72.5</td>
<td>47.85</td>
</tr>
<tr>
<td>Silk</td>
<td>32.5</td>
<td>90.39 (&gt;1 log10)</td>
</tr>
<tr>
<td>Polyester</td>
<td>75</td>
<td>NA</td>
</tr>
</tbody>
</table>

Though the antimicrobial mechanism of zinc nanoparticles is not clearly studied, reports from previous studies have suggested that their photocatalytic properties generate hydrogen peroxide (H\(_2\)O\(_2\)) through a series of reactions in the presence of H\(_2\)O. These hydrogen peroxide molecules are toxic to the microbial cells as they can penetrate the cell
membrane and kill them [198]. There have been fewer studies on the antimicrobial efficacy of these textiles on fungal pathogens. However, the results obtained here indicate that these novel nanocomposite textiles can also be used for antifungal applications.

<table>
<thead>
<tr>
<th></th>
<th>Nylon cotton</th>
<th>Silk</th>
<th>Polyester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (Gram -)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Methicillin resistant Staphylococcus aureus (Gram +)</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 46.** Selected photos of cell culture plates used in antimicrobial testing via cell counting for samples eluted after 24 hours with $10^5 X$ dilution.

7.3.2.2 After-wash antibacterial test

7.3.2.2.1 Third-party wash durability analysis

Zinc nanocomposite polyester cotton was machine washed 50 times per AATCC LP1: Home Laundering method and the antibacterial properties were assessed separately by a third-party testing through Vartest Laboratories LLC. The bacterial reduction was found to be 0% for both *Klebsiella pneumoniae* (*KP*, Gram-negative) and *Staphylococcus aureus* (*SA*, Gram-positive) indicating that the nanocomposite textile did not exhibit antimicrobial properties during the 0-hour (immediate elution) process after 50 wash cycles (See Table 7). However, the same material exhibited antimicrobial properties even after 50 wash cycles with more than 99.999% (5 log10) bacterial reduction for both *Klebsiella pneumoniae* and *Staphylococcus aureus* after 24-hour incubation (See Table 7). While there have been some studies in the past on the application of zinc oxide
nanoparticles for the fabrication of antimicrobial textiles, most of these show much lower microbial reduction percentages than the desired 99.999% (5 log10) range for samples washed over 20 cycles [199, 200]. The zinc nanocomposite textiles prepared using the novel Crescoating technique on the other hand could retain their antimicrobial properties even after being reused over 50 times repeatedly. Preliminary tests on samples washed for 100 cycles (see next section) also show promising antibacterial activity. Therefore, these fabrics can be successfully applied to manufacture antimicrobial textiles in the healthcare industry.

Table 7. Microbial reduction (%) of *Klebsiella pneumoniae* (KP) and *Staphylococcus aureus* (SA) after 0 hours of incubation (immediate elution) and after 24 hours with zinc nanocomposite textiles subjected to 50 wash cycles. Results obtained from third party independent testing (Independent evaluation conducted by Vartest Laboratories LLC).

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Reduction after 0 hours</th>
<th>% Reduction after 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KP</td>
<td>SA</td>
</tr>
<tr>
<td>Polycotton</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

7.3.2.2.2 Laboratory wash durability analysis

Zinc nanocomposite cotton fabric was washed 100 times by following the AATCC LP1: Home Laundering method. These samples were then tested against the Gram-positive *Staphylococcus aureus* bacteria according to AATCC test method 100. The results from the 24-hour elution samples show >99.999% (>5 log10) reduction indicating that the nanocomposite fabrics can retain their antimicrobial behavior even after several washes. This means that the novel nanocomposite fabrics have better longevity and durability when compared to other antimicrobial textiles previously studied [199, 200].
7.3.3 Safety and skin irritation test

To determine whether the antimicrobial nanocomposite fabrics were safe to use as clothing, the Human Repeat Insult Patch Test (HRIPT) was conducted by Evalulabs LLC. The tests were conducted after obtaining informed consent from 50 human subjects and were carried out under the supervision of a licensed dermatologist. The protocol was approved by the Ethics Committee and the Evalulab LLC Independent Ethics Committee (IEC) prior to the tests to protect the rights of the human participants and the tests were performed in accordance with the relevant guidelines and regulations. The results indicated that the fabrics were non-irritating and hypoallergenic to human skin.

7.4 Conclusions

A novel Crescoating technology has been applied as a promising technique to synthesize highly efficient antimicrobial nanocomposite fabrics. Antimicrobial tests conducted on zinc nanocomposite textiles against Gram-positive and Gram-negative bacteria and fungi showed >99.999% (>5 log10) microbial reduction. The fabrics are also safe, highly durable and can be reused over 100 wash/dry cycles without loss of their functionality. Third party dermatological tests showed that the nanocomposite fabric materials are non-irritating and hypoallergenic to human skin. Therefore, these fabrics can be successfully used as medical textiles such as hospital linen and surgical gowns, which can aid in the fight against nosocomial infections and disease transmission in healthcare settings.
Chapter 8. Conclusions

The goal of this research is to develop a rapid onsite nucleic acid based diagnostic assay for the early detection of microbial pathogens with high sensitivity, specificity, and accuracy. For this purpose, LAMP assay was explored due to its ease of operation and the flexibility to optimize the detection process. For the first time LAMP primers were designed for the specific detection of the oak wilt fungus, *Bretziella fagacearum* in red oak, with 100% sensitivity and specificity. To overcome the challenges associated with the current methods used to detect LAMP products, a naked eye visualization method based on nanoparticle assembly was successfully developed. The novel method was validated using traditional techniques, fluorescence spectroscopy and gel electrophoresis.

**Fundamental Aspect:** The work described here is built upon the foundational knowledge on nanomaterial properties and nucleic acid synthesis. The diagnostic approach relies on the optical properties of functionalized gold nanoparticles (AuNPs). The self-assembly of these nanoparticles with the nucleic acids was used as a visual indicator of the LAMP amplicons in the sample. In addition, this study has enhanced our fundamental understanding of the behaviour of highly concentrated nucleic acids in solution and their behaviour with AuNPs.

**Impact and Prospects:** The LAMP assay combined with the novel visualization method potentially achieves the ASSURED standard set by WHO for diagnostic assays with its cost-effective and field-friendly model. It provides the flexibility to design the assay for any pathogen, expanding its application into clinical diagnostics and in low-
resource settings. Further field tests would benefit from end-users’ feedback for optimization and scale-up.
Bibliography


126


[168] How can nursing homes protect residents from infection? follow the research [Internet]. [cited 2024 Feb 28]. Available from: https://www.michiganmedicine.org/health-lab/how-can-nursing-homes-protect-residents-infection-follow-research


Appendix I: PhD outcomes and achievements

PATENTS


GRANTS

PI: Abdennour Abbas, Minnesota Invasive Terrestrial Plants and Pests Center, $466,000, 3 years (started January 2024), Portable diagnostic platform for early detection of plant pathogens. (Grant based on work described here)

PUBLICATIONS

• **Novi VT**, Aboubakr HA, Moore MJ, Juzwik J, Abbas A. A rapid LAMP assay for the diagnosis of oak wilt with the naked eye. Preprint: [https://doi.org/10.21203/rs.3.rs-3960787/v1](https://doi.org/10.21203/rs.3.rs-3960787/v1). Under Review. BMC Plant Methods.

PRESENTATIONS, POSTERS, TALKS

• Invited oral presentation at Virtual 2024 Invasive Species Forum, February 14, 2024
• Presented poster at Department of BBE Showcase, UMN-TC, Saint Paul, MN, October 26, 2023
• Oral presentation at American Chemical Society (ACS) Fall 2023 Meeting, Moscone Center, San Francisco, CA, August 13-17, 2023
• Participated in the Science in Seconds Competition by CFANS, October 17, 2022
• Presented poster at Department of BBE Showcase, UMN-TC, Saint Paul, MN, October 13, 2022
• Presented poster as coauthor at American Fisheries Society 151st Annual Meeting, Baltimore, Maryland, November 6-10, 2021
• Participated in the Science in Seconds Competition by CFANS, October 18, 2021
• Presented poster at 31st Anniversary World Congress on Biosensors, July 27, 2021
• Oral presentation at Upper Midwest Invasive Species Conference (UMISC), November 1, 2020
• Presented poster at Department of BBE Showcase, UMN-TC, Saint Paul, MN, October 23, 2019

HONORS AND AWARDS

• Awarded full fellowship offered by Schwan’s Company for the entire PhD program.
• Published article, "Naked-eye visualization of nucleic acid amplicons using hierarchical nanoassembly" selected for front cover in Analytical Methods, 28 September 2023, Issue 36, Page 4619 to 4788.
• Saint Paul Science in Seconds People’s Choice Award, UMN – TC, Saint Paul, MN, October 17, 2022.
• Invited and presented thesis in 3 minutes at the CFANS Dean’s Circle Forum student research presentations, January 27, 2022.