The Antibacterial Properties of Brookite Phase Titanium Dioxide Nanoparticles Against Methicillin-Resistant Staphylococcus aureus

Rupal Shah

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THE ANTIBACTERIAL PROPERTIES OF BROOKITE PHASE TITANIUM DIOXIDE NANOPARTICLES AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Microbiology

by
Rupal Ramesh Shah
August 2007

Accepted by:
Dr. Jeremy Tzeng, Committee Chair
Dr. Burtrand Lee
Dr. Lesly Temesvari
Dr. Pengju Luo
ABSTRACT

*Staphylococcus aureus* (*S. aureus*), a major human pathogen, is a common cause of infections worldwide due to its high virulence intensity. By adapting to rapidly changing and uniformly hostile environments, strains of *S. aureus* acquire resistance to antimicrobial agents shortly after their exposure. For example, within a year of its introduction, *S. aureus* developed resistance to methicillin which triggered the development of other antimicrobial treatments. In spite of the various antibiotics currently used to treat methicillin-resistant *S. aureus* (MRSA) infections, antimicrobial resistance is an unavoidable consequence due to the selective pressure of antibiotic exposure. Thus, other prevention modalities are warranted to prevent MRSA transmission.

Titanium dioxide (*TiO$_2$*) nanoparticles decompose organic compounds by the formation and constant release of hydroxyl radicals and superoxide ions when exposed to non-lethal ultraviolet (UV) light of 365nm at 370$\mu$w/cm$^2$. Commercially available anatase phase *TiO$_2$* nanoparticles can serve as antimicrobial agents via UV light activation. However, brookite phase nanoparticles, due to their smaller particle size, may increase the efficiency of *TiO$_2$* nanoparticles to inhibit bacterial growth by promoting a greater surface area contact ratio which subsequently causes cell death in less time than anatase phase nanoparticles.

Both the *TiO$_2$*-free suspension and drop-coated slide bioassays were conducted to determine the effects of UV light activated *TiO$_2$* nanoparticles on gram-negative, *Escherichia coli*, and gram-positive, *S. aureus*, cells and the results revealed non-
selective killing properties of the nanoparticles. Furthermore, UV light activated brookite nanoparticles (1mg/mL) caused a 100% reduction in MRSA cell growth within 30 minutes while anatase nanoparticles, under the same conditions, required approximately 75 minutes for such complete cell death. Additionally, physical damage to the cells by UV light activated TiO$_2$ nanoparticles was confirmed by scanning electron microscopy images.

Due to MRSA’s ability to acquire resistance to antibiotics, these agents remain a temporary solution for the treatment of such pathogenic infections. In contrast, brookite phase TiO$_2$ nanoparticles offer promise for the prevention of MRSA due to their physical, non-selective inhibitory effects on cells. Additionally, the utilization of TiO$_2$ nanoparticles, as a means to prevent transmission could further reduce the emergence of multiple drug-resistant bacteria.

The long term goal of this research is to develop visible light activated surface coatings of TiO$_2$ nanoparticles that could be used in clinical settings to reduce the transmission of bacterial infections. Therefore, visible light activation of brookite nanoparticles for practical usage was also evaluated.
DEDICATION

This thesis is dedicated to my parents, Ramesh and Jayshree, my sister, Sonal, and my brother, Sagar, who offered me unconditional love, guidance, and support throughout the course of my graduate studies. The accomplishments of this thesis are as much yours as they are mine. Thank you for being by my side every step of the way and for encouraging and reminding me that with faith in God, I am capable of accomplishing anything that I put my mind and efforts into. Thank you for all your affection and care.
ACKNOWLEDGEMENTS

From the experimental stages of this thesis to its completion, I owe an immense debt of gratitude to my advisor and mentor, Dr. Jeremy Tzeng. Dr. Tzeng’s constant guidance, support, and encouragement have been invaluable as I have attempted to complete my graduate studies. I have learned and grown so much and this is all due to Dr. Tzeng’s confidence in my abilities. Thank you very much for such an amazing experience throughout the past year.

I would like to thank Dr. Burtrand Lee, Dr. Lesly Temesvari, and Dr. Pengju Luo for serving on my committee and for their invaluable support throughout the course of this project. Thank you for your time, assistance, and cooperation.

I would like to thank Mr. Darryl Krueger in the Jordan Hall Microscopy Facility for his assistance and advice for the microscopy portions of this project. I would also like to express my appreciation for the staff at the Clemson University Electron Microscopy Facility, especially Mr. Dayton Cash and Dr. Joan Hudson for their unlimited services and access to their equipment.

I would also like to recognize my lab mates, John Abercrombie, Donna Weinbrenner, and Jyothi Raganini for their constant support and assistance with materials needed in the laboratory. My special thanks are reserved for Donna Weinbrenner for her constant and honest feedback during the compilation of my thesis. I wish to thank my peers in the Materials Science and Engineering Department, Sujaree Kaewgun and Chris Nolph, for constantly supplying materials needed for my experiments and for always answering my TiO₂-related questions. I would also like to recognize Radhika Bhave in
the Materials Science and Engineering Department for providing me with TiO$_2$ samples at the very beginning of this research study. I would like to acknowledge the undergraduates, Jennifer Odle, Brittany Adams, and Brittany Taylor, who have assisted in a portion of the experiments presented in this thesis.

I would also like to acknowledge Oconee Memorial Hospital, Mr. Mark Teal, the Vice President of Performance and Safety, and Mrs. Joyce Lawhorne, the Infection Control Manager, for providing valuable MRSA statistics for my thesis. I would, especially, like to acknowledge Mrs. Joyce Lawhorne for giving me a better understanding of MRSA. Last but not least, I would like to thank the Biological Sciences Department for giving me the opportunity to conduct this research and for funding my graduate studies.
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CHAPTER 1

LITERATURE REVIEW

1.1 Staphylococcus aureus

1.1.1 Morphology and Characteristics

*Staphylococcus aureus* (*S. aureus*) has been recognized as a major human pathogen since it was discovered by Sir Alexander Ogston in the 1880s as the major cause of wound suppuration (Archer 1998). *S. aureus* is a gram-positive coccus, approximately 0.5 to 1.5μm in diameter (*Figure 1A*) and appears as grape-like clusters (*Figure 1B*) when viewed under a microscope. *S. aureus* forms large, round, golden-yellow colonies, often with β-hemolysis, when grown on blood agar plates (Carr *et al*., 2005; Holt *et al*., 1994; Kaiser, 1999).

![Staphylococcus aureus Images](image)

*Figure 1: Images of Staphylococcus aureus* in (A) Scanning Electron Microscopy and (B) Gram Stain (Carr *et al*., 2005; Kaiser, 1999)
The golden appearance is the etymological root of the bacteria’s name. *S. aureus* are non-motile and non-spore forming facultative anaerobes that grow by aerobic respiration or by fermentation that yields lactic acid (Holt *et al*., 1994). The bacteria can grow at temperature ranges of 15 to 45ºC and at sodium chloride concentrations as high as 15%. *S. aureus* is catalase positive and therefore, able to convert hydrogen peroxide to water and oxygen. Also, it is coagulase positive and thus, able to coagulate blood serum into a clot (Holt *et al*., 1994).

### 1.1.2 Pathogenesis

Due to its virulence intensity, *S. aureus*, is a common cause of infections not only in the United States, but in countries all over the world (Archer, 1998). Its resistance to antibiotics is currently increasing which indicates that its prevalence will continue to rise. Due to the high occurrences and severity of *S. aureus* infections, it is necessary to understand the pathogenesis of *S. aureus* (Archer, 1998).

The five stages in the pathogenesis of *S. aureus* infections are (a) colonization, (b) local infection, (c) systemic dissemination and/or sepsis, (d) metastatic infection, and (e) toxinosis. *S. aureus* can be asymptotically carried for weeks or months on mucous membranes and intact skin (Archer, 1998). *S. aureus* mainly colonizes the nasal passages but it may also be found in other anatomical locales (Archer, 1998). The colonization may be transient and may spread faster during times of upper respiratory tract infections.

Staphylococcal infections usually remain localized at the port of entry due to the normal host defenses (Todar, 2005). Colonization precedes infection (Archer, 1998). The
localized host response to staphylococcal infections is inflammation, characterized by an
elevation of temperature at the site, swelling, accumulation of pus, and necrosis of tissue
(Todar, 2005). Around the inflamed area, a fibrin clot may form, walling off the bacteria,
as a pus-filled boil or abscess (Figure 2) (Todar, 2005). Thereafter, superficial skin
lesions such as boils, styes, and furunculosis (Figure 2) can occur (Archer, 1998). The
infection can spread locally or can gain access to the blood. If it spreads locally, it can
progress to pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections
(Figure 2) (Archer 1998). In the blood, the organism spreads widely to peripheral sites in
distant organs and septic shock can occur. Due to hematogenous dissemination, a number
of specific staphylococcal infections can result such as endocarditis, osteomyelitis, renal
carbuncle, septic arthritis, or epidural abscess (Figure 2). Finally, even if the organism
does not invade the bloodstream, syndromes can result from the local or systemic effects
of specific toxins such as toxic shock syndrome, scalded skin syndrome, and food-borne
gastroenteritis (Figure 2) (Archer, 1998).
Figure 2: Sites of Infection and Diseases Caused by *Staphylococcus aureus* (Archer, 1998)

1.1.3 Virulence Factors

The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are due to its virulence factors (Archer, 1998). According to Archer (1998), *S. aureus* displays a wider variety of virulence mechanisms than any other human pathogen (Archer, 1998). Specific factors are present that allow the organism to thwart opsonophagocytosis. These factors include invasion through the tissue from the initial site of infection inducing sepsis syndrome by promoting a massive cytokine release. Opsonophagocytosis can also be avoided by extravasation into the endothelial cells and underlying tissues or by the production and excretion of toxins (Archer, 1998).

*S. aureus* expresses (a) membrane-damaging toxins (hemolysins, kinases, and leukocidins) that invade tissues and promote bacterial spread, (b) surface factors (adhesins, microcapsules, and proteins) that promote colonization of host tissues, inhibit
phagocytic engulfment, and thwart host defenses, and (c) exoproteins (enterotoxins, toxic shock syndrome toxin-1, and exfoliative toxins) (Figure 3) that induce specific toxinosis (Quinn, 2006; Todar, 2005).

![Figure 3: Virulence Factors of Staphylococcus aureus (Quinn, 2006)](image)

*S. aureus* produces the hemolytic toxins (Figure 3), α-, β-, γ-, and δ- (Dinges et al., 2000; Todar, 2005). Platelets and monocytes are sensitive to α-toxin. Susceptible cells have a specific receptor for α-toxin which allows the toxin to bind causing small pores through which monovalent cations can pass. After binding the toxin, a series of reactions ensue, causing a release of cytokines which generate the production of inflammatory mediators. These events cause the symptoms of septic shock that occur during severe infections (Dinges et al., 2000; Todar, 2005). The α-hemolysin is the most toxic *S. aureus* hemolysin (Quinn, 2006). The β-toxin is a sphingomyelinase which is produced by approximately 20% of all *S. aureus* isolates, is toxic to monocytes, and is
commonly found in strains causing mastitis (Dinges et al., 2000; Quinn, 2006; Todar, 2005). The γ-toxin affects neutrophils and macrophages and is capable of lysing various mammalian erythrocytes (Dinges et al., 2000; Todar, 2005). The small peptide, δ-toxin, is capable of lysing erythrocytes, mammalian cells, and subcellular structures such as membrane-bound organelles, spheroplasts, and protoplasts (Dinges et al., 2000; Todar, 2005).

Furthermore, many strains of *S. aureus* express a plasminogen activator protein called staphylokinase (Figure 3). It consists of 136 amino acids and lyases fibrin (Dinges et al., 2000; Todar, 2005). Staphylokinase is carried by a converting phage that inserts into the β-hemolysin structural gene. Expression of staphylokinase is positively regulated by the accessory gene regulator (Agr) and negatively regulated by the staphylococcal accessory gene regulator (Sar). The complex formed between staphylokinase and plasminogen initiates plasmin-like proteolytic activity which causes dissolution of fibrin clots. This localized fibrinolysis may aid in bacterial spreading and invasion of host tissues (Dinges et al., 2000, Jin et al., 2004; Todar, 2005).

Another membrane-damaging toxin, Panton-Valentine Leukocidin (PVL) (Figure 3), is a member of the synergohymenotropic toxin family that induces pores in the membranes of cells (Bradley, 2005). Pairs of secretory proteins, S and F, work synergistically on cell membranes as superantigens which release intracellular interleukin-8, leukotrienes, proteases, and oxygen metabolites. This leads to chemotaxis, vasodilation, tissue necrosis, and death of neutrophils. SF protein pairs composed of S and F PVL-associated proteins (LukSPV + LukFPV) and S and F α-hemolysin-associated
proteins, HIgA (class S), HIgB (class F), HIgC (class S), have been discovered in various combinations of necrotizing pneumonia (Bradley, 2005). Panton-Valentine Leukocidin-positive \textit{S. aureus} strains belong to the 4 Agr allele type. The Agr locus controls the expression of most virulence factors in \textit{S. aureus}. It encodes a two-component signaling pathway whose activating ligand is a density-sensing peptide also encoded by Agr (Dufour, 2002). Panton-Valentine Leukocidin has been linked to infections such as furunculosis, cellulitis, and abscesses (Dinges \textit{et al.}, 2000; Dufour, 2002; Todar, 2005).

Surface proteins (Figure 3) promote attachment to host proteins such as laminin and fibronectin which form the extracellular matrix in epithelial and endothelial surfaces (Todar, 2005). Most strains of \textit{S. aureus} express fibronectin and fibrinogen-binding proteins which promote attachment to blood clots and traumatized tissue. An adhesin that promotes attachment to collagen has been found in strains of \textit{S. aureus} that cause osteomyelitis and septic arthritis. In addition, interaction with collagen is important in promoting bacterial attachment to damaged tissue when the underlying layers of tissue have been exposed (Todar, 2005).

The extracellular capsule (CP) (Figure 3) of \textit{S. aureus} consists of polysaccharides and enhances virulence by allowing the bacteria to resist phagocytosis and killing by polymorphonuclear phagocytes (Karakawa \textit{et al.}, 1982; Koenig, 1962; Nilsson \textit{et al.}, 1997; Peterson \textit{et al.}, 1978). So far, eleven capsular serotypes have been identified. \textit{S. aureus} expressing CP type 5 or CP type 8 polysaccharides account for 80% to 85% of clinical blood isolates in septicemia (Fattom \textit{et al.}, 1990; Fournier, 1990; Karakawa \textit{et al.}, 1982; Nilsson \textit{et al.}, 1997). The capsule impedes the interaction between cell wall-bound
C3b and immunoglobulin on the phagocytic cells (O’ Riordan et al., 2004). As a result, the bacteria evade phagocytic uptake.

Another surface factor, Protein A (**Figure 3**), consists of a single polypeptide chain with a molecular weight of 42kDa, containing four repetitive domains rich in aspartic and glutamic acids but devoid of cysteine. It contains little or no carbohydrates, four tyrosine residues, and no tryptophans (Bjork et al., 1972; Boyle et al., 1987; Goden, 1978). It is a surface protein that binds immunoglobulins, especially IgG molecules by their Fc region. The IgG binding domain of Protein A consists of three anti-parallel α-helices, the third of which is disrupted when the protein is complexed with the Fc region of the immunoglobulins (Boyle et al., 1987). The binding event prevents the IgG molecules from reacting with the Fc receptor on phagocytes (Quinn, 2006; Todar, 2005). In serum, the bacteria will bind IgG molecules in the wrong orientation on their surface which disrupts opsonization and phagocytosis (Todar, 2005).

Enterotoxins (**Figure 3**) are a series of extracellular monomeric proteins that were first discovered in 1959 (Bergdoll, 1959; Munson et al., 1998; Su et al., 1995). According to serological classification, six enterotoxins have been recognized: A, B, C, D, E, and H. These enterotoxins are small peptides, with sizes ranging from 26kDa to 29kDa, and have a great deal of similarity at the amino acid level (Marrack et al., 1990; Mehrotra et al., 2000; Schmitz et al., 1997; Vannuffel et al., 1995). Enterotoxins cause staphylococcal food poisoning (Bergdoll, 1983; Bohach et al., 1990; Munson et al., 1998).

Toxic Shock Syndrome Toxin-1 (TSST-1) (**Figure 3**) is a 22kDa peptide expressed systemically and is the cause of toxic shock syndrome (TSS) (Quinn, 2006;
Todar, 2005). This toxin may induce the production of interleukin-1B and tumor necrosis factor by monocytes which have a negative effect on neutrophil chemotactic function (Fast et al., 1989; Todar, 2005). Toxic Shock Syndrome Toxin-1 is responsible for 75% of all TSS cases, including all menstrual cases. Toxic Shock Syndrome can occur as a sequel to any staphylococcal infection if an enterotoxin or TSST-1 is released systemically and the host lacks appropriate neutralizing antibodies (Todar, 2005).

There are two antigenically distinct forms of exfoliative toxins (Figure 3), ETA and ETB. Exfoliative toxin A is chromosomally encoded and ETB is plasmid encoded (Quinn, 2006; Todar, 2005). These toxins possess the same biological activity but they differ in amino acid composition and amino acid sequences. ETA consists of 242 amino acid residues while ETB consists of 246 amino acid residues (Lee, 1987). The ETA and ETB toxins have specific esterase and protease activity. Therefore, the toxins may target a specific protein (Todar, 2005). The exfoliative toxins cause blistering and loss of the epidermis which eventually persist to scalded-skin syndromes (Todar, 2005).

Enterotoxins, TSST-1, and exfoliative toxins are also known as superantigens (Lowy, 1998; Quinn, 2006). They belong to a group of polypeptide products that can activate subsets of T lymphocytes to liberate cytokines, leading to major systemic effects such as fever, hypotension, skin lesions, shock, multiorgan failure, and death (Lowy, 1998; Quinn, 2006). A superantigen (Figure 4) is a molecule that binds with high affinity to MHC class II receptors of monocytes and macrophages at sites distinct from the classic antigen binding groove. This complex (Figure 4) is recognized by the variable region, Vβ, of the T lymphocytes. This action causes prolific activation or sometimes the
apoptosis of T cells because of the recognition of the Vβ chains by the toxin (Lowy, 1998; Quinn, 2006).

**Figure 4:** Superantigens and the Non-Specific Stimulation of T Lymphocytes (Quinn, 2006)

### 1.2 Emergence of Antibiotic-Resistant *Staphylococcus aureus*

The introduction of large quantities of diverse antimicrobial agents into the human environment in the past century has presented a new set of challenges to pathogens such as *S. aureus*. Effective lineages of pathogens must excel in several capacities. They must be able to acquire resistant genes in order to survive against antibiotics. In the environment, the resistance determinants must find their way into genetic backgrounds that assure the capacity to compete with other bacteria. Finally, the pathogens must be able to spread, establish ecological reservoirs, colonize, and cause disease.
Most *S. aureus* strains are opportunistic pathogens that can colonize individuals, with or without symptoms, for either short or extended periods of time, causing disease when the immune system becomes compromised (Oliveira *et al.*, 2002). *S. aureus* has affected human beings and caused high mortality rates before the antibiotic era. *S. aureus* is unsurpassed by any other human pathogen due to its flexibility in pathogenic strategies, numbers of virulence factors, and capacity to survive and multiply in a wide range of environments (Oliveira *et al.*, 2002). The ability of *S. aureus* to adapt to rapidly changing and uniformly hostile environments has repeatedly been shown by the emergence of strains that acquire resistance to any antimicrobial agent shortly after its introduction (Oliveira *et al.*, 2002).

*S. aureus* like all bacterial cells grow and divide, replicating repeatedly to reach the large numbers present during an infection in the body. Antimicrobial agents interfere with specific processes (Figure 5) that are essential for growth and division which is lethal to the cells or inhibitory to their growth (Lambert, 2005; Neu *et al.*, 2004).
Figure 5: Sites of Action for Antimicrobial Agents (Neu et al., 2004)

The various antimicrobial agents can be separated into groups such as inhibitors of nucleic acid synthesis, inhibitors of ribosome function, inhibitors of cytoplasmic membranes, inhibitors of folate metabolism, and inhibitors of bacterial cell wall synthesis (Table 1) (Neu et al., 2004). Antimicrobial agents may be either bactericidal, killing the target bacterium or bacteriostatic, inhibiting the growth of microorganisms (Neu et al., 2004).
Antibiotics can interfere with nucleic acid synthesis at several different levels. They can inhibit nucleotide synthesis by interfering with purine or pyrimidine synthesis or with the interconversion of nucleotides. Some antimicrobial agents can act as nucleotide analogs that are incorporated into polynucleotides. Other drugs bind to DNA by intercalation and inhibit nucleic acid synthesis (Neu et al., 2004).

Several antimicrobial agents act by inhibiting ribosome function. Bacterial ribosomes contain two subunits, 50S and 30S. Antibiotics that can act on the 30S subunit, bind to specific proteins in the ribosomal subunit which causes the ribosome to misread
the genetic code. Some antibiotics can combine with binding sites on the ribosomes and kill the bacteria by inducing the formation of aberrant, nonfunctional complexes. Antibiotics that act on the 50S subunit inhibit peptide bond formation by binding to a peptidyltransferase enzyme on the 50S ribosome (Neu et al., 2004).

A number of antimicrobial agents can cause disorganization of the membrane. These agents can be divided into cationic, anionic, and neutral agents. Certain drugs disorganize the permeability of membranes so that nucleic acids and cations leak out which eventually results in death. Other antibiotics act by producing aqueous pores in the membranes (Neu et al., 2004). Some drugs interfere with folate metabolism by blocking the biosynthesis of tetrahydrofolate, which acts as a carrier of one carbon fragments and is necessary for the ultimate synthesis of DNA, RNA, and bacterial cell wall proteins (Neu et al., 2004).

Drugs such as β-lactams affect bacterial cell wall synthesis. Bacterial cell walls contain a peptidoglycan layer (Figure 6) which is the critical attack site of anti-cell wall synthesis agents. Peptidoglycan, also known as murein, serves a structural role in the bacterial cell wall, providing the wall shape and structural strength, as well as counteracting the osmotic pressure of the cytoplasm (Neu et al., 2004). The peptidoglycan layer is substantially thicker in Gram-positive bacteria than in Gram-negative bacteria (Neu et al., 2004). The Gram-positive cell wall consists of a single 20nm to 80nm thick homogenous peptidoglycan layer lying outside the plasma membrane while the Gram-negative cell wall consists of a 2nm to 7nm peptidoglycan layer surrounded by a 7nm to 8nm thick outer membrane. Due to this thickness, the walls
of Gram-positive bacteria are stronger and can, therefore, protect the bacteria from toxic substances with more ease than Gram-negative bacteria (Prescott et al., 2002).

The peptidoglycan layer in the bacterial cell wall is a crystal lattice structure formed from linear chains of two alternating amino sugars, namely N-acetyl muramic acid (MurNAc or NAM) and N-acetyl glucosamine (GLcNAc or NAG) (Figure 6). The alternating sugars are connected by a β-(1, 4)-glycosidic bond. Each MurNAc is attached to a short (4 to 5 residue) amino acid chain, normally containing D-alanine, D-glutamic acid, and mesodiaminopimelic acid. These three amino acids do not occur in proteins and help protect against attacks by most peptidases. Cross-linking between amino acids in different linear amino sugar chains by an enzyme known as transpeptidase results in a peptidoglycan layer that is strong and rigid (Neu et al., 2004).

Figure 6: The Peptidoglycan Layer of Bacterial Cell Walls (Prescott et al., 2002)
1.2.1 Penicillin-Resistant *Staphylococcus aureus*

The introduction of benzylpenicillin (also known as penicillin G) into chemotherapy in the early 1940s found *S. aureus* fully susceptible and several of the first successes of penicillin therapy were related to the cure of formerly untreatable staphylococcal diseases (Abraham *et al*., 1941; Oliveira *et al*., 2002).

Penicillin refers to a group of β-lactam antibiotics used in the treatment of bacterial infections usually caused by susceptible Gram-positive organisms. It was Alexander Fleming who, in 1928, discovered that the mold, *Penicillium notatum*, produced a diffusible substance, under certain circumstances, which inhibited the growth of some bacterial species. This substance was named penicillin. Very little was done with this substance in the subsequent years, probably because it was found to be unstable (Harrison, 2007).

In 1939, Chain and Florey, as part of a comprehensive program of research on antibacterial substances, began work on penicillin at Oxford and established the efficacy of penicillin as a chemotherapeutic agent. The choice of penicillin was, for Chain, mainly determined by the challenges posed by its instability, and, for Florey, by the fact that it was the only substance at that time that might be effective against *S. aureus* (Abraham *et al*., 1941). In order to strengthen the chemical side of the work, Florey attracted Abraham who set about the difficult task of purifying penicillin and then determining its structure. Abraham was successful in both these aims, and was the first to propose the correct chemical structure for penicillin. Abraham's structure of penicillin involved the novel β-lactam ring (Abraham *et al*., 1941; Harrison, 2007).
β-lactam antibiotics are a broad class of antibiotics that include penicillin derivatives (Figure 7A) such as methicillin, cloxacillin, oxacillin, and fludoxacillin; cephalosporins (Figure 7B) including oxacephams and cephamycins; monobactems (Figure 7C) such as aztreonam; carbapenams (Figure 7D) such as thienamycins, meropenem, ertapenem, faropenem, and doripenem; and other β-lactam antibiotics such as clavulanic acid (Figure 7E), tazobactam, and sulbactam. These antibiotics share a three carbon and one nitrogen structure known as the β-lactam ring (Harrison, 2007; Neu et al., 2004). An intact β-lactam ring is required for these antibiotics to effectively exert their bactericidal activity (Harrison, 2007).

Figure 7: β-Lactam Ring Structure in (A) Penicillin; (B) Cephalosporin; (C) Monobactem; (D) Carbapenam; and (E) Clavulanic Acid (Neu et al., 2004)

R and R’ represent carbon groups. X represents hydrogen or a methoxy group.

β-lactam antibiotics act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The final transpeptidation step in the synthesis of the peptidoglycan layers of bacterial cell walls is facilitated by transpeptidases known as penicillin binding proteins (PBPs). β-lactam antibiotics are analogs of D-alanyl-D-alanine, the terminal
amino acid residues on the precursor NAM/NAG peptide subunits of the peptidoglycan layer (Harrison, 2007). The structural similarity between β-lactam antibiotics and D-alanyl-D-alanine facilitates their binding to the active site of PBPs. The β-lactam nucleus of the molecule irreversibly binds to the Ser_{403} residue of the PBP active site. This irreversible inhibition of the PBPs prevents the final crosslinking of the nascent peptidoglycan layer, disrupting cell wall synthesis. Therefore, the effectiveness of these antibiotics relies on their ability to reach the PBP intact and their ability to bind to the PBP (Harrison, 2007).

However, by the mid 1950s, the number of *S. aureus* clinical isolates showing high levels of resistance to penicillin rapidly increased to such an extent that penicillin ceased to be a useful therapeutic agent against staphylococcal infections (Oliveira et al., 2002). One study indicated that within approximately 6 years after the introduction of penicillin, 25% of hospital strains were resistant to penicillin (Chambers, 2001). Today, virtually all strains of *S. aureus* are resistant to natural penicillins, aminopenicillins, and antipseudomonal penicillins. The mechanism of penicillin resistance involved the acquisition of a plasmid-borne, β-lactamase enzyme, penicillinase, (Figure 8) which was capable of breaking down the β-lactam ring of penicillin thus, degrading the antibiotic before it could reach its cellular targets (Neu et al., 2004).
The genes encoding these enzymes may be inherently present on the bacterial chromosome or may be acquired by plasmid transfer (Jessen et al., 1969; Oliveira et al., 2002). The effects of this plasmid epidemic were first seen on hospital isolates of *S. aureus*, which rose dramatically from 1946 to 1958, but soon afterwards the penicillinase-based mechanisms found their way into the community (Figure 9) (Chambers, 2001; Jessen et al., 1969; Oliveira et al., 2002). Penicillin-resistant community-acquired strains of *S. aureus* were not reported until 1949, when the rate of penicillin resistance in hospitals approached 50%. The prevalence of penicillin-resistant *S. aureus* in communities continued to increase throughout the next 20 years (Figure 9), finally approaching resistance rates close to those seen among hospital strains (Chambers, 2001).
Currently most *S. aureus* isolates that cause disease or colonize healthy individuals are resistant to penicillin. A study of *S. aureus* colonizing flora of 1001 healthy volunteers showed that 97% of the *S. aureus* isolates recovered carried the penicillin-resistant trait (Oliveira et al., 2002; Sa-Leon et al., 2001). Bacteria resistant to a particular β-lactam antibiotic may sometimes remain sensitive to other antibiotics. Therefore, once penicillin-resistant strains came about, other antibiotics started being utilized against *S. aureus*.

However, the power of *S. aureus* against various antibiotics, besides penicillin is also evident. Records of the Danish Health Board registered the years of introduction of various antimicrobials into clinical practice, beginning with penicillin in 1945-1946, streptomycin in 1948, tetracycline in 1950, and erythromycin in 1953 (Jessen et al., 1969; Oliveira et al., 2002). The same records indicate that *S. aureus* bloodstream isolates
resistant to penicillin, streptomycin, tetracycline, and erythromycin were recovered as early as 1957. The introduction of methicillin into clinical practice in 1959 was followed by the appearance, in 1961, of the first bloodstream isolate of *S. aureus* that was resistant not only to penicillin, streptomycin, tetracycline, and erythromycin but to methicillin as well (Oliveira et al., 2002).

### 1.2.2 Methicillin-Resistant *Staphylococcus aureus*

Methicillin, originally called celbenine, is a semisynthetic derivative of penicillin, chemically modified to withstand the degradative action of penicillinase. The drug was introduced into therapy in Europe in 1959-1960 and in the United States in 1961 (Oliveira et al., 2002; Rice, 2006).

The first cases of Methicillin-Resistant *Staphylococcus aureus* (MRSA) were reported in the United Kingdom in 1961, followed soon by reports in other European countries, Japan, and Australia (Palavecino, 2004; Rice, 2006). The first report of MRSA in the United States appeared in 1968 (Barrett et al., 1968; Rice, 2006). Periodic outbreaks of MRSA were observed in various countries throughout the world in the 1970s, but it was not until the 1980s that MRSA became a significant problem in United States hospitals (Palavecino, 2004; Rice, 2006).

The emergence of MRSA is due to the *mecA* gene, a 2.1kb stretch of DNA that is not native to *S. aureus* and is embedded in 60kb of additional DNA called the *mec* element or staphylococcal chromosomal cassette (SCC*mec*), which is incorporated into the *S. aureus* chromosome at a site-specific location (Beck et al., 1986; Ito et al., 1999;
Ito et al., 2001; Katayama et al., 2000; Oliveira et al., 2002). The mecA gene encodes for a 78kDa alternative penicillin binding protein (PBP2a), which has a very low affinity for β-lactam antibiotics. β-lactams cannot bind as effectively to these PBP2as, and as a result, the β-lactams are ineffective at disrupting cell wall synthesis (Oliveira et al., 2002).

According to Enright and colleagues (2002), evolutionarily, the mec genes that are responsible for SCCmec typing are thought to have initially been introduced into coagulase-negative Staphylococci from an unknown source, where deletion of the mec regulatory genes occurred, and then they were introduced into S. aureus. The Staphylococcal species that donated SCCmec found in MRSA today is unknown but their patterns of presence indicates multiple introductions into S. aureus and that horizontal gene transfer of mec is possible in S. aureus (Enright et al., 2002).

### 1.2.3.1 Incidence of Hospital-Associated Methicillin-Resistant Staphylococcus aureus

A survey of 500 women attending prenatal clinics in London in 1989 to 1990 found 2% of the staphylococcal strains isolated were MRSA (Abudu, 2001). Since that time, the epidemiology of MRSA in the United Kingdom has been changing with the number of hospitals in England and Wales reporting MRSA incidents increasing each year. Over a third of staphylococcal bloodstream infections in England and Wales in early 1999 were due to MRSA compared to 8% in 1994 (Abudu, 2001). A survey undertaken in nursing homes in Birmingham, UK (1996) found a 17% prevalence of MRSA colonization which is markedly higher than the 4% prevalence found in a survey

According to the Centers for Disease Control and Prevention (CDC), the National Nosocomial Infections Surveillance System reported the increase of MRSA incidence (Figure 10) from 1995 to 2004 in Intensive Care Units (ICU) in the United States (Centers for Disease Control and Prevention, 2005). In 2004, the report identified methicillin resistance in 59.5% of S. aureus infections in ICU patients. This represented an 11% increase in resistance compared with rates during 1998 to 2002 (Chambers, 2001).

![Figure 10: Methicillin-Resistant Staphylococcus aureus in Intensive Care Unit Patients (Centers for Disease Control and Prevention, 2005)](image-url)
In 2005, to assess the frequency of hospital-associated MRSA (HA-MRSA) infections among dialysis patients in the United States, data was analyzed from the Active Bacterial Core Surveillance System (Collins et al., 2005). A report that summarizes the results of that analysis, estimated that, in 2005, the incidence of MRSA infections among dialysis patients was 45.2 cases per population of 1,000 (Collins et al., 2005).

In 2005, nine states in the United States, Connecticut, California, Colorado, Georgia, Maryland, Minnesota, New York, Oregon, and Tennessee, monitored MRSA infections (Collins et al., 2005). Of the 5,287 cases of invasive MRSA reported from the sites, a total of 813 (15.4%) occurred in dialysis patients. The majority (86%) of the infections were bloodstream infections, identified via positive blood cultures. About 90% of those patients required hospitalization. The mortality rate was 17% (Collins et al., 2005).

At Oconee Memorial Hospital (OMH), a rural 160-bed hospital in Seneca, South Carolina, data has shown that in a total number of MRSA cases, a significant number of them are hospital-acquired (Figure 11). The rates of hospital-acquired MRSA cases was increasing until 1999 when the figures started declining (Figure 11) due to strict preventative measures implemented within the hospital. These preventative measures include strict hand-washing and hand-sanitizing policies.
Figure 11: Incidence of Facility-Acquired Methicillin-Resistant *Staphylococcus aureus* Compared to Total Methicillin-Resistant *Staphylococcus aureus* Cases (Oconee Memorial Hospital, 2007)

1.2.3.2 Incidence of Community-Associated Methicillin-Resistant

*Staphylococcus aureus*

Initially, MRSA was only observed in the hospital setting, but it is now clear that MRSA may infect people in the community without identifiable risk factors (Rice, 2006). According to Graffunder and Venezia (2002), several reports have suggested an increase in the appearance and number of cases of MRSA among patients in the community. Chambers (2001) suggests that the epidemiology of *S. aureus* is now changing in order to adapt to the community.
Several research studies of community-associated MRSA (CA-MRSA) revealed their prevalence in children attending day care centers. In 1999, Shahin and researchers reported CA-MRSA in a 2-year old child from Toronto, Canada, and transmission via a day care center. A survey of two day care centers in Dallas, Texas, revealed that 3% and 24% of the children were colonized with isolates that were susceptible to multiple antibiotics, which is in contrast to the typical (multiple drug-resistant) MDR HA-MRSA. From that group of children, 40% had no previous contact with a health care facility, which suggests that transmission and colonization of MRSA in those children occurred in the community (Chambers, 2001).

Herold and colleagues (1998) reviewed cases of MRSA from pediatric inpatients at the University of Chicago Children's Hospital in Chicago, Illinois. From 1988 through 1990, 8 of 32 (25%) cases of MRSA were community-acquired. From 1993 through 1995, 35 of 52 (67%) cases of MRSA were community-acquired and 10 of these 35 patients had an identified exposure (Herold et al., 1998). In the second part of this study (1993-1995) of the 52 pediatric patients hospitalized with MRSA, 25 (48%) patients had CA-MRSA without an identified exposure (Herold et al., 1998). Those strains illustrated susceptibility to multiple antibiotics and under pulsed field gel electrophoresis (PFGE) revealed patterns that were distinct from nosocomial isolates (Chambers, 2001).

Finally, the deaths of four children from rural Minnesota and North Dakota caused by infection with CA-MRSA attracted national attention in 1999. Out of those children two died from necrotizing pneumonia and severe sepsis (Rice, 2006). Those children lacked risk factors for MRSA colonization and the infections were caused by
strains susceptible to all antibiotics, except β-lactams. The PFGE patterns of these strains also indicated that they were related to one another but different from hospital isolates (Chambers, 2001).

The reports of strains of MRSA in children provided compelling evidence that MRSA strains, like penicillinase-producing strains almost 30 years ago, were now prevalent in the community (Chambers, 2001). MRSA is currently recognized as a major problem in both hospitalized patients and in healthy persons within the community (Diekema et al., 2001; Nilsson et al., 1997; Rice, 2006). The increased morbidity, mortality, and costs associated with MRSA infections are major incentives to control the spread of this organism within health care facilities and in the community (Brumfitt et al., 1990; L’Heriteau et al., 1999; Warshawsky et al., 2000).

1.2.3.3 Hospital-Associated Methicillin-Resistant *Staphylococcus aureus* versus Community-Associated Methicillin-Resistant *Staphylococcus aureus*

The classical definition from the CDC of nosocomially-acquired versus community-acquired infections has been based on the presumed site of acquisition of the infection (Bradley, 2005). According to the CDC, a nosocomial infection is an infection that develops in the hospital and was not incubating at the time of admission while a community-acquired infection is one that is incubating at the time of admission and was not caused by an organism acquired during previous health care (Salgado et al., 2003).

There are plenty of reasons for the incidence of HA-MRSA and these reasons can be attributed to hospitalization within the past 12 months, admission into the intensive
care unit, previous surgery, enteral feedings, prolonged use of antibiotics, indwelling medical devices (such as dialysis tubing, catheters), and proximity to an infected or colonized patient (Brumfitt et al., 1990; L’Heriteau et al., 1999; Salgado et al., 2003; Warshawsky et al., 2000). Previous hospitalization and longer length of stay before infection may represent a chronic illness and indicates exposure to antibiotics which increases the opportunities to be colonized with antibiotic-resistant microorganisms (Asensio et al., 1996; Ayliffe, 1997; Boyce et al., 1981; Graffunder et al., 2002). Prior surgery may represent a breakdown of the normal host defenses, surgical techniques, or post-operative care. Enteral feedings may also serve as a portal of entry for MRSA (Asensio et al., 1996; Ayliffe, 1997; Boyce et al., 1981; Graffunder et al., 2002).

In general, HA-MRSA is MDR. Results from a recent study in the United Kingdom examining a new epidemic strain of MRSA, EMRSA-17, illustrated this characteristic of multiple drug resistance in HA-MRSA (Aucken et al., 2002; Rice, 2006). In particular, fluoroquinolone resistance is a hallmark of nosocomial MRSA, although this was not always the case. When ciprofloxacin, a fluoroquinolone, was first licensed, it was recommended as the first orally administered treatment effective against MRSA. However, within one year many hospitals observed dramatic increases in the rate of ciprofloxacin resistance in MRSA. In one study, high levels of ciprofloxacin resistance were observed within 3 months of ciprofloxacin introduction, and within one year, 70% of all MRSA from hospitalized patients exhibited resistance (Blumberg et al., 1991; Rice, 2006).
Fluoroquinolones act by inhibiting DNA gyrase and topoisomerase IV, two enzymes involved in bacterial DNA synthesis (Lambert, 2005). DNA gyrase is comprised of two GyrA and two GyrB subunits, encoded by the \textit{gyrA} and \textit{gyrB} genes, respectively. DNA gyrase is responsible for introducing negative superhelical twists in the bacterial DNA double helix ahead of the replication fork (Lambert, 2005). Topoisomerase IV is comprised of two GrlA and two GrlB subunits encoded by the \textit{grlA} and \textit{grlB} genes, respectively. Topoisomerase IV is responsible for the decatenation of interlinked daughter chromosomes produced at the end of a round of replication. Fluoroquinolones interact with the complexes formed between DNA and DNA gyrase or topoisomerase IV enzymes creating conformational changes that result in the inhibition of normal enzyme activity which leads to breaks in the double strands of DNA (Lambert, 2005). Resistance to fluorquinolones can result from chromosomal mutations in DNA gyrase and/or topoisomerase IV enzymes (Lambert, 2005).

Nosocomial MRSA is remarkable for its clonal pattern of spread. A recent study looking at 359 MRSA isolates collected from 20 countries from 1961 to 1999 identified 11 major MRSA clones within 5 groups of related genotypes (Enright \textit{et al.}, 2002; Rice, 2006). Similarly, Oliveira and colleagues (2002) used molecular typing techniques to identify 5 major MRSA clones that accounted for approximately 70% of more than 3000 MRSA isolates obtained primarily from hospitals in the United States, South America, and Europe. The major reason for this sort of clonal spread is thought to be infection control lapses by healthcare practitioners or other persons who become colonized with \textit{S. aureus} and then have contact with hospitalized patients (Lowy, 1998).
There is, now, an increase of MRSA in the community among people without typical hospital-associated risk factors for MRSA acquisition (Chambers, 2001). Community-associated MRSA infections are prevalent among young and healthy individuals who do not have a history of recent hospitalization, residence in health care facilities, surgery, or dialysis (Salgado et al., 2003). Community-associated MRSA infections are usually observed in children and young adults. Clusters of CA-MRSA infections have also been reported in prisoners and athletes (Rice, 2006). In addition, crowded living conditions and poor access to sanitation facilities by street and shelter dwellers place them at a high risk for CA-MRSA (Charlebois et al., 2002). Intravenous drug users are also on the list of populations at risk for CA-MRSA. In addition, there have been several reports that show that CA-MRSA has been occurring in closed populations such as the Canadian and Australian aboriginal communities and the Pacific Islanders (Salgado et al., 2003).

Unlike nosocomial strains which are resistant to multiple antibiotics, CA-MRSA strains tend to be susceptible to other antibacterial drugs and are only resistant to β-lactam antibiotics (Chambers, 2001). They tend to be more susceptible than hospital strains to tetracycline and trimethoprim-sulfamethoxazole (Rice, 2006). The lack of resistance to multiple antibiotics suggests a community origin because antibiotic selective pressure is much lower within the community than in hospitals and the survival advantage of multiple drug resistance is lower (Chambers, 2001).

Community-associated MRSA strains are different from hospital strains in that they tend to cause infections at a higher rate and some of these infections can be severe
(Rice, 2006). Skin and soft tissue infections and furunculosis are the most common manifestations of CA-MRSA. Community-associated MRSA may also be associated with life-threatening infections such as necrotizing pneumonia (Rice, 2006). The high rates of CA-MRSA may be associated with risk factors for spread in the community, such as overcrowding, high rates of skin infections, and the frequent use of antibiotics (Salgado et al., 2003).

A high percentage of CA-MRSA strains carry genes for PVL, the cytotoxin that causes leukocyte destruction and tissue necrosis (Rice, 2006). Lina et al., (1999) screened isolates from patients in France with S. aureus infections and identified PVL in 93% of the cases of furunculosis and 85% of the cases of necrotic hemorrhagic pneumonia. More recently, Francis and colleagues (2005) described 4 cases of previously healthy adult patients in the United States who developed severe necrotizing pneumonia caused by MRSA-carrying PVL genes. One patient died after 2 days, while the other three survived. All of the survivors experienced very prolonged hospitalizations. According to a study conducted by Dufour and colleagues (2002), PVL genes were detected in 93% of strains associated with furunculosis, 55% of strains associated with cellulitis, 50% of strains associated with cutaneous abscess, and 13% of strains associated with finger-pulp infection, but these genes were absent in strains associated with superficial folliculitis and impetigo. Therefore, the PVL genes may be responsible for causing more disease in people infected with CA-MRSA than HA-MRSA.

Besides being acquired in the community there are a number of other differences between HA-MRSA and CA-MRSA. Both HA-MRSA and CA-MRSA are resistant to
methicillin and other β-lactams due to the presence of the meca gene (Rice, 2006). However, the genetic environment of the meca gene differs in hospital-acquired and community-acquired isolates (Rice, 2006). The meca gene which is carried on the mobile genetic element known as SCCmec is divided into 4 types designated I to IV, which differ in size and the presence of additional resistance genes. Types I, II, and III of SCCmec are relatively large, about 34kb to 67kb in size, and contain significant quantities of DNA in addition to the basic components of meca, its regulators, and the ccrAB genes that confer mobility. In some cases, the functions encoded by the additional DNA are unknown; in other instances, further antimicrobial resistance determinants are included through the insertion of small plasmids or transposons. Types I, II, and III in SCCmec are common in HA-MRSA strains (Rice, 2006). In contrast, type IV SCCmec is relatively small containing only the basic components of SCCmec. Type IV SCCmec is estimated to be 20kb long and is a typical feature of the CA-MRSA strains (Rice, 2006).

The small size and lack of resistance genes besides meca have been connected in the non-MDR nature of CA-MRSA, whose drug-susceptibility profile is characterized by resistance to methicillin and by susceptibility to non-β-lactam drugs (Rice, 2006). However, there are exceptions, some CA-MRSA strains exhibit resistance to a few non-β-lactam drugs, probably due to the acquisition of resistance via other mechanisms (Rice, 2006).

The rapid spread and polyclonal nature of CA-MRSA has raised the intriguing question of whether methicillin resistance is transferable from these strains (Rice, 2006). The small size of SCCmec type IV would allow its incorporation into a bacteriophage
head (an option not available to the larger types of SCCmec), implying that transduction could be responsible for the spread of the determinant between strains. Studies have shown that the presence of type IV SCCmec in CA-MRSA is the only thing that distinguishes it from methicillin-susceptible S. aureus (MSSA), suggesting that CA-MRSA may have spread into the community when type IV SCCmec was transferred into MSSA strains (Rice, 2006).

1.2.3.4 Current Approach for the Treatment of Methicillin-Resistant Staphylococcus aureus

For many years, vancomycin, a glycopeptide, was the only effective treatment for MRSA infections. Glycopeptides (Table 1) are drugs that exhibit antibacterial effects by combining with cell wall substrates (Bohach et al., 1999). Vancomycin (Figure 12) prevents the NAM/NAG peptide subunits from being incorporated into the peptidoglycan matrix (Neu et al., 2004). The large hydrophilic molecule is able to form hydrogen bonds with terminal D-alanyl-D-alanine moieties of the NAM/NAG peptides. This binding of vancomycin to the D-alanyl-D-alanine moieties prevents the incorporation of the peptide subunits into the peptidoglycan matrix, and thus, prevents cell wall synthesis (Neu et al., 2004).
Figure 12: Structure of the Vancomycin Glycopeptide (Neu et al., 2004)

*S. aureus* strains with intermediate resistance to vancomycin were eventually observed in 1997. The first case occurred in a hospitalized patient in Japan (Hiramatsu et al., 1997; Rice, 2006). In the United States, the first 4 cases of vancomycin intermediate-resistant *S. aureus* (VISA) were reported between 1997 and 1999. In each instance, emergence of VISA was associated with extensive exposure to vancomycin, ranging from 25 days to 18 weeks. These were patients, often on dialysis, who were exposed to large amounts of vancomycin to treat MRSA infections (Rice, 2006; Sieradzki et al., 1999; Smith et al., 1999).

The mechanism of resistance in VISA has been linked to cell wall thickening, which may cause vancomycin molecules to become trapped in the outer layers of the cell wall, thereby limiting access to the cytoplasmic membrane where the functional
transglycosylate targets of vancomycin are located (Cui et al., 2003; Lambert, 2005; Rice, 2006). Recently, vancomycin-resistant S. aureus (VRSA) strains were isolated from 3 patients in the United States. The mechanism for the high level of vancomycin resistance involves the horizontal transfer of a transposon containing vanA and associated genes from vancomycin-resistant Enterococcus (Chang et al., 2003; Rice, 2006; Tenover et al., 2004; Weigel et al., 2003). Certain transposable genetic elements also encode special cell wall synthesizing enzymes which change the structure of the normal D-alanyl-D-alanine side chains in the peptidoglycan assembly pathway (Neu et al., 1999). The altered side chain does not bind vancomycin and allows normal peptidoglycan polymerization to occur in the presence of the drug (Neu et al., 1999).

Thus, in the past few years, new agents with anti-MRSA activity have been introduced such as linezolid, quinupristin-dalfopristin, daptomycin, rifampin, and tigecycline (Drew, 2007). Due to the rapid emergence of resistance to rifampin, it can never be used as a single agent to treat MRSA infections (Drew, 2007). At OMH, HA-MRSA is usually treated with vancomycin or linezolid. On the other hand, CA-MRSA is usually treated with trimethoprim-sulfamethoxazole or rifampin. Of note is that these drugs affect MRSA via different mechanisms (Table 1). In addition, at OMH, HA-MRSA is always treated using multiple drug therapy due to the high rates of resistance to multiple antibiotics while CA-MRSA is treated using monotherapy.

There are several ways to reduce the incidence of S. aureus infections and to treat infections caused by MRSA, especially the ones that are caused by strains that are resistant to other antibiotics (Archer, 1998). The first way is to establish more effective
infection control and prevention practices. This includes improving hygiene practices among people who are colonized and susceptible to MRSA. The second method is by the development of \textit{S. aureus} vaccines (Archer, 1998). Although there has been little success with vaccination for preventing human infections, a conjugate vaccine with \textit{S. aureus} types 5 and 8 capsular polysaccharide coupled to \textit{Pseudomonas} exotoxin A has been shown to be immunogenic in humans and provides some protection. Studies of additional antigens, including toxoids, are needed (Archer, 1998). The third option is the development of new or improved antimicrobial agents (Archer, 1998). With the increase in the prevalence of MDR isolates, the pharmaceutical industry is responding by modifying existing compounds to broaden their spectra of novel compounds. Therefore, the synthesis of unique compounds that attack new targets may work against MRSA (Archer, 1998).

1.3 Nanotechnology as a Treatment Modality

Nanotechnology is an emerging field of research which consists of the study of functional structures with dimensions in the 1-100nm range (Jianrong \textit{et al.}, 2004). Nanotechnology impacts a wide variety of disciplines, from materials science to engineering to biology, and has a wide variety of effects in every industry, from aerospace to medicine to agriculture.

A nanometer is one-billionth of a meter, which is the width of about three to five atoms. Nanotechnology provides the ability to engineer the properties of materials by controlling their size. In the nanoscale range, bulk materials exhibit specific physical,
chemical, and biological properties such as changes in conductivity and changes in surface-to-weight ratios (Ebbesen et al., 2007; Luo et al., 2007, In Press).

For years, chemists have designed and fabricated nanomaterials via chemical synthesis. Indeed, during the last decade, developments in the areas of nanotechnology have evolved to provide outstanding capabilities for understanding, fabricating, and manipulating structures at the atomic level. The US Business Weekly listed nanotechnology as one of the key areas of focus in the 21st century. In addition, in 1999, the government of the United States classified nanometer technology as one of the eleven important research areas in the 21st century (Markoff, 2000). In February 2000, Bill Clinton, former president of the United States, announced that the government would invest and allocate funds to promote research in nanometer technology (Markoff, 2000).

Currently, nanotechnology is utilized in a range of applications from water and air purification using nanofilters to clothing with stain-resistant nanofibers treated with fluorinated nanopolymers to food storage incorporating the antibacterial effects of nanoparticles (Luo et al., 2007, In Press). In addition, nanoscience and nanotechnology also has a broad range of applications in the fields of biomedicine and biotechnology. Among these are drug delivery, cancer detection and diagnosis, labeling, biosensors, and several others. One of the most studied aspects of nanotechnology nowadays is their ability to offer the opportunity to fight microbial infections via the synthesis of nanoparticles (Luo et al., 2007, In Press).

As described earlier, antibiotic resistance is rampant. Due to the clinical misuse, the administration of low doses of antibiotics, and the changes in the target receptors of
drugs, antibiotic resistance is a major problem (Luo et al., 2007, In Press). The mechanisms by which antibiotics prevent bacterial growth vary from the mechanisms by which nanoparticles inhibit microbial growth. Therefore, nanoparticles have the potential to serve as an alternative to antibiotics and to control microbial infections such as those caused by MRSA (Luo et al., 2007, In Press).

In the review paper by Luo and colleagues (2007), various applications of nanoparticles as antibacterial agents are listed. These include the usage of sugar nanoparticles for anti-adhesion therapy, the usage of metallic nanoparticles to produce modified antibiotics, and the utilization of silver and gold nanoparticles, chitosan polysaccharides, and metal oxide nanoparticles such as magnesium oxide, zinc oxide, and titanium dioxide for antimicrobial therapy (Luo et al., 2007, In Press).

1.4 Titanium Dioxide

1.4.1 Structure and Characteristics

William Gregor discovered titanium as a mineral in 1791 while he was studying mineralogy in England and named it menachanite. Martin Klaproth later recognized that there was a new chemical element in this mineral and he later renamed it titanium after the Titans. However, Klaporth was only able to produce titanium dioxide (TiO₂) rather than the pure element, titanium.

Titanium dioxide, also known as titanium (IV) oxide or titania, is the naturally occurring oxide of titanium. There are several different crystalline and amorphous forms of TiO₂ (Bokhimi et al., 2004). Titanium dioxide mainly exists in three crystalline
polymorphs, namely rutile, anatase, and brookite forms. These three polymorphs are expressed using the same chemical formula, but have different crystalline structures (Watson et al., 2004). All the phases contain a TiO$_6^{2-}$ (Figure 13) octahedra (Bhave, 2007). The octahedral structure has titanium as the center atom and is surrounded by six oxygen atoms (Figure 13).

![Figure 13: Structure of the TiO$_6^{2-}$ Octahedra (Bhave, 2007)](image)

During the formation of a TiO$_2$ crystal (Figure 14A), two octahedrons condense to form a bond and the orientation of a third octahedron determines the phase of TiO$_2$ that will be formed (Bhave, 2007). Rutile phase has a tetragonal structure (Three Bond Technical News, 2004; Watson et al., 2004). In rutile structures, two of the twelve edges of the octahedron are shared forming a linear chain (Figure 14B). The linear chains are
joined by the sharing of corner oxygen atoms to form the overall structure of rutile particles. The linear arrangement in the rutile structure is the most stable since the electrostatic repulsive energy is minimized thus, thermodynamically this structure is most favored (Watson et al., 2004). When materials are thermodynamically stable, their phase, structure, and physical and chemical properties are not altered when exposed to extremely high temperatures. Anatase phase of TiO₂ also has a tetragonal structure (Three Bond Technical News, 2004; Watson et al., 2004). Anatase phase has no corner oxygen sharing and has four edges shared per octahedron (Figure 14C). In contrast to rutile and anatase, brookite phase has an orthorhombic structure (Three Bond Technical News, 2004; Watson et al., 2004). In brookite particles, three edges are shared per octahedron (Figure 14D). Although brookite particles are less stable than rutile and anatase particles, the phase and structure of brookite particles does remain the same at room temperature conditions, which indicates that brookite particles may still be used in practical applications.
Figure 14: Condensation of (A) Titanium Dioxide Crystal; (B) Rutile Titanium Dioxide; (C) Anatase Titanium Dioxide; and (D) Brookite Titanium Dioxide (Watson et al., 2004)

The three forms of TiO$_2$ can be synthesized by various techniques such as dry methods like flame synthesis, chemical vapor deposition and wet chemistry methods such as the chloride method, hydrothermal processing, and the alkoxide method also known as the sol-gel process (Watson et al., 2004). Rutile, anatase, and brookite forms can be formed at room temperature conditions; however, anatase and brookite phases are transformed into thermodynamically stable rutile phases at higher temperatures (Jagtap et al., 2005). The properties of pure brookite phase particles are scarcely known, because of the difficulty in preparing samples in pure brookite phases at low temperatures. It is, however, often synthesized as a second phase, when other phases of TiO$_2$ are produced. For example, during the synthesis of anatase particles, brookite particles frequently
appear as a secondary minority phase (Bokhimi et al., 2004). An exact explanation for its appearance has not been elucidated. Studies have shown that brookite phase \( \text{TiO}_2 \) can be carefully prepared at low temperatures by thermolysis of strongly acidic solutions or by the hydrothermal treatment of basic solutions. However, care has to be exercised to make sure that the phases formed are not mixtures of brookite and anatase types (Bokhimi et al., 2004). Studies have shown that heat treatment of amorphous \( \text{TiO}_2 \) suggests a closer relation to the brookite phase than to the other phases of \( \text{TiO}_2 \) (Zallen et al., 2006). However, the exact relationship is yet to be determined.

When amorphous \( \text{TiO}_2 \) is crystallized, the original bonding in the amorphous particles is modified to form new bonding for the various phases. During crystallization these bonds are broken, resulting in the deformation of particles and fine crystal forms into the interstices among the original amorphous structure. Depending on the temperature of synthesis, various phases and corresponding crystalline structures (Figure 15) are formed. At low reaction temperatures, brookite phase is produced while at high reaction temperatures anatase and rutile phases are formed (Bhave, 2007; Jagtap et al., 2005; Three Bond Technical News, 2004; Watson et al., 2004).
The band gap values are almost the same for each crystalline structure. At room temperature, the band gap values for anatase and rutile phases are approximately 3.3eV and 3.06eV, respectively (Degussa Corporation, 2005; Zallen et al., 2006). The exact band gap for brookite particles is unknown. However, studies have shown that the band gap for brookite phase is similar to anatase and rutile forms at about 3eV. According to Li et al., (2004), the density of rutile, anatase, and brookite TiO$_2$ structures is 4.26g/cm$^3$, 3.84g/cm$^3$, and 4.11g/cm$^3$, respectively. Therefore, it can be deduced that the physical properties of rutile, anatase, and brookite forms of TiO$_2$ are similar.

Due to their band gap, the TiO$_2$ phases mentioned can only absorb light in the ultraviolet (UV) range. For rutile structures, the absorption band is less than 415nm and for anatase types, the absorption band is less than 385nm (Degussa Corporation, 2005). Rutile phases may absorb rays that are slightly closer to visible light rays. Since the rutile type can absorb light in a wider range, it would be hypothesized that the rutile type is most suitable for use as a photocatalyst. However, studies have demonstrated that the
anatase form exhibits higher photocatalytic activity and stability than the rutile phase (Jagtap et al., 2005; Three Bond Technical News, 2004).

Several theories have been suggested for the better photocatalytic activity and stability of anatase phases of TiO$_2$. In both rutile and anatase types of TiO$_2$, the position of the valence band and the resulting positive holes exhibit sufficient oxidative power. However, the conduction band is positioned near the oxidation-reduction potential of the hydrogen, indicating that both types are relatively weak in terms of reducing power. The conduction band in anatase forms is closer to the negative position than in rutile phases. The difference in the positions of the conduction band allows anatase structures of TiO$_2$ to exhibit stronger photocatalytic activity than rutile phases (Three Bond Technical News, 2004). The stronger photoactivity of anatase particles can also be explained by the longer lifetime of the excited state in anatase phases and the better adsorption of oxygen in anionic form at the anatase structure surface (Degussa Corporation, 2005).

1.4.2 Properties as a Nanoparticle

Typical TiO$_2$ pigments are characterized by a primary particle size of between 200nm and 250nm. In contrast to TiO$_2$ pigments, nanoscaled TiO$_2$ products can be produced at a size of less than 50nm.

It is well known that the average size of TiO$_2$ nanoparticles increases with the increase of synthesis temperatures. A key difference between the brookite phase from rutile and anatase phases is the particle size and surface area. Rutile and anatase phases are synthesized at higher temperatures and result in the production of slightly larger
particles than brookite phase nanoparticles that are synthesized at lower temperatures. An advantage for brookite phase is that the decreased size of the particles and the increased surface area which make up a given weight will lead to the increase in the number of particles represented by the same weight. This means a larger amount of brookite nanoparticles will occupy the same volume than a smaller amount of rutile and anatase particles will occupy and therefore it is hypothesized that the smaller particle size results in an increase in the rate of reaction because the surface area of the brookite catalysts have been increased.

Titanium dioxide nanoparticles are widely studied as semiconductor photocatalysts due to their chemical stability, availability at a reasonable cost, and capability of repeated usage without substantial loss of catalytic activity. Also, TiO$_2$ does not produce hazardous waste neither does it require the addition of consumable chemicals during photocatalysis (Srinivasan et al., 2003).

1.4.3 Properties as a Semiconductor

The path that an electron travels in the atoms is referred to as an orbit. The number of electrons that can occupy one orbit is limited. Electrons in the outermost orbit are referred to as valence electrons (Three Bond Technical News, 2004). Valence electrons are responsible for bonding between elements. When there are a few atoms, the energy values of electrons in orbits are scattered. However, when the number of atoms increases, the values become consistent within a certain range. This range is referred to as an energy band. There is an area between the two energy bands, valence and conduction
bands, where there is no electron energy and this is referred to as the forbidden band. In the bands filled with electrons, the one with the lowest energy level is found in the electron orbit farthest from the nucleus and is referred to as the valence band and the band outside this valence band is the conduction band (Three Bond Technical News, 2004).

The energy width of the forbidden band, between the valence band and the conduction band is referred to as the band gap. The band gap acts as a wall that electrons must leap over in order to become free. The amount of energy required to leap over the wall is referred to as the band gap energy (Three Bond Technical News, 2004). Only electrons that can leap over the wall and enter the conduction band can move freely. As the electrons jump to the conduction band positive holes are created in their place (Three Bond Technical News, 2004).

The valence band of TiO₂ is comprised of the 2p orbital of oxygen, while the conduction band is comprised of the 3d orbital of titanium. In a semiconductor with a large band gap, electrons in the valence band cannot jump up to the conduction band unless energy is applied externally which causes the electrons in the valence band to leap to the conduction band. As a result, electron holes are created in the place of the electrons that move up to the conduction band. This is similar to the concept of movement of electrons from the bonding orbital to the antibonding orbital (Three Bond Technical News, 2004).

The creation of the positive holes affects the photoexcited state of a semiconductor and makes it unstable. However, TiO₂ remains stable even when it is
photoexcited thus, making it an excellent semiconductor (Three Bond Technical News, 2004).

**1.4.4 Properties as a Photocatalyst**

Since the discovery of photocatalytic cleavage of water on TiO$_2$ electrodes by Fujishima and Honda (1972), interest in the photocatalytic properties of TiO$_2$ has increased. Photocatalysis is a reaction that uses light to activate a substance in order to improve the rate of a reaction without being degraded (Three Bond Technical News, 2004).

When light is absorbed by TiO$_2$, two carriers, electrons (e$^-$) and positive holes (h$^+$) are formed. The positive holes exhibit greater decomposing power than the electrons excited to the conduction band (Three Bond Technical News, 2004). The surface of a photocatalyst contains water known as adsorbed water. When the adsorbed water is oxidized by positive holes, hydroxyl radicals (·OH) (**Figure 16**) are formed which have strong oxidizing power and can damage organic material (Srinivasan *et al.*, 2003; Three Bond Technical News, 2004).

If oxygen is present when photocatalysis takes place, the intermediate radicals in the organic compounds and oxygen molecules can undergo chain reactions with the radicals and consume oxygen in some cases. If oxygen is consumed, the organic matter eventually decomposes and releases carbon dioxide and water.
Additionally, the electrons promoted from the valence band to the conduction band are available for transfer and causes the reduction of oxygen in the air. This occurs as a pairing reaction which results in the formation of superoxide anions ($O_2^-$) (Srinivasan et al., 2003; Three Bond Technical News, 2004). Superoxide anions (Figure 16) attach to the intermediate product in the oxidative reactions, forming peroxide or changing to hydrogen peroxide and then to water (Three Bond Technical News, 2004).

The $e^-$ and $h^+$ in TiO$_2$ nanoparticles do not recombine as quickly as they do in other substances. This low percentage of recombination has a major effect on the efficiency of the photocatalytic surface reactions of TiO$_2$ (Three Bond Technical News, 2004).
1.4.5 Ultraviolet Light Activation

In photocatalytic reactions, the band gap energy determines which wavelength of light is most effective (Three Bond Technical News, 2004). The band gap energy of anatase TiO\textsubscript{2} particles is equivalent to a wavelength of 388nm, which implies that anatase phase TiO\textsubscript{2} is a photocatalyst under UV light (Three Bond Technical News, 2004).

Ultraviolet light (Figure 17) is electromagnetic radiation with a wavelength shorter than that of visible light, but longer than soft x-rays. It can be subdivided into near UV (400nm to 200nm) wavelength, far or vacuum UV (200nm to 10nm) wavelength, and extreme or deep UV (31nm to 1nm) wavelength. When considering the effect of UV light radiation on human health and the environment, the range of UV wavelengths is often subdivided into UVA (400nm to 320nm), also called long wave or black light; UVB (320nm to 280nm), also called medium wave light, and UVC (less than 280nm), also called short wave or germicidal light.

![Electromagnetic Spectrum](image)

**Figure 17:** Electromagnetic Spectrum (Gerrity, 2005)
Shorter wavelengths of light have a greater frequency and more energy than longer wavelengths. This means that they release more energy in a shorter time and that their energy is likely to meet or exceed the required activation energy for molecules. Thus, they cause photochemical deterioration to occur more quickly and they are extremely damaging. As wavelengths become longer, toward the red end of the spectrum, they have less energy, lower frequency, and a reduced capacity to excite molecules.

An advantage of TiO₂ photocatalysts is that they do not require UV light that has a high energy level such as 254nm which is used in germicidal lamps. This wavelength of UV light is often used to inhibit bacterial growth and disinfect materials. This wavelength of UV light is utilized because it damages the DNA of living organisms and is thus, also proven to be hazardous to humans. Fortunately, TiO₂ initiates reactions when exposed to near UV light of greater than 350nm with relatively long wavelengths emitted from fluorescent lamps (Srinivasan et al., 2003; Three Bond Technical News, 2004).

1.4.6 Visible Light Activation

The activation of TiO₂ nanoparticles utilizing UV light exposure is not practical for daily usage. Since TiO₂ nanoparticles require UV light exposure to effectively release radicals and anions to destruct organic substances, using TiO₂ nanoparticles without UV light is not effective. Therefore, the utilization of TiO₂ nanoparticles under visible light would allow the nanoparticles to be used for practical applications as coatings on surfaces such as walls and tables or even doorknobs and computer keyboards in hospitals, clinics, or household settings where visible light is already available.
The development of a visible light photocatalyst may be highly desired as an alternative to using UV light, but no substance superior to TiO₂ has yet been discovered. One of the reasons is that a semiconductor with a smaller band gap than that of TiO₂ results in autolysis if it receives light in the presence of water (Three Bond Technical News, 2004).

However, it is believed that under conditions where the positive holes are sufficiently consumed, electrons transferring to oxygen molecules on the reduction side of the photocatalytic reaction determine the outcome of the entire reaction (Three Bond Technical News, 2004). Therefore, by enabling easier transfer of electrons to oxygen molecules, the efficiency of photocatalytic reactions can be improved. This can be done by the addition of metals such as silver, iron, and copper and non-metals such as nitrogen and carbon, which would increase the absorption edge to the visible light region by forming a donor or an acceptor level in the forbidden band (Pan et al., 2006; Umebayashi et al., 2002). Sometimes, the addition of these substances inhibits the recombination of carriers and improves their photocatalytic efficiency by ensuring the stable formation of radicals (Three Bond Technical News, 2004).

According to Machida and colleagues (2005), metal ions such as silver, copper, and zinc have antibacterial capabilities, a phenomenon known as oligodynamic effect. If such metals are coupled with TiO₂, the metal ions would exhibit antibacterial properties in the dark and the TiO₂ photocatalyst would exhibit antibacterial properties under UV light (Machida et al., 2005). Anpo et al., (2001) substituted transition metals such as chromium (Cr³⁺) and vanadium into the lattice of TiO₂ by the ion implantation method.
This shifted the absorption band of Cr\textsuperscript{3+}-TiO\textsubscript{2} to the visible light region of greater than 450 nm. Chromium doped into the TiO\textsubscript{2} lattice was responsible for the visible light photocatalytic activity because of the isolated narrow bands formed in the band gap thus, lowering the photon energy required to excite the electrons (Pan \textit{et al.}, 2006). In most cases, with increased dopant concentration, the absorption edge shifts to a longer wavelength and the band gap energy is reduced. Another research study showed that gold-capped TiO\textsubscript{2} nanocomposites and vanadium-doped TiO\textsubscript{2} nanoparticles exhibit antibacterial activity against \textit{Escherichia coli} (\textit{E. coli}) and \textit{Bacillus megaterium} under indoor ambient light. According to the researcher, this may be due to the increased amount of active sites for catalytic reactions to occur (Fu \textit{et al.}, 2005). Finally, another study released last year indicated that InVO\textsubscript{4}-TiO\textsubscript{2} thin films extended the edge of radiation absorption towards the visible light region (Ge \textit{et al.}, 2006).

1.4.7 Applications

1.4.7.1 White Pigment Properties

Due to its brightness, high refractive index, and white color, TiO\textsubscript{2} provides an excellent reflective optical coating for dielectric mirrors. Titanium dioxide is also an effective opacifier in powder form, where it is employed as a pigment to provide whiteness and opacity to products such as paints, coatings, papers, inks, foods, and most toothpastes (Chen \textit{et al.}, 2006). The pigment is also used in plastics for its UV light-resistant properties where it acts as a UV light absorber (Chen \textit{et al.}, 2006).
Titanium dioxide is also currently being used as a pigment and thickener in skin care products and cosmetics. In many sunscreen lotions with a physical blocker, TiO$_2$ is found because of its refractive index and its resistance to discoloration under UV light. This advantage increases its stability and ability to protect the skin from harmful UV light.

1.4.7.2 Photomineralization

Activation of TiO$_2$ with UV light causes the complete decomposition of phenol, cholorophenols, nitroaromates, aromatic amines, agricultural effluents, and crude oil in water. Due to this TiO$_2$ nanoparticles have been used for outdoor and indoor air and water purification (Chen et al., 2006).

The purification of water by TiO$_2$ is attracting attention from water purification companies because the minerals it produces are harmless to the environment, the process of photomineralization can be turned on and off by activating or not activating with UV light, and there is a possibility that this technology can be incorporated into existing UV light water purification systems (Mill et al., 1993).

1.4.7.3 Photoinduced Hydrophilicity

Various glass products, such as mirrors and glasses, can now be imparted with anti-fogging, self-cleaning, and stain-proof capabilities with TiO$_2$ thin films (Chen et al., 2006). After enough UV light exposure, the TiO$_2$ surface reaches super hydrophilicity, due to the adsorption of water and the occurrence of hydroxylation. In other words, it
ceases to repel water so that the water spreads flat on the surface of the photocatalyst rather than forming a drop. Water contact angles of less than 5° can be measured and such surfaces are considered as being super hydrophilic. This process is reversed in the dark with the surface turning hydrophobic (Degussa Corporation, 2005). The hydrophilic property of TiO₂ also makes it hard for mold to stay on surfaces treated with TiO₂ coatings so surfaces sanitize themselves (Chen et al., 2006).

1.4.7.4 Photoelectrode Properties

Titanium dioxide has the potential for use in energy production. Titanium dioxide is also used as a hydrolysis catalyst in the Graetzel cell, a type of chemical solar cell. When TiO₂ absorbs light, it can convert solar energy into electrical energy which can be used for various energy applications (Chen et al., 2006). As a photocatalyst it carries out hydrolysis to produce hydrogen and oxygen and if the hydrogen were collected, it could be used as a source of fuel. Using TiO₂ nanomaterials as active photoelectrode materials for the production of electricity and/or hydrogen is one of the most important research areas for future clean energy applications (Chen et al., 2006).

1.4.7.5 Photosterilization

The photocatalytic properties can be useful for self-sterilizing materials. Titanium dioxide can also be added to paints, cements, windows, tiles, and other similar products for sterilizing, deodorizing, and anti-fouling purposes (Chen et al., 2006). The hydroxyl
radicals produced kill and decompose bacteria and odor-causing organic compounds as can be concluded from the following studies.

In 1985, Matsunaga et al., reported that *Lactobacillus acidophilus*, *Saccharomyces cerevisiae*, and *E. coli* were sterilized when incubated with platinum-loaded 1mg/mL concentrations of TiO$_2$ nanoparticles under a metal halide lamp irradiated for 60 to 120 minutes. In another study on solar-assisted water disinfection systems, Wei and colleagues (1994) established that irradiation of suspensions of *E. coli* and TiO$_2$ with UV light of about 380nm resulted in the complete killing of cells within minutes. In another study the survival of *E. coli* in a liquid film decreased when exposed to UV light (Srinivasan et al., 2003). These studies demonstrate the adverse effects of free radicals on bacterial viability.

Another study proved that the antibacterial effects of TiO$_2$-coated materials were not a simple bacteriostatic action, but rather a bactericidal action that involves the decomposition of the cell wall (Srinivasan et al., 2003). Regardless of the initial cell concentration, the damage of cells involved two steps, an initial lower rate photokilling step followed by a higher rate photokilling step (Srinivasan et al., 2003). The mechanism of photokilling involves decomposition of the outer membrane by the reactive species and the decomposition of the membrane allows the permeability of reactive species to easily reach the cytoplasmic membrane. When the cytoplasmic membrane is attacked, it leads to the peroxidation of lipids in the membrane. Lipid peroxidation causes structural and functional disorders of the cytoplasmic membrane that causes cell wall decomposition and thus, the loss of cell viability (Srinivasan et al., 2003).
CHAPTER 2
CURRENT RESEARCH RATIONALE

2.1 Overview

In spite of the various antibiotics currently used to treat MRSA infections, antimicrobial resistance is an unavoidable consequence due to the selective pressure of antibiotic exposure. Although the epidemiology of the various MRSA strains may differ, the ability to develop resistance to the antibiotics is the same. Due to the mechanisms by which bacteria develop resistance to antibiotics, other prevention methods such as TiO$_2$ nanoparticles that inhibit viable cell counts by different means than antibiotics, may be used to prevent MRSA infections.

Previous research has been conducted on commercially produced anatase phase TiO$_2$ nanoparticles due to their ease of production, high stability, and small particle size (Kim et al., 2003; Kuhn et al., 2003; Maness et al., 1999). The studies have concluded that UV light activated anatase nanoparticles can successfully inhibit bacterial growth. However, for several studies, there is a lack of appropriate controls and thus, a need for an experimental methodology with appropriate controls.

Due to the difficulty in brookite production, brookite nanoparticles are not commercially available and consequently, there is a lack of research on brookite nanoparticles as antimicrobial agents. However, when produced, brookite nanoparticles are smaller in size than anatase nanoparticles which promote a larger number of brookite nanoparticles to contact the surface of bacterial cells, than anatase nanoparticles. The
primary objective of this study was to evaluate the effects of brookite nanoparticles on various S. aureus strains and to compare the lethality of UV light activated brookite nanoparticles with that of UV light activated anatase nanoparticles, by implementing appropriate controls.

2.2 Research Objectives

Objective I: Evaluate the Antibacterial Effects of Activated Anatase and Brookite Phase Nanoparticles on MRSA

Hypothesis: Due to their larger surface area, brookite phase nanoparticles (diameter = 10nm to 15nm) when compared to anatase phase nanoparticles (diameter = 40nm) provide a higher surface area and approximately three to four times the number of particles per weight than anatase phase nanoparticles, causing an increase in the production of radicals which attack bacterial cells. This difference in surface area implies that brookite phase nanoparticles may inhibit MRSA growth within a lesser amount of time than larger anatase phase nanoparticles at similar concentrations.

Experimental Plan: A free suspension and drop-coated slide bioassay will be utilized to test the antibacterial properties of anatase versus brookite phase nanoparticles at specific time intervals under UV light activation. Appropriate controls that show the effects of UV light alone and nanoparticles alone will also be utilized.

Objective II: Determine the Optimum Concentration of UV Light Irradiated Brookite Phase Nanoparticles that Effectively Reduces Viable Cell Counts within 30 Minutes
**Hypothesis:** Previous studies have indicated that 1mg/mL concentration of anatase phase nanoparticles successfully inhibit bacterial growth within 30 minutes (Maneerat et al., 2005). Therefore, brookite phase nanoparticles may exhibit such antibacterial activities at similar concentrations. Furthermore, brookite phase nanoparticles may be ineffective at extremely high or low concentrations, as are anatase phase nanoparticles (Zhang et al., 2004).

**Experimental Plan:** Various concentrations of brookite phase nanoparticles will be tested via the free suspension and the drop-coated slide bioassays to determine the optimum concentration that will reduce MRSA cell viability when activated with UV light.

**Objective III:** Evaluate the Toxicity of Brookite Phase Nanoparticle Coatings without UV Light Irradiation

**Hypothesis:** When activated under UV light, TiO₂ nanoparticles release hydroxyl radicals which have strong oxidizing power and cause the destruction of organic compounds. Since TiO₂ nanoparticles are photocatalysts, brookite nanoparticles should not exhibit antibacterial effects in the absence of UV light activation.

**Experimental Plan:** To study the activities of non-activated nanoparticles, the free-suspension bioassay will be used without any source of light. Various concentrations of brookite phase nanoparticles will be tested to determine the degree of toxicity as concentrations change.
**Objective IV:** Investigate whether TiO$_2$ nanoparticles need to Undergo Continuous UV Light Activation to Prevent MRSA Cell Growth

**Hypothesis:** Researchers have indicated that TiO$_2$ nanoparticles result in damage to organic compounds due to the active formation and constant release of ·OH radicals and O$_2^-$ ions under UV light exposure (Srinivasan *et al.*, 2003; Three Bond Technical News, 2004). These nanoparticles must be under continuous UV light exposure while in concurrent contact with the object of killing, such as bacterial cells, in order to exhibit a significant amount of reduction in viable cell counts. Thus, the extent of the nanoparticles’ antibacterial effects may not persist without steady UV light exposure.

**Experimental Plan:** The drop-coated slide bioassay will be utilized to ascertain the effects of pre-exposed nanoparticle-coated slides on MRSA cell growth. Post exposure, slides will be incubated for various time periods in order to recover undamaged cells.

**Objective V:** Qualitatively Examine the Morphological Changes in Bacterial Cells Post Treatment with Activated TiO$_2$ Nanoparticles

**Hypothesis:** Past studies have indicated that exposure to UV light activated nanoparticles cause cells to develop abnormal and irregular shaped cells (Amezaga-Madrid *et al.*, 2003; Kuhn *et al.*, 2003). In contrast, untreated cells have uniform composition without any defects. Hence, brookite phase nanoparticles may cause similar damages to bacteria at an optimum concentration and time when activated by UV light.
**Experimental Plan:** Scanning Electron Microscopy (SEM) will be utilized to determine the changes in bacteria shape, structure, and size. Higher magnifications will provide details such as the extent of interactions between bacterial cells and nanoparticles.

**Objective VI:** Determine the Antibacterial Effects of Visible Light Activated TiO$_2$ Nanoparticles on MRSA Viable Cell Counts

**Hypothesis:** In order to exhibit antibacterial properties under visible light (400nm to 700nm), TiO$_2$ nanoparticles must be doped with another element. Since these TiO$_2$ nanoparticles do not contain other elements, visible light activation may not cause any significant changes in the viable cell counts of MRSA.

**Experimental Plan:** A free suspension bioassay will be utilized to test the antibacterial properties of anatase and brookite phase nanoparticles under visible light activation for 8 hours. Appropriate controls that show the effects of visible light activation alone and nanoparticles alone will be utilized.
CHAPTER 3
MATERIALS AND METHODS

3.1 Bacterial Strains

Two gram-negative bacterial strains were utilized to establish the protocols utilized in this study. The wild-type strain, *E. coli* B, was purchased from the American Tissue Culture Collection (ATCC) and is referred to as *E. coli* ATCC 23848. *E. coli* DH10B/pK21 is a mutant strain that was graciously provided by Dr. Glenn Kaatz from Wayne State University School of Medicine. *E. coli* DH10B/pK21 contains the plasmid, pK21, which encodes the *S. aureus* norA1199 gene that is responsible for the efflux of fluoroquinolones from *E. coli* cells (Kaatz et al., 1993).

Three gram-positive bacterial strains, *S. aureus* ATCC 25923, *S. aureus* 1199, and *S. aureus* 1199B, were utilized in the experiments to obtain a brief understanding of the overall effects on MRSA. *S. aureus* ATCC 25923 is a clinical isolate. *Staphylococcus aureus* 1199 is a methicillin- and fluoroquinolone-susceptible isolate, and *S. aureus* 1199B is its methicillin- and ciprofloxacin-resistant derivative (Dickson et al., 2007; Gibbons et al., 2003; Huang et al., 2004, Kaatz et al., 1997). *S. aureus* 1199B has shown resistance to multiple drugs while *S. aureus* 1199 is not an MDR strain. The two strains, *S. aureus* 1199 and *S. aureus* 1199B, were also courteously provided by Dr. Kaatz and were recovered from the blood and cardiac vegetations of rabbits that had experimental endocarditis (Kaatz et al., 1997).
3.2 Culture Media

Stock cultures were stored at -80°C in tryptase soy broth (TSB) (CASO Broth, VWR Scientific International) with 10% glycerol. Media was sterilized by autoclaving at 121°C. Tryptic soy agar (TSA) (CASO Agar, VWR Scientific International) was poured into 100mm X 15mm polystyrene Petri dishes and refrigerated at 4°C until ready for use.

Stock cultures were thawed, isolated on TSA plates using sterile techniques, and incubated at 37°C for 24 hours. When preparing for experiments, a single colony was used to inoculate 10mL of TSB and incubated at 37°C for 24 hours in a shaking incubator. Overnight cultures were utilized for all experiments.

Antibiotic solutions of ampicillin (Sigma-Aldrich) at 25µg/mL concentrations were filter sterilized by a 0.45µm filter and added to autoclaved media after cooling to 45°C. This antibiotic was utilized for the isolation of the mutant, MDR E. coli DH10B/pK21.

3.3 Free Suspensions of Nanoparticles

Two various types of TiO$_2$ photocatalysts were utilized in this study, namely P25 and Br200. Free suspensions of P25 nanoparticles consist of 79% anatase phase and 21% rutile phase and was the commercial source of TiO$_2$ from Degussa Corporation (Coleman et al., 2005; Degussa Corporation, 2005). Degussa’s TiO$_2$ samples are produced by the Aerosil fumed silica process by utilizing TiO$_2$ as a raw material and by utilizing titanium tetrachloride (TiCl$_4$), a high-purity liquid, which is vaporized and mixed with air and hydrogen (Degussa Corporation, 2005). Immediately thereafter, the gases are reacted at
temperatures between 1000ºC and 2400 ºC in a burner leading to the formation of pure and nanoscaled TiO₂ according to the following reaction:

\[ \text{TiCl}_4 + 2\text{H}_2 + \text{O}_2 \rightarrow \text{TiO}_2 + 4\text{HCl} \]

The P25 nanoparticles have a mean diameter of approximately 40nm. The small particle size and high density of approximately 3.71g/cm³ lead to a specific surface area of approximately 56.3m²/g. (Degussa Corporation, 2005). The P25 sample was utilized as a reference for all the experiments performed.

Free suspensions of the Br200 brookite nanoparticles, which consist of 10% rutile phase and 90% brookite phase nanoparticles, were synthesized and also graciously supplied by Dr. Lee’s Lab. The particles were prepared under ambient condition sol (Figure 18) process (Bhave, 2007). Titanium tetrachloride (Sigma Aldrich) was used as the precursor in water with the co-solvent, isopropanol (Alfa Aesar), in high concentrations of hydrochloric acid (HCl) (Alfa Aesar) which was employed as the reaction catalyst. The formed gel mass was peptized and crystallized under refluxing conditions. A refluxing temperature of 83°C with a refluxing time period of 15 hours led to the formation of brookite phase nanoparticles. In the last step, the sample was dried at 100°C, in a vacuum oven (Napco, Model 5831) for a maximum of 20 hours, to remove excess water (Bhave, 2007).
The synthesized brookite nanoparticles have a mean diameter of approximately 10nm to 15nm. The particles have a density of 3.85g/cm$^3$ and a surface area of 156m$^2$/g (Bhave, 2007). Finally, both the P25 and the Br200 samples were calcined in a calcination oven (Thermolyne Oven) at high temperatures of 200°C for 2 hours in order to remove any impurities from the sample and to increase the physical stability and absorbent properties of the sample.

The densities and surface area of the calcined samples were calculated to obtain slightly different values than the uncalcined nanoparticles. The Brunauer-Emmett-Teller surface area and density were determined by nitrogen physisorption at -196 and 200°C (Micromeritics Automated System, Model ASAP 2020). The density and surface area of the calcined P25 sample was 3.63g/cm$^3$ and 57.1m$^2$/g, respectively while the density and
surface area of the calcined Br200 was 3.68g/cm³ and 163m²/g, respectively. Synthesized nanoparticles were stored in sterile plastic vials at room temperature in the dark.

### 3.4 Free Suspension Bioassay

A free suspension bioassay was established in order to evaluate the antibacterial properties of the TiO₂ nanoparticles via cell viability calculations. Bacterial cells were harvested overnight, centrifuged at 7,000xg in a microcentrifuge (Eppendorf Centrifuge, Model 5417R), washed with phosphate buffered saline (PBS) (MP Biomedicals) three times, and resuspended in distilled water. The cells were standardized to 10⁸ colony forming units (CFU) by spectrophotometrical (UV Visible Reading SmartSpec, Model 3000) examination. Preliminary experiments and calculations indicated that an optical density of approximately 0.2 at 600nm equals to roughly 10⁸CFU/mL. Titanium dioxide suspensions in distilled water at concentrations of 100, 10, 1, 0.1, 0.01, and 0.001mg/mL were prepared while exercising sterile techniques.

Six-well polystyrene plates were utilized for the experiments and each well contained a mixture of 6mL TiO₂ suspensions or distilled water plus 10⁸ total cells. Distilled water was used as a medium for exposure with cells in order to observe the effects of UV light alone or visible light alone, without TiO₂ nanoparticles.

The bioassay apparatus (Figure 19) consisted of a microplate mixer (Microplate Genie, Model SI-0400), which exerts an orbit of motion and speed to allow consistent mixing within all microplate wells. The plates were always placed on the mixer during experimentation for constant mixing of nanoparticles and cells.
The experimental apparatus also consisted of a multiple ray lamp (MRL, Model 58; Fisher Scientific) (Figure 19) with various tubes consisting of shortwave germicidal UV light (254nm), longwave unfiltered UV light (365nm), or a cool white light. The energy intensity of the light source utilized was measured and stabilized with a photoradiometer (UVX Digital Radiometer, Model 97-0015-02; Fisher Scientific).

![Figure 19: Free Suspension Bioassay Apparatus](image)

**Figure 19:** Free Suspension Bioassay Apparatus (A) Light Source; (B) Six-well Polystyrene Plate; and (C) Microplate Mixer

In the samples incubated in the dark, the light source was absent and the sample was covered with aluminum foil. Distance between the light source and the six-well polystyrene plate was based on the energy intensity of the light source utilized for each experiment.

The mixtures were exposed vertically to the various sources of light as well as incubated in the dark without any light source as a control. Appropriate dilutions of the sample mixtures (Table 2) were plated uniformly on TSA plates using a spiral plater (Spiral Biotech Autoplate, Model 4000). For UV light activation, appropriate dilutions of the sample mixtures were plated at time intervals of 0, 30, 75, and 120 minutes. For visible light activation, appropriate dilutions of the sample mixtures were plated
uniformly on TSA plates after 4 and 8 hours post activation. Samples were plated in triplicates.

**Table 2:** Treatments via Free Suspension Bioassay

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Exposure Time in Minutes</th>
<th>TiO$_2$ Concentrations in Mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>UVLA</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>UVLA-P25</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>UVLA-Br200</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>P25</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Br200</td>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>

**Control** represents cells incubated in the dark, without UV light exposure, **UVLA** represents cells under UV light exposure without nanoparticles, **UVLA-P25** represents cells exposed to UV light activated P25 nanoparticles, **UVLA-Br200** represents cells exposed to UV light activated Br200 nanoparticles, **P25** represents cells with nanoparticles incubated in the dark, without UV light exposure, and **Br200** represents cells with nanoparticles incubated in the dark, without UV light exposure. All samples were tested at all time points. For Control and UVLA samples, distilled water was utilized as the medium of treatment, instead of nanoparticle suspensions.

The TSA plates were incubated at 37°C for 24 hours. Viable cell counts at various time intervals were obtained by quantifying the number of colonies on TSA plates post 24 hours of incubation. Cell viability calculations were based on respective dilution factors. The experiments were duplicated at least four times to confirm that the results are consistent. Results were plotted in line graphs of time versus cell viability to compare the effects of various treatments at various concentrations. Changes in viable cell counts at various time points were calculated as percentages by the following formula:

\[
\frac{\text{Change in Number of Viable Cells}}{\text{Original Cell Concentration}} \times 100\%
\]
3.5 Drop-Coated Slides of Nanoparticles

Apart from their usage as nanoparticles, TiO\textsubscript{2} photocatalysts can be utilized for coatings on inanimate objects. Studies have shown that TiO\textsubscript{2} coatings are important for microbiologically sensitive environments such as medical and sanitary facilities that have to be kept clean from contamination (Fu et al., 2005; Machida et al., 2005). Therefore, TiO\textsubscript{2} coatings could be utilized on hospital and clinic walls to combat the high rates of MRSA and other bacterial cells. A variety of coating techniques have been developed to produce photocatalytic TiO\textsubscript{2} surfaces. In this study, the drop-coating technique was employed.

Glass slides of size 25mm X 75mm and 1mm in thickness were coated with P25 anatase and Br200 brookite nanoparticles. An inorganic binder was produced with 3 mol of 3-glycidoxypropyl-trimethoxysilane (Gelest) and 5 mol of Tetramethyl orthosilicate (Alfa Aesar). The two solutions were prehydrolyzed with ethanol (Alfa Aesar) at room temperature for 3 hours under continuous stirring. After 5 minutes, acetic acid was added to maintain the pH at 4. The nanoparticles were added to the inorganic solution which was then ultrasonicated for 1 hour and later stirred with a magnetic rod for at least 12 hours. The P25 and Br200 nanoparticle solutions were drop-coated on glass slides in concentrations of 0.0125g/5mL of binder, 0.0375g/5mL of binder, and 0.075g/5mL of binder. These concentrations correspond to 2.5, 7.5, and 15mg/mL. Slides coated with plain binder at similar concentrations were employed as controls. The coatings were then cured at 110°C for 4 hours in a constant temperature drying oven (Baxter Scientific Products, Model DN43). The process of creating drop-coated slides (Figure 20) is
summarized below. Prepared slides were stored at room temperature in the dark until ready to use.

![Figure 20: Titanium Dioxide Slide Coating Process (Bhave, 2007)](image)

### 3.6 Drop-Coated Slide Bioassay

A film analysis bioassay was established in order to evaluate the antibacterial properties of the TiO$_2$-coated slides. Bacterial cells were harvested overnight, centrifuged at 7,000xg in a microcentrifuge, washed with PBS three times, and resuspended in distilled water. The cells were standardized to $10^8$ CFU by spectrophotometrical examination. Preliminary experiments indicated that an optical density of approximately 0.2 at 600nm equals to roughly $10^8$ CFU/mL.

Sterile polystyrene 150mm X 15mm Petri dishes were utilized to hold slides during UV light exposure. The energy intensity of the UV light utilized was measured and stabilized with a photoradiometer.
Various treatments were conducted on the slides that were exposed. Slides were either initially or continuously exposed to UV light for 30 and 60 minutes. An amount of $2 \times 10^5$ cells from the bacterial culture was placed on each slide. The effects of TiO$_2$ nanoparticles alone were determined by placing the slides in the dark without any UV light exposure. All cells were recovered by incubating the slides for 30 minutes in 35mL of PBS. Appropriate dilutions of the sample mixtures (Table 3) were plated uniformly on TSA plates using a spiral plater. Samples were plated in triplicates.

Table 3: Treatments via Drop-Coated Slide Bioassay

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No Exposure in Minutes</th>
<th>Exposure Time in Minutes</th>
<th>TiO$_2$ Concentrations in mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 30 60</td>
<td>30 60 60</td>
<td>2.5 7.5 15</td>
</tr>
<tr>
<td>Binder</td>
<td>0 30 60</td>
<td>30 60 60</td>
<td>2.5 7.5 15</td>
</tr>
<tr>
<td>P25</td>
<td>0 30 60</td>
<td>30 60 60</td>
<td>2.5 7.5 15</td>
</tr>
<tr>
<td>Br200</td>
<td>0 30 60</td>
<td>30 60 60</td>
<td>2.5 7.5 15</td>
</tr>
</tbody>
</table>

*Control* slide represents plain, uncoated slides, *Binder* slide represents slides coated with the inorganic binder, *P25* slide represents slides coated with P25 nanoparticles, and *Br200* slide represents slides coated with Br200 nanoparticles. All samples were tested at all time points. Initial exposure indicates that the slides were pre-exposed to UV light before contact with bacterial cells. Constant exposure indicates that the slides were continuously exposed to UV light while in contact with bacterial cells.

The TSA plates were incubated at 37°C for 24 hours. Viable cell counts at various time intervals were obtained by quantifying the number of colonies on TSA plates post 24 hours of incubation. Cell viability calculations were based on respective dilution factors. The experiments were replicated at least three times to confirm that the results are consistent. Results were plotted in bar graphs to determine the cell viability at various
concentrations and at various time points. Changes in viable cell counts at various time points were calculated as percentages by the following formula:

\[
\frac{\text{Change in Number of Viable Cells}}{\text{Original Cell Concentration}} \times 100\%
\]

3.7 Scanning Electron Microscopy

Scanning electron microscopy was used to evaluate the interactions of TiO\textsubscript{2} nanoparticles with the various cells. Bacterial cells were harvested, centrifuged at 7,000xg in a microcentrifuge, washed with PBS three times, and resuspended in distilled water. The cells were standardized to 10\textsuperscript{9}CFU/mL as described previously. To prepare the cells for imaging, 10mL of the 10\textsuperscript{9}CFU/mL bacterial samples were utilized. In addition, the concentration of TiO\textsubscript{2} nanoparticles varied according to the imaging needs of the samples.

Samples of the untreated cells and treated cells were obtained at various time points (0, 30, and 180 minutes) during the free suspension bioassay. Titanium dioxide nanoparticle suspensions were sonicated at maximum setting for 4 minutes (Banson Ultrasonicator Bath, Model 200) in order to disperse them. The mixtures were centrifuged and after the removal of the supernatants, the samples were fixed with 1mL of Karnowsky’s fixative for 4 hours at 4°C. Karnowsky’s fixative at a pH of 7.2 was prepared by mixing 10mL of 25% glutaraldehyde (Electron Microscopy Sciences), 20mL of 16% formaldehyde (Electron Microscopy Sciences), 50mL of Gomori phosphate buffer (VWR International), and 2.4g of sucrose (VWR International). After 4 hours, the
samples were rinsed three times with PBS. Thereafter, the samples were post fixed with 1mL of 1% osmium tetroxide (Electron Microscopy Sciences) at 4°C. After 6 hours, the samples were dehydrated using an ascending series of 30%, 75%, and 100% ethanol. Following the last wash, the sample was resuspended in 1mL of fresh 100% ethanol. A 100μl droplet of each sample was mounted on 200mesh, formvar coated, carbon stabilized copper grids (Electron Microscopy Sciences). After air drying the grids for 10 minutes the samples were ready for imaging.

High resolution images at various magnifications were derived from the scanning mode of a Hitachi HD2000 Scanning Transmission Electron Microscope System to observe the surface morphology of the bacterial cells and the destruction due to the action of radicals released by activated TiO₂ nanoparticles. It is pertinent to note that images in the same treatment group may not be of the same scale. The images have been presented in order to observe the differences between the damaged and the undamaged cells.

3.8 Statistical Analysis

All subsequent data, for a specified group size (n), are expressed as mean ± standard error. Most graphs are plotted in logarithmic scale to determine the changes in CFU/mL over time. The changes in CFU/ml over time were compared to the original CFU/ml obtained at the beginning of the study. In general, standard errors were too small to be witnessed on logarithmic scale graphs. Statistical analyses of the data were performed by analysis of variance (single factor) and probability values (P) for
significance were calculated using the two-tailed student’s t-test. *P* values of less than 0.05 were considered statistically significant.
CHAPTER 4
RESULTS

4.1 Preliminary Experiments

Previous studies have indicated that a wavelength of 365nm and an energy intensity of 400μw/cm² effectively activates anatase phase TiO₂ nanoparticles (Kim et al., 2003). Preliminary studies were conducted to determine the optimum wavelength and energy of UV light required for the activation of nanoparticles with non-drug-resistant E. coli 23848. At wavelengths of 254nm, viable cell counts were completely reduced within 5 minutes in the absence of TiO₂ nanoparticles. Hence, a longer wavelength of 365nm was utilized for the activation of TiO₂ nanoparticles under UV light, which caused a minimal reduction in the number of viable cells without the usage of TiO₂ nanoparticles. Various experiments were conducted to determine the optimum energy output required to activate nanoparticles. Finally, UV light at an intensity of 370μw/cm² was utilized to establish relevant bioassay protocols. At this energy, UV light alone caused a minimal decrease in viable cell counts during 120 minutes of exposure.

In addition, visible light activation (VLA) of nanoparticles was performed using a cool white light at a wavelength of between 400nm and 700nm. The energy intensity was stabilized at 140μw/cm². At that energy level, visible light activation alone caused a minimal decrease in viable cell counts.
4.2 Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Nanoparticles

4.2.1 *Escherichia coli* ATCC 23848

To compare the effects of UV light activated P25 and Br200 nanoparticles on non-drug-resistant *E. coli* ATCC 23848, various concentrations of 10, 1, and 0.1mg/mL P25 and Br200 nanoparticles were tested via the free suspension bioassay. Ultraviolet light (Figure 21 and 22) alone (UVLA) caused a steady decline in viable cell counts over time. It is important to note that UV light alone caused an approximate one log reduction after 120 minutes of exposure. The decrease from 0 to 30 minutes was not statistically significant (*P* = 0.4321). However, the decrease from 0 to 75 minutes was statistically significant at concentrations of 10mg/mL (*P* = 0.00002068), 1mg/mL (*P* = 0.00004249), and 0.1mg/mL (*P* = 0.0008669). Cells (control) incubated at similar experimental conditions in the dark, away from any light source, did not show any significant increase or decrease in the number of viable cells.

The figure below (Figure 21) is a representation of the Petri dishes that illustrates the difference in colony growth over time in various samples, including the appropriate controls and various concentrations of TiO₂ nanoparticles, containing non-drug-resistant *E. coli* ATCC 23848.
Figure 21: Viable Cell Counts of *Escherichia coli* ATCC 23848 on Petri Dishes at
(A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL

Concentrations of Nanoparticle Suspensions

Samples were plated at 0, 30, 75, and 120 minutes. The following samples were plated: (a) **Control**: Cells incubated in the dark, without UV light exposure; (b) **UVLA**: Cells under UV light exposure without nanoparticles; (c) **UVLA-P25**: Cells exposed to UV light activated P25 nanoparticles; (d) **UVLA-Br200**: Cells exposed to UV light activated Br200 nanoparticles; (e) **P25**: Cells with nanoparticles incubated in the dark, without UV light exposure; and (f) **Br200**: Cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions.
Figure 22: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Nanoparticles on *Escherichia coli* ATCC 23848 at concentrations of (A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL

Samples were plated at 0, 30, 75, and 120 minutes. **Control** represents cells incubated in the dark, without UV light exposure; **UVLA** represents cells under UV light exposure without nanoparticles; **UVLA-P25** represents cells exposed to UV light activated P25 nanoparticles; **UVLA-Br200** represents cells exposed to UV light activated Br200 nanoparticles; **P25** represents cells with nanoparticles incubated in the dark, without UV light exposure; and **Br200** represents cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.
At the concentration of 10mg/mL (Figure 22A), there was a seven log statistically significant reduction in viable cell counts within 30 minutes of contact with UV light activated P25 and Br200 nanoparticles ($P = 0.0001281$ and $P = 0.0001402$, respectively). Similarly, at the concentration of 1mg/mL (Figure 22B), there was a seven log statistically significant reduction in viable cell counts within 30 minutes of contact with UV light activated P25 and Br200 nanoparticles ($P = 0.0002352$ and $P = 0.0006764$, respectively).

However, at the 0.1mg/mL concentration (Figure 22C), it was evident that P25 and Br200 nanoparticles showed different antibacterial effects than at 10 and 1mg/mL concentrations. Within 120 minutes P25 nanoparticles only caused a four log reduction while activated Br200 nanoparticles resulted in a seven log reduction. The activated P25 nanoparticles did not result in a seven log reduction at any time point. However, after 30 minutes activated P25 nanoparticles did show a statistically significant difference in growth ($P = 0.0001574$) as did Br200 nanoparticles after 30 minutes ($P = 0.0003308$).

Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 22) without UV light. At the concentration of 10mg/mL (Figure 22A), non-activated P25 nanoparticles exhibited a one log statistically significant decrease ($P = 0.00006130$) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.0006105$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200
nanoparticles caused a half log statistically significant reduction \((P = 0.001589)\) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes \((P = 0.4993)\). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes \((P = 0.00006129)\) of incubation.

The trends observed at the 10mg/mL concentration were also observed at the 1mg/mL concentration (Figure 22B). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease \((P = 0.0001294)\) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease \((P = 0.001040)\) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction \((P = 0.0009927)\) while while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes \((P = 0.6290)\). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes \((P = 0.0001294)\) of incubation.

Non-activated nanoparticles exhibited similar effects at the concentration of 0.1mg/mL (Figure 22C). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease \((P = 0.0001085)\) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease \((P = 0.00004256)\) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log
statistically significant reduction ($P = 0.001906$) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes ($P = 0.2907$). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes ($P = 0.0001084$) of incubation.

### 4.2.2 *Escherichia coli* DH10B/pK21

To compare the effects of UV light activated P25 and Br200 nanoparticles on MDR *E. coli* DH10B/pK21, various concentrations of 10, 1, and 0.1mg/mL P25 and Br200 nanoparticles were tested via the free suspension bioassay. Ultraviolet light (Figure 23 and 24) alone (UVLA) caused a steady decline in viable cell counts over time. It is important to note that UV light alone caused an approximate one log reduction after 120 minutes of exposure. The decrease from 0 to 30 minutes was not statistically significant ($P = 0.2101$). However, the decrease from 0 to 75 minutes was statistically significant at concentrations of 10mg/mL ($P = 0.004075$), 1mg/mL ($P = 0.0005914$), and 0.1mg/mL ($P = 0.001189$). Cells (control) incubated at similar experimental conditions in the dark, away from any light source, did not show any significant increase or decrease in viable cell counts.

The figure below (Figure 23) is a representation of the Petri dishes that illustrates the difference in colony growth over time in various samples, including the appropriate controls and various concentrations of TiO$_2$ nanoparticles, containing MDR *E. coli* DH10B/pK21.
Figure 23: Viable Cell Counts of *Escherichia coli* DH10B/pK21 on Petri Dishes at
(A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL

Concentrations of Nanoparticle Suspensions

Samples were plated at 0, 30, 75, and 120 minutes. The following samples were plated:
(a) Control: Cells incubated in the dark, without UV light exposure;
(b) UVLA: Cells under UV light exposure without nanoparticles;
(c) UVLA-P25: Cells exposed to UV light activated P25 nanoparticles;
(d) UVLA-Br200: Cells exposed to UV light activated Br200 nanoparticles;
(e) P25: Cells with nanoparticles incubated in the dark, without UV light exposure; and
(f) Br200: Cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions.
Figure 24: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Nanoparticles on *Escherichia coli* DH10B/pK21 at Concentrations of (A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL

Samples were plated at 0, 30, 75, and 120 minutes. **Control** represents cells incubated in the dark, without UV light exposure; **UVLA** represents cells under UV light exposure without nanoparticles; **UVLA-P25** represents cells exposed to UV light activated P25 nanoparticles; **UVLA-Br200** represents cells exposed to UV light activated Br200 nanoparticles; **P25** represents cells with nanoparticles incubated in the dark, without UV light exposure; and **Br200** represents cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.
At the concentration of 10mg/mL (Figure 24A), there was a seven log statistically significant reduction in viable cell counts within 30 minutes of contact with UV light activated P25 and Br200 nanoparticles ($P = 0.0009050$ and $P = 0.0004853$, respectively). Similarly, at the concentration of 1mg/mL (Figure 24B), there was a seven log statistically significant reduction in viable cell counts within 30 minutes of contact with UV light activated P25 and Br200 nanoparticles ($P = 0.0003140$ and $P = 0.0001999$, respectively).

However, at the 0.1mg/mL concentration (Figure 24C), it was evident that P25 and Br200 nanoparticles showed different antibacterial effects than at 10 and 1mg/mL concentrations. Within 120 minutes P25 nanoparticles only caused a four log reduction while activated Br200 nanoparticles resulted in a seven log reduction. The activated P25 nanoparticles did not result in a seven log reduction at any time point. However, after 30 minutes activated P25 nanoparticles did show a statistically significant difference in growth ($P = 0.001175$) as did Br200 nanoparticles after 30 minutes ($P = 0.0001222$).

Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 24) without UV light. At the concentration of 10mg/mL (Figure 24A), non-activated P25 nanoparticles exhibited a one log statistically significant decrease ($P = 0.00009869$) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.0006506$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200
nanoparticles caused a half log statistically significant reduction \( (P = 0.0001567) \) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes \( (P = 0.2622) \). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes \( (P = 0.0001083) \) of incubation.

The trends observed at the 10mg/mL concentration were also observed at the 1mg/mL concentration (Figure 24B). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease \( (P = 0.00005695) \) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease \( (P = 0.0006207) \) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction \( (P = 0.005259) \) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes \( (P = 0.1728) \). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes \( (P = 0.00005695) \) of incubation.

Non-activated nanoparticles exhibited similar effects at the concentration of 0.1mg/mL (Figure 24C). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease \( (P = 0.00005695) \) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease \( (P = 0.002439) \) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log
statistically significant reduction \((P = 0.03314)\) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes \((P = 0.2186)\). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes \((P = 0.0008739)\) of incubation.

### 4.2.3 *Staphylococcus aureus* ATCC 25923

To compare the effects of UV light activated P25 and Br200 nanoparticles on non-drug-resistant *S. aureus* ATCC 25923, various concentrations of 10, 1, and 0.1mg/mL P25 and Br200 nanoparticles were tested via the free suspension bioassay. Ultraviolet light (Figure 25 and 26) alone (UVLA) caused a steady decline in viable cell counts over time. It is important to note that UV light alone caused an approximate one log reduction after 120 minutes of exposure. The decrease from 0 to 30 minutes was not statistically significant \((0.07621)\). However, the decrease from 0 to 75 minutes was statistically significant at concentrations of 10mg/mL \((P = 0.005428)\), 1mg/mL \((P = 0.03766)\), and 0.1mg/mL \((P = 0.002150)\). Cells (control) incubated at similar experimental conditions in the dark, away from any light source, did not show any significant increase or decrease in viable cell counts.

The figure below (Figure 25) is a representation of the Petri dishes that illustrates the difference in colony growth over time in various samples, including the appropriate controls and various concentrations of TiO2 nanoparticles, containing non-drug-resistant *S. aureus* ATCC 25923.
Sample plating was conducted at 0, 30, 75, and 120 minutes. The following samples were plated:

- **Control**: Cells incubated in the dark, without UV light exposure;
- **UVLA**: Cells under UV light exposure without nanoparticles;
- **UVLA-P25**: Cells exposed to UV light activated P25 nanoparticles;
- **UVLA-Br200**: Cells exposed to UV light activated Br200 nanoparticles;
- **P25**: Cells with nanoparticles incubated in the dark, without UV light exposure; and
- **Br200**: Cells with nanoparticles incubated in the dark, without UV light exposure.

For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions.
Figure 26: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Nanoparticles on *Staphylococcus aureus* ATCC 25923 at Concentrations of (A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL

Samples were plated at 0, 30, 75, and 120 minutes. Control represents cells incubated in the dark, without UV light exposure; UVLA represents cells under UV light exposure without nanoparticles; UVLA-P25 represents cells exposed to UV light activated P25 nanoparticles; UVLA-Br200 represents cells exposed to UV light activated Br200 nanoparticles; P25 represents cells with nanoparticles incubated in the dark, without UV light exposure; and Br200 represents cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.
At the concentration of 10mg/mL (Figure 26A), there was a three log statistically significant reduction ($P = 0.0001156$) in viable cell counts within 30 minutes of contact with UV light activated P25 which led to a statistically significant seven log reduction ($P = 0.0001154$) post 75 minutes of exposure. In contrast, Br200 nanoparticles caused a seven log reduction within 30 minutes of exposure which was statistically significant ($P = 0.0002023$).

At the concentration of 1mg/mL (Figure 26B), there was a statistically significant two log reduction ($P = 0.0008179$) in viable cell counts within 30 minutes of contact with UV light activated P25 which led to a seven log reduction after 75 minutes of exposure. Br200 nanoparticles caused a seven log reduction within 30 minutes of exposure which was statistically significant ($P = 0.0003792$) when compared to the original sample.

At the 0.1mg/mL concentration (Figure 26C), it was evident that P25 and Br200 nanoparticles showed different antibacterial effects than at 10 and 1mg/mL concentrations. Within 120 minutes P25 nanoparticles only caused a two log reduction while activated Br200 nanoparticles resulted in a three log reduction. The activated P25 and Br200 nanoparticles did not result in a seven log reduction at any time point. However, after 30 minutes activated P25 nanoparticles did show a statistically significant difference in growth ($P = 0.0004298$) as did Br200 nanoparticles after 30 minutes ($P = 0.0004094$).

Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 26) without UV light. At the
concentration of 10mg/mL (Figure 26A), non-activated P25 nanoparticles exhibited a one log statistically significant decrease ($P = 0.0002713$) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.0006540$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction ($P = 0.02206$) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes ($P = 0.5122$). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes ($P = 0.0001336$) of incubation.

The trends observed at the 10mg/mL concentration were also observed at the 1mg/mL concentration (Figure 26B). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease ($P = 0.00005384$) in cell growth after 120 minutes while Non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.0001252$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction ($P = 0.001706$) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes ($P = 0.06228$). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes ($P = 0.0005429$) of incubation.
Non-activated nanoparticles exhibited similar effects at the concentration of 0.1mg/mL (Figure 26C). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease ($P = 0.001013$) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.0007249$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction ($P = 0.01354$) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes ($P = 0.3295$). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes ($P = 0.0005564$) of incubation.

4.2.4 *Staphylococcus aureus* 1199

To compare the effects of UV light activated P25 and Br200 nanoparticles on non-drug-resistant *S. aureus* 1199, various concentrations of 10, 1, and 0.1mg/mL P25 and Br200 nanoparticles were tested via the free suspension bioassay. Ultraviolet light (Figure 27 and 28) alone (UVLA) caused a steady decline in viable cell counts over time. It is important to note that UV light alone caused an approximate one log reduction after 120 minutes of exposure. The decrease from 0 to 30 minutes was not statistically significant (0.07706). However, the decrease from 0 to 75 minutes was statistically significant at concentrations of 10mg/mL ($P = 0.002199$), 1mg/mL ($P = 0.03044$), and 0.1mg/mL ($P = 0.0003151$). Cells (control) incubated at similar experimental conditions
in the dark, away from any light source, did not show any significant increase or decrease in viable cell counts.

The figure below (Figure 27) is a representation of the Petri dishes that illustrates the difference in colony growth over time in various samples, including the appropriate controls and various concentrations of TiO\textsubscript{2} nanoparticles, containing non-drug-resistant *S. aureus* 1199.

![Figure 27: Viable Cell Counts of *Staphylococcus aureus* 1199 on Petri Dishes at (A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL Concentrations of Nanoparticle Suspensions](image)

Samples were plated at 0, 30 75, and 120 minutes. The following samples were plated: (a) Control: Cells incubated in the dark, without UV light exposure; (b) UVLA: Cells under UV light exposure without nanoparticles; (c) UVLA-P25: Cells exposed to UV light activated P25 nanoparticles; (d) UVLA-Br200: Cells exposed to UV light activated Br200 nanoparticles; (e) P25: Cells with nanoparticles incubated in the dark, without UV light exposure; and (f) Br200: Cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions.
Figure 28: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Nanoparticles on *Staphylococcus aureus* 1199 at Concentrations of 
(A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL

Samples were plated at 0, 30, 75, and 120 minutes. Control represents cells incubated in the dark, without UV light exposure; UVLA represents cells under UV light exposure without nanoparticles; UVLA-P25 represents cells exposed to UV light activated P25 nanoparticles; UVLA-Br200 represents cells exposed to UV light activated Br200 nanoparticles; P25 represents cells with nanoparticles incubated in the dark, without UV light exposure; and Br200 represents cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.
At the concentration of 10mg/mL (Figure 28A), there was a three log statistically significant reduction ($P = 0.0004186$) in viable cell counts within 30 minutes of contact with UV light activated P25 which led to a statistically significant seven log reduction ($P = 0.0004178$) post 75 minutes of exposure. In contrast, Br200 nanoparticles caused a seven log reduction within 30 minutes of exposure which was statistically significant ($P = 0.0001877$).

At the concentration of 1mg/mL (Figure 28B), there was a statistically significant two log reduction ($P = 0.0006872$) in viable cell counts within 30 minutes of contact with UV light activated P25 which led to a seven log reduction after 75 minutes of exposure. Br200 nanoparticles caused a seven log reduction within 30 minutes of exposure which was statistically significant ($P = 0.001402$).

At the 0.1mg/mL concentration (Figure 28C), it was evident that P25 and Br200 nanoparticles showed different antibacterial effects than at 10 and 1mg/mL concentrations. Within 120 minutes P25 nanoparticles only caused a two log reduction while activated Br200 nanoparticles resulted in a three log reduction. The activated P25 and Br200 nanoparticles did not result in a seven log reduction at any time point. However, after 30 minutes activated P25 nanoparticles did show a statistically significant difference in growth ($P = 0.00004064$) as did Br200 nanoparticles after 30 minutes ($P = 0.0004960$).

Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 28) without UV light. At the concentration of 10mg/mL (Figure 28A), non-activated P25 nanoparticles exhibited a
one log statistically significant decrease \((P = 0.0007937)\) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease \((P = 0.0001077)\) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction \((P = 0.003927)\) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes \((P = 0.8632)\). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes \((P = 0.0002654)\) of incubation.

The trends observed at the 10mg/mL concentration were also observed at the 1mg/mL concentration (Figure 28B). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease \((P = 0.0002840)\) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease \((P = 0.001269)\) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction \((P = 0.008369)\) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes \((P = 0.7331)\). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes \((P = 0.0002961)\) of incubation.

Non-activated nanoparticles exhibited similar effects at the concentration of 0.1mg/mL (Figure 28C). Non-activated P25 nanoparticles exhibited a one log
statistically significant decrease ($P = 0.0004390$) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.000004798$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction ($P = 0.00006649$) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes ($P = 0.8521$). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes ($p = 0.0003256$) of incubation.

### 4.2.5 Staphylococcus aureus 1199B

To compare the effects of UV light activated P25 and Br200 nanoparticles on MDR *S. aureus* 1199B, various concentrations of 10, 1, and 0.1mg/mL P25 and Br200 nanoparticles were tested via the free suspension bioassay. Ultraviolet light (Figure 29 and 30) alone (UVLA) caused a steady decline in viable cell counts over time. It is important to note that UV light alone caused an approximate one log reduction after 120 minutes of exposure. The decrease from 0 to 30 minutes was not statistically significant ($P = 0.07823$). However, the decrease from 0 to 75 minutes was statistically significant at concentrations of 10mg/mL ($P = 0.004927$), 1mg/mL ($P = 0.04845$), and 0.1mg/mL ($P = 0.001755$). Cells (control) incubated at similar experimental conditions in the dark, away from any light source, did not show any significant increase or decrease in viable cell counts.
The figure below (Figure 29) is a representation of the Petri dishes that illustrates the difference in colony growth over time in various samples, including the appropriate controls and various concentrations of TiO₂ nanoparticles, containing MDR *S. aureus* 1199B.

**Figure 29:** Viable Cell Counts of *Staphylococcus aureus* 1199B on Petri Dishes

(A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL

Concentrations of Nanoparticle Suspensions

Samples were plated at 0, 30, 75, and 120 minutes. The following samples were plated: (a) **Control:** Cells incubated in the dark, without UV light exposure; (b) **UVLA:** Cells under UV light exposure without nanoparticles; (c) **UVLA-P25:** Cells exposed to UV light activated P25 nanoparticles; (d) **UVLA-Br200:** Cells exposed to UV light activated Br200 nanoparticles; (e) **P25:** Cells with nanoparticles incubated in the dark, without UV light exposure; and (f) **Br200:** Cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions.
**Figure 30:** Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Nanoparticles on *Staphylococcus aureus* 1199B at Concentrations of 

(A) 10mg/mL; (B) 1mg/mL; and (C) 0.1mg/mL

Samples were plated at 0, 30, 75, and 120 minutes. **Control** represents cells incubated in the dark, without UV light exposure; **UVLA** represents cells under UV light exposure without nanoparticles; **UVLA-P25** represents cells exposed to UV light activated P25 nanoparticles; **UVLA-Br200** represents cells exposed to UV light activated Br200 nanoparticles; **P25** represents cells with nanoparticles incubated in the dark, without UV light exposure; and **Br200** represents cells with nanoparticles incubated in the dark, without UV light exposure. For Control and UVLA samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.
At the concentration of 10mg/mL (Figure 30A), there was a three log statistically significant reduction \((P = 0.0001250)\) in viable cell counts within 30 minutes of contact with UV light activated P25 which led to a statistically significant four log reduction \((P = 0.0001248)\) post 75 minutes of exposure and finally caused a seven log reduction in 120 minutes, which was also statistically significant \((P = 0.0001248)\). In contrast, Br200 nanoparticles caused a seven log reduction within 30 minutes of exposure which was statistically significant \((P = 0.0003557)\).

At the concentration of 1mg/mL (Figure 30B), there was a statistically significant two log reduction \((P = 0.00003103)\) in viable cell counts within 30 minutes of contact with UV light activated P25 which led to a statistically significant three log reduction \((P = 0.00003076)\) after 75 minutes of exposure, and finally a complete seven log reduction in viable cell counts, which was statistically significant \((P = 0.00003071)\). Br200 nanoparticles caused a seven log reduction within 30 minutes of exposure which was statistically significant \((P = 0.001040)\).

At the 0.1mg/mL concentration (Figure 30C), it was evident that P25 and Br200 nanoparticles showed different antibacterial effects than at 10 and 1mg/mL concentrations. Within 120 minutes P25 nanoparticles only caused a two log reduction while activated Br200 nanoparticles resulted in a three log reduction. The activated P25 and Br200 nanoparticles did not result in a seven log reduction at any time point. However, after 30 minutes activated P25 nanoparticles did show a statistically significant difference in growth \((P = 0.007128)\) as did Br200 nanoparticles after 30 minutes \((P = 0.001520)\).
Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 30) without UV light. At the concentration of 10mg/mL (Figure 30A), non-activated P25 nanoparticles exhibited a one log statistically significant decrease ($P = 0.00001734$) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.0003354$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction ($P = 0.01079$) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes ($P = 0.6439$). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes ($P = 0.0002375$) of incubation.

The trends observed at the 10mg/mL concentration were also observed at the 1mg/mL concentration (Figure 30B). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease ($P = 0.0001991$) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.0002318$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction ($P = 0.008171$) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes ($P = \ldots$)
0.5399). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes ($P = 0.00007632$) of incubation.

Non-activated nanoparticles exhibited similar effects at the concentration of 0.1mg/mL (Figure 30C). Non-activated P25 nanoparticles exhibited a one log statistically significant decrease ($P = 0.0001250$) in cell growth after 120 minutes while non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.00005513$) in cell growth after 120 minutes. It was evident that non-activated Br200 nanoparticles caused a much larger decrease in viable cell counts than non-activated P25 nanoparticles. Within 30 minutes non-activated Br200 nanoparticles caused a half log statistically significant reduction ($P = 0.04477$) while P25 nanoparticles did not cause a statistically significant reduction within 30 minutes ($P = 0.7762$). In fact, non-activated P25 nanoparticles did not cause a statistically significant reduction until after 120 minutes ($P = 0.0000963$) of incubation.

### 4.3 Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Coatings

#### 4.3.1 *Escherichia coli* ATCC 23848

The effects of UV light activated P25 and Br200 nanoparticles on non-drug-resistant *E. coli* ATCC 23848 were tested via the drop-coated slide bioassay, utilizing slides of 15, 7.5, and 2.5mg/mL concentrations (Figure 31). Uncoated (plain) slides containing cells when exposed to UV light (Figure 31) alone, caused a steady decline in viable cell counts over time. Evidently, UV light alone caused an approximate one log
reduction after 30 minutes of exposure and an approximate two log reduction after 60 minutes of exposure. Both decreases were statistically significant at all concentrations of 15mg/mL ($P = 0.002054$ and $P = 0.001723$, respectively), 7.5mg/mL ($P = 0.0003591$ and $P = 0.0004189$, respectively), and 2.5mg/mL ($P = 0.0006596$ and $P = 0.0006352$, respectively).

Binder slides (Figure 31) containing cells when exposed to UV light did not cause a statistically significant reduction in cell growth after 30 minutes of exposure ($P = 0.43354$) but resulted in almost a one log reduction after 60 minutes of exposure, which was statistically significant at slide concentrations of 15mg/mL ($P = 0.00009274$), 7.5mg/mL ($P = 0.0002270$), and 2.5mg/mL ($P = 0.0002925$). Slides incubated at similar experimental conditions in the dark (Figure 31), away from any light source, did not show any significant increase or decrease in viable cell counts after 30 and 60 minutes.
Figure 31: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Coatings on *Escherichia coli* ATCC 23848 at Concentrations of (A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. **Control** slide represents plain, uncoated slides; **Binder** slide represents slides coated with the inorganic binder; **P25** slide represents slides coated with P25 nanoparticles; and **Br200** slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (**UV LIGHT**) represent samples that were treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 31A), activated P25 and Br200 slides showed the same decrease in viable cell counts as seen at the 7.5 and 2.5mg/mL concentrations. Activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0009152$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0009782$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.001266$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.001258$).

At the concentration of 7.5mg/mL (Figure 31B), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.001364$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.001308$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0009901$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0009841$).

At the concentration of 2.5mg/mL (Figure 31C), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.001094$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.001156$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0005331$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0005289$).
4.3.2 *Escherichia coli* DH10B/pK21

The effects of UV light activated P25 and Br200 nanoparticles on MDR *E. coli* DH10B/pK21 were tested via the drop-coated slide bioassay, utilizing slides of 15, 7.5, and 2.5mg/mL concentrations (Figure 32). Uncoated (plain) slides containing cells when exposed to UV light (Figure 32) alone, caused a steady decline in viable cell counts over time. Evidently, UV light alone caused an approximate one log reduction after 30 minutes of exposure and an approximate two log reduction after 60 minutes of exposure. Both decreases were statistically significant at all concentrations of 15mg/mL (*P* = 0.001873 and *P* = 0.001630, respectively), 7.5mg/mL (*P* = 0.0003867 and *P* = 0.0003943, respectively), and 2.5mg/mL (*P* = 0.002558 and *P* = 0.002302, respectively).

Binder slides (Figure 32) containing cells when exposed to UV light did not cause a statistically significant reduction in cell growth after 30 minutes of exposure (*P* = 0.5321) but resulted in almost a one log reduction after 60 minutes of exposure, which was statistically significant at slide concentrations of 15mg/mL (*P* = 0.001028), 7.5mg/mL (*P* = 0.002275), and 2.5mg/mL (*P* = 0.0004695). Slides incubated at similar experimental conditions in the dark (Figure 32), away from any light source, did not show any significant increase or decrease in viable cell counts after 30 and 60 minutes.
Figure 32: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Coatings on *Escherichia coli* DH10B/pK21 at Concentrations of

(A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. **Control** slide represents plain, uncoated slides; **Binder** slide represents slides coated with the inorganic binder; **P25** slide represents slides coated with P25 nanoparticles; and **Br200** slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (**UV LIGHT**) represent samples that were treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 32A), activated P25 and Br200 slides showed the same decrease in viable cell counts as seen at the 7.5 and 2.5mg/mL concentration. Activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0001738$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0002975$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.000004830$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.000007178$).

At the concentration of 7.5mg/mL (Figure 32B), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0002310$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0002342$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0002191$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0002174$).

At the concentration of 2.5mg/mL (Figure 32C), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.001672$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.001672$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0004261$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0004222$).
4.3.3 *Staphylococcus aureus* ATCC 25923

The effects of UV light activated P25 and Br200 nanoparticles on non-drug-resistant *S. aureus* ATCC 25923 were tested via the drop-coated slide bioassay, utilizing slides of 15, 7.5, and 2.5mg/mL concentrations ([Figure 33](#)). Uncoated (plain) slides containing cells when exposed to UV light ([Figure 33](#)) alone caused a steady decline in viable cell counts over time. Evidently, UV light alone caused an approximate one log reduction after 30 minutes of exposure and an approximate two log reduction after 60 minutes of exposure. Both decreases were statistically significant at all concentrations of 15mg/mL ($P = 0.0001026$ and $P = 0.0001737$, respectively), 7.5mg/mL ($P = 0.0009727$ and $P = 0.0009671$, respectively), and 2.5mg/mL ($P = 0.00001842$ and $P = 0.0001238$, respectively).

Binder slides ([Figure 33](#)) containing cells when exposed to UV light did not cause a statistically significant reduction in cell growth after 30 minutes of exposure ($p=0.3577$) but resulted in almost a one log reduction after 60 minutes of exposure, which was statistically significant at slide concentrations of 15mg/mL ($P = 0.0001408$), 7.5mg/mL ($P = 0.0007286$), and 2.5mg/mL ($P = 0.0002857$). Slides incubated at similar experimental conditions in the dark ([Figure 33](#)), away from any light source, did not show any significant increase or decrease in viable cell counts after 30 minutes and 60 minutes.
Figure 33: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Coatings on *Staphylococcus aureus* ATCC 25923 at Concentrations of (A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. Control slide represents plain, uncoated slides; Binder slide represents slides coated with the inorganic binder; P25 slide represents slides coated with P25 nanoparticles; and Br200 slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (UV LIGHT) represent samples that were treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 33A), activated P25 and Br200 slides showed the same decrease in viable cell counts as seen at the 7.5 and 2.5mg/mL concentration. Activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant \( (P = 0.00003285) \) and a two log reduction after 60 minutes of exposure which was statistically significant \( (P = 0.00004834) \). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant \( (P = 0.0004397) \) and a three log reduction after 60 minutes of exposure which was statistically significant \( (P = 0.0004365) \).

At the concentration of 7.5mg/mL (Figure 33B), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant \( (P = 0.0005458) \) and a two log reduction after 60 minutes of exposure which was statistically significant \( (P = 0.0005384) \). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant \( (P = 0.0004468) \) and a three log reduction after 60 minutes of exposure which was statistically significant \( (P = 0.0004432) \).

At the concentration of 2.5mg/mL (Figure 33C), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant \( (P = 0.0008246) \) and a two log reduction after 60 minutes of exposure which was statistically significant \( (P = 0.0007964) \). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant \( (P = 0.0006490) \) and a three log reduction after 60 minutes of exposure which was statistically significant \( (P = 0.0006397) \).
4.3.4 Staphylococcus aureus 1199

The effects of UV light activated P25 and Br200 nanoparticles on non-drug-resistant S. aureus 1199 were tested via the drop-coated slide bioassay, utilizing slides of 15, 7.5, and 2.5mg/mL concentrations (Figure 34). Uncoated (plain) slides containing cells when exposed to UV light (Figure 34) alone caused a steady decline in viable cell counts over time. Evidently, UV light alone caused an approximate one log reduction after 30 minutes of exposure and an approximate two log reduction after 60 minutes of exposure. Both decreases were statistically significant at all concentrations of 15mg/mL \((P = 0.0005566 \text{ and } P = 0.0004861, \text{ respectively})\), 7.5mg/mL \((P = 0.0003396 \text{ and } P = 0.0004600, \text{ respectively})\), and 2.5mg/mL \((P = 0.0005357 \text{ and } P = 0.0005555, \text{ respectively})\).

Binder slides (Figure 34) containing cells when exposed to UV light did not cause a statistically significant reduction in cell growth after 30 minutes of exposure \((P = 0.05672)\) but resulted in almost a one log reduction after 60 minutes of exposure, which was statistically significant at slide concentrations of 15mg/mL \((P = 0.001276)\), 7.5mg/mL \((P = 0.0001494)\), and 2.5mg/mL \((P = 0.0003594)\). Slides incubated at similar experimental conditions in the dark (Figure 34), away from any light source, did not show any significant increase or decrease in viable cell counts after 30 and 60 minutes.
Figure 34: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Coatings on *Staphylococcus aureus* 1199 at Concentrations of

(A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. Control slide represents plain, uncoated slides; Binder slide represents slides coated with the inorganic binder; P25 slide represents slides coated with P25 nanoparticles; and Br200 slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (UV LIGHT) represent samples that were treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 34A), activated P25 and Br200 slides showed the same decrease in viable cell counts as seen at the 7.5 and 2.5mg/mL concentration. Activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0006371$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0006910$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.001127$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.001114$).

At the concentration of 7.5mg/mL (Figure 34B), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.001561$) and a two log reduction after 60 minutes of exposure which was statistically significant ($p =0.001393$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.001231$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.001216$).

At the concentration of 2.5mg/mL (Figure 34C), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.003555$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.003093$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0001229$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0001225$).
4.3.5 *Staphylococcus aureus* 1199B

The effects of UV light activated P25 and Br200 nanoparticles on MDR *S. aureus* 1199B were tested via the drop-coated slide bioassay, utilizing slides of 15, 7.5, and 2.5mg/mL concentrations (Figure 35). Uncoated (plain) slides containing cells when exposed to UV light (Figure 35) alone caused a steady decline in viable cell counts over time. Evidently, UV light alone caused an approximate one log reduction after 30 minutes of exposure and an approximate two log reduction after 60 minutes of exposure. Both decreases were statistically significant at all concentrations of 15mg/mL (*P* = 0.0007823 and *P* = 0.0007012 respectively), 7.5mg/mL (*P* = 0.001168 and *P* = 0.001048, respectively), and 2.5mg/mL (*P* = 0.002536 and *P* = 0.002180, respectively).

Binder slides (Figure 35) containing cells when exposed to UV light did not cause a statistically significant reduction in cell growth after 30 minutes of exposure (*P* = 0.6520) but resulted in almost a one log reduction after 60 minutes of exposure, which was statistically significant at slide concentrations of 15mg/mL (*P* = 0.001811), 7.5mg/mL (*P* = 0.00007400), and 2.5mg/mL (*P* = 0.0003291). Slides incubated at similar experimental conditions in the dark (Figure 35), away from any light source, did not show any significant increase or decrease in viable cell counts after 30 and 60 minutes.
Figure 35: Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Coatings on *Staphylococcus aureus* 1199B at Concentrations of

(A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. **Control** slide represents plain, uncoated slides; **Binder** slide represents slides coated with the inorganic binder; **P25** slide represents slides coated with P25 nanoparticles; and **Br200** slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (**UV LIGHT**) represent samples that were treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 35A), activated P25 and Br200 slides showed the same decrease in viable cell counts as seen at the 7.5 and 2.5mg/mL concentration. Activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0002560$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0002553$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.001609$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.001590$).

At the concentration of 7.5mg/mL (Figure 35B), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0006068$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0006297$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0001142$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0001147$).

At the concentration of 2.5mg/mL (Figure 35C), activated P25 slides caused a one log reduction after 30 minutes of exposure which was statistically significant ($P = 0.0001292$) and a two log reduction after 60 minutes of exposure which was statistically significant ($P = 0.0001243$). In contrast, activated Br200 slides caused a two log reduction after 30 minutes of exposure which was statistically significant ($P = 0.00005662$) and a three log reduction after 60 minutes of exposure which was statistically significant ($P = 0.00005735$).
4.4 Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Nanoparticles

4.4.1 *Escherichia coli* ATCC 23848

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties 100, 10, 1, 0.1, 0.01, and 0.001mg/mL concentrations (Figure 36) of Br200 nanoparticles were tested against non-drug-resistant *E. coli* ATCC 23848 under UV light via the free suspension bioassay. These concentrations were tested against a control sample of bacterial cells under UV light without Br200 nanoparticles. Under UV light alone (Figure 36) without Br200 nanoparticles, there was an 8.59% reduction within 30 minutes of exposure which was not statistically significant (*P* = 0.06721). However, at 75 minutes there was a statistically significant reduction of 21.41% (*P* = 0.04075) which led to a statistically significant 91.82% decrease (*P* = 0.0002954) after 120 minutes of exposure.
Figure 36: Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Nanoparticles on *Escherichia coli* ATCC 23848

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of UV light activated Br200 nanoparticles were tested and compared with one another and with the effects of UV light exposure (distilled water) alone. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (Figure 36), activated Br200 nanoparticles caused a statistically significant 72.87% reduction ($P = 0.0002970$) after 30 minutes of exposure, a statistically significant 97.28% reduction ($P = 0.001326$) after 75 minutes of exposure, and a statistically significant 99.71% reduction ($P = 0.001415$) after 120 minutes of exposure. At 10 and 1mg/mL concentrations (Figure 36), activated Br200 nanoparticles caused a larger reduction of 100% within 30 minutes of exposure.

At the 0.1mg/mL concentration (Figure 36), there was a gradual decrease of 99.00% within 30 minutes of exposure and 100% within 75 minutes of exposure and 120 minutes of exposure. Even though there wasn’t a complete 100% reduction until 75 minutes, the difference after 30 minutes was statistically significant ($P = 0.001784$). At 0.01 and 0.001mg/mL concentrations (Figure 36), there was no statistically significant
reduction at any time point. The number of cells remained consistent throughout the experiment for 120 minutes.

4.4.2 *Escherichia coli DH*$_{10}$B/pK21

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties 100, 10, 1, 0.1, 0.01, and 0.001mg/mL concentrations (Figure 37) of Br200 nanoparticles were tested against MDR *E. coli DH*$_{10}$B/pK21 under UV light via the free suspension bioassay. These concentrations were tested against a control sample of bacterial cells under UV light without Br200 nanoparticles. Under UV light alone (Figure 37) without Br200 nanoparticles, there was an 8.22% reduction within 30 minutes of exposure which was not statistically significant (*P* = 0.06554). However, at 75 minutes there was a statistically significant reduction of 17.40% (*P* = 0.005914) which led to a statistically significant 91.81% decrease (*P* = 0.0003775) after 120 minutes of exposure.
Figure 37: Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Nanoparticles on *Escherichia coli* DH10B/pK21

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of UV light activated Br200 nanoparticles were tested and compared with one another and with the effects of UV light exposure (distilled water) alone. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (Figure 37), activated Br200 nanoparticles caused a statistically significant 78.10% reduction (*P* = 0.0001249) after 30 minutes of exposure, a statistically significant 96.96% reduction (*P* = 0.001308) after 75 minutes of exposure, and a statistically significant 99.72% reduction (*P* = 0.001415) after 120 minutes of exposure. At 10 and 1mg/mL concentrations (Figure 37), activated Br200 nanoparticles caused a larger reduction of 100% within 30 minutes of exposure.

At the 0.1mg/mL concentration (Figure 37), there was a gradual decrease of 98.97% within 30 minutes of exposure and 100% within 75 minutes of exposure and 120 minutes of exposure. Even though there wasn’t a complete 100% reduction until 75 minutes, the difference after 30 minutes was statistically significant (*P* = 0.001786). At
0.01 and 0.001mg/mL concentrations (Figure 37), there was no statistically significant reduction at any time point. The number of cells remained consistent throughout the experiment for 120 minutes.

### 4.4.3 Staphylococcus aureus ATCC 25923

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties 100, 10, 1, 0.1, 0.01, and 0.001mg/mL concentrations (Figure 38) of Br200 nanoparticles were tested against non-drug-resistant *S. aureus* ATCC 25923 under UV light via the free suspension bioassay. These concentrations were tested against a control sample of bacterial cells under UV light without Br200 nanoparticles. Under UV light alone (Figure 38) without Br200 nanoparticles, there was an 8.22% reduction within 30 minutes of exposure which was not statistically significant ($P = 0.08621$). However, at 75 minutes there was a statistically significant reduction of 17.16% ($P = 0.01037$) which led to a statistically significant 92.23% decrease ($P = 0.0003500$) after 120 minutes of exposure.
**Figure 38:** Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Nanoparticles on *Staphylococcus aureus* ATCC 25923

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of UV light activated Br200 nanoparticles were tested and compared with one another and with the effects of UV light exposure (distilled water) alone. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (**Figure 38**), activated Br200 nanoparticles caused a statistically significant 70.46% reduction ($P = 0.0001631$) after 30 minutes of exposure, a statistically significant 96.88% reduction ($P = 0.001416$) after 75 minutes of exposure, and a statistically significant 98.37% reduction ($P = 0.001448$) after 120 minutes of exposure. At 10 and 1mg/mL concentrations (**Figure 38**), activated Br200 nanoparticles caused a larger reduction of 100% within 30 minutes of exposure.

At the 0.1mg/mL concentration (**Figure 38**), there was a gradual decrease of 96.89% within 30 minutes of exposure, 99.03% within 75 minutes of exposure, and 99.92% within 120 minutes of exposure. Even though there wasn’t a complete 100% reduction, the difference after 30 minutes was statistically significant ($P = 0.001821$). At
0.01 and 0.001mg/mL concentrations (Figure 38), there was no statistically significant reduction at any time point. The number of cells remained consistent throughout the experiment for 120 minutes.

4.4.4 *Staphylococcus aureus* 1199

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties, 100, 10, 1, 0.1, 0.01, and 0.001mg/mL concentrations (Figure 39) of Br200 nanoparticles were tested against non-drug-resistant *S. aureus* 1199 under UV light via the free suspension bioassay. These concentrations were tested against a control sample of bacterial cells under UV light without Br200 nanoparticles. Under UV light alone (Figure 39) without Br200 nanoparticles, there was an 8.46% reduction within 30 minutes of exposure which was not statistically significant ($P = 0.06512$). However, at 75 minutes there was a statistically significant reduction of 20.54% ($P = 0.01348$) which led to a statistically significant 92.12% decrease ($P = 0.0002832$) after 120 minutes of exposure.
Figure 39: Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Nanoparticles on *Staphylococcus aureus* 1199

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of UV light activated Br200 nanoparticles were tested and compared with one another and with the effects of UV light exposure (distilled water) alone. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (Figure 39), activated Br200 nanoparticles caused a statistically significant 69.31% reduction \((P = 0.0002580)\) after 30 minutes of exposure, a statistically significant 97.09% reduction \((P = 0.001457)\) after 75 minutes of exposure, and a statistically significant 98.40% reduction \((P = 0.001443)\) after 120 minutes of exposure. At 10 and 1mg/mL concentrations (Figure 39), activated Br200 nanoparticles caused a larger reduction of 100% within 30 minutes of exposure.

At the 0.1mg/mL concentration (Figure 39), there was a gradual decrease of 97.19% within 30 minutes of exposure, 99.09% within 75 minutes of exposure, and 99.91% within 120 minutes of exposure. Even though there wasn’t a complete 100% reduction, the difference after 30 minutes was statistically significant \((P = 0.001848)\). At
0.01 and 0.001mg/mL concentrations (Figure 39), there was no statistically significant reduction at any time point. The number of cells remained consistent throughout the experiment for 120 minutes.

4.4.5 *Staphylococcus aureus* 1199B

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties, 100, 10, 1, 0.1, 0.01, and 0.001mg/mL concentrations (Figure 40) of Br200 nanoparticles were tested against MDR *S. aureus* 1199B under UV light via the free suspension bioassay. These concentrations were tested against a control sample of bacterial cells under UV light without Br200 nanoparticles. Under UV light alone (Figure 40) without Br200 nanoparticles, there was a 7.92% reduction within 30 minutes of exposure which was not statistically significant ($P = 0.07231$). However, at 75 minutes there was a statistically significant reduction of 17.40% ($P = 0.005914$) which led to a statistically significant 92.54% decrease ($P = 0.0004464$) after 120 minutes of exposure.
Figure 40: Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Nanoparticles on *Staphylococcus aureus* 1199B

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of UV light activated Br200 nanoparticles were tested and compared with one another and with the effects of UV light exposure (*distilled water*) alone. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (*Figure 40*), activated Br200 nanoparticles caused a statistically significant 74.31% reduction (*P* = 0.0001328) after 30 minutes of exposure, a statistically significant 97.09% reduction (*P* = 0.001420) after 75 minutes of exposure, and a statistically significant 98.50% reduction (*P* = 0.001448) after 120 minutes of exposure. At 10 and 1mg/mL concentrations (*Figure 40*), activated Br200 nanoparticles caused a larger reduction of 100% within 30 minutes of exposure.

At the 0.1mg/mL concentration (*Figure 40*), there was a gradual decrease of 96.73% within 30 minutes of exposure, 99.13% within 75 minutes of exposure, and 99.92% within 120 minutes of exposure. Even though there wasn’t a complete 100% reduction, the difference after 30 minutes was statistically significant (*P* = 0.001844). At 0.01 and 0.001mg/mL concentrations (*Figure 40*), there was no statistically significant
reduction at any time point. The number of cells remained consistent throughout the experiment for 120 minutes.

4.5 Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Coatings

4.5.1 *Escherichia coli* ATCC 23848

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties, 15, 7.5, and 2.5mg/mL concentrations (Figure 41) of Br200 slides were also tested against non-drug-resistant *E. coli* ATCC 23848 under UV light via the drop-coated slide bioassay. These concentrations were tested against a control slide of bacterial cells under UV light without any Br200 nanoparticle coatings. On the uncoated slides, under UV light alone (Figure 41), there was a 90.65% reduction after 30 minutes and a 99.06% reduction after 60 minutes, both of which were statistically significant ($P = 0.0003591$ and $P = 0.0004189$, respectively).
Figure 41: Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Coatings on *Escherichia coli* ATCC 23848

Samples were plated at 0, 30, and 60 minutes. Various concentrations of Br200 nanoparticle slide coatings were tested and compared with one another and with the effects of the plain control slides under UV light activation without any coatings. The data represents the mean ± standard error of 3 experiments.

As noted on the slides, there was a similar cell growth reduction in the 15 and 7.5mg/mL concentration slides. On the 15mg/mL concentration slide (Figure 41), there was a 99.62% reduction after 30 minutes of exposure and a 99.96% reduction after 60 minutes of exposure. Both of which were statistically significant reductions ($P = 0.001266$ and $P = 0.001258$, respectively). Similarly, on the 7.5mg/mL concentration slide (Figure 41), there was a 99.54% reduction after 30 minutes of exposure and a 99.96% reduction after 60 minutes of exposure. Both of these values were also statistically significant ($P = 0.0009900$ and $P = 0.0009841$, respectively).

On the 2.5mg/mL concentration slide (Figure 41), there was a slightly lower decrease noted than in the 15 and 7.5mg/mL concentration slides. However, the decrease was still statistically significant. Within 30 minutes of exposure, there was a 99.35%
decrease ($P = 0.0005331$) and within 60 minutes of exposure, there was a 99.94% decrease ($P = 0.0005289$) in viable cell counts.

4.5.2 *Escherichia coli* DH10B/pK21

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties, 15, 7.5, and 2.5mg/mL concentrations (Figure 42) of Br200 slides were also tested against MDR *E. coli* DH10B/pK21 under UV light via the drop-coated slide bioassay. These concentrations were tested against a control slide of bacterial cells under UV light without any Br200 nanoparticle coatings. On the uncoated slides, under UV light alone (Figure 42), there was a 90.66% reduction after 30 minutes and a 99.08% reduction after 60 minutes, both of which were statistically significant ($P = 0.0003791$ and $P = 0.0004189$, respectively).
Figure 42: Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Coatings on *Escherichia coli* DH10B/pK21

Samples were plated at 0, 30, and 60 minutes. Various concentrations of Br200 nanoparticle slide coatings were tested and compared with one another and with the effects of the plain control slides under UV light activation without any coatings. The data represents the mean ± standard error of 3 experiments.

As noted on the slides, there was a similar cell growth reduction in the 15 and 7.5mg/mL concentration slides. On the 15mg/mL concentration slide (Figure 42), there was a 99.56% reduction after 30 minutes of exposure and a 99.95% reduction after 60 minutes of exposure. Both of which were statistically significant reductions ($P = 0.000004830$ and $P = 0.000007178$, respectively). Similarly, on the 7.5mg/mL concentration slide (Figure 42), there was a 99.54% reduction after 30 minutes of exposure and a 99.96% reduction after 60 minutes of exposure. Both of these values were also statistically significant ($P = 0.0002191$ and $P = 0.0002174$, respectively).

On the 2.5mg/mL concentration slide (Figure 42), there was a slightly lower decrease noted than in the 15 and 7.5mg/mL concentration slides. However, the decrease was still statistically significant. Within 30 minutes of exposure, there was a 99.35%
decrease ($P = 0.0004261$) and within 60 minutes of exposure, there was a 99.94% decrease ($P = 0.0004222$) in viable cell counts.

### 4.5.3 *Staphylococcus aureus ATCC 25923*

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties, 15, 7.5, and 2.5mg/mL concentrations (Figure 43) of Br200 slides were also tested against non-drug-resistant *S. aureus ATCC 25923* under UV light via the drop-coated slide bioassay. These concentrations were tested against a control slide of bacterial cells under UV light without any Br200 nanoparticle coatings. On the uncoated slides, under UV light alone (Figure 43), there was a 91.06% reduction after 30 minutes and a 99.05% reduction after 60 minutes, both of which were statistically significant ($P = 0.0004763$ and $P = 0.0004197$, respectively).
Figure 43: Antibacterial Effects of Various Concentrations of Ultraviolet Activated Light Brookite Coatings on *Staphylococcus aureus* ATCC 25923

Samples were plated at 0, 30, and 60 minutes. Various concentrations of Br200 nanoparticle slide coatings were tested and compared with one another and with the effects of the plain control slides under UV light activation without any coatings. The data represents the mean ± standard error of 3 experiments.

As noted on the slides, there was a similar cell growth reduction in the 15 and 7.5mg/mL concentration slides. On the 15mg/mL concentration slide (Figure 43), there was a 99.34% reduction after 30 minutes of exposure and a 99.94% reduction after 60 minutes of exposure. Both of which were statistically significant reductions ($P = 0.0004397$ and $P = 0.0004365$, respectively). Similarly, on the 7.5mg/mL concentration slide (Figure 43), there was a 99.37% reduction after 30 minutes of exposure and a 99.94% reduction after 60 minutes of exposure. Both of these values were also statistically significant ($P = 0.0004468$ and $P = 0.0004433$, respectively).

On the 2.5mg/mL concentration slide (Figure 43), there was a slightly lower decrease noted than in the 15 and 7.5mg/mL concentration slides. However, the decrease was still statistically significant. Within 30 minutes of exposure, there was a 99.17%
decrease ($P = 0.0006490$) and within 60 minutes of exposure, there was a 99.92% decrease ($P = 0.0006397$) in viable cell counts.

4.5.4 *Staphylococcus aureus 1199*

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties, 15, 7.5, and 2.5mg/mL concentrations (*Figure 44*) of Br200 slides were also tested against non-drug-resistant *S. aureus* 1199 under UV light via the drop-coated slide bioassay. These concentrations were tested against a control slide of bacterial cells under UV light without any Br200 nanoparticle coatings. On the uncoated slides, under UV light alone (*Figure 44*), there was a 90.12% reduction after 30 minutes and a 99.06% reduction after 60 minutes, both of which were statistically significant ($P = 0.0003544$ and $P = 0.0004182$, respectively).
Samples were plated at 0, 30, and 60 minutes. Various concentrations of Br200 nanoparticle slide coatings were tested and compared with one another and with the effects of the plain control slides under UV light activation without any coatings. The data represents the mean ± standard error of 3 experiments.

As noted on the slides, there was a similar cell growth reduction in the 15 and 7.5mg/mL concentration slides. On the 15mg/mL concentration slide (Figure 44), there was a 99.32% reduction after 30 minutes of exposure and a 99.93% reduction after 60 minutes of exposure. Both of which were statistically significant reductions ($P = 0.001127$ and $P = 0.001114$, respectively). Similarly, on the 7.5mg/mL concentration slide (Figure 44), there was a 99.32% reduction after 30 minutes of exposure and a 99.94% reduction after 60 minutes of exposure. Both of these values were also statistically significant ($P = 0.001231$ and $P = 0.001216$, respectively).

On the 2.5mg/mL concentration slide (Figure 44), there was a slightly lower decrease noted than in the 15 and 7.5mg/mL concentration slides. However, the decrease was still statistically significant. Within 30 minutes of exposure, there was a 99.11%
decrease ($P = 0.0001229$) and within 60 minutes of exposure, there was a 99.92% decrease ($P = 0.0001225$) in viable cell counts.

4.5.5 *Staphylococcus aureus* 1199B

To determine the minimum concentration at which activated Br200 nanoparticles exhibit antibacterial properties, 15, 7.5, and 2.5mg/mL concentrations ([Figure 45](#)) of Br200 slides were also tested against MDR *S. aureus* 1199B under UV light via the drop-coated slide bioassay. These concentrations were tested against a control slide of bacterial cells under UV light without any Br200 nanoparticle coatings. On the uncoated slides, under UV light alone ([Figure 45](#)), there was a 90.66% reduction after 30 minutes and a 99.06% reduction after 60 minutes, both of which were statistically significant ($P = 0.0003590$ and $P = 0.0004288$, respectively).
Figure 45: Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Coatings on Staphylococcus aureus 1199B

Samples were plated at 0, 30, and 60 minutes. Various concentrations of Br200 nanoparticle slide coatings were tested and compared with one another and with the effects of the plain control slides under UV light activation without any coatings. The data represents the mean ± standard error of 3 experiments.

As noted on the slides, there was a similar cell growth reduction in the 15 and 7.5mg/mL concentration slides. On the 15mg/mL concentration slide (Figure 45), there was a 99.32% reduction after 30 minutes of exposure and a 99.94% reduction after 60 minutes of exposure. Both of which were statistically significant reductions ($P = 0.001609$ and $P = 0.001590$, respectively). Similarly, on the 7.5mg/mL concentration slide (Figure 45), there was a 99.35% reduction after 30 minutes of exposure and a 99.94% reduction after 60 minutes of exposure. Both of these values were also statistically significant ($P = 0.0001142$ and $P = 0.0001147$, respectively).

On the 2.5mg/mL concentration slide (Figure 45), there was a slightly lower decrease noted than in the 15 and 7.5mg/mL concentration slides. However, the decrease was still statistically significant. Within 30 minutes of exposure, there was a 99.23%
decrease ($P = 0.00005662$) and within 60 minutes of exposure, there was a 99.92% decrease ($P = 0.00005735$) in viable cell counts.

4.6 Toxic Effects of Non-Activated Brookite Nanoparticles

4.6.1 *Escherichia coli* ATCC 23848

To examine the potency of non-activated Br200 nanoparticles at various concentrations of 100, 10, 1, 0.1, 0.01, and 0.001mg/mL (Figure 46), the free suspension bioassay was utilized against non-drug-resistant *E. coli* ATCC 23848. The various concentrations of nanoparticles were tested against a control sample of bacterial cells in the dark, without any light source, by substituting nanoparticles with distilled water. In the dark (Figure 46), without Br200 nanoparticles, there was no statistically significant reduction at any time point. The number of cells remained constant throughout the experiment for 120 minutes.
Figure 46: Toxic Effects of Non-Activated Brookite Nanoparticles on *Escherichia coli* ATCC 23848

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of non-activated Br200 nanoparticles were tested and compared with one another and with the effects of cells incubated in the dark (distilled water) without any UV light exposure. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (Figure 46), non-activated Br200 nanoparticles caused a statistically significant reduction of 70.76% (*P* = 0.0001913) within 30 minutes, a statistically significant reduction of 97.15% (*P* = 0.001472) within 75 minutes, and a statistically significant reduction of 99.73% (*P* = 0.001415) within 120 minutes. At the 10mg/mL concentration (Figure 46), non-activated Br200 nanoparticles demonstrated lower death rates but similar trends. Within 30 minutes there was a statistically significant reduction of 56.07% (*P* = 0.0007662) in cell growth, within 75 minutes there was a statistically significant reduction of 79.20% (*P* = 0.0009387) in cell growth, and
within 120 minutes there was a statistically significant reduction of 99.08% ($P = 0.001406$) in cell growth.

At the 1mg/mL concentration (Figure 46), non-activated Br200 nanoparticles presented an even lower death rate but similar trends. After 30 minutes, there was a statistically significant reduction of 39.80% ($P = 0.001522$), after 75 minutes, there was a statistically significant reduction of 52.45% ($P = 0.001224$), and after 120 minutes, there was a statistically significant reduction of 98.66% ($P = 0.001309$).

At the 0.1mg/mL concentration (Figure 46), non-activated Br200 nanoparticles resulted in a statistically significant decrease of 24.77% ($P = 0.01346$) post 30 minutes of contact, a statistically significant decrease of 43.64% ($P = 0.003478$) post 75 minutes of contact, and a statistically significant decrease of 97.55% ($P = 0.001696$) post 120 minutes of contact. At 0.01 and 0.001mg/mL concentrations (Figure 46), non-activated Br200 nanoparticles did not cause any significant changes in viable cell counts over time.

### 4.6.2 *Escherichia coli* DH10B/pK21

To examine the potency of non-activated Br200 nanoparticles at various concentrations of 100, 10, 1, 0.1, 0.01, and 0.001mg/mL (Figure 47), the free suspension bioassay was utilized against MDR *E. coli* DH10B/pK21. The various concentrations of nanoparticles were tested against a control sample of bacterial cells incubated in the dark, without any light source, by substituting nanoparticles with distilled water. In the dark (Figure 47), without Br200 nanoparticles, there was no statistically significant reduction
at any time point. The number of cells remained constant throughout the experiment for 120 minutes.

**Figure 47:** Toxic Effects of Non-Activated Brookite Nanoparticles on *Escherichia coli DH*$_{10}$B/pK21

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of non-activated Br200 nanoparticles were tested and compared with one another and with the effects of cells incubated in the dark (distilled water) without any UV light exposure. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (**Figure 47**), non-activated Br200 nanoparticles caused a statistically significant reduction of 72.14% ($P = 0.0002688$) within 30 minutes, a statistically significant reduction of 97.61% ($P = 0.001358$) within 75 minutes, and a statistically significant reduction of 99.76% ($P = 0.001414$) within 120 minutes. At the 10mg/mL concentration (**Figure 47**), non-activated Br200 nanoparticles demonstrated lower death rates but similar trends. Within 30 minutes there was a statistically
significant reduction of 55.47% \((P = 0.0009584)\) in cell growth, within 75 minutes there was a statistically significant reduction of 76.99% \((P = 0.0003103)\) in cell growth, and within 120 minutes there was a statistically significant reduction of 98.90% \((P = 0.001395)\) in cell growth.

At the 1mg/mL concentration (Figure 47), non-activated Br200 nanoparticles presented an even lower death rate but similar trends. After 30 minutes, there was a statistically significant reduction of 35.59% \((P = 0.003285)\), after 75 minutes, there was a statistically significant reduction of 56.30% \((P = 0.0003380)\), and after 120 minutes, there was a statistically significant reduction of 98.79% \((P = 0.001303)\).

At the 0.1mg/mL concentration (Figure 47), non-activated Br200 nanoparticles resulted in a statistically significant decrease of 25.38% \((P = 0.02178)\) post 30 minutes of contact, a statistically significant decrease of 37.34% \((P = 0.008181)\) post 75 minutes of contact, and a statistically significant decrease of 97.84% \((P = 0.001807)\) post 120 minutes of contact. At 0.01 and 0.001mg/mL concentrations (Figure 47), non-activated Br200 nanoparticles did not cause any significant changes in viable cell counts over time.

**4.6.3 Staphylococcus aureus ATCC 25923**

To examine the potency of non-activated Br200 nanoparticles at various concentrations of 100, 10, 1, 0.1, 0.01, and 0.001mg/mL (Figure 48), the free suspension bioassay was utilized against non-drug-resistant S. aureus ATCC 25923. The various concentrations of nanoparticles were tested against a control sample of bacterial cells incubated in the dark, without any light source, by substituting nanoparticles with
distilled water. In the dark (Figure 48), without Br200 nanoparticles, there was no statistically significant reduction at any time point. The number of cells remained constant throughout the experiment for 120 minutes.

![Graph](image.png)

**Figure 48:** Toxic Effects of Non-Activated Brookite Nanoparticles on *Staphylococcus aureus* ATCC 25923

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of non-activated Br200 nanoparticles were tested and compared with one another and with the effects of cells incubated in the dark (distilled water) without any UV light exposure. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (Figure 48), non-activated Br200 nanoparticles caused a statistically significant reduction of 72.86% ($P = 0.0001934$) within 30 minutes, a statistically significant reduction of 97.38% ($P = 0.001451$) within 75 minutes, and a statistically significant reduction of 98.36% ($P = 0.001445$) within 120 minutes. At the 10mg/mL concentration (Figure 48), non-activated Br200 nanoparticles demonstrated
lower death rates but similar trends. Within 30 minutes there was a statistically significant reduction of 21.64% \((P = 0.0231)\) in cell growth, within 75 minutes there was a statistically significant reduction of 36.40% \((P = 0.006410)\) in cell growth, and within 120 minutes there was a statistically significant reduction of 97.94% \((P = 0.001448)\) in cell growth.

At the 1mg/mL concentration (Figure 48), non-activated Br200 nanoparticles presented an even lower death rate but similar trends. After 30 minutes, there was a statistically significant reduction of 19.16% \((P = 0.01790)\), after 75 minutes, there was a statistically significant reduction of 36.52% \((P = 0.006462)\), and after 120 minutes, there was a statistically significant reduction of 97.30% \((P = 0.001303)\).

At the 0.1mg/mL concentration (Figure 48), non-activated Br200 nanoparticles resulted in a statistically significant decrease of 17.80% \((P = 0.03178)\) post 30 minutes of contact, a statistically significant decrease of 32.00% \((P = 0.007193)\) post 75 minutes of contact, and a statistically significant decrease of 97.26% \((P = 0.001783)\) post 120 minutes of contact. At 0.01 and 0.001mg/mL concentrations (Figure 48), non-activated Br200 nanoparticles did not cause any significant changes in viable cell counts over time.

4.6.4 Staphylococcus aureus 1199

To examine the potency of non-activated Br200 nanoparticles at various concentrations of 100, 10, 1, 0.1, 0.01, and 0.001mg/mL (Figure 49), the free suspension bioassay was utilized against non-drug-resistant \textit{S. aureus} 1199. The various concentrations of nanoparticles were tested against a control sample of bacterial cells
incubated in the dark, without any light source, by substituting nanoparticles with distilled water. In the dark (Figure 49), without Br200 nanoparticles, there was no statistically significant reduction at any time point. The number of cells remained constant throughout the experiment for 120 minutes.

Figure 49: Toxic Effects of Non-Activated Brookite Nanoparticles on Staphylococcus aureus 1199

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of non-activated Br200 nanoparticles were tested and compared with one another and with the effects of cells incubated in the dark (distilled water) without any UV light exposure. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (Figure 49), non-activated Br200 nanoparticles caused a statistically significant reduction of 70.22% ($P = 0.0001789$) within 30 minutes, a statistically significant reduction of 97.09% ($P = 0.001485$) within 75 minutes, and a statistically significant reduction of 98.42% ($P = 0.001446$) within 120 minutes. At the
10mg/mL concentration (Figure 49), non-activated Br200 nanoparticles demonstrated lower death rates but similar trends. Within 30 minutes there was a statistically significant reduction of 23.85% \((P = 0.008689)\) in cell growth, within 75 minutes there was a statistically significant reduction of 38.43% \((P = 0.002238)\) in cell growth, and within 120 minutes there was a statistically significant reduction of 97.61% \((P = 0.001426)\) in cell growth.

At the 1mg/mL concentration (Figure 49), non-activated Br200 nanoparticles presented an even lower death rate but similar trends. After 30 minutes, there was a statistically significant reduction of 19.65% \((P = 0.01450)\), after 75 minutes, there was a statistically significant reduction of 37.26% \((P = 0.001660)\), and after 120 minutes, there was a statistically significant reduction of 97.71% \((P = 0.001301)\).

At the 0.1mg/mL concentration (Figure 49), non-activated Br200 nanoparticles resulted in a statistically significant decrease of 19.24% \((P = 0.04026)\) post 30 minutes of contact, a statistically significant decrease of 38.30% \((P = 0.007884)\) post 75 minutes of contact, and a statistically significant decrease of 97.34% \((P = 0.001845)\) post 120 minutes of contact. At 0.01 and 0.001mg/mL concentrations (Figure 49), non-activated Br200 nanoparticles did not cause any significant changes in viable cell counts over time.

**4.6.5 Staphylococcus aureus 1199B**

To examine the potency of non-activated Br200 nanoparticles at various concentrations of 100, 10, 1, 0.1, 0.01, and 0.001mg/mL (Figure 50), the free suspension bioassay was utilized against MDR \(S.\) aureus 1199B. The various concentrations of
nanoparticles were tested against a control sample of bacterial cells incubated in the dark, without any light source, by substituting nanoparticles with distilled water. In the dark (Figure 50), without Br200 nanoparticles, there was no statistically significant reduction at any time point. The number of cells remained constant throughout the experiment for 120 minutes.

![Figure 50: Toxic Effects of Non-Activated Brookite Nanoparticles on Staphylococcus aureus 1199B](image)

Samples were plated at 0, 30, 75, and 120 minutes. Various concentrations of non-activated Br200 nanoparticles were tested and compared with one another and with the effects of cells incubated in the dark (distilled water) without any UV light exposure. The data represents the mean ± standard error of 3 experiments.

At the 100mg/mL concentration (Figure 50), non-activated Br200 nanoparticles caused a statistically significant reduction of 70.10% ($P = 0.0001938$) within 30 minutes, a statistically significant reduction of 96.85% ($P = 0.001441$) within 75 minutes, and a
statistically significant reduction of 98.43% ($P = 0.001448$) within 120 minutes. At the 10mg/mL concentration (Figure 50), non-activated Br200 nanoparticles demonstrated lower death rates but similar trends. Within 30 minutes there was a statistically significant reduction of 21.88% ($P = 0.01492$) in cell growth, within 75 minutes there was a statistically significant reduction of 39.21% ($P = 0.001859$) in cell growth, and within 120 minutes there was a statistically significant reduction of 97.21% ($P = 0.001287$) in cell growth.

At the 1mg/mL concentration (Figure 50), non-activated Br200 nanoparticles presented an even lower death rate but similar trends. After 30 minutes, there was a statistically significant reduction of 15.87% ($P = 0.02847$), after 75 minutes, there was a statistically significant reduction of 37.63% ($P = 0.001744$), and after 120 minutes, there was a statistically significant reduction of 97.60% ($P = 0.001340$).

At the 0.1mg/mL concentration (Figure 50), non-activated Br200 nanoparticles resulted in a statistically significant decrease of 18.52% ($P = 0.03277$) post 30 minutes of contact, a statistically significant decrease of 35.06% ($P = 0.003346$) post 75 minutes of contact, and a statistically significant decrease of 97.40% ($P = 0.001836$) post 120 minutes of contact. At 0.01 and 0.001mg/mL concentrations (Figure 50), non-activated Br200 nanoparticles did not cause any significant changes in viable cell counts over time.
4.7. Antibacterial Effects of Pre-Ultraviolet Light Exposed Coatings

4.7.1 *Escherichia coli* ATCC 23848

To ascertain the effects of UV light on nanoparticles and whether continuous activation with UV light is required in order for nanoparticles to exhibit antibacterial properties, the drop-coated slide bioassay was employed. Various concentrations of drop-coated slides were tested, namely 15, 7.5, and 2.5mg/mL (Figure 51) against non-drug-resistant *E. coli* ATCC 23848.

As controls, various slides containing bacterial samples were incubated in the dark, without any UV light activation for 0, 30, and 60 minutes. The viable cell counts did not change on the unexposed slides (Figure 51) as there was no significant increase or decrease noted after recovery. In addition, as observed (Figure 51) there was no significant change on the control and binder when they were pre-exposed to UV light for 30 minutes. When exposed for a longer amount of time for 60 minutes (Figure 51) there was still no difference on the control and binder slides.
Figure 51: Antibacterial Effects of Pre-Ultraviolet Light Exposed Coatings on *Escherichia coli* ATCC 23848 at Concentrations of

(A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. Control slide represents plain, uncoated slides; Binder slide represents slides coated with the inorganic binder P25 slide represents slides coated with P25 nanoparticles; and Br200 slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (UV LIGHT) represent samples that were pre-treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 51A), there was no statistically significant decrease in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.3662$) and 60 minutes ($P = 0.5650$). Likewise, there was no statistically significant decrease in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.9265$) and 60 minutes ($P = 0.4803$).

In addition, the amount of bacterial cells remained the same even when cells were exposed to slides with lower concentrations of nanoparticle coatings. At the concentration of 7.5mg/mL (Figure 51B), there was no statistically significant reduction in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.5562$) and 60 minutes ($P = 0.9680$). Comparably, there was no statistically significant reduction in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.7168$) and 60 minutes ($P = 0.5565$).

Furthermore, at the concentration of 2.5mg/mL (Figure 51C), there was no statistically significant difference in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.8476$) and 60 minutes ($P = 0.5586$). Similarly, there was no statistically significant difference in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.2620$) and 60 minutes ($P = 0.1118$).

### 4.7.2 Escherichia coli DH10B/pK21

To ascertain the effects of UV light on nanoparticles and whether continuous activation with UV light is required in order for nanoparticles to exhibit antibacterial properties, the drop-coated slide bioassay was employed. Various concentrations of drop-
coated slides were tested, namely 15, 7.5, and 2.5mg/mL (Figure 52) against MDR *E. coli* DH10B/pK21.

As controls, various slides containing bacterial samples were incubated in the dark, without any UV light activation, for 0, 30, and 60 minutes. The viable cell counts did not change on the unexposed slides (Figure 52) as there was no significant increase or decrease noted after recovery. In addition, as observed (Figure 52) there was no significant change on the control and binder when they were pre-exposed to UV light for 30 minutes. When exposed for a longer amount of time for 60 minutes (Figure 52) there was still no difference on the control and binder slides.
Figure 52: Antibacterial Effects of Pre-Ultraviolet Light Exposed Coatings on *Escherichia coli* DH10B/pK21 at Concentrations of 

(A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. **Control** slide represents plain, uncoated slides; **Binder** slide represents slides coated with the inorganic binder; **P25** slide represents slides coated with P25 nanoparticles; and **Br200** slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (**UV LIGHT**) represent samples that were pre-treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 52A), there was no statistically significant decrease in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.3768$) and 60 minutes ($P = 0.2222$). Likewise, there was no statistically significant decrease in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.05183$) and 60 minutes ($P = 0.9306$).

In addition, the amount of bacterial cells remained the same even when cells were exposed to slides with lower concentrations of nanoparticle coatings. At the concentration of 7.5mg/mL (Figure 52B), there was no statistically significant reduction in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.6176$) and 60 minutes ($P = 0.7476$). Comparably, there was no statistically significant reduction in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.5345$) and 60 minutes ($P = 0.7399$).

Furthermore, at the concentration of 2.5mg/mL (Figure 52C), there was no statistically significant difference in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.8825$) and 60 minutes ($P = 0.4947$). Similarly, there was no statistically significant difference in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.2506$) and 60 minutes ($P = 0.3466$).

4.7.3 Staphylococcus aureus ATCC 25923

To ascertain the effects of UV light on nanoparticles and whether continuous activation with UV light is required in order for nanoparticles to exhibit antibacterial properties, the drop-coated slide bioassay was employed. Various concentrations of drop-
coated slides were tested, namely 15, 7.5, and 2.5mg/mL (Figure 53) against non-drug-resistant *S. aureus* ATCC 25923.

As controls, various slides containing bacterial samples were incubated in the dark, without any UV light activation, for 0, 30, and 60 minutes. The viable cell counts did not change on the unexposed slides (Figure 53) as there was no significant increase or decrease noted after recovery. In addition, as observed (Figure 53) there was no significant change on the control and binder when they were pre-exposed to UV light for 30 minutes. When exposed for a longer amount of time for 60 minutes (Figure 53) there was still no difference on the control and binder slides.
Figure 53: Antibacterial Effects of Pre-Ultraviolet Light Exposed Coatings on *Staphylococcus aureus* ATCC 25923 at Concentrations of

(A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. **Control** slide represents plain, uncoated slides; **Binder** slide represents slides coated with the inorganic binder; **P25** slide represents slides coated with P25 nanoparticles; and **Br200** slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (**UV LIGHT**) represent samples that were pre-treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 53A), there was no statistically significant decrease in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.4386$) and 60 minutes ($P = 0.4240$). Likewise, there was no statistically significant decrease in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.2103$) and 60 minutes ($P = 0.6232$).

In addition, the amount of bacterial cells remained the same even when cells were exposed to slides with lower concentrations of nanoparticle coatings. At the concentration of 7.5mg/mL (Figure 53B), there was no statistically significant reduction in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.8040$) and 60 minutes ($P = 0.5866$). Comparably, there was no statistically significant reduction in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.6160$) and 60 minutes ($P = 0.2592$).

Furthermore, at the concentration of 2.5mg/mL (Figure 53C), there was no statistically significant difference in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.9229$) and 60 minutes ($P = 0.1448$). Similarly, there was no statistically significant difference in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.4332$) and 60 minutes ($P = 0.6613$).

4.7.4 *Staphylococcus aureus* 1199

To ascertain the effects of UV light on nanoparticles and whether continuous activation with UV light is required in order for nanoparticles to exhibit antibacterial properties, the drop-coated slide bioassay was employed. Various concentrations of drop-
coated slides were tested, namely 15, 7.5, and 2.5mg/mL (Figure 54) against non-drug-resistant S. aureus 1199.

As controls, various slides containing bacterial samples were incubated in the dark, without any UV light activation, for 0, 30, and 60 minutes. The viable cell counts did not change on the unexposed slides (Figure 54) as there was no significant increase or decrease noted after recovery. In addition, as observed (Figure 54) there was no significant change on the control and binder when they were pre-exposed to UV light for 30 minutes. When exposed for a longer amount of time for 60 minutes (Figure 54) there was still no difference on the control and binder slides.
Figure 54: Antibacterial Effects of Pre-Ultraviolet Light Exposed Coatings on *Staphylococcus aureus* 1199 at Concentrations of

(A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. Control slide represents plain, uncoated slides; Binder slide represents slides coated with the inorganic binder; P25 slide represents slides coated with P25 nanoparticles; and Br200 slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (UV LIGHT) represent samples that were pre-treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 54A), there was no statistically significant decrease in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.8917$) and 60 minutes ($P = 0.2402$). Likewise, there was no statistically significant decrease in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.6831$) and 60 minutes ($P = 0.9591$).

In addition, the amount of bacterial cells remained the same even when cells were exposed to slides with lower concentrations of nanoparticle coatings. At the concentration of 7.5mg/mL (Figure 54B), there was no statistically significant reduction in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.8404$) and 60 minutes ($P = 0.8231$). Comparably, there was no statistically significant reduction in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.3810$) and 60 minutes ($P = 0.8354$).

Furthermore, at the concentration of 2.5mg/mL (Figure 54C), there was no statistically significant difference in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.8091$) and 60 minutes ($P = 0.5972$). Similarly, there was no statistically significant difference in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.1485$) and 60 minutes ($P = 0.4993$).

### 4.7.5 *Staphylococcus aureus* 1199B

To ascertain the effects of UV light on nanoparticles and whether continuous activation with UV light is required in order for nanoparticles to exhibit antibacterial properties, the drop-coated slide bioassay was employed. Various concentrations of drop-
coated slides were tested, namely 15, 7.5, and 2.5mg/mL (Figure 55) against MDR S. aureus 1199B.

As controls, various slides containing bacterial samples were incubated in the dark, without any UV light activation, for 0, 30, and 60 minutes. The viable cell counts did not change on the unexposed slides (Figure 55) as there was no significant increase or decrease noted after recovery. In addition, as observed (Figure 55) there was no significant change on the control and binder when they were pre-exposed to UV light for 30 minutes. When exposed for a longer amount of time for 60 minutes (Figure 55) there was still no difference on the control and binder slides.
Figure 55: Antibacterial Effects of Pre-Ultraviolet Light Exposed Coatings on *Staphylococcus aureus* 1199B at Concentrations of

(A) 15mg/mL; (B) 7.5mg/mL; and (C) 2.5mg/mL

Samples were plated at 0, 30, and 60 minutes. Control slide represents plain, uncoated slides; Binder slide represents slides coated with the inorganic binder; P25 slide represents slides coated with P25 nanoparticles; and Br200 slide represents slides coated with Br200 nanoparticles. The first 3 time intervals of 0, 30, and 60 minutes represent controls, without UV light activation. The next 2 time intervals of 30 and 60 minutes (UV LIGHT) represent samples that were pre-treated under UV light activation. The data represents the mean ± standard error of 3 experiments.
At the concentration of 15mg/mL (Figure 55A), there was no statistically significant decrease in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.6382$) and 60 minutes ($P = 0.8892$). Likewise, there was no statistically significant decrease in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.2997$) and 60 minutes ($P = 0.8515$).

In addition, the amount of bacterial cells remains the same even when cells were exposed to slides with lower concentrations of nanoparticle coatings. At the concentration of 7.5mg/mL (Figure 55B), there was no statistically significant reduction in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.3815$) and 60 minutes ($P = 0.7495$). Comparably, there was no statistically significant reduction in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.7157$) and 60 minutes ($P = 0.5545$).

Furthermore, at the concentration of 2.5mg/mL (Figure 55C), there was no statistically significant difference in the P25 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.4781$) and 60 minutes ($P = 0.06642$). Similarly, there was no statistically significant difference in the Br200 drop-coated slides when pre-exposed to UV light for 30 minutes ($P = 0.6127$) and 60 minutes ($P = 0.1269$).
4.8 Morphological Effects of Ultraviolet Light Activated Anatase and Brookite Nanoparticles on Bacterial Cells

4.8.1 Escherichia coli ATCC 23848

Non-activated P25 and Br200 nanoparticles alone could be seen in clusters and grouped together (Figure 56 and 57). It was obvious that P25 nanoparticles (Figure 56) appeared larger than Br200 nanoparticles (Figure 57). Control bacteria, i.e., bacteria that were not in contact with activated or non-activated nanoparticles or UV light, had well preserved cells (Figure 58). The cells demonstrated typical thick, uniform, and rod-shaped characteristics (Figure 58).

Figure 56: Scanning Electron Microscopy Images of Unexposed P25 Anatase Nanoparticles (10mg/mL) Magnified at (A) 100,000X and (B) 100,000X
Figure 57: Scanning Electron Microscopy Images of Unexposed Br200 Nanoparticles (10mg/mL) Magnified at (A) 150,000X and (B) 350,000X

Figure 58: Scanning Electron Microscopy Images of Unexposed Escherichia coli Cells (10mL of $10^9$ CFU/mL) Magnified at (A) 13,000X and (B) 18,000X
Images of the bacterial cells mixed with TiO₂ nanoparticles were obtained at 0 minutes before any incubation (Figure 59). It was obvious that nanoparticles aggregated and formed clusters around the bacterial cells. Exposure to UV light activated nanoparticles for 30 minutes did not cause any significant damage to the cells (Figure 60). Exposure of cells to UV light activated nanoparticles for 180 minutes (Figure 61) caused a significant amount of damage to the cells. The damage could be seen as tiny indentions (Figure 61), which caused the deformities observed in cell shapes. In addition, untreated cells presented smooth and rod-shaped cell surfaces (Figure 58) while treated cells presented rough and crinkled surfaces (Figure 61). Of note is that the cells exposed to UV light alone did not reveal a significant amount of damage. There were more undamaged cells present in the sample then damaged cells.
Figure 59: Scanning Electron Microscopy Images of Unexposed *Escherichia coli* Cells (10mL of 10^9 CFU/mL) with

(A) P25 Nanoparticles (3mL of 10mg/mL) Magnified at 15,000X;
(B) P25 Nanoparticles (3mL of 10mg/mL) Magnified at 20,000X;
(C) Br200 Nanoparticles (5mL of 0.1mg/mL) Magnified at 20,000X; and
(D) Br200 Nanoparticles (3mL of 0.1mg/mL) Magnified at 20,000X
Figure 60: Scanning Electron Microscopy Images of *Escherichia coli* Cells (10mL of 10⁹ CFU/mL) Post 30 Minutes Exposure to Ultraviolet Light Activated

(A) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 8,000X;  
(B) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 20,000X;  
(C) Br200 Nanoparticles (5mL of 0.1mg/mL) Magnified at 18,000X; and  
(D) Br200 Nanoparticles (3mL of 0.1mg/mL) Magnified at 20,000X
Figure 61: Scanning Electron Microscopy Images of *Escherichia coli* Cells (10mL of 10⁹ CFU/mL) Post 180 Minutes Exposure to Ultraviolet Light Activated

(A) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 20,000X; 
(B) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 30,000X; 
(C) Br200 Nanoparticles (5mL of 0.1mg/mL) Magnified at 20,000X; and 
(D) Br200 Nanoparticles (3mL of 0.1mg/mL) Magnified at 35,000X

Bacterial cells that were incubated in the dark without any UV light exposure did not reveal any damage after 180 minutes (Figure 62). However, after 180 minutes of UV
light exposure, damage on the cells was evident (Figure 63). The physical damage was similar to the damage observed due to the activated TiO$_2$ nanoparticles although the amount of damaged cells on the TiO$_2$ activated samples was greater than the amount of damaged cells on the UV light exposed samples.

**Figure 62:** Scanning Electron Microscopy Images of *Escherichia coli* Cells (10mL of $10^9$ CFU/mL)
Post 180 Minutes Incubation in the Dark, Without Ultraviolet Light Activation Magnified at (A) 13,000X and (B) 13,000X
Figure 63: Scanning Electron Microscopy Images of *Escherichia coli* Cells (10mL of 10⁹ CFU/mL) Post 180 Minutes Exposure to Ultraviolet Light Magnified at (A) 11,000X and (B) 15,000X

4.8.2 *Staphylococcus aureus 1199B*

Control bacteria, i.e., bacteria that were not in contact with activated or non-activated nanoparticles or UV light, had well preserved cells (Figure 64). The cells demonstrated typical thick, uniform, and rounded characteristics (Figure 64).
Figure 64: Scanning Electron Microscopy Images of Unexposed *Staphylococcus aureus* Cells (10mL of $10^9$ CFU/mL) Magnified at (A) 11,000X and (B) 20,000X

Images of the bacterial cells mixed with TiO$_2$ nanoparticles were obtained at 0 minutes before any incubation (Figure 65). It was obvious that nanoparticles aggregated and formed clusters around the bacterial cells. Similar to the *E. coli* cells, exposure to UV light activated nanoparticles for 30 minutes did not cause any significant damage to the cells of MDR *S. aureus* 1199B (Figure 66). Exposure of cells to UV light activated nanoparticles for 180 minutes (Figure 67) caused a significant amount of damage to the cells. The damage could be seen as malformations in the circular shapes (Figure 67) of the cells. Apparently, untreated cells presented smooth surfaces (Figure 64) while treated cells presented irregular-shaped surfaces (Figure 67). It is important to note that cells
exposed to UV light alone did not reveal a significant amount of damage. There were
more undamaged cells present in the sample than damaged cells.

**Figure 65:** Scanning Electron Microscopy Images of
Unexposed *Staphylococcus aureus* Cells (10mL of $10^9$ CFU/mL) with
(A) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 11,000X;
(B) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 15,000X,
(C) Br200 Nanoparticles (5mL of 0.1mg/mL) Magnified at 35,000X; and
(D) Br200 Nanoparticles (3mL of 0.1mg/mL) Magnified at 45,000X
Figure 66: Scanning Electron Microscopy Images of *Staphylococcus aureus* Cells (10mL of 10^9 CFU/mL)

Post 30 Minutes Exposure to Ultraviolet Light Activated

(A) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 15,000X;
(B) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 30,000X;
(C) Br200 Nanoparticles (5mL of 0.1mg/mL) Magnified at 35,000X; and
(D) Br200 Nanoparticles (3mL of 0.1mg/mL) Magnified at 35,000X
Figure 67: Scanning Electron Microscopy Images of *Staphylococcus aureus* Cells (10mL of 10⁹ CFU/mL) Post 180 Minutes Exposure to Ultraviolet Light Activated

(A) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 30,000X; 
(B) P25 Nanoparticles (5mL of 0.1mg/mL) Magnified at 45,000X; 
(C) Br200 Nanoparticles (5mL of 0.1mg/mL) Magnified at 40,000X; and 
(D) Br200 Nanoparticles (3mL of 0.1mg/mL) Magnified at 45,000X
Bacterial cells that were incubated in the dark without any UV light exposure did not reveal any damage after 180 minutes (Figure 68). However, after 180 minutes of UV light exposure, damage on the cells was evident (Figure 69). The physical damage was similar to the damage observed due to the activated TiO$_2$ nanoparticles although the amount of damaged cells on the TiO$_2$ activated samples was greater than the amount of damaged cells on the UV light exposed samples.

**Figure 68:** Scanning Electron Microscopy Images of *Staphylococcus aureus* Cells (10mL of $10^9$ CFU/mL)

Post 180 Minutes Incubation in the Dark,
Without Ultraviolet Light Activation Magnified at (A) 13,000X and (B) 15,000X
Figure 69: Scanning Electron Microscopy Images of
Staphylococcus aureus Cells (10mL of $10^9$ CFU/mL)
Post 180 Minutes Exposure to Ultraviolet Light Magnified
at (A) 25,000X and (B) 45,000X
4.9 Antibacterial Effects of Visible Light Activated Anatase and Brookite Nanoparticles

4.9.1 *Escherichia coli* ATCC 23848

Visible light activation alone (Figure 70) did not cause any increase or decrease in viable cell counts over time. In addition, cells incubated in the dark without any light source did not illustrate any increase of decrease in viable cell counts over time.

![Graph](image)

**Figure 70:** Antibacterial Effects of Visible Light Activated Anatase and Brookite Nanoparticles on *Escherichia coli* ATCC 23848

Samples were plated at 0, 4, and 8 hours. The following samples were plated: **Control:** Cells incubated in the dark, without visible light exposure; **VLA:** Cells under visible light exposure without nanoparticles; **VLA-P25:** Cells exposed to visible light activated P25 nanoparticles; **VLA-Br200:** Cells exposed to visible light activated Br200 nanoparticles; **P25:** Cells with nanoparticles incubated in the dark, without visible light exposure; and **Br200:** Cells with nanoparticles incubated in the dark, without visible light exposure. For P25 and Br200 samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.
At the concentration of 10mg/mL (Figure 70), after exposure of cells to activated P25 nanoparticles there was a less than three log reduction in cell growth after 4 hours. This reduction was statistically significant \((P = 0.0007254)\). After 8 hours of exposure to activated P25 nanoparticles there was a three log reduction in cell growth, which was statistically significant \((P = 0.0007237)\). Similarly, there was a three log reduction in cell growth after 4 hours of exposure to visible light activated Br200 nanoparticles, which eventually led to a five log reduction after 8 hours of exposure to visible light activated Br200 nanoparticles. Both these reductions were also statistically significant \((P = 0.0006960\) and \(P = 0.0006946\), respectively).

Appropriate controls were established which represented the action of TiO2 nanoparticles on the cells incubated in the dark (Figure 70) without visible light activation. At the concentration of 10mg/mL, non-activated P25 nanoparticles exhibited one log statistically significant decrease \((P = 0.001143)\) in cell growth after 4 hours of incubation which led to a two log reduction after 8 hours. Non-activated Br200 nanoparticles exhibited caused a greater amount of decrease in cell growth compared to non-activated P25 nanoparticles. At the concentration of 10mg/mL, non-activated Br200 nanoparticles exhibited a two log statistically significant decrease \((P = 0.0007342)\) in cell growth after 4 hours of incubation which led to a three log reduction after 8 hours.
4.9.2 *Escherichia coli* DH10B/pK21

Visible light activation alone (Figure 71) did not cause any increase or decrease in viable cell counts over time. In addition, cells incubated in the dark without any light source did not illustrate any increase of decrease in viable cell counts over time.

![Figure 71: Antibacterial Effects of Visible Light Activated Anatase and Brookite Nanoparticles on *Escherichia coli* DH10B/pK21](image)

Samples were plated at 0, 4, and 8 hours. The following samples were plated: **Control**: Cells incubated in the dark, without visible light exposure; **VLA**: Cells under visible light exposure without nanoparticles; **VLA-P25**: Cells exposed to visible light activated P25 nanoparticles; **VLA-Br200**: Cells exposed to visible light activated Br200 nanoparticles; **P25**: Cells with nanoparticles incubated in the dark, without visible light exposure; and **Br200**: Cells with nanoparticles incubated in the dark, without visible light exposure. For P25 and Br200 samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.

At the concentration of 10mg/mL (Figure 71), after exposure of cells to activated P25 nanoparticles there was a less than three log reduction in cell growth after 4 hours. This reduction was statistically significant ($P = 0.002838$). After 8 hours of exposure to
activated P25 nanoparticles there was a three log reduction in cell growth, which was statistically significant ($P = 0.002831$). Similarly, there was a three log reduction in cell growth after 4 hours of exposure to visible light activated Br200 nanoparticles, which eventually led to a five log reduction after 8 hours of exposure to visible light activated Br200 nanoparticles. Both these reductions were also statistically significant ($P = 0.001837$ and $P = 0.001834$, respectively)

Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 71) without visible light activation. At the concentration of 10mg/mL, non-activated P25 nanoparticles exhibited one log statistically significant decrease ($P = 0.002524$) in cell growth after 4 hours of incubation which led to a two log reduction after 8 hours. Non-activated Br200 nanoparticles exhibited caused a greater amount of decrease in cell growth compared to non-activated P25 nanoparticles. At the concentration of 10mg/mL, non-activated Br200 nanoparticles exhibited a two log statistically significant decrease ($P = 0.001336$) in cell growth after 4 hours of incubation which led to a three log reduction after 8 hours.

4.9.3 Staphylococcus aureus 25923

Visible light activation alone (Figure 72) did not cause any increase or decrease in viable cell counts over time. In addition, cells incubated in the dark without any light source did not illustrate any increase of decrease in viable cell counts over time.
Figure 72: Antibacterial Effects of Visible Light Activated Anatase and Brookite Nanoparticles on *Staphylococcus aureus* ATCC 25923

Samples were plated at 0, 4, and 8 hours. The following samples were plated: **Control**: Cells incubated in the dark, without visible light exposure; **VLA**: Cells under visible light exposure without nanoparticles; **VLA-P25**: Cells exposed to visible light activated P25 nanoparticles; **VLA-Br200**: Cells exposed to visible light activated Br200 nanoparticles; **P25**: Cells with nanoparticles incubated in the dark, without visible light exposure; and **Br200**: Cells with nanoparticles incubated in the dark, without visible light exposure. For P25 and Br200 samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.

At the concentration of 10mg/mL (Figure 72), after exposure of cells to activated P25 nanoparticles there was a less than one log reduction in cell growth after 4 hours. However, this reduction was statistically significant ($P = 0.006424$). After 8 hours of exposure to activated P25 nanoparticles there was an almost two log reduction in cell growth, which was statistically significant ($P = 0.0002743$). Similarly, there was a one log reduction in cell growth after 4 hours of exposure to visible light activated Br200 nanoparticles, which eventually led to a four log reduction after 8 hours of exposure to
visible light activated Br200 nanoparticles. Both these reductions were also statistically significant ($P = 0.001105$ and $P = 0.001100$, respectively).

Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 72) without visible light activation. At the concentration of 10mg/mL, non-activated P25 nanoparticles exhibited a less than one log statistically significant decrease ($P = 0.04080$) in cell growth after 4 hours of incubation which led to a one log reduction after 8 hours. Non-activated Br200 nanoparticles exhibited caused a greater amount of decrease in cell growth compared to non-activated P25 nanoparticles. At the concentration of 10mg/mL, non-activated Br200 nanoparticles exhibited one log statistically significant decrease ($P = 0.002524$) in cell growth after 4 hours of incubation which led to a two log reduction after 8 hours.

4.9.4 *Staphylococcus aureus 1199*

Visible light activation alone (Figure 73) did not cause any increase or decrease in viable cell counts over time. In addition, cells incubated in the dark without any light source did not illustrate any increase of decrease in viable cell counts over time.
Samples were plated at 0, 4, and 8 hours. The following samples were plated: **Control**: Cells incubated in the dark, without visible light exposure; **VLA**: Cells under visible light exposure without nanoparticles; **VLA-P25**: Cells exposed to visible light activated P25 nanoparticles; **VLA-Br200**: Cells exposed to visible light activated Br200 nanoparticles; **P25**: Cells with nanoparticles incubated in the dark, without visible light exposure; and **Br200**: Cells with nanoparticles incubated in the dark, without visible light exposure. For P25 and Br200 samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.

At the concentration of 10mg/mL (**Figure 73**), after exposure of cells to activated P25 nanoparticles there was a less than one log reduction in cell growth after 4 hours. However, this reduction was statistically significant ($P = 0.008238$). After 8 hours of exposure to activated P25 nanoparticles there was an almost two log reduction in cell growth, which was statistically significant ($P = 0.001078$). Similarly, there was a one log reduction in cell growth after 4 hours of exposure to visible light activated Br200 nanoparticles, which eventually led to a four log reduction after 8 hours of exposure to
visible light activated Br200 nanoparticles. Both these reductions were also statistically significant ($P = 0.002029$ and $P = 0.001797$, respectively).

Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 73) without visible light activation. At the concentration of 10mg/mL, non-activated P25 nanoparticles exhibited a less than one log statistically significant decrease ($P = 0.03133$) in cell growth after 4 hours of incubation which led to a one log reduction after 8 hours. Non-activated Br200 nanoparticles exhibited caused a greater amount of decrease in cell growth compared to non-activated P25 nanoparticles. At the concentration of 10mg/mL, non-activated Br200 nanoparticles exhibited one log statistically significant decrease ($P = 0.002945$) in cell growth after 4 hours of incubation which led to a two log reduction after 8 hours.

### 4.9.5 *Staphylococcus aureus 1199B*

Visible light activation (Figure 74) alone did not cause any increase or decrease in viable cell counts over time. In addition, cells incubated in the dark without any light source did not illustrate any increase of decrease in viable cell counts over time.
Samples were plated at 0, 4, and 8 hours. The following samples were plated: **Control**: Cells incubated in the dark, without visible light exposure; **VLA**: Cells under visible light exposure without nanoparticles; **VLA-P25**: Cells exposed to visible light activated P25 nanoparticles; **VLA-Br200**: Cells exposed to visible light activated Br200 nanoparticles; **P25**: Cells with nanoparticles incubated in the dark, without visible light exposure; and **Br200**: Cells with nanoparticles incubated in the dark, without visible light exposure. For P25 and Br200 samples, distilled water was utilized as the medium of treatment instead of nanoparticle suspensions. The data represents the mean ± standard error of 3 experiments.

At the concentration of 10mg/mL (**Figure 74**), after exposure of cells to activated P25 nanoparticles there was a less than one log reduction in cell growth after 4 hours. However, this reduction was statistically significant ($P = 0.01314$). After 8 hours of exposure to activated P25 nanoparticles there was an almost two log reduction in cell growth, which was statistically significant ($P = 0.0001969$). Similarly, there was a one log reduction in cell growth after 4 hours of exposure to visible light activated Br200 nanoparticles, which eventually led to a four log reduction after 8 hours of exposure to
visible light activated Br200 nanoparticles. Both these reductions were also statistically significant ($P = 0.001632$ and $P = 0.001595$, respectively).

Appropriate controls were established which represented the action of TiO$_2$ nanoparticles on the cells incubated in the dark (Figure 74) without visible light activation. At the concentration of 10mg/mL, non-activated P25 nanoparticles exhibited a less than one log statistically significant decrease ($P = 0.009952$) in cell growth after 4 hours of incubation which led to a one log reduction after 8 hours. Non-activated Br200 nanoparticles exhibited caused a greater amount of decrease in cell growth compared to non-activated P25 nanoparticles. At the concentration of 10mg/mL, non-activated Br200 nanoparticles exhibited one log statistically significant decrease ($P = 0.002510$) in cell growth after 4 hours of incubation which led to a two log reduction after 8 hours.
5.1 Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Nanoparticles

Ultraviolet light alone, at a wavelength of 365nm with an energy level of 370\( \mu \)w/cm\(^2\) exhibited limited bactericidal properties as expected. There was a slight decrease in viable cell counts over time. After 30 minutes of exposure, UV light alone caused minimal reduction. After 120 minutes of exposure, UV light alone caused a one log reduction, which was less efficient than photocatalysis with nanoparticles. This was consistent with previous studies which have demonstrated that UV light alone at a wavelength of 365nm did not cause a significant decrease in cell growth.

At concentrations of 10 and 1mg/mL, UV light activated anatase and brookite nanoparticles exhibited similar antibacterial properties on both strains of \( E. \) coli, namely non-drug-resistant \( E. \) coli ATCC 23848 and MDR \( E. \) coli DH\(_{10}\)B/pK21. Both P25 and Br200 nanoparticles caused a 100% decline in \( E. \) coli cell growth within 30 minutes of exposure. However, at similar concentrations, Br200 nanoparticles caused a 100% reduction of all \( S. \) aureus cells within 30 minutes of exposure to UV light while P25 nanoparticles did not. Instead, at 10 and 1mg/mL concentrations, activated P25 nanoparticles caused a 100% decrease in non-drug-resistant \( S. \) aureus cell counts after 75 minutes of exposure and a 100% decrease in MDR \( S. \) aureus cell counts after 120
minutes of exposure. This implies that there was a difference in the antibacterial effects of P25 and Br200 nanoparticles against *S. aureus*.

The reason that Br200 nanoparticles exhibited stronger antibacterial activities than P25 nanoparticles may be due to the higher specific surface area of the Br200 nanoparticles. Brookite nanoparticles have a specific surface area that is almost three times larger than that of anatase nanoparticles. Due to the higher specific surface area, there is an increase in active sites which increases the probability of electrons and holes to reach the surface and react thus, leading to a faster degradation process.

The Br200 nanoparticles exhibited stronger bactericidal effects on *E. coli* cells than on *S. aureus* cells. The difference in susceptibility may be due to the cell wall structures of the gram-negative *E. coli* and gram-positive *S. aureus*. Since gram-positive cell walls are thicker than gram-negative cell walls, it may be more feasible for the radicals and anions to damage the thinner cell walls. Additionally, gram-positive cell walls contain teichoic acids which are not present in gram-negative cell walls. Teichoic acids are polymers of glycerol or ribitol connected by phosphate groups. Amino acids such as D-alanine or sugars like glucose are attached to the glycerol and ribitol groups. Teichoic acids appear to extend to the surface of the peptidoglycan layer and past studies have indicated that teichoic acids may be important in maintaining the structure of the cell walls of gram-positive bacteria such as *S. aureus*. Furthermore, even though the cell and nanoparticle mixtures were thoroughly mixed on a vortex, one of the characteristics of *S. aureus* is to form clumps. The clumping of *S. aureus* may have provided some level of protection to the cells.
UV light activated brookite nanoparticles resulted in a reduction in viable cell counts within 30 minutes, at concentrations of 10 and 1mg/mL. Although it was obvious that complete killing was achieved within 30 minutes, the exact time required to achieve complete killing is not known since the first set of samples for the free suspension bioassay were plated at 0 minutes and the second set of samples were plated at 30 minutes. The second set of samples were not obtained in less than 30 minutes due to the processing time required for the number of samples plated on TSA plates. For future studies, 0 minute samples may be omitted and instead samples may be obtained at another time interval of less than 30 minutes in order to determine the exact time required to achieve complete killing with brookite nanoparticles.

The control, non-activated nanoparticles, caused a slight reduction in cell growth. However, activated nanoparticles caused a greater reduction of seven logs in cell growth within 30 minutes. The reason for the effects of the non-activated nanoparticles may be due to the toxicity present in the samples which is further explained in Section 5.5.

**5.2 Antibacterial Effects of Ultraviolet Light Activated Anatase versus Brookite Coatings**

Plain, uncoated slides exposed to UV light alone at a wavelength of 365nm with an energy level of 370μw/cm² demonstrated antibacterial properties as predicted. There was a consistent decrease in viable cell counts over time. After 30 minutes of exposure, UV light alone, caused a one log reduction and a two log reduction after 60 minutes of exposure, which was less efficient than the antibacterial properties exhibited by the
nanoparticle coatings. These results were consistent with past studies that have demonstrated that UV light alone at a wavelength of 365nm does not cause a significant decrease in viable cell counts.

Slides coated with the binder exhibited minimal antibacterial properties after 30 minutes of UV light exposure. However, after 60 minutes of UV light exposure the slides resulted in an almost one log reduction in cell viability. The reason for the slower decrease in viable cell counts may have been due to the texture of the coatings. The coatings on the slide did not allow the bacterial culture to spread evenly on the surface. Instead, the bacterial culture formed droplets on the slide which in turn may have protected the cells from UV light exposure until the UV light penetrated through the droplet due to prolonged exposure, i.e., 60 minutes of exposure, which eventually resulted in a further decrease in viable cell counts.

At concentrations of 15, 7.5, and 2.5mg/mL, P25 coatings resulted in a one log reduction post 30 minutes of UV light exposure and a two log reduction post 60 minutes of UV light exposure with *E. coli* and *S. aureus* cells. In contrast, Br200 coatings resulted in a greater decrease, a two log reduction after 30 minutes of exposure and a three log reduction after 60 minutes of exposure with *E. coli* and *S. aureus* cells.

It was obvious that the coatings did not cause a complete 100% reduction in cell growth at any time point and this may have been due to the nature of the coatings. The coatings were exposed to UV light constantly but due to the droplets that were formed on the coated slides rather than the smooth spreading of the bacterial culture, UV light may not have been able to penetrate in all areas of the coatings effectively. This may have
prevented the complete killing of the bacterial cells found on the surface. However, it can be accurately confirmed that activated Br200 coatings were more effective at killing bacterial cells than P25 coatings which caused a one log reduction in viable cell counts compared to the two log reductions caused by Br200 coatings within 30 minutes.

**5.3 Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Nanoparticles**

At 10 and 1mg/mL concentrations, Br200 nanoparticles exhibited similar antibacterial properties when exposed to UV light. At extreme concentrations of 100, 0.1, 0.01, and 0.001mg/mL, the effectiveness of the activated nanoparticles was reduced. There was a consistent decrease in cell growth at 100 and 0.1mg/mL concentrations within 120 minutes of UV light exposure, although neither concentration led to a 100% reduction in cell growth within 120 minutes of UV light exposure. On the other hand, concentrations of 0.01 and 0.001mg/mL did not cause any significant decrease or increase in cell growth at any exposure times, which indicates that extremely low concentrations are ineffective as photocatalysts within 120 minutes.

A possible explanation for nanoparticle concentrations greater than 10mg/mL to result in a decreased killing efficiency may have been related to the fact that there were too many TiO₂ nanoparticles which did not allow UV light illumination to reach evenly throughout all the nanoparticles. Therefore, there may have been a decrease in UV light absorption due to the large amount of particle to particle contact. Similarly, at a concentration lower than 1mg/mL such as 0.1mg/mL, the killing efficiency was reduced.
because there were too few nanoparticles for reactions to occur even after consistent UV light illumination for 120 minutes. Extremely low concentrations of 0.01mg/mL and 0.001mg/mL did not result in any cell death, even after 120 minutes of UV light exposure. This suggested that brookite nanoparticles at such low concentrations protected the bacterial cells from UV light exposure since the decrease seen when cells were exposed to UV light was not seen at these concentrations of brookite nanoparticles. Scanning electron microscopy images have also revealed that the brookite nanoparticles aggregate around the bacterial cells which may protect them from exposure to UV light.

As a result, the minimum concentration which was optimum for obtaining successful killing with Br200 nanoparticles within 30 minutes of UV light exposure was 1mg/mL. Of particular note was the effect of UV light irradiation alone, which was not as effective in causing cell death as the activated Br200 nanoparticles were, especially the 10 and 1mg/mL concentrations of Br200 nanoparticles, which caused a 100% decrease within 30 minutes of UV light illumination.

5.4 Antibacterial Effects of Various Concentrations of Ultraviolet Light Activated Brookite Coatings

Slides coated with Br200 concentrations of 15, 7.5, and 2.5mg/mL depicted similar antibacterial properties within 30 and 60 minutes of UV light exposure. Since changes in viable cell counts were not observed among the different concentrations, the optimum concentration of coatings required to obtain maximum killing could not be
stated. A larger range of higher and lower concentrations of brookite coatings should be tested in order to determine the optimum concentration of coatings.

At higher concentrations of coatings, there may be too many nanoparticles clustered together during the preparation of the slides which will not allow UV light exposure to activate all nanoparticles on the coatings effectively. Similarly, at lower concentrations of coatings, there may not be enough nanoparticles in the coatings to cause a reduction in cell counts once activated by UV light.

Therefore, an optimum concentration of coatings that can effectively, when activated, exhibit antibacterial properties, needs to be determined. Of particular note was the effect of UV light irradiation alone, which resulted in a one log decrease in viable cell counts within 30 minutes but was not as effective in causing cell death as the activated Br200 coatings were. The 7.5mg/mL concentration of Br200 nanoparticle coatings caused a two log reduction within 30 minutes of UV light illumination.

5.5 Toxic Effects of Non-Activated Brookite Nanoparticles

Bacterial cells incubated with distilled water in the dark did not cause any reduction in cell growth which represented an excellent control for this toxicity study since it proved that the distilled water alone was not the cause of the reductions in viable cell counts. Non-activated Br200 nanoparticles, obviously, resulted in toxicity. At concentrations of 100, 10, 1, and 0.1mg/mL, nanoparticles exhibited decreasing levels of toxicity. In addition, longer times of contact led to more toxic effects of the nanoparticles. At low concentrations of 0.01 and 0.001mg/mL, the nanoparticles did not exhibit any
toxicity. At higher concentrations of up to 100mg/mL, there was a larger degree of
toxicity than at lower concentrations of 0.1mg/mL because there were a greater number
of nanoparticles in the higher concentrations and a lesser number of nanoparticles in the
lower concentrations.

Br200 nanoparticles may have exhibited toxicity due to the contamination or
impurities present in the sample which caused the decomposition of organic substances.
The synthesis of Br200 nanoparticles involves the utilization of the compound, TiCl4,
which generates HCl upon reactions with water and produces TiO2. During the sample
preparation, residual HCl may have persisted in the Br200 samples that caused the
samples to become acidic. Due to this presumption, the pH of the Br200 nanoparticles
was measured at various concentrations. Distilled water, which was the medium used to
suspend the nanoparticles had a pH of 6.82. At concentrations of 10mg/mL Br200
nanoparticles had a pH of 2.00, at concentrations of 1mg/mL Br200 nanoparticles had a
pH of 2.98, and at concentrations of 0.1mg/mL Br200 nanoparticles had a pH of 4.10.
This confirmed the speculation that due to the sample preparation the Br200
nanoparticles may have exhibited toxicity effects of outstanding acids, which may have
resulted in some direct damage to the cells.

In addition, TiO2 as a chemical may possess inherent toxicity due to its properties
which may cause the decline in cell growth over time. Additionally, bacterial cell counts
may have decreased due to the changes in their natural environment caused by TiO2
nanoparticles.
It is important to note that the decrease in viable cell counts when nanoparticles were activated under UV light may have been partially due to the effects of toxicity. However, when the nanoparticles were activated under UV light, there was a 100% reduction in viable cell counts within 30 minutes, which was significantly lower than the viable cell counts obtained when nanoparticles were non-activated.

5.6 Antibacterial Effects of Pre-Ultraviolet Light Exposed Coatings

Uncoated slides pre-exposed to UV light and then incubated with cells did not exhibit any changes in viable cell counts within 30 or 60 minutes. This was consistent with other studies because when cells were not directly exposed to UV light, there was no decrease in viable cell counts.

At concentrations of 15, 7.5, and 2.5mg/mL of nanoparticle coatings, there was no reduction in cell growth after pre-exposure to UV light for 30 or 60 minutes. The viable cell counts remained the same throughout the experiment.

As expected pre-exposure of nanoparticle coatings to UV light did not cause a decrease in viable cell counts. This was due to the concept that nanoparticles need to be under constant UV light activation in order to release reactive radicals that destroy organic compounds. Therefore, it can be concluded that it is the simultaneous action of the TiO$_2$ photocatalysts and UV light irradiation that has a synergistic effect on organic substances. Subsequently, when there is no UV light exposure, no antibacterial activity will be observed from the nanoparticles.
This also confirms that the released radicals are short-lived and do not extend their effects for a long period of time. They exhibit a limited amount of decomposing effects which do not last further without UV light activation.

The nanoparticle coatings did not indicate toxic effects since the slides did not inhibit bacterial growth when unexposed to UV light. As previously seen the nanoparticle cell suspensions resulted in a decrease in viable cell counts when incubated in the dark. The reason the coatings did not cause a reduction in viable cell counts in the dark may be due to the preparation of the slides with the inorganic binder. The effects of the binder may have inhibited the toxic effects of the brookite nanoparticles or may have, somehow, protected the cells from the toxic effects of the brookite nanoparticles in the coatings.

5.7 Morphological Effects of Ultraviolet Light Activated Anatase and Brookite Nanoparticles on Bacterial Cells

The SEM images revealed the effects of activated nanoparticles on bacterial cells. After \(10^{10}\) CFU of bacterial cells were exposed for 180 minutes to 0.1mg/mL concentrations of nanoparticles, there was a significant amount of damage to the bacterial cells as could be observed by the abnormal cell shapes and structures in the SEM images.

Evidently, there was an increase in abnormal-shaped cells and a decrease in normal-shaped cells in the sample, compared to the images obtained after UV light irradiation alone. Ultraviolet light exposure at the same time without TiO\(_2\) nanoparticles did not cause a significant amount of damage to the cells as bacteria with intact cells were consistently observed while imaging the UV light exposed bacterial samples. There was a
significantly greater amount of damaged cells in the activated TiO$_2$-treated sample than the UV light-treated sample.

Also, as can be noted, there was no difference in the sizes of the cells post treatment with activated TiO$_2$ nanoparticles. Although the shapes of the cells were different post treatment with TiO$_2$ nanoparticles, there was no evidence of cell lysis. Due to the processing methodology for obtaining SEM images, the smaller cell debris might not have been recovered. Overall, the cells lost their original shapes but the sizes remained the same.

Thus, it can be concluded, that the action of the photocatalysts along with UV light irradiation causes physical damage to the bacterial cells, as could be noted by the abnormal cell shapes. It can also be implied that at higher concentrations of 1 or 10mg/mL, and the same amount of bacterial cells and exposure times, a significant amount of damage may occur on the activated nanoparticle-treated cells compared to the cells treated with UV light alone.

5.8 Antibacterial Effects of Visible Light Activated Anatase and Brookite Nanoparticles

Cells incubated with distilled water under visible light did not result in any reduction in viable cell counts over time which indicates that visible light activation alone does not cause a reduction in viable cell counts. Similarly, the control cells, incubated in the dark, away from all light sources did not show any reduction in viable cell counts over time.
However, there was a decrease in viable cell counts post exposure to visible light activated nanoparticles at a concentration of 10mg/mL. When tested against *E. coli* cells, visible light activated anatase nanoparticles caused a three log reduction after 8 hours of exposure while brookite nanoparticles caused a greater decrease of five logs after 8 hours of exposure. The cells incubated with non-activated nanoparticles also caused a statistically significant decrease over time. After 8 hours of exposure with non-activated P25 nanoparticles, there was a two log reduction and after 8 hours of exposure with non-activated Br200 nanoparticles, there was a three log reduction.

When tested against *S. aureus* cells, visible light activated anatase nanoparticles induced a two log reduction after 8 hours of exposure while brookite nanoparticles induced a greater decrease of four logs after 8 hours of exposure. The cells incubated with non-activated nanoparticles also caused a statistically significant decrease over time. After 8 hours of exposure with non-activated P25 nanoparticles, there was a one log reduction and after 8 hours of exposure with non-activated Br200 nanoparticles, there was a two log reduction.

The nanoparticles utilized in this study did not contain any other elements hence they could function efficiently under UV light exposure. They could function less efficiently under visible light (400nm to 700nm) exposure due to the current band gap energy of the nanoparticles which under optimal conditions are activated via a wavelength of 365nm at an energy intensity of 370µw/cm². However, the experiments in this study indicated that there was a reduction in viable cell counts when activated under visible light at a nanoparticle concentration of 10mg/mL for an extended period of time.
The decrease in viable cell counts may be due to the longer exposure times. Since the energy intensity of the light source utilized for the experiments was low, the activation time under visible light was extended. This infers that if the nanoparticles are doped with an element that results in a lower band gap energy, shorter times of exposure to visible light may be sufficient to inhibit growth. The reduction may also have been partially due to the toxicity of the nanoparticles or the chemical nature of the nanoparticles which may have inhibited the growth of the bacterial cells, as observed by the results of the non-activated nanoparticles.

Similar studies, with lower concentrations of nanoparticles and more time intervals should be conducted. If samples are obtained at several time intervals within 8 hours, it would be possible to see a gradual decline in cell growth over time. Finally, it was evident that Br200 nanoparticles have stronger antibacterial properties than P25 nanoparticles which suggests that brookite nanoparticles may also be more efficient under visible light activation than anatase nanoparticles.
CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Despite many advances with antibiotics and their increased production, the treatment of MRSA with antibiotics still remains a temporary solution or a solution that can be utilized only until MRSA expresses resistance to those certain antibiotics. Due to the high rates of resistance, the use of a treatment different from antibiotics is being tested by many researchers.

Nanoparticles, in particular TiO$_2$ nanoparticles, offer promise for the prevention and treatment of MRSA infections. The experiments conducted in this study have shown that TiO$_2$ nanoparticles, in both the anatase and brookite forms, caused death of bacterial cells. *E. coli* cells were more susceptible to the decomposing effects of TiO$_2$ nanoparticles than *S. aureus* cells. Collectively all strains of bacteria tested showed a significant reduction in viable cell counts at brookite nanoparticle concentrations of 1mg/mL which strongly suggests that brookite may be used against bacteria where multiple drug resistance is evident.

This study concludes that TiO$_2$ nanoparticles under UV light irradiation, particularly the brookite forms, have stronger antibacterial effects than UV light or TiO$_2$ nanoparticles alone. Within 30 minutes of exposure to a 1mg/mL concentration of activated brookite nanoparticles, there was a 100% reduction in MDR *S. aureus*, while activated anatase nanoparticles at the same concentration required longer exposure times.
Brookite phase nanoparticles exhibited antibacterial properties that were effective as suspensions and as coatings. The effective concentration of the suspensions was approximately 1mg/mL while the effective concentration of the coatings is yet to be determined. Non-activated brookite nanoparticles exhibited some level of toxicity. However, the decrease in viable cell counts due to non-activated brookite nanoparticles was significantly less than that caused by activated brookite nanoparticles. In addition, constant activation under UV light was required in order for the nanoparticles to exhibit antibacterial properties. It could also be concluded that activated TiO₂ nanoparticles resulted in bacterial cell damage, as observed on the SEM images. Finally, visible light activation caused a decrease in viable cell counts when exposure times were increased to 8 hours.

To validate the widespread use of these nanoparticles and coatings, as antimicrobial agents, it will be necessary to validate their effects on other microorganisms such as viruses and fungi. It would also be important to determine the safety of the long term usage of activated nanoparticles. Coatings of nanoparticles may be utilized as antimicrobial agents on inanimate objects. Eventually, suspensions of nanoparticles may be used as antimicrobial agents for the elimination of infections on body surfaces. Currently, there are no reports of bacterial resistance to nanoparticle coatings since the nanoparticles result in physical damage to the cells. However, the emergence of a mutant strain of bacteria that may develop resistance to the physical damage caused by nanoparticles is a possibility.
6.2 Recommendations for Future Studies

Titanium dioxide exhibits excellent photocatalytic properties under UV light activation because of its high reactivity and chemical stability. In this study, brookite nanoparticles exhibited more destruction to cells than anatase nanoparticles.

The ultimate aim of this research is to develop nanoparticles that are active under visible light (400nm to 700nm). If these nanoparticles can be activated under white light, their utilization under solar beams will increase (Umebayashi et al., 2003). Once activated under white light, nanoparticles can be used for applications in clinics and hospitals where MRSA is prevalent. The applications could be in the form of paints or coatings which can be applied onto surfaces such as bench tops and walls, medical equipment, and various other materials used in clinics and hospitals.

The development of TiO₂ nanoparticles that can yield high reactivity under visible light activation (smaller than a band gap of 3.2eV or greater than an absorption wavelength of 380nm) would eventually lead to the production of doped-TiO₂ nanoparticles (Asahi et al., 2001). Studies have shown that doping transition metals such as Cr and Co extends the spectral response of TiO₂ into the visible light region by inducing optical transitions from d level electrons of the transition metal ions to the conduction bands of TiO₂ (Morikawa et al., 2001). Other researchers have shown that the substitution of a non-metal atom such as nitrogen and fluorine for oxygen may shift the optical absorption edge to a lower energy, thereby increasing the reactivity of the nanoparticles in the visible light region (Umebayashi et al., 2003). Therefore, nanoparticles with dopants would lead to the practical utilization of these nanoparticles.
Next, this study indicates that UV light activated brookite phase TiO\textsubscript{2} nanoparticles can effectively inhibit bacterial growth. Further studies should be performed to determine the effectiveness of brookite nanoparticles against cancer and tumor cells. In one study, Zhang and colleagues (2004) performed experiments to determine the effects of TiO\textsubscript{2} nanoparticles on Ls-174-t human colon carcinoma cells. They concluded that in the presence of 1mg/ml TiO\textsubscript{2}, 44% of cells were killed within 10 minutes of UVA irradiation and 88% of cells were killed after 30 minutes of irradiation.

Studies utilizing cancer cell lines such as MCF7 (human breast cancer), SiHa (human cervical cancer), HT29 (human colon cancer), and HepG2 (Human hepatocellular liver carcinoma) can be conducted to determine the effects of TiO\textsubscript{2} nanoparticles on eukaryotic cells. The photocatalytic killing of human cancer cells by TiO\textsubscript{2} nanoparticles would imply that TiO\textsubscript{2} nanoparticles together with light irradiation may be used to treat various types of cancer. This mechanism could be adapted to an anticancer therapy by the local or regional treatment of cancer or tumor with TiO\textsubscript{2} nanoparticles, followed by light irradiation.

Free radicals are cells that have lost an electron, thus, are unstable cells. These free radicals basically steal electrons from other cells, ultimately creating new radicals in the process. By stealing electrons, radicals can cause DNA damage, possibly leading to the development of cancer. Therefore, the release of radicals from the TiO\textsubscript{2} nanoparticles may result in the development of cancer. If TiO\textsubscript{2} nanoparticles will be used for treatment against cancer and tumor cells, the release of radicals and their potential to cause DNA damage will need to be addressed.
In conclusion, by activating TiO$_2$ nanoparticles under visible light, the practical usage of nanoparticles is possible. Furthermore, if the activated nanoparticles display killing effects on cancerous and tumor cells, there is promise for use as an anti-cancer and an anti-tumor treatment modality.
CHAPTER 7
REFERENCES


